Risk Assessment of Exposure to Organic Chemicals in Children and Mothers using Human Biomonitoring

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Risk Assessment of Exposure to Organic Chemicals in Children and Mothers using Human Biomonitoring

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Sandra Fernández Fernández, Graduada en Química por la Universitat de València, ha estado trabajando bajo nuestra dirección en la elaboración de la tesis **"Risk Assessment of Exposure to Organic Chemicals in Children and Mothers using Human Biomonitoring"**, y autorizan su presentación para optar al título de Doctora con Mención Internacional por la Universitat de València.

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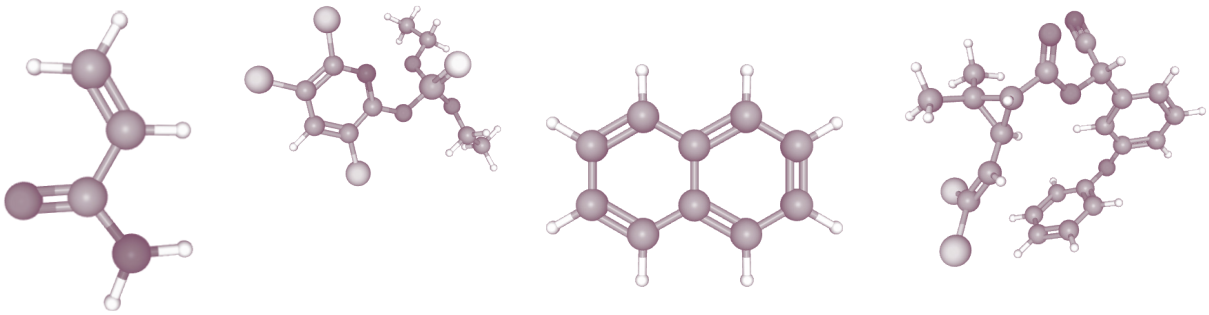
“To keep the body in good health is a duty, otherwise we shall not be able to keep our mind strong and clear”

— Buddha (566 - 486 B.C.)



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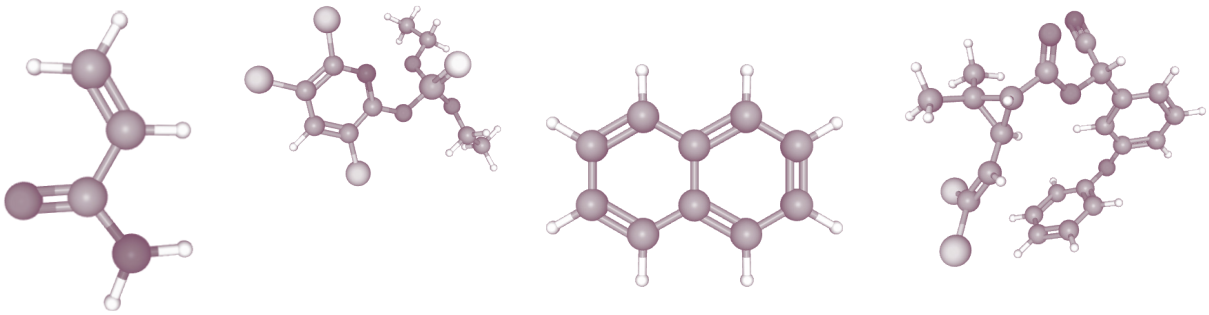
A mis tías, mi fuente de energía positiva



RESUMEN AMPLIO

INTRODUCCIÓN

En la actualidad, la producción mundial de productos químicos aumenta cada año, lo que hace que la contaminación ambiental y la exposición humana a sustancias químicas sea un grave problema de salud pública vinculado al desarrollo de muchas enfermedades crónicas. Se estima que aproximadamente el 70 % de las sustancias químicas consumidas en Europa en 2019 pueden generar algún efecto nocivo adverso en la salud humana. Estas sustancias pueden entrar en la cadena alimentaria y permanecer en el medio ambiente, provocando que los seres humanos estén expuestos a ellas principalmente por ingestión, inhalación o exposición dérmica. Por lo tanto, la exposición a sustancias químicas debe controlarse continuamente para garantizar la protección de la salud de los/as ciudadanos/as. Esto puede realizarse de dos formas:

i) estudiando su presencia en compartimentos ambientales, como el agua, los alimentos, el suelo o el aire, mediante la vigilancia ambiental, y

ii) determinando su presencia en muestras biológicas, como la orina, la sangre, el pelo, etc., mediante la biomonitorización humana (HBM). A diferencia de los estudios de vigilancia ambiental, los datos derivados de estudios de HBM reflejan la carga corporal total que integra todas las potenciales vías de exposición a sustancias químicas, siendo un componente clave para guiar la toma de decisiones en políticas medioambientales y sanitarias.

A pesar del gran potencial de los estudios de HBM, solo un número reducido de países en el mundo presentan actualmente programas nacionales de HBM. Entre ellos se encuentran Estados Unidos (EE.UU.), Canadá, Alemania y Bélgica, cuyos gobiernos financian, coordinan y gestionan estos programas de HBM regularmente con el fin de generar datos de los niveles de exposición interna a una amplia variedad de sustancias químicas en una muestra representativa de la población,

y así controlar y evaluar la exposición a lo largo del tiempo. En España, existen muchos estudios de HBM a nivel regional, pero actualmente no existe un programa nacional que cubra una muestra representativa de la población. Por esta razón, el Ministerio de Sanidad y el Ministerio para la Transición Ecológica y el Reto Demográfico han contemplado en el «Plan Estratégico de Salud y Medio Ambiente 2022 – 2026», la creación urgente de un nodo nacional para la realización de campañas periódicas de HBM en España.

En el diseño de un estudio de HBM y en la interpretación de los datos resultantes se deben tener en consideración varios factores. En primer lugar, es necesario seleccionar la combinación biomarcador(es)-matriz que mejor refleje la exposición a la sustancia o grupos de sustancias en estudio. Además, el procedimiento de muestreo debe ser sólido y reproducible para obtener datos comparables, y la matriz y los biomarcadores seleccionados deben ser estables durante el muestreo, el transporte, el procesamiento y el análisis. En este sentido, las matrices más estudiadas son la orina y la sangre. El análisis de orina proporciona información sobre la exposición reciente a sustancias no persistentes e hidrófilas, como los bisfenoles, los parabenos y los ftalatos. En el caso de contaminantes persistentes como las dioxinas o algunos metales, la sangre es la matriz más común y representa la exposición a largo plazo, ya que está en contacto continuo con todos los órganos y tejidos donde se depositan estas sustancias.

Por otro lado, también es importante la elección del método analítico adecuado, que debe ser lo suficientemente sensible y selectivo, pues los biomarcadores de exposición suelen estar presentes en bajas concentraciones en las muestras biológicas. Las técnicas más empleadas para la determinación de biomarcadores de exposición a sustancias orgánicas en HBM son la cromatografía líquida (LC) y gaseosa (GC) acopladas a espectrometría de masas en tándem (MS/MS). En este sentido, la metodología analítica debe ser robusta y cumplir ciertos criterios de funcionamiento en el rango de concentración previsto, especialmente en cuanto a precisión y exactitud. En consecuencia, la validación del método, así como el frecuente control de la calidad tanto externa, mediante participación en ejercicios interlaboratorio, como interna de todo el proceso analítico, es vital para garantizar la fiabilidad y comparabilidad de los datos generados, con el fin de ser interpretados y empleados en posteriores evaluaciones del riesgo.

En un contexto de evaluación del riesgo, existen dos estrategias para evaluar la exposición humana a sustancias químicas utilizando datos de HBM: la dosimetría directa y la dosimetría inversa. En la primera, se comparan directamente los datos obtenidos con un valor guía basado en salud (HBGV), que se corresponde con la concentración del biomarcador en la muestra biológica de interés en relación con un determinado efecto adverso sobre la salud, como el *Biomonitoring Equivalent* (BE). Sin embargo, actualmente muchas sustancias químicas no presentan un HBGV equivalente para poder emplear este método. En estos casos se suele utilizar la dosimetría inversa, en la que se necesita información toxicocinética para transformar la concentración del biomarcador en la matriz estudiada en una ingesta diaria estimada (EDI), que luego se compara con el HBGV adecuado, como la ingesta diaria admisible (ADI), el

nivel sin efecto adverso observado (NOAEL), la dosis de referencia (RfD) o el límite mínimo de confianza para la dosis de referencia ($BMDL_{10}$). En general, para la evaluación del riesgo, se utiliza el cociente de la concentración del biomarcador en la matriz analizada (dosimetría directa) o la EDI (dosimetría inversa), y el HBGV correspondiente, conocido como cociente de peligrosidad (HQ), o la inversa del mismo, llamado margen de exposición (MOE). Para el cálculo del riesgo asociado a la exposición a mezclas con el mismo modo de acción o que provocan el mismo efecto sobre la salud, se suele emplear la suma de los HQ, llamado índice de peligrosidad (HI), o el margen de exposición total (MOET), como la suma de la inversa de los MOEs, respectivamente. Sin embargo, todavía existen muchas lagunas de conocimiento en este ámbito.

En la presente tesis se estudió la exposición de población infantil y de madres lactantes, por ser consideradas poblaciones más vulnerables, a los siguientes grupos de sustancias químicas: plaguicidas, hidrocarburos aromáticos policíclicos (PAHs) y acrilamida, mediante estudios de HBM. La selección de estos compuestos se basó en los objetivos de la iniciativa europea *Human Biomonitoring for Europe* (HBM4EU), de la Agencia Española de Seguridad Alimentaria y Nutrición, y del «Plan Nacional de Control Oficial de la Cadena Alimentaria» en España. Los siguientes apartados contienen información relevante acerca de las aplicaciones, efectos sobre la salud, legislación, exposición y metabolismo de cada grupo de sustancias estudiadas.

PLAGUICIDAS

Los plaguicidas son sustancias químicas que se utilizan para controlar insectos, roedores, hongos y malas hierbas no deseadas, o enfermedades transmitidas por vectores, así como para proteger las plantas durante la producción, el almacenamiento y el transporte, con el objetivo de lograr mayores rendimientos. Además, estas sustancias también pueden utilizarse ocasionalmente con fines domésticos y en lugares públicos, como parques y jardines. En Europa, los principales tipos de plaguicidas utilizados en 2020 fueron los insecticidas, como los organofosforados (OPs) y los piretroides (Pyr), algunos herbicidas (Herb), como las cloroacetanilidas o las triazinas, y varios grupos de fungicidas y bactericidas.

Algunos de estos plaguicidas tienen propiedades neurotóxicas y de alteración endocrina pudiendo afectar a la fauna y la salud humana. Por ejemplo, el clorpirifos, uno de los insecticidas OPs más utilizados en Europa en los últimos años, ha sido recientemente prohibido y clasificado como categoría 1B (‘tóxico para la reproducción’) por la Agencia Europea de Seguridad Alimentaria (EFSA). Otros plaguicidas han sido clasificados en los grupos 2A (‘probablemente cancerígenas para los seres humanos’) y 2B (‘posiblemente cancerígenas para los seres humanos’) por la Agencia Internacional para la Investigación del Cáncer (IARC). De hecho, la exposición a plaguicidas se ha relacionado con diversos problemas de salud, como cáncer, Parkinson, Alzheimer, trastornos reproductivos, etc., así como alteraciones en el desarrollo de

los/las niños/as. Por tanto, la exposición a estas sustancias durante el embarazo, la lactancia y la infancia es especialmente preocupante.

Debido a los posibles efectos nocivos de los plaguicidas sobre la salud, su uso está legislado desde 1979 en la Unión Europea (UE). Así, las autoridades de salud pública controlan la presencia de plaguicidas para garantizar una baja exposición de los consumidores en diversos productos finales de consumo, como alimentos y piensos de origen vegetal y animal (Reglamento de la Comisión Europea, CE, 396/2005), productos alimentarios destinados a lactantes y niños/as pequeños/as (Reglamento (UE) 2016/127), y aguas (Directiva 2006/118/CE).

Los OPs, Pyr y Herb son plaguicidas no persistentes, en consecuencia no se bioacumulan en el organismo y se excretan principalmente por la orina en menos de 72 horas tras la exposición en forma libre o conjugada (glucurónidos o sulfatos). Por tanto, el análisis de los mismos y/o de sus metabolitos en la orina proporciona información sobre la exposición reciente a estas sustancias. Los productos de biotransformación de los plaguicidas pueden ser metabolitos inespecíficos (comunes a una familia de plaguicidas), como los dialquil fosfatos (DAPs), o específicos, como el 3,5,6-tricloro-2-piridinol (TCPY), que solamente proporciona información sobre la exposición a clorpirifos.

Dado que los plaguicidas están presentes no solo en la cadena alimentaria, sino también en el suelo, el agua y el aire, la población general está expuesta a sus residuos a través de varias vías. La principal vía de exposición a plaguicidas es la ingestión, principalmente del consumo de frutas y verduras, y la ingesta de agua. No obstante, la inhalación y el contacto dérmico por contacto directo o el polvo también pueden desempeñar un papel importante. Por tanto, el desarrollo de estudios de HBM que den información acerca de la exposición global a estas sustancias, teniendo en cuenta los posibles efectos acumulativos y sinérgicos, es de vital importancia.

De los países europeos, España es uno de los principales productores agrícolas, siendo además el segundo país con mayores ventas de plaguicidas (según datos de 2018). Así, la población española podría estar altamente expuesta a estas sustancias, y por tanto se considera necesario llevara a cabo estudios de HBM centrados en la evaluación de la exposición a plaguicidas en este país.

HIDRUROS AROMÁTICOS POLICÍCLICOS

Los PAHs son compuestos orgánicos altamente lipofílicos formados por dos o más anillos aromáticos, que se liberan al medio ambiente durante la combustión incompleta de la materia orgánica procedente de fuentes naturales (p. ej., erupciones volcánicas, incendios forestales) y de actividades antropogénicas e industriales, como el humo del tabaco, los escapes de los

vehículos, quema de combustibles, así como de algunas prácticas culinarias (asado, fritura y ahumado de alimentos). Además, se utilizan en la fabricación de medicamentos, plastificantes, pigmentos, agentes secantes y algunos plaguicidas.

En los últimos años, la exposición a PAHs se ha convertido en una preocupación para la salud pública, ya que algunos de ellos se han clasificado como Grupo 1 ('carcinógeno para los seres humanos') o Grupo 2B por la IARC. Además, la Agencia de Protección Ambiental (EPA) de EE.UU. y la Agencia para las Sustancias Tóxicas y el Registro de Enfermedades (ATSDR) han incluido varios PAHs en su lista de sustancias prioritarias para su seguimiento, ya que se consideran amenazantes para la salud debido a su potencial toxicidad y la alta frecuencia de exposición entre la población. De hecho, numerosos estudios epidemiológicos han identificado algunos PAHs como sustancias genotóxicas, carcinógenas, mutagénicas y teratógenas, ya que pueden causar daños en el ADN, las proteínas y los lípidos a través del estrés oxidativo. Asimismo, la exposición humana a PAHs se ha relacionado con el desarrollo de muchas enfermedades crónicas como el cáncer, la diabetes, las enfermedades cardiovasculares y la hipertensión. Además, también pueden provocar efectos adversos sobre los sistemas inmunológico y reproductor, así como en el desarrollo de los seres humanos, por lo que la población infantil y las mujeres embarazadas/lactantes se consideran grupos especialmente vulnerables en este contexto.

Para controlar y mitigar la exposición a PAHs en la población general, la UE ha puesto en marcha varias medidas reguladoras de la presencia de PAHs en los alimentos (Reglamento 1881/2006) y el medio ambiente, como el agua (Directivas 2000/60) y el aire (Directiva 2016/2284/CE). Además, su contenido en cauchos y componentes plásticos de artículos de consumo está restringido bajo el Reglamento de Registro, evaluación, autorización y restricción de sustancias químicas (REACH) y el Reglamento 1272/2013, respectivamente.

Una vez que se produce la exposición a PAHs, estos se absorben rápidamente en el tracto gastrointestinal, se distribuyen en los diferentes tejidos del cuerpo y se metabolizan en el cuerpo humano, excretándose como metabolitos hidroxilados (OHPAHs) conjugados con ácido glucurónico o sulfato en la orina (PAHs de bajo peso molecular) o en las heces (PAHs de alto peso molecular). Sin embargo, debido a sus características lipofílicas, muchos PAHs también pueden encontrarse en la sangre, la leche materna o el tejido adiposo.

En general, la principal vía de exposición humana a PAHs es la ingestión de alimentos. Sin embargo, la inhalación del humo del tabaco y el contacto dérmico también contribuyen de forma significativa a la exposición a PAHs en personas fumadoras, aquellas que viven en zonas cercanas a fuentes de combustión intensas y en las que están expuestas laboralmente. Por tanto, la amplia exposición de la población a PAHs y su toxicidad ha despertado el interés en evaluar la carga corporal interna de PAHs proveniente de todas las fuentes y vías posibles

a través de estudios de HBM. Entre todos los OHPAHs, el metabolito urinario del pireno, el 1-hidroxipireno (1OHPYR), es el biomarcador más comúnmente utilizado para evaluar la exposición general a PAHs. Sin embargo, dado que los seres humanos están expuestos a una mezcla de PAHs y no a un único compuesto, el HBM de otros metabolitos de PAHs junto con el 1OHPYR proporciona una evaluación global más precisa de la exposición a PAHs. En concreto, en España existe una escasez de datos de HBM sobre la exposición a PAHs en la población.

ACRILAMIDA

La acrilamida (AA) es un compuesto orgánico de bajo peso molecular, clasificado como probable cancerígeno para los seres humanos (grupo 2A) por la IARC. Comercialmente, tiene muchas aplicaciones industriales, ya que se utiliza como monómero en la síntesis de polímeros de poliacrilamida, que se emplean en las industrias del papel y textil, el tratamiento del agua, la construcción, los cosméticos y el envasado de alimentos. Además, también está presente en el humo del tabaco.

En 2002, el descubrimiento de la formación de AA en alimentos ricos en hidratos de carbono a temperaturas superiores a 120 °C y en condiciones de baja humedad (p. ej., durante la fritura, el asado y el horneado) a través de la reacción de Maillard dio la voz de alarma entre la sociedad científica y las autoridades de salud pública. En consecuencia, debido a su potencial amenaza para la salud, en Europa se han adoptado diferentes medidas reguladoras para mitigar la exposición humana a AA mediante la ingesta de alimentos (Reglamento (UE) 2017/2158) y agua potable (Directiva 98/83/CE), además se ha restringido su uso en productos cosméticos (Reglamento (CE) 1223/2009) y materiales en contacto con alimentos (Reglamento (UE) 10/2011).

Diversos estudios *in vivo* e *in vitro* han demostrado que la AA produce efectos neurotóxicos, carcinógenos, mutagénicos, genotóxicos e inmunotóxicos. En humanos, se ha relacionado con el desarrollo de enfermedades cardiovasculares y daños por estrés oxidativo. Además, la exposición a AA se ha asociado con efectos negativos en el desarrollo de los/as niños/as, ya que es capaz de transferirse al feto a través de la placenta y de las barreras hematoencefálicas, y a los bebés lactantes a través del consumo de leche materna, concluyendo que la población infantil, las embarazadas y las madres lactantes son grupos de población de alto riesgo en este sentido. Por otro lado, aunque el último informe de la EFSA aporta nuevas evidencias de sus propiedades genotóxicas y cancerígenas, según diversos estudios epidemiológicos todavía no hay pruebas consistentes de su relación con el riesgo de padecer cáncer en humanos.

Una vez se produce la exposición humana a AA, esta se absorbe rápidamente y se distribuye a todos los tejidos del organismo. Alrededor del 50 % de la AA ingerida se excreta tras 24

h en orina como N-acetil-S-(2-carbamoiletil)-L-cisteína (AAMA), N-acetil-S-(2-carbamoil-2-hidroxietil)-L-cisteína (GAMA3), y acetil-3-[(3-amino-3-oxopropil)sulfinil]-L-alanina (AAMA-Sul). Por tanto, estos tres metabolitos son ampliamente utilizados como biomarcadores de exposición reciente a AA en orina.

Se considera que la dieta es la principal fuente de exposición a AA en la población general no fumadora, aunque existen algunas vías de exposición no dietéticas, como la inhalación en personas fumadoras o la absorción dérmica en población ocupacionalmente expuesta. Una forma de tener en cuenta todas las posibles vías y fuentes de exposición a AA en un contexto de evaluación del riesgo es mediante la aplicación de programas de HBM, complementando así a los tradicionales estudios de dieta total que solo tienen en consideración la contribución del consumo de alimentos. En Europa, se han notificado muy pocos estudios sobre la exposición interna a AA en la población general y, especialmente, en grupos vulnerables. Por lo tanto, teniendo en cuenta la potencial actividad carcinogénica de la AA y la escasez de estudios en Europa, especialmente en España, la obtención de datos de HBM sobre la exposición interna a AA es de gran interés desde la perspectiva de salud pública.

OBJETIVOS

El objetivo de esta tesis doctoral fue desarrollar diferentes herramientas analíticas para su aplicación a proyectos de HBM, y diseñar y realizar estudios específicos de HBM en población infantil y madres lactantes centrados en plaguicidas no persistentes, PAHs de bajo peso molecular y acrilamida. En concreto, los objetivos eran:

i) Desarrollar, optimizar y validar una nueva metodología para analizar seis metabolitos inespecíficos de plaguicidas organofosforados en orina mediante LC-MS/MS (**Artículo I.1**).

ii) Desarrollar un estudio de HBM sobre la exposición a plaguicidas no persistentes en madres lactantes (**Artículo I.2**) y en población infantil (**Artículo I.3**): determinación de niveles urinarios, estimación de la evaluación del riesgo y análisis estadístico.

iii) Realizar un estudio de HBM sobre la exposición a PAHs de bajo peso molecular en madres lactantes (**Artículo II.1**) y población infantil (**Artículo II.1**): descripción de los niveles urinarios, evaluación de los riesgos asociados e identificación de los posibles determinantes de la exposición.

iv) Realizar un estudio de HBM sobre la exposición a acrilamida en madres lactantes (**Artículo III.1**) y población infantil (**Artículo III.2**): determinación de las concentraciones de los metabolitos de acrilamida en orina, evaluación de la exposición en un contexto de riesgo e identificación de posibles fuentes de exposición.

MATERIALES Y MÉTODOS

POBLACIÓN ESTUDIADA

Los dos grupos de población estudiados en la presente tesis fueron las madres lactantes y los/as niños/as del proyecto BETTERMILK y el programa BIOVAL de la Comunidad Valenciana, respectivamente. En el proyecto BETTERMILK, el reclutamiento tuvo lugar en 2015 en el Hospital Universitario y Politécnico La Fe (Valencia, España), periodo en el que las mujeres participantes acudieron al hospital para dar a luz. Un total de 120 mujeres (20 – 45 años) cumplieron los criterios de selección y se ofrecieron a participar en el estudio. El programa BIOVAL, llevado a cabo por la Dirección de Salud Pública de la Generalitat Valenciana (España), se desarrolló en 2016 y, en el contexto del mismo, se reclutó una muestra representativa aleatoria de 666 niños/as (5 – 12 años) de 25 escuelas primarias ubicadas en 16 municipios de todo el territorio valenciano. Cada participante dio su consentimiento informado por escrito, una muestra de orina y un cuestionario sobre sus costumbres dietéticas, estilo de vida y características sociodemográficas.

DETERMINACIÓN DE METABOLITOS DE PLAGUICIDAS EN ORINA

En la presente tesis se ha desarrollado y optimizado un nuevo método para la determinación en orina de seis DAPs: dimetil fosfato (DMP), dietil fosfato (DEP), dimetil tiofosfato (DMTP), dietil tiofosfato (DETP), dimetil ditiofosfato (DMDTP), y dietil ditiofosfato (DEDTP). Para ello, se probaron tres métodos de preparación de muestras, *dilute-and-shoot*, *QuEChERS* ('rápido, fácil, barato, eficaz, resistente y seguro') con diferentes sales, disolventes y sorbentes de limpieza, y extracción en fase sólida (SPE) con cartuchos *STRATA X-AW* (fase polimérica de intercambio aniónico débil) en diferentes condiciones. Para la separación cromatográfica, se evaluaron las siguientes columnas HILIC (cromatografía de interacción hidrófila): *KINETEX HILIC* e *Hypersil GOLD HILIC* de las mismas dimensiones (2.1 × 100 mm, 3 µm). También se probaron varios pH y modificadores de la fase móvil A (agua) y fase móvil B (acetonitrilo). Para maximizar la señal de los DAPs en el espectrómetro de masas, los parámetros de la fuente de ionización de electroespray (ESI) se optimizaron mediante un diseño de experimentos (DoE) que consistía en un *Plackett-Burman* de dos niveles seguido de un *Central Composite Design*.

El método que proporcionó resultados óptimos consiste en lo siguiente: a 4 ml de muestra de orina se le adiciona la solución de trabajo de patrones internos (IS) a una concentración de 5 $\mu g \cdot ml^{-1}$ y HCl 6 M. A continuación, la mezcla se centrifuga y se transfiere a un tubo de polipropileno, donde se añade acetato de etilo y éter dietílico para la extracción. Después de enfriar en un baño de hielo, se añade un paquete de extracción *QuEChERS Original* (4 g $MgSO_4$

y 1 g NaCl) y un homogeneizador de cerámica, y se agita la mezcla enérgicamente. La capa orgánica total se separa por centrifugación y se recoge en un tubo limpio, donde luego se evapora bajo una corriente de N_2. El residuo seco se disuelve en fase móvil (agua:acetonitrilo con acetato amónico 200 mM al 5 %), se centrifuga, se transfiere a un vial de inyección que se analiza por LC-MS/MS, en un sistema *Finnigan Surveyor* HPLC (Cromatografía líquida de alta eficacia), equipado con una columna *KINETEX HILIC* (2.1 × 100 mm, 3 µm). La detección de los DAPs se realiza en un espectrómetro de masas *Finnigan TSQ Quantum Ultra* de triple cuadrupolo equipado con una fuente ESI en modo negativo. El límite de cuantificación (LoQ) del procedimiento es de 0.5 $ng \cdot ml^{-1}$ para todos los DAPs.

DETERMINACIÓN DE METABOLITOS DE PAHs EN ORINA

El análisis de los 11 metabolitos urinarios de PAHs, 1-hidroxinaftaleno (1OHNAP), 2-hidroxinaftaleno (2OHNAP), 2-hidroxifluoreno (2OHFLU), 3-hidroxifluoreno (3OHFLU), 1-hidroxifenantreno (1OHPHEN), 2-hidroxifenantreno (2OHPHEN), 3-hidroxifenantreno (3OHPHEN), 4-hidroxifenantreno (4OHPHEN), 9-hidroxifenantreno (9OHPHEN), 1OHPYR y 3-hidroxibenzo(a)pireno (3OHBAP), se realizó aplicando el procedimiento desarrollado por *Lankova et al. (2016)* pero con algunas modificaciones. La desconjugación de los derivados glucurónidos y sulfatados de los OHPAHs se optimizó aplicando dos enzimas diferentes, β-Glucuronidasa/Arilsulfatasa de *Helix pomatia* y β-Glucuronidasa de *Escherichia coli*, a diferentes tiempos de incubación (30 min, 2 h, 6 h y 17 h), utilizando muestras de orina y un material de referencia. También se testaron dos tipos de limpieza para los extractos, basados en la adición de Z-Sep y eliminación de la enzima por filtración. Los parámetros de la fuente de ionización ESI fueron optimizados mediante la aplicación de un DoE.

El método final consiste en lo siguiente: 2.5 ml de orina se mezclan con la solución de trabajo de IS a concentraciones entre 1.5 y 3 $ng \cdot ml^{-1}$. Esta mezcla se somete a un paso de desconjugación enzimática utilizando β-Glucuronidasa/Arilsulfatasa de *Helix pomatia* a 37 ºC durante 15 h, seguido de una extracción líquido-líquido con acetato de etilo y una limpieza con *Supel™ QuE Z-Sep (*300 mg Z-Sep y 900 mg $MgSO_4$). A continuación, el extracto limpio se evapora y se reconstituye en fase móvil (agua:metanol al 0.1 % de ácido acético). Los OHPAHs en el extracto final se determinan utilizando un sistema HPLC *Vanquish* equipado con una columna *KINETEX Core-shell technology F5* (2.1 x 150 mm, 2.6 µm). Su detección se realiza en un espectrómetro de masas *Finnigan TSQ Quantum Ultra* de triple cuadrupolo equipado con una fuente ESI en modo negativo. El LoQ del procedimiento es de 0.05 $ng \cdot ml^{-1}$ para el 3OHFLU y el 3OHBAP, y de 0.01 $ng \cdot ml^{-1}$ para el resto de los OHPAHs.

DETERMINACIÓN DE METABOLITOS DE AA EN ORINA

Se utilizó un método de *dilute-and-shoot* basado en *Brisson et al. (2014)* para analizar AAMA, GAMA3 y AAMA-Sul en las muestras de orina. Para la optimización del método, se ensayaron dos factores de dilución diferentes (1:2 y 1:5), así como dos estrategias de reducción de interferencias (centrifugación y filtración). La optimización de los parámetros de la fuente de ionización por electroespray térmico (HESI) se llevó a cabo utilizando un DoE.

Brevemente, la metodología seleccionada consiste en lo siguiente: a 200 µl de la muestra de orina homogeneizada se le adiciona la solución de trabajo de IS a 25 $ng \cdot ml^{-1}$ y se disuelve en fase móvil (ácido acético al 0.08 %) hasta 1 ml. A continuación, se agita la mezcla y se inyecta en el sistema de LC-MS/MS. La separación cromatográfica de los tres metabolitos AA se realiza en un sistema HPLC *Vanquish* equipado con una columna *Atlantis dC18* (150 x 2.1 mm, 2.6 µm, 100 Å). La detección se realiza en un espectrómetro de masas de triple cuadrupolo *TSQ Altis*™ equipado con una fuente de ionización HESI en modo negativo. El LoQ del procedimiento es de 0.25 $ng \cdot ml^{-1}$ para los tres analitos.

VALIDACIÓN, ASEGURAMIENTO Y CONTROL DE LA CALIDAD

Las metodologías de validación y control de calidad para la cuantificación de DAPs, OHPAHs y metabolitos de AA en orina se basaron en la *Bioanalytical Method Validation Guidance for Industry* de la Administración de Alimentos y Medicamentos (FDA) de EE.UU., y en la *SANTE/11813*. Se redactó un procedimiento normalizado de trabajo para cada procedimiento analítico que contiene una descripción detallada de la metodología, así como procedimientos específicos para controlar los criterios de funcionamiento críticos durante la validación y el análisis de las muestras: linealidad, exactitud, precisión, sensibilidad y especificidad. Asimismo, como evaluación externa de la calidad de algunos de los biomarcadores analizados, el laboratorio participó en los programas de intercomparación alemanes *G-EQUAS,* y en las cinco rondas del programa de intercomparación organizado por la iniciativa HBM4EU. Además, también se evaluó la calidad de forma interna mediante el análisis de materiales de referencia y el uso de controles de calidad internos en cada secuencia con el fin de asegurar la calidad de los resultados a lo largo del tiempo.

TRATAMIENTO DE DATOS Y ANÁLISIS ESTADÍSTICO

En primer lugar, se utilizó el método de estimación de máxima verosimilitud propuesto por la EFSA para imputar estadísticamente las concentraciones de analitos no detectados o detectados por debajo del LoQ. Para la construcción del modelo de regresión lineal, aquellos metabolitos que provenían del mismo compuesto se sumaron para evaluarse en conjunto. Los modelos de regresión lineal se construyeron utilizando el logaritmo neperiano de las concentraciones (en $ng \cdot ml^{-1}$ o $nmol \cdot l^{-1}$), y aplicando el procedimiento de selección de variables *Forward Stepwise Regression* ('paso a paso hacia delante') basado en el criterio de información de Akaike corregido, o *Backward Stepwise Regression* ('paso a paso hacia atrás') basado en el criterio de Error de Predicción Final Robusto. Un valor de significación menor o igual a 0.05 se consideraba estadísticamente significativo.

EXPOSICIÓN Y EVALUACIÓN DEL RIESGO

Con el fin de estimar la exposición a plaguicidas, PAHs y AA, se utilizó principalmente el enfoque de la dosimetría inversa. Para ello, se calculó la EDI a partir de los niveles urinarios de los metabolitos analizados. Para evaluar el riesgo asociado a estas EDIs, se aplicó el concepto de HQ para los tres grupos de compuestos, y de MOE para los plaguicidas y la AA, mediante la comparación con el correspondiente valor de referencia basado en salud: ADI (para el HQ) y NOAEL (para el MOE) en el caso de los plaguicidas, RfD para el cálculo del HQ en los PAHs, y $BMDL_{10}$ (MOE) para la AA. Para la evaluación del riesgo acumulado, se estimó el HI para las sustancias con el mismo modo de acción (OPs y PAHs, por separado), y el MOET para los plaguicidas que causan alteraciones del sistema motor ($MOET_{motor}$) y los que afectan a la actividad de la acetil colinesterasa ($MOET_{AChE}$). En el caso de la AA, también se utilizó el enfoque de la dosimetría directa comparando los niveles de AAMA en orina directamente con el BE mediante diagramas de priorización y el cálculo del HQ.

RESULTADOS Y DISCUSIÓN

CAPÍTULO I

BIOMONITORIZACIÓN DE PLAGUICIDAS NO PERSISTENTES EN POBLACIÓN INFANTIL Y MADRES: NIVELES URINARIOS, PREDICTORES DE EXPOSICIÓN Y EVALUACIÓN DEL RIESGO

En la primera parte del capítulo I, se desarrolló y validó un método basado en QuEChERS y LC-MS/MS para el análisis simultáneo de seis metabolitos no específicos de OPs (DAPs) en orina (**Artículo I.1**).

De los métodos de preparación de muestra testados, se seleccionó finalmente el método basado en QuEChERS (4 g $MgSO_4$ y 1 g NaCl), y 8 ml de una mezcla de éter dietílico:acetato de etilo (1:1), sin necesidad de una limpieza adicional, debido a que las recuperaciones observadas empleando esta metodología fueron las más altas para todos los analitos logrando el LoQ más bajo (0.5 $ng \cdot ml^{-1}$) para todos ellos. Para la separación cromatográfica, se seleccionó la columna *Hypersil GOLD HILIC* (2.1 × 100 mm, 3 µm), ya que la resolución era mayor para los 6 DAPs y el tiempo de análisis era tres veces menor que para la columna *KINETEX HILIC*. Las fases móviles consistían en agua y acetonitrilo con acetato de amonio 200 mM (pH = 6.3).

El método seleccionado se validó en términos de linealidad, exactitud, precisión y selectividad a tres niveles de concentración, y utilizando el *G-EQUAS 60/2017* como material de referencia. El rango dinámico lineal era de 0.5 – 50 $ng \cdot ml^{-1}$ con coeficientes de correlación lineal (R^2) superiores a 0.98 y residuales inferiores al 20 %. La exactitud (%R, porcentaje de recuperación relativa) y precisión (%CV, porcentaje de coeficiente de variación) oscilaban entre el 82 % (DMP, a 5 $ng \cdot ml^{-1}$) y el 117 % (DMP y DEDTP, al LoQ), y entre el 3 % (DMP, al LoQ) y el 20 % (DMDTP, a 5 $ng \cdot ml^{-1}$), respectivamente, con selectividades (entendida como presencia de interferencias) menores al 30 %. Además, se obtuvieron Z-scores ≤ 2 en el análisis del *G-EQUAS/60-2017*. Así pues, el método analítico propuesto cumplió todos los criterios de garantía de calidad que se definieron previamente.

En comparación con otras metodologías de análisis de DAPs basadas en LC-MS previamente publicadas en la bibliografía, el LoQ obtenido en este método era el más bajo hasta el momento. Además, este mostraba recuperaciones considerablemente mejores (82 – 117 % vs 13 – 122 % en otros estudios) que la gran mayoría, principalmente para DEDTP y DMP. Además, los %CV máximos obtenidos para DEDTP (11 %), DMP (12 %), DMTP (19 %) y DMDTP (20 %) son muy inferiores a los observados en otros métodos, que eran de hasta el 24 %, 70 %, 34 % y 30 %, respectivamente. Hasta el momento, la estrategia desarrollada ofrecía los resultados

más precisos y exactos de todas las metodologías basadas en LC-MS. Es cierto que existen métodos basados en GC con menores LoQs, pero estos necesitan un paso de derivación complejo y tiempos de análisis más elevados, lo que conlleva un mayor coste de análisis.

El método desarrollado para la determinación de DAPs, junto con otros 20 metabolitos de OPs, piretroides y herbicidas, se aplicó para el análisis de orina de 116 madres (Artículo I.2) y 568 niños y niñas (Artículo I.3) de los proyectos BETTERMILK y BIOVAL llevados a cabo en España.

En el 99 % de las madres se detectó al menos uno de los DAPs analizados. Las frecuencias de detección (DF) de DAPs oscilaron entre el 3 % (DEDTP) y el 91 % (DEP). La mayor media geométrica (GM) se obtuvo para el DEP (1.9 $ng{\cdot}ml^{-1}$). Por otro lado, el 98 % de las muestras de orina analizadas tenían al menos un metabolito específico de plaguicidas: el TCPY fue el detectado con mayor frecuencia (85 %, GM = 1.4 $ng{\cdot}ml^{-1}$), seguido del p-nitrofenol (PNP), metabolito específico del paratión (84 %, GM = 0.8 $ng{\cdot}ml^{-1}$). El metabolito inespecífico de Pyr, el ácido 3-fenoxibenzoico (PBA), fue el más frecuentemente detectado de este grupo (65 %), con una GM de 1.4 $ng{\cdot}ml^{-1}$. En el caso de la población infantil, el 85 % de las muestras de orina presentaban cinco o más metabolitos de plaguicidas. De hecho, se detectó al menos un metabolito de OPs y Pyr en el 97 % y el 83 % de los/las niños/as en estudio, respectivamente. La mayor DF y GM se obtuvo para el DEP (92 %, 1.5 $ng{\cdot}ml^{-1}$), seguido por el DMP (65 %, 0.9 $ng{\cdot}ml^{-1}$). Por otro lado, los metabolitos PNP, TCPY y PBA se encontraron en más del 74 % de las muestras con GMs superiores a 1 $ng{\cdot}ml^{-1}$. En un contexto global, la población española objeto de estudio parecía estar más expuesta a los Pyr, ya que presentaba mayores niveles de PBA en orina en comparación con otros países.

El análisis estadístico mostró que la cercanía a las actividades agrícolas estaba relacionada con mayores niveles urinarios de TCPY, y aquellas mujeres con jardín/plantas en el hogar presentaban mayores niveles de DAPs en orina, posiblemente debido al uso de OPs en estas áreas/zonas del hogar. En el caso del PNP, aquellas mujeres que vivían en zonas urbanas, parecían tener mayores concentraciones en orina. Sin embargo, hay que tener en cuenta que el PNP es posible que sea metabolito de otros compuestos químicos diferentes al paratión, ya que este plaguicida lleva prohibido desde el siglo XX. En población infantil, se observó que el consumo de verduras, y de legumbres y cereales estaba significativamente asociado con niveles más altos de PBA y DAPs en orina, respectivamente. Además, la población infantil de menor edad parecía presentar mayores concentraciones de DAPs en orina, lo que podía estar relacionado con la mayor ingesta comparada con el peso corporal de esta población.

Para la evaluación del riesgo, se calcularon las EDIs para ambas poblaciones. En el caso de las madres, las medias oscilaban entre 0.09 (dimetotato) y 0.40 $\mu g{\cdot}kg^{-1}{\cdot}día^{-1}$ (clorpirifos), y para la población infantil, entre 0.08 (clorpirifos) y 1.62 $\mu g{\cdot}kg^{-1}{\cdot}día^{-1}$ (λ-cihalotrina). En el percentil

95 (P95), se observaron las EDIs más altas para la deltametrina (4.73 μg·kg^{-1}·día^{-1}) en el caso de las madres, y para la λ-cihalotrina (1.62 μg·kg^{-1}·día^{-1}) en el caso de la población infantil, ambos pertenecientes a la familia de los Pyr. La evaluación del riesgo dio valores medios de HQ entre 0.01 (clorpirifos) y 0.05 (dimetoato) en el caso de las madres, y entre 0.01 (clorpirifos) y 0.08 (λ-cihalotrina) en el caso de la población infantil estudiada, con MOEs entre »240000 (dimetoato) y 2300 (paratión). El riesgo acumulado por exposición a OPs calculado como HI estaba muy por debajo de 1, con valores de 0.09 y 0.33 en las madres lactantes, y de 0.06 y 0.69 en la población infantil, par la GM y el P95, respectivamente. Los MOETs para este último grupo de población estaban alrededor de 2200 en la GM y de 300 en el P95, ambos por encima del límite marcado por la EFSA (125).

Ante estos resultados, se pudo afirmar que existía un riesgo relativamente bajo para la salud debido a la exposición a los plaguicidas analizados en ambos grupos de población. Sin embargo, debido al amplio uso de plaguicidas en España, es altamente aconsejable realizar un seguimiento continuo de las tendencias temporales de la exposición de la población española a estas sustancias.

CAPÍTULO II

BIOMONITORIZACIÓN DE HIDROCARBUROS AROMÁTICOS POLICÍCLICOS EN POBLACIÓN INFANTIL Y MADRES: NIVELES URINARIOS, PREDICTORES DE EXPOSICIÓN Y EVALUACIÓN DEL RIESGO

En este capítulo, se desarrolló y validó un método basado en una extracción líquido-líquido seguida de la detección por LC-MS/MS de 11 metabolitos de PAHs, que se aplicó a muestras de orina de madres españolas (n = 110) y población infantil (n = 566) (**Artículos II.1** y **II.2**, respectivamente).

En el estudio BETTERMILK, el 2OHNAP y el 1OHPYR, metabolitos urinarios del naftaleno y del pireno, respectivamente, fueron los compuestos más abundantes encontrados en el 100 % de las madres. Otro metabolito del naftaleno, el 1OHNAP, se detectó en el 96 % de las muestras, mientras que los OHFLUs se detectaron en más del 70 % de las muestras, y los OHPHENs entre el 90 % (9OHPHEN) y el 99 % (3OHPHEN) de las mismas. Por otro lado, el 3OHBAP, solamente se encontró en una muestra. En cuanto a las concentraciones, los niveles más altos se encontraron para el 1OHNAP y el 2OHNAP, con GMs de 0.8 y 7.1 ng·ml^{-1}, y P95 de 5.7 y 31.2 ng·ml^{-1}, respectivamente. Para el resto de metabolitos, se observaron niveles medios entre 0.04 (4OHPHEN) y 0.17 ng·ml^{-1} (2OHFLU). Cabe destacar que el 100 % de las mujeres presentaban siete o más metabolitos de PAHs en sus muestras de orina a niveles cuantificables. En la población infantil del programa BIOVAL, los niveles más altos se encontraron igualmente para los metabolitos del naftaleno, 1OHNAP y 2OHNAP, con GMs de 0.3 y 10 ng·ml^{-1}, respectivamente. Por el contrario, los metabolitos del fenantreno 4OHPHEN, 9OHPHEN y 2OHPHEN tuvieron las

GM más bajas, entre 0.02 y 0.04 ng·ml^{-1}. El resto de OHPAHs oscilaban entre 0.08 (3OHPHEN) y 0.17 ng·ml^{-1} (2OHFLU), mientras que los P95 estaban en el rango de 0.11 (4OHPHEN) a 53 ng·ml^{-1} (2OHNAP). En este caso, más de siete metabolitos urinarios de PAHs fueron detectados y cuantificados en el 90 % de los/as niños/as en estudio. En general, la población española parecía estar más expuesta al naftaleno que otras nacionalidades según sus niveles urinarios de 2OHNAP.

De acuerdo con el análisis de regresión múltiple, las madres fumadoras presentaban mayores niveles de PAHs en orina comparando con las no fumadoras. Además, la ingesta de aceites y grasas, y de cereales y café parecía estar relacionado con mayores niveles de OHNAPs y OHFLUs en orina, respectivamente. Por el contrario, el consumo de vegetales se relacionó con una menor exposición a OHNAPs, y mayores concentraciones de OHNAPs y 1OHPYR fueron asociados a un mayor consumo de pescado ahumado en las madres españolas. En la población infantil estudiada, el consumo de patatas fritas y de alimentos envasados en plástico, se asociaron significativamente con mayores niveles urinarios de OHNAPs, y el resto de OHPAHs, respectivamente. En la literatura se encontraron estudios que apoyaban las asociaciones encontradas en la presente investigación.

La exposición a PAHs se evaluó mediante el cálculo de la EDI, que se estaban entre 9 (fluoreno) y 446 ng·kg^{-1}·día^{-1} (naftaleno) en las madres, y entre 5 (fluoreno) y 204 ng·kg^{-1}·día^{-1} (naftaleno) en la población infantil estudiada. En un contexto de evaluación del riesgo, no se observó ningún potencial riesgo basado en efectos no cancerígenos asociados a la exposición acumulada a PAHs en ninguno de los estudios, ya que los valores para el HQ eran muy inferiores a 0.1, y el P95 para el HI oscilaba entre 0.04 y 0.11. Sin embargo, debería prestarse más atención a la exposición al naftaleno por sus potenciales efectos cancerígenos sobre los seres humanos, ya que fue el mayor contribuyente a la exposición acumulada a PAHs en este estudio.

CAPÍTULO III

BIOMONITORIZACIÓN DE ACRILAMIDA EN NIÑOS Y MADRES: NIVELES URINARIOS, PREDICTORES DE EXPOSICIÓN Y EVALUACIÓN DEL RIESGO

En el último capítulo, los tres biomarcadores de exposición a AA en orina, AAMA, GAMA3 y AAMA-Sul, se analizaron mediante una metodología rápida y validada basada en *dilute-and-shoot* y posterior determinación mediante LC-MS/MS. Este método se aplicó a muestras de orina de 114 madres lactantes (**Artículo III.1**) y 612 niños/as (**Artículo III.2**) de los proyectos BETTERMILK y BIOVAL.

En el caso de las madres lactantes, todos los metabolitos de AA estaban por encima del LoQ en el 100 % de las orinas analizadas. Las concentraciones más altas se encontraron para

el AAMA, el principal biomarcador urinario de AA, con una GM y un P95 de 70 y 266 $ng \cdot ml^{-1}$, respectivamente. Para el AAMA-Sul y GAMA3, se observaron valores más bajos, con GMs de 33 y 15 $ng \cdot ml^{-1}$, respectivamente. En el estudio BIOVAL, los tres metabolitos fueron también cuantificados en el 100 % de las muestras. Los niveles medios de los metabolitos de AA fueron del siguiente orden: 79 $ng \cdot ml^{-1}$ (AAMA), 28 $ng \cdot ml^{-1}$ (AAMA-Sul) y 18 $ng \cdot ml^{-1}$ (GAMA3). En comparación con otros estudios a nivel mundial, los niveles de exposición encontrados en esta tesis eran similares, lo que demuestra la amplia exposición a AA en poblaciones de todo el mundo.

El análisis estadístico sugería que el consumo de tabaco y la ingesta de café eran los principales contribuyentes a la exposición a AA en las mujeres del proyecto BETTERMILK. También se observó una relación significativa entre el consumo de pan y productos precocinados, y niveles más altos de AA en orina en estas mujeres. En cuanto a la población infantil estudiada, aquellos/as niños/as que consumían mayores cantidades de patatas fritas y galletas, parecían presentar mayores niveles urinarios de AA. Además, la población infantil de menor edad parecía estar más expuesta que la de mayor edad, por la misma razón comentada anteriormente en el caso de los plaguicidas. Las observaciones obtenidas en los modelos de regresión de exposición a AA estaban en línea con otros estudios previamente publicados en la bibliografía.

La media de las EDIs de AA en madres lactantes estaba entre 1.2 y 1.9 $\mu g \cdot kg^{-1} \cdot día^{-1}$, y en el caso de la población infantil, entre 1.2 y 1.5 $\mu g \cdot kg^{-1} \cdot día^{-1}$. En un contexto de riesgo, los valores medios obtenidos para el MOE estaban entre 349 y 227 para las madres, y entre 364 y 283 para la población infantil. Solo en el caso del P95 se observaron MOEs por debajo de 125 (límite fijado por la EFSA) en ambas poblaciones. Sin embargo, si se utilizaba la dosimetría directa, se podía observar que los valores medios de AAMA en ambas poblaciones eran muy superiores al BE, superando el BE del punto de partida (BE_{POD}) en el P95 en ambos casos. Esto revelaba que no se podría descartar una preocupación sanitaria con respecto a la toxicidad generada por la exposición a AA en la población española. Por lo tanto, es necesario realizar más actividades de monitorización, regulación y gestión de riesgos, para controlar, mitigar y reducir la exposición a AA en los hogares españoles, así como en otros países.

CONCLUSIONES

METODOLOGÍAS DE ANÁLISIS

Las metodologías analíticas basadas en LC-MS/MS desarrolladas en la presente tesis se han aplicado con éxito en dos estudios de HBM (BETTERMILK y BIOVAL) para determinar las concentraciones urinarias de metabolitos de plaguicidas no persistentes, PAHs de bajo peso molecular y acrilamida. Las tres estrategias analíticas resultantes han sido satisfactoriamente validadas en términos de exactitud, precisión, selectividad, linealidad y sensibilidad, demostrando la aplicabilidad de las mismas para determinar la exposición interna a estas sustancias mediante el análisis de sus metabolitos en orina. Además, en muchos de los analitos analizados se ha podido asegurar y controlar la calidad de los resultados mediante la participación exitosa en ejercicios interlaboratorio y el uso de materiales de referencia. En el caso del benzo(a)pireno, se considera que deberían utilizarse otras matrices, como las heces o la sangre, para poder evaluar la exposición interna a este contaminante.

Por otro lado, se ha evidenciado la necesidad de armonizar las estrategias de muestreo, análisis y control de calidad en este campo. Además, los métodos multianalito que comprenden diferentes grupos químicos, así como las estrategias de *suspect screening* ('análisis de sospechosos') y *non-target* ('análisis no dirigido'), deberían implementarse en los proyectos de HBM, ya que son enfoques prometedores y rápidos para la detección simultánea de un gran número de sustancias.

NIVELES DE EXPOSICIÓN

En lo referente a la exposición a plaguicidas, las DF más elevadas se observaron para el DEP (metabolito inespecífico de OPs), TCPY (metabolito de clorpirifos), PNP (metabolito de paratión) y PBA (metabolito de diversos Pyr), con GM entre 0.8 y 1.9 $ng{\cdot}ml^{-1}$ en las madres, y de 1.0 a 1.5 $ng{\cdot}ml^{-1}$ en la población infantil. Por otro lado, de los 11 metabolitos de PAHs analizados, se detectaron 9 biomarcadores del naftaleno, fluoreno, fenantreno y pireno en más del 78 % de las muestras analizadas en ambas poblaciones. El biomarcador más abundante fue el 2OHNAP, con una GM de 7 y 10 $ng{\cdot}ml^{-1}$ para las madres y la población infantil estudiadas, respectivamente. En el caso de la acrilamida, se observó una DF del 100 % para sus 3 metabolitos, con unas GM en el rango de 18 (GAMA3) – 79 $ng{\cdot}ml^{-1}$ (AAMA) en la población infantil, y 15 (GAMA3) – 70 $ng{\cdot}ml^{-1}$ (AAMA) en mujeres lactantes. En comparación con otros países, la población española en estudio parecía presentar mayores niveles urinarios de PBA y de 2OHNAP que otras nacionalidades.

En general, los resultados y conclusiones obtenidas en esta tesis contribuyen en gran medida al conocimiento de la exposición a plaguicidas no persistentes, PAHs y acrilamida en España, siendo de los primeros datos sobre exposición interna a estas sustancias químicas que se publican en este país. Debido a las altas DF obtenidas, se ha evidenciado la amplia exposición de la población infantil y las madres españolas a estas sustancias químicas, principalmente a OPs, Pyr, al naftaleno y a la acrilamida, que debe de seguir monitorizándose con el tiempo, sirviendo de guía para el diseño de futuros proyectos de HBM.

DETERMINANTES DE LA EXPOSICIÓN

Algunos determinantes de la exposición pudieron ser potencialmente identificados, siendo los relacionados con el consumo de alimentos los más relevantes, seguidos por el hábito de fumar y los factores ambientales. En concreto, la cercanía a las actividades agrícolas, el lugar de residencia y la presencia de jardín/plantas en el hogar podrían ser factores importantes que contribuyen a la exposición a plaguicidas en las madres objeto de estudio. En población infantil, el consumo de verduras, legumbres y cereales parecían ser factores significativamente determinantes de la exposición a estas sustancias. En el caso de PAHs y acrilamida, las madres fumadoras y los/as niños/as más pequeños se relacionaron con mayores niveles de sus correspondientes metabolitos en orina. Además, algunos hábitos dietéticos, como una mayor ingesta de aceites y grasas, pescado ahumado y café en el caso de las mujeres lactantes, así como de patatas fritas y alimentos envasados en plástico para la población infantil estudiada, se asociaron con una mayor exposición a PAHs. Por otro lado, el análisis estadístico realizado para la acrilamida sugería que la ingesta de café, pan y productos precocinados en las madres lactantes, así como de patatas fritas y galletas en los/as niños/as objeto de estudio, podrían estar asociados a niveles más altos de acrilamida en orina.

De este modo, se ha comprobado que los estudios de HBM son herramientas útiles para identificar potenciales determinantes de exposición que pueden guiar las políticas de salud pública en el diseño de estrategias de mitigación con el objetivo de reducir la exposición a estas sustancias entre la población.

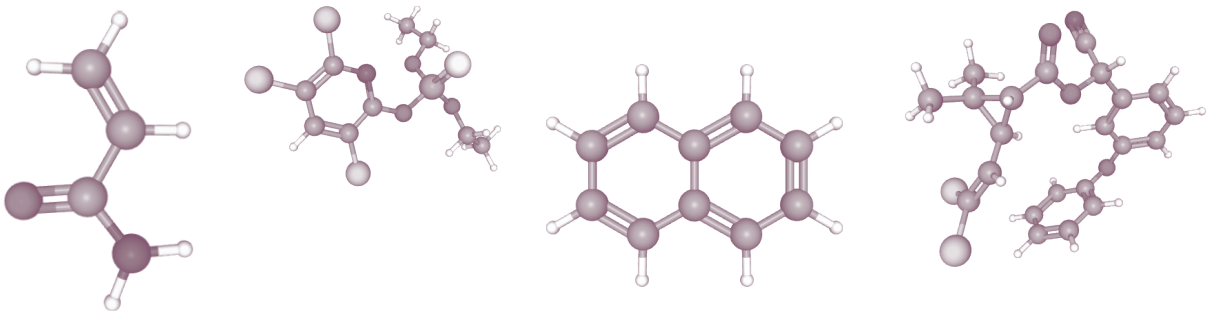
EVALUACIÓN DEL RIESGO

En términos de riesgo, las estimaciones realizadas en la presente tesis mostraron un riesgo para la salud relativamente bajo debido a la exposición a plaguicidas y PAHs en las poblaciones estudiadas. En el caso de los plaguicidas, los HQ y HI medios eran menores a 0.10 en todos los casos, con valores en el P95 entre 0.33 (madres) y 0.69 (población infantil). En el estudio BIOVAL, tanto los MOEs como los MOETs eran superiores a 100, con valores medios superiores a 2200, y alcanzando valores alrededor de 300 en el P95. En el estudio de exposición a PAHs

se obtuvieron HQ y HI menores a 0.11 en todos los casos. Por el contrario, debería prestarse más atención a la acrilamida, ya que podría suponer una preocupación para un porcentaje significativo de la población española, ya que se encontraron niveles medios de AAMA en orina que cuadriplicaban el BE, superando el límite superior (BE_{POD}) en el P95 en ambos estudios, siendo clasificada como de prioridad media-alta. Por el contrario, la evaluación del riesgo para la exposición a acrilamida calculada como MOE, solamente indicaba un riesgo significativo en el P95. Debido a que todavía existe mucha incertidumbre en la evaluación de riesgos empleando datos de HBM, es necesario seguir investigando en este ámbito con el fin de armonizar las metodologías empleadas y poder obtener estimaciones más precisas.

COMUNICACIÓN Y DIFUSIÓN

Si se realiza una interpretación y difusión eficaz de los datos de HBM, los estudios de HBM podrían ser poderosas herramientas complementarias a los programas de monitorización ambiental, siendo extremadamente útiles para evaluar y orientar estrategias de prevención, intervención y reducción de la exposición química en el contexto de las políticas de protección de la salud y del medio ambiente. En este sentido, los hábitos saludables y una dieta equilibrada son la clave para reducir sustancialmente la exposición humana a sustancias nocivas, como en el caso de la acrilamida, así como la probabilidad de desarrollar enfermedades crónicas, por lo que debería fomentarse en gran medida la comunicación de recomendaciones dietéticas y la promoción de estilos de vida saludables entre la población basadas en la evidencia científica.



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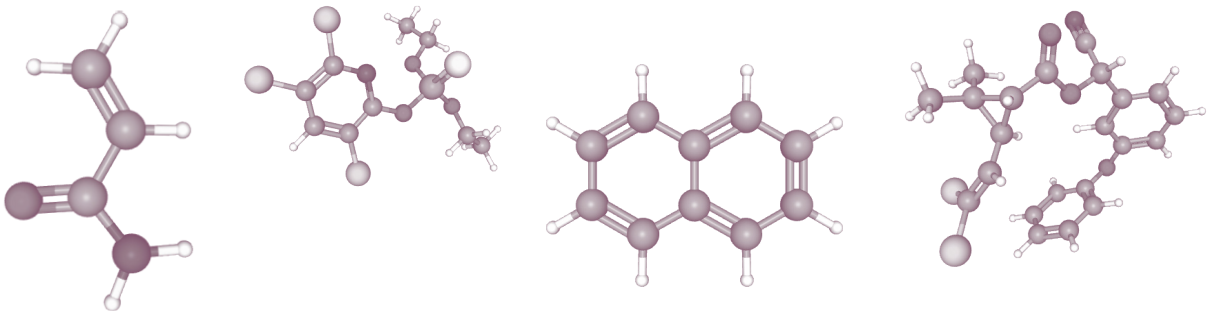


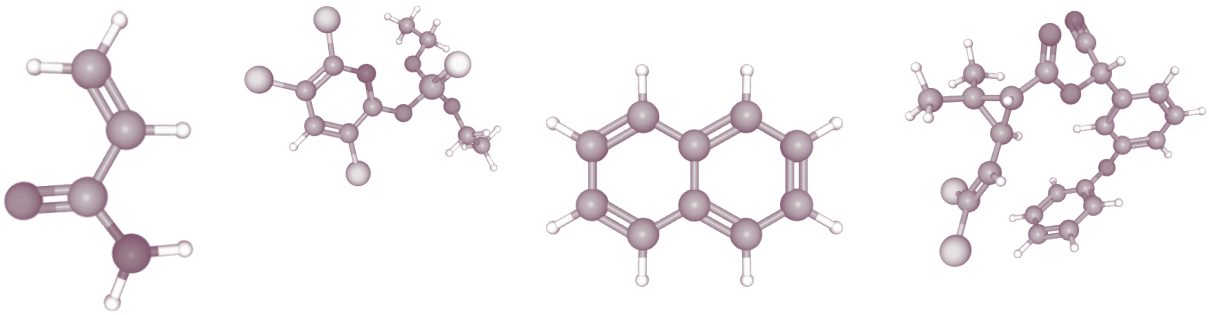
ABBREVIATIONS

%CV	Percentage of coefficient of variation
%R	Percentage of relative recovery
%RR	Percentage of relative ion ratio
%RSD	Percentage of residual standard deviations
%S	Percentage of specificity
%SFA	Saturated fatty acids energy
|Z|	Z-Score absolute value
1OHNAP	1-Hydroxynaphthalene
1OHPHEN	1-Hydroxyphenanthrene
1OHPYR	1-Hydroxypyrene
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
2OHFLU	2-Hydroxyfluorene
2OHNAP	2-Hydroxynaphthalene
2OHPHEN	2-Hydroxyphenanthrene
3OHBAP	3-Hydroxybezon(a)pyrene
3OHFLU	3-Hydroxyfluorene
3OHPHEN	3-Hydroxyphenanthrene
4OHPHEN	4-Hydroxyphenanthrene
9OHPHEN	9-Hydroxyphenanthrene
a.u.	Arbitrary units
AA	Acrylamide
AAMA	N-acetyl-S-(2-carbamoylethyl)-L-cysteine
AAMA-Sul	Acetyl-3-[(3-amino-3-oxopropyl) sulfinyl]-L-alanine
AAVal	Acrylamide hemoglobin adduct
ACEP	Acephate
AChE	Acetylcholinesterase
ADI	Acceptable daily intake
AF	Adjustment factor
AG	Auxiliary gas
ALAM	Alachlor mercapturate
AM	Arithmetic mean
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
ATSDR	Agency for Toxic Substances and Disease Registry
ATZM	Atrazine mercapturate
BE	Biomonitoring equivalent
BE_{POD}	Biomonitoring equivalent directly derived from a point of departure
BMD	Benchmark dose
$BMDL_{10}$	Lower bound of the benchmark dose associated with a 10 % extra risk of adverse effect
BW	Body weight

CAG	Cumulative assessment group
CAG-AChE	CAG of pesticides affecting the acetylcholinesterase activity
CAG-motor	CAG of pesticides causing alterations of the motor system
CCD	Central composite design
CDC	Centers for Disease Control and Prevention
CE	Collision energy
CECs	Chemicals of emerging concern
CGP	Collision gas pressure
CHMS	Canadian Health Measures Survey
CI	Confidence interval
CMHC	3-Chloro-7-hydroxy-4-methylcoumarin
CONTAM panel	Panel on Contaminants in the Food Chain
COPHES/DE-MOCOPHES	Consortium to Perform Human Biomonitoring on a European Scale/Demonstration of a Study to Coordinate and Perform Human biomonitoring on a European Scale
Cre	Creatinine
Cre_{24h}	Total urinary creatinine excreted within 24h
CRMs	Certified reference materials
CT	Capillary temperature
CZ	Czech Republic
D&S	'Dilute-and-shoot'
D1	Journal Impact Factor 1st Decile
DAPs	Dialkyl phosphates
DBCA	2,2-Dibromovinyl-2,2-dimethylcyclopropane-1-carboxylic acid
DBP	Dibutyl phosphate
DCCA	2,2-Dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid
DEAMPY	2-Diethylamino-6-methyl-4-pyrimidinol
DEDTP	Diethyl dithiophosphate
DEP	Diethyl phosphate
DETP	Diethyl thiophosphate
DF	Detection frequency
DIMET	Dimethoate
DMDTP	Dimethyl dithiophosphate
DMP	Dimethyl phosphate
DMTP	Dimethyl thiophosphate
DoE	Design of experiments
EC	European Commission
ECHA	European Chemicals Agency
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EQUAS	External quality assessment schemes
ESI	Electrospray ionization
EU	European Union
FDA	U.S. Food and Drug Administration
FLEHS	Flemish Environment and Health Survey
FPBA	4-Fluoro-3-phenoxybenzoic acid
F_{UE}	Urinary excretion factor
G-EQUAS	German External QUality Assessment Scheme
GA	Glycidamide
GAMA3	N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine
GAVal	GA hemoglobin adduct
GC	Gas chromatography
GC-MS	Gas chromatography coupled to mass spectrometry
GC-MS/MS	Gas chromatography coupled to tandem mass spectrometry
GerES	German Environmental Surveys
GM	Geometric mean

HÄMI	Health-related environmental monitoring of the Swedish Environmental Protection Agency program area
HBGV	Health-based guidance value
HBM	Human biomonitoring
HBM-I	German HBM control value
HBM-II	German HBM action value
HCl	Hydrochloric acid
Herb	Herbicides
HESI	Heated electrospray ionization source
HFRs	Halogenated flame retardants
HI	Hazard index
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
HRMS	High-resolution mass spectrometry
IARC	International Agency for Research on Cancer
IMPY	2-Isopropyl-4-methyl-6-hydroxypyrimidine
IS	Internal standards
ISCIII	*Carlos III* Institute of Health
ITT	Ion transfer tube temperature
JECS	Japan Environment and Children's Study
JRC	Joint Research Centre
KoNHES	Korean National Environmental Health Survey
LC	Liquid chromatography
LC-MS	Liquid chromatography coupled to mass spectrometry
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LLE	Liquid-liquid extraction
LOAEL	Lowest-observed-adverse-effect
LoQ	Limit of quantification
m/z	Mass-to-charge ratio
max.	Maximum value
MDA	Malathion dicarboxylic acid
Med-DQI	Adherence to the Mediterranean diet
METHA	Methamidophos
METM	Metolachlor mercapturate
min.	Minimum value
MLs	Maximum levels
MNP	3-Methyl-4-nitrophenol
MoA	Mode of action
MOE	Margin of exposure
MOET	Total margin of exposure
MOS	Margin of safety
MS/MS	Tandem mass spectrometry
MW	Molecular weight
n.r.	not reported
NHANES	U.S. National Health and Nutrition Examination Survey
NIST	National Institute of Standards and Technology of the U.S. Department of Commerce
no.	Number of
NOAEL	No-observed-adverse-effect level
OHPAHs	Hydroxylated PAHs
OMET	Omethoate
OPs	Organophosphate pesticides
P-B	Placket-Burman design
P25	25th percentile
P50	50th percentile
P75	75th percentile
P95	95th percentile
PAHs	Polycyclic aromatic hydrocarbons
PAR	Pesticide residue analysis
PARC	European Partnership for the Assessment of Risk from Chemicals

PBA	3-Phenoxybenzoic acid
PCBs	Polychlorinated biphenyls
PFAS	Per- and polyfluoroalkyl substances
PFRs	Organophosphorus flame retardants
PNBS	French national biomonitoring program
PNP	p-Nitrophenol
POD	Point of departure
POPs	Persistent organic pollutants
Pyr	Pyrethroids
Q (Q1, q2, Q3)	Quadrupole (1st, 2nd and 3rd quadrupole)
Q ion	Quantification ion
q ion	Confirmation ion
Q2	Journal Impact Factor 2nd Quartile
QA	Quality assurance
QC	Quality control
QuEChERS	Quick, Easy, Cheap, Effective, Rugged, Safe
R^2	Coefficient of determination
REACH	Registration, Evaluation, Authorization and Restriction of Chemicals
RF	Radio frequency
RfC	Reference concentration
RfD	Reference dose
RP	Reverse-phased
RT	Retention time
RV_{95}	Reference value at P95
S/N	Signal-to-noise ratio
SD	Standard deviation
SEHM	Slovenian national HBM program
SG	Sheath gas
SOP	Standard operating procedure
SPE	Solid-phase extraction
SRM	Selected reaction monitoring
SV	Spray voltage
SwG	Sweep gas
TCPY	3,5,6-Trichloro-2-pyridinol
TDI	Tolerable daily intake
TEF	Toxic equivalency factor
TK	Toxicokinetic data
TL	Tube lens
U.S./USA	United States of America
UBA	German Environment Agency
UF	Uncertainty factors
UF_A	Interspecies uncertainty factors
UF_H	Intraspecies uncertainty factors
UHPLC	Ultra-high performance liquid chromatography
V_{24h}	Total urinary volume excreted within 24h
VOCs	Volatile organic compounds
VT	Vaporizer temperature
WHO	World Health Organization
ΣDAPs	Sum of all dialkyl phosphates (DMP, DMTP, DMDTP, DEP, DETP and DEDTP)
ΣDEPs	Sum of diethyl phosphates (DEP, DETP and DEDTP)
ΣDMPs	Sum of dimethyl phosphates (DMP, DMTP and DMDTP)
ΣOHFLUs	Sum of hydroxyfluorene isomers (2OHFLU and 3OHFLU)
ΣOHNAPs	Sum of hydroxynaphthalene isomers (1OHNAP and 2OHNAP)
ΣOHPHENs	Sum of hydroxyphenanthrene isomers (1OHPHEN, 2OHPHEN, 3OHPHEN, 4OHPHEN and 9OHPHEN)
ΣUAAM	Sum of urinary acrylamide metabolites (AAMA, GAMA3 and AAMA-Sul)



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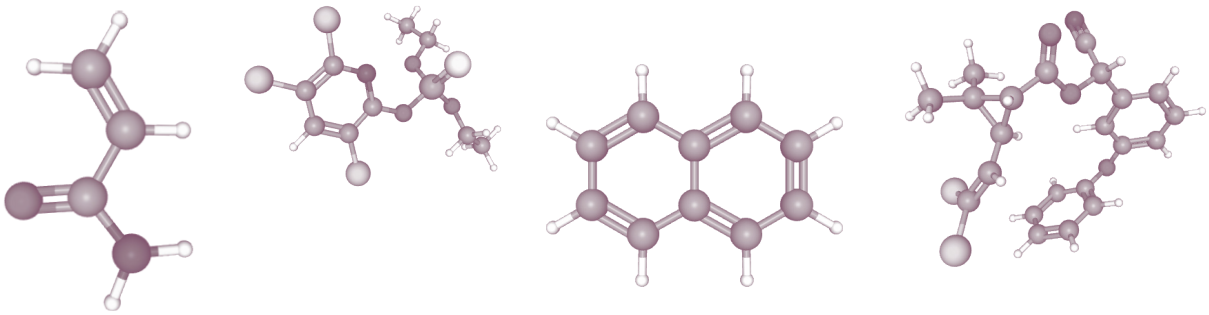

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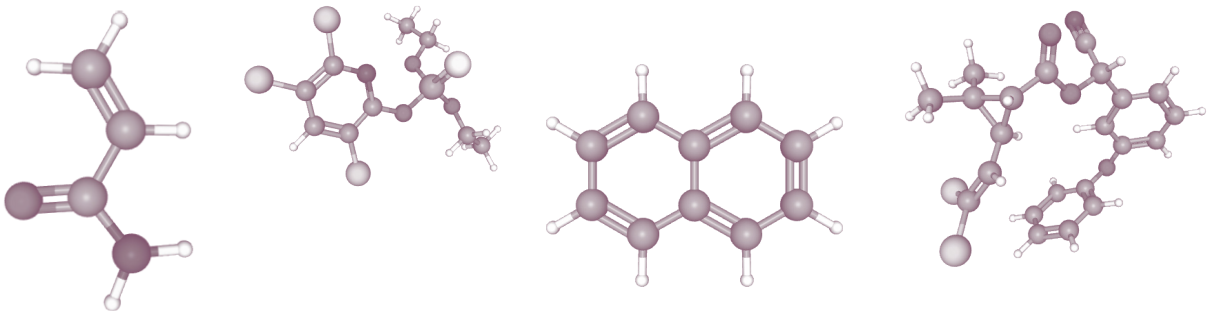


LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the scientific articles listed below, which are referred to in the text by the Roman numeral of the chapter (I, II or III) followed by a numeric value (1, 2 or 3) according to their chronological order of publication date:

		Scientific articles	Journal	JIF[a]
CHAPTER I	I.1.	S. F. Fernández, A. Pastor, V. Yusà, L. Montesinos, O. Pardo, *Development of a novel methodology for determination of dialkyl phosphates in human urine using liquid chromatography-tandem mass spectrometry*, J. Chromatogr. B. (2019) 121810. doi:10.1016/J.JCHROMB.2019.121810.	Journal of Chromatography B	Q2[b]
	I.2.	S. F. Fernández, O. Pardo, I. Adam-Cervera, L. Montesinos, F. Corpas-Burgos, M. Roca, A. Pastor, M. Vento, M. Cernada, V. Yusà, *Biomonitoring of non-persistent pesticides in urine from lactating mothers: Exposure and risk assessment*, Sci. Total Environ. 699 (2020). doi:10.1016/J.SCITOTENV.2019.134385.	Science of the Total Environment	D1[c]
	I.3.	S. F. Fernández, O. Pardo, F. Corpas-Burgos, V. Yusà, *Exposure and cumulative risk assessment to non-persistent pesticides in Spanish children using biomonitoring*, Sci. Total Environ. 746 (2020) 140983. doi:10.1016/j.scitotenv.2020.140983.	Science of the Total Environment	D1[c]

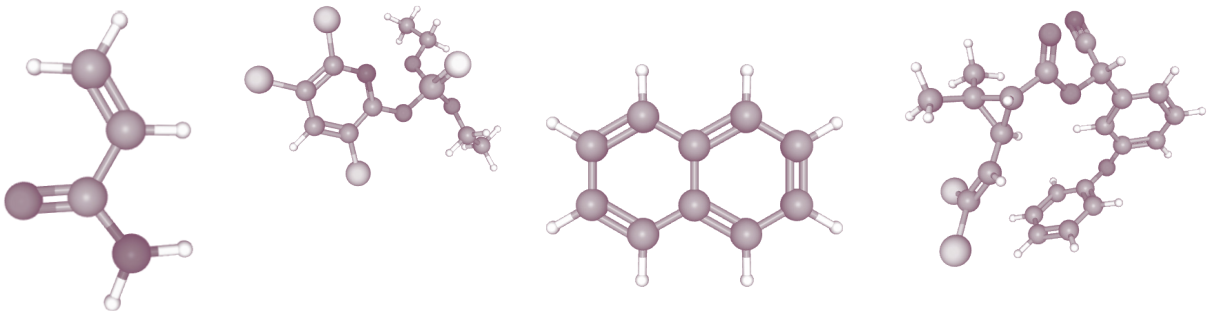
		Scientific articles	Journal	JIF[a]
CHAPTER II	II.1.	S. F. Fernández, O. Pardo, A. Pastor, V. Yusà, M. Vento, M. Cernada, M. Gormaz, J. Kuligowski, A. Sánchez, E. Torres, P. Correcher, A. Núñez, A. Parra, A. Ramón, *Biomonitoring of polycyclic aromatic hydrocarbons in the urine of lactating mothers: Urinary levels, association with lifestyle factors, and risk assessment*, Environ. Pollut. 268 (2021). doi:10.1016/j.envpol.2020.115646.	Environmental Pollution	D1[c]
	II.2.	S. F. Fernández, O. Pardo, C. S. Hernández, B. Garlito, V. Yusà, *Children's exposure to polycyclic aromatic hydrocarbons in the Valencian Region (Spain): Urinary levels, predictors of exposure and risk assessment*, Environ. Int. 153 (2021). doi:10.1016/j.envint.2021.106535.	Environment International (Open Access)	D1[c]
CHAPTER III	III.1.	S. F. Fernández, O. Pardo, C. Coscollà, V. Yusà, *Exposure assessment of Spanish lactating mothers to acrylamide via human biomonitoring*, Environ. Res. 203 (2022). doi:10.1016/j.envres.2021.111832.	Environmental Research	D1[d]
	III.2.	S. F. Fernández, O. Pardo, C. Coscollà, V. Yusà, *Risk assessment of the exposure of Spanish children to acrylamide using human biomonitoring*, Environ. Pollut. 305 (2022). doi:10.1016/j.envpol.2022.119319.	Environmental Pollution	D1[c]

[a]JIC: Journal Citation Reports dataset (updated Oct 25, 2021); Q2: 2nd quartile; D1: 1st decile

[b]Category: "Analytical Chemistry"

[c]Category: "Environmental Sciences"

[d]Category: "Public, Environmental & Occupational Health"



ABSTRACT

Today, global chemical manufacturing is increasing each year, making environmental pollution and exposure to chemicals a serious public health problem linked to the development of many non-genetic diseases. Approximately, 70 % of chemicals consumed in Europe in 2019 were hazardous for human health. These harmful substances can enter to the food chain and remain in the environment, making humans exposed to them mainly by ingestion, inhalation or dermal exposure. Consequently, exposure to chemicals should be continuously monitored for assuring health protection of citizens by two ways:

i) studying their presence in environmental compartments, such as water, food, soil or air, by environmental monitoring, and

ii) determining their presence in biological samples, such as urine, blood, hair, etc., by human biomonitoring (HBM). In contrast to environmental monitoring studies, HBM data reflect the total body burden integrating all potential pathways of exposure to chemicals.

The overall aim of this doctoral thesis was to develop different target analytical tools for their application to HBM projects, and to design and carry out some specific HBM studies on urine samples of children (5 – 11 years old) and lactating mothers of the BIOVAL and BETTERMILK projects, respectively, focusing on non-persistent pesticides, polycyclic aromatic hydrocarbons (PAHs), and acrylamide (AA), which are priority substances in public health and food safety.

In Chapter I, a method based on QuEChERS and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) was developed and validated for the simultaneous analysis of six non-specific metabolites of organophosphate pesticides (OPs), known as dialkyl phosphates (DAPs), in urine (Article I.1). Urinary levels of DAPs, together with other 20 metabolites of OPs, pyrethroids and herbicides, were analyzed in 116 mothers (Article I.2) and 568 children (Article I.3) living in Spain. The highest detection frequencies (DFs) were observed for diethyl phosphate (non-specific metabolite of OPs), 3,5,6-trichloro-2-pyridinol (a specific metabolite

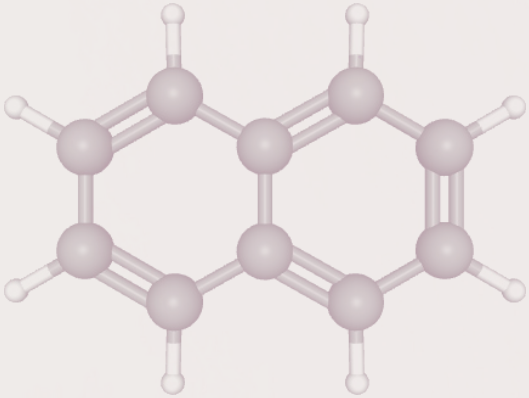
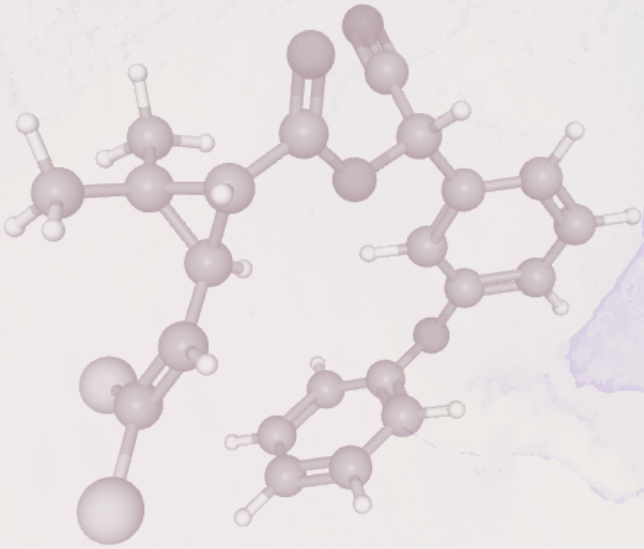
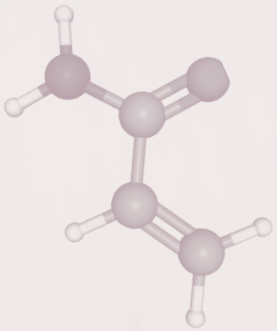
of chlorpyrifos), p-nitrophenol (specific metabolite of parathion), and 3-phenoxybenzoic acid (PBA, a non-specific metabolite of pyrethroids), with geometric means (GMs) ranging from 0.8 and 1.9 $ng \cdot ml^{-1}$ in mothers, and from 1.03 to 1.53 $ng \cdot ml^{-1}$ in children. In a global context, the Spanish population under study seemed to be at higher exposure to pyrethroids, since they presented higher urinary levels of PBA compared to other countries. The statistical analysis showed that closeness to farming activities, place of residence and presence of garden/plants at home might be important contributors to pesticide exposure in the mothers under study. In children, the consumption of vegetables, legumes and cereals seemed to be significant predictors of exposure to these substances. Mean estimated daily intakes (EDIs) were also calculated, ranging from 0.08 (chlorpyrifos) to 1.62 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (λ-cyhalothrin) in children, and from 0.09 (dimethoate) to 0.40 $\mu g \cdot kg^{-1} \cdot day^{-1}$ (chlorpyrifos) in mothers. Risk assessment revealed a relatively low health risk of exposure to the analyzed pesticides in both target population groups. However, due to the extensive use of pesticides in Spain, it is highly advisable to continuously monitor time trends in the Spanish population's exposure.

In **Chapter II,** the development and validation of a liquid-liquid extraction method followed by LC-MS/MS detection of 11 urinary PAH metabolites (OHPAHs) was performed and applied to urine samples of Spanish mothers (n = 110) and children (n = 566) (**Article II.1** and **Article II.2**, respectively). In total, 9 hydroxy-metabolites of naphthalene, fluorene, phenanthrene and pyrene were detected in more than 78 % of the analyzed samples. The most abundant biomarker was 2-hydroxynaphthalene (2OHNAP), with a GM of 7 and 10 $ng \cdot ml^{-1}$ for mothers and children, respectively. In general, Spanish children and mothers under study were at greater exposure to naphthalene than those populations from other countries based on their urinary levels of 2OHNAP. According to the multiple regression analysis, smoker mothers and young children were observed to be at higher exposure to PAHs. In addition, some dietary habits, such as intake of oils and fats, smoked fish and coffee, as well as, potato fried products and plastic-packed foods, were statistically associated with a higher PAH exposure in lactating women and children, respectively. Exposure to PAHs was evaluated by calculating EDIs, which were between 9 (fluorene) and 446 $ng \cdot kg^{-1} \cdot day^{-1}$ (naphthalene) in mothers, and between 5 (fluorene) and 204 $ng \cdot kg^{-1} \cdot day^{-1}$ (naphthalene). In a risk context, no potential non-cancer risk associated to cumulative PAH exposure was observed. However, more attention should be paid to exposure to naphthalene, considered as a Group 2B (Possibly carcinogenic to humans), since it was by far the highest contributor to cumulative PAH exposure in this study.

In **Chapter III**, N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA3) and N-Acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine (AAMA-Sul), which are exposure biomarkers of AA in urine, were analyzed by a fast and validated methodology based on 'dilute-and-shoot' and LC-MS/MS determination. This method was applied to urine samples of 114 lactating mothers (**Article III.1**) and 612 children (**Article III.2**), resulting in DFs of 100 % with GMs in the range of 18 (GAMA3) – 79 $ng \cdot ml^{-1}$

(AAMA) in children, and 15 (GAMA3) – 70 ng·ml^{-1} (AAMA) in women. Comparing with previous studies, similar exposure levels were found in other countries, showing the widespread AA exposure in many populations around the world. Statistical analysis suggested that intake of fried potato products and biscuits, as well as coffee, bread and precooked food products, could be associated with higher levels of urinary AA in children and mothers, respectively. Mean EDIs for AA in the target populations were between 1.2 – 1.9 µg·kg^{-1}·day^{-1}, and risk assessment estimations showed that exposure levels were above current guidance values, revealing that a health concern with respect to non-neoplastic toxicity of AA could not be discarded in the Spanish population. Therefore, there is a need of further regulation and risk management activities, such as promotion of mitigation measures of AA exposure at households and a risk assessment follow-up, to minimize its exposure in Spain and other countries.

In conclusion, three LC-MS/MS methods validated under a high-quality control/quality assurance system with low limits of quantification were developed to determine urinary biomarkers of exposure to pesticides, PAHs and AA in HBM studies. Their applicability has been successfully tested by the analysis of urines of lactating mothers and children of the BETTERMILK and BIOVAL studies, and it allowed to potentially identify determinants of exposure and estimate the associated risks. Therefore, HBM studies are shown to be powerful tools to guide and evaluate strategies for prevention, intervention, and reduction of chemical exposure in general and the most vulnerable population.

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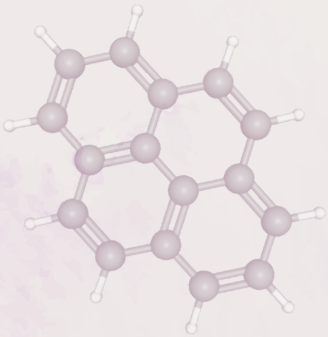
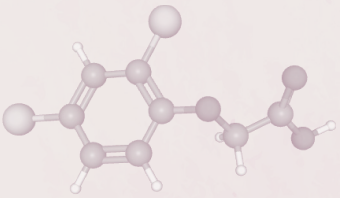
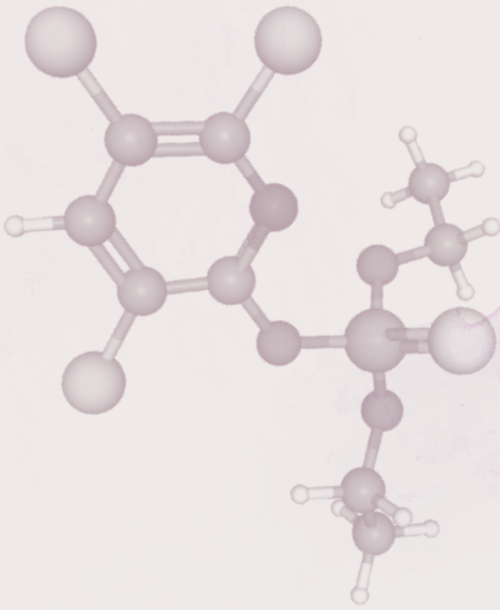
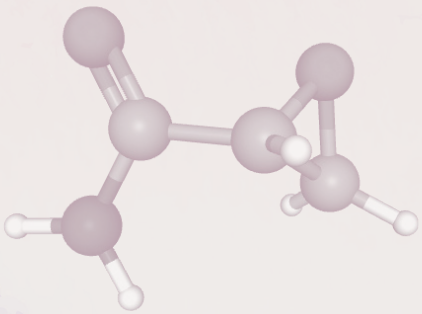
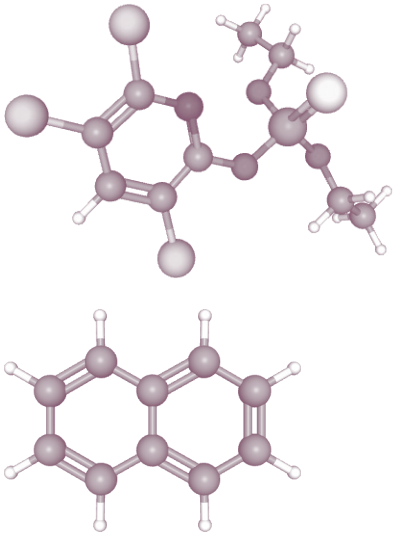
INTRODUCTION



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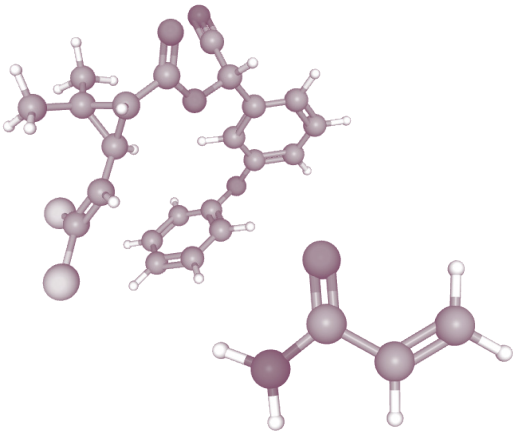


INTRODUCTION

1.1 CHEMICALS, ENVIRONMENT, AND HEALTH

Today, several hundreds of million tones of chemical compounds are continuously produced worldwide, and the number of new chemicals is rising each year[1]. In fact, chemical manufacturing is the fourth largest industry in the European Union (EU) with ~30,000 companies, employing approximately 5 million people[2]. This made it the world's second biggest chemical producer in 2020[3]. Hence, the chemical industry makes a vital contribution to the EU's economy in terms of trade and employment[2].

In modern society, many chemicals are widely used in several industrial applications, such as in the production of medicines, textiles, electronic devices, personal care products, etc. Others may be by-products of these manufacturing activities or be formed during processing or storage, e.g. in foodstuffs[4]. As a result of such intensive use, these substances can be found in the environment, most commonly in soil, air, water, food, and feed[5,6]. Therefore, they can enter to the food chain, making humans exposed to a wide variety of chemicals and environmental pollutants through many pathways. While not all hazardous substances raise the same concerns, exposure to high levels of some chemicals, such as organophosphorus flame retardants (PFRs), polychlorinated biphenyls (PCBs), pesticides, toxic metals (Hg, Pb, As), dioxins, acrylamide, bisphenol A, and polycyclic aromatic hydrocarbons (PAHs), have been associated to the development of chronic diseases, such as, cancer, diabetes, asthma, birth disorders, etc[5,7]. Therefore, some of the substances present in products that are daily used or highly consumed may negatively affect ecosystems and health of living beings. In 2019, approximately 70 and 25 % of the chemicals consumed in the EU were hazardous to health and the environment, respectively, and 13 % of the total amount of produced chemicals were carcinogenic, mutagenic and/or toxic for the sexual function and fertility[8]. Consequently, a large majority of European citizens are worried about the impact of chemical substances on their health, as well as, on the environment[9].

To address this issue, several European action plans have been implemented by the European Commission (EC). In 2019, the European Green Deal was launched for reducing net greenhouse gas emissions by at least 55 % by 2030, and by 100 % in 2050. To this end, several actions on climate, energy, agriculture, industry, environment, transport, research and innovation, as well as finance and regional development were proposed to improve the well-being and health of citizens and future generations[10]. One of its key commitments, the *Zero pollution ambition*, was proposed to improve air, water and soil quality, as well as to avoid the loss of biodiversity by reducing pollution of natural ecosystems[11]. In addition, as a part of the EU's zero pollution action plan, a new long-term vision approach to chemicals' management and policy in Europe, the *EU Chemicals Strategy for Sustainability*, was published in 2020[2]. The main objective of this strategy is to take actions to better protect citizens and the environment from harmful substances by boosting innovation of safe and sustainable chemicals and production methods[2]. As a result, these EU strategies aim to contribute to the green and digital transition, and a circular economy for a toxic-free environment.

According to the World Health Organization (WHO), environmental pollution is responsible for 23 % of global mortality, leading to 12.6 million deaths each year[12]. In total, 65 % of these deaths are caused by non-genetic diseases[12]. Therefore, environmental factors are considered the main cause of global disease and death, representing between 2 and 7 % of annual health spending worldwide[6]. These statistics highlight the need to integrate the genetic research ('genomics') together with the study and characterization of environmental exposure from a broad and quantitative perspective, investigating any contribution to the development of adverse human health effects[13]. In this sense, Christopher Wild proposed the 'exposome' concept, which is defined as "a compilation of every exposure to which an individual is subjected from conception to death" (Figure 1)[13,14]. Thus, the 'exposome' complements the 'genome' by providing a complete description of lifetime exposure history to better understand the causes of human diseases and how to prevent them[13,14].

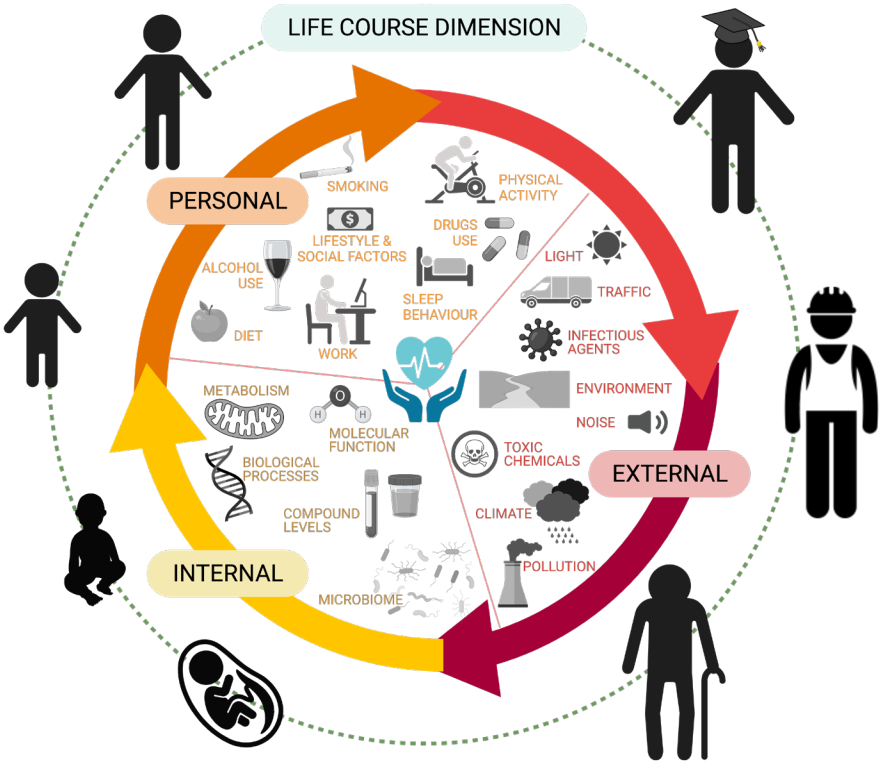


Figure 1. The 'exposome' concept: combination of every non-genetic exposure, including internal, external, and personal factors, during daily life activities over the lifetime of an individual which may contribute to the development and progression of diseases[13,14].

1.2 HUMAN EXPOSURE TO CHEMICALS: 'DECODING THE EXPOSOME'

The objective of the 'exposome decoding' is to gain knowledge about a wide range of individual exposures. This new concept is in line with one of the major goals of public health authorities: to evaluate human exposure to chemicals and environmental pollutants in order to adopt appropriate risk management activities to better protect human health[2,15]. To address this issue, two complementary strategies, environmental monitoring and human biomonitoring, can be used[15].

1.2.1 EXTERNAL EXPOSURE AND ENVIRONMENTAL MONITORING

The principal pathway of human exposure to chemicals and environmental pollutants is the ingestion of foodstuffs. Indeed, from a policy-maker perspective, the control of residue levels of chemicals and environmental contaminants in these goods, known as environmental monitoring, is the common approach to follow-up the human exposure to these substances[15].

In this sense, several mitigation measures have been adopted. For example, maximum levels (MLs) of certain contaminants, including mycotoxins, metals, dioxins, PCBs, PAHs, among others, in some foodstuffs have been set by the Commission Regulation (EC) No 1881/2006[16].

According to the *Codex Alimentarius*, the risk analysis process should be used to ensure human health protection in food safety. The three main components of risk analysis are risk assessment, risk management and risk communication, which should be established as integral parts of the national food safety systems[17]. In this context, the risk assessment is one of the most relevant parts of the risk analysis process since it is the basis for decision making in food safety and nutrition, involving many scientific and government experts from public health systems. It is composed by four steps: i) hazard identification, ii) hazard characterization, iii) exposure assessment, and iv) risk characterization[17].

In a European level, the Panel on Contaminants in the Food Chain (CONTAM panel) of the European Food Safety Authority (EFSA) evaluates and provides scientific opinion on contaminants and other undesirable substances in the food chain based on the four steps of the risk assessment process[18]. This approach involves the combination of the occurrence of hazardous substances in foodstuffs and the consumption data of different population groups to obtain the resulting exposure that can be compared with a health-based guidance value[4]. In a national level, the existence of a multi-annual control plan is mandatory in all EU Member States, according to Article 109 of Regulation (EU) 2017/625 *on official controls and other official activities performed to ensure the application of food and feed law, rules on animal health and welfare, plant health and plant protection products*[19]. As an example, the Spanish Agency for Food Safety & Nutrition (AESAN) together with the Ministry of Consumer Affairs coordinate the official control programs conducted by the different competent authorities at state, regional and local level to verify that business operators' activities and goods placed on the market comply with EU food safety regulations in Spain[20].

Other exposure media to these substances in humans are ambient air, drinking water and other consumer products, such as cosmetics. In Europe, maximum values for certain substances in these compartments have been also set by the EC to protect public health. For example, i) a target value for the protection of health of 1 ng benzo(a)pyrene·m^{-3} of air should not be exceeded in one calendar year in agreement to the Commission Implementing Decision of 12th December, 2011[21]; ii) the Directive (EU) 2020/2184 sets an arsenic maximum concentration of 10 µg·l^{-1} in water of human consumption[22]; and, iii) according to the Regulation (EC) No 1223/2009, more than 0.8 % of paraben mixtures are not allowed to be used as a preservative in cosmetic preparations, and they are totally banned in products designed for the nappy area of children under 3 years old[23].

Therefore, all European public health national systems have implemented long-term monitoring programs to control the presence of some hazardous substances in food, air, water, and other consumer products. Additionally, the Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation establishes procedures for collecting and assessing information on the properties and hazards of substances[24]. Following these procedures, manufacturers must register their substances and send them to the European Chemical Agency (ECHA) for evaluation. Then, the EU Member States assess the potential concerns for human health and for the environment, as well as whether the associated risks can be managed[24].

However, it should be noted that the body burden of a specific chemical or environmental contaminant is determined not only by its concentration in different environmental media, but also by its physical and chemical properties, timing of exposure, as well as individual factors, such as uptake, metabolism, and excretion rates[25]. Thus, the risk assessment through environmental monitoring is not the most accurate approach, since the presence of these substances in the environment does not necessarily reflect the real exposure of the population[15]. As a result, human biomonitoring is being implemented in many countries around the world since it takes all these factors into account without having to identify potential pathways of exposure. Therefore, although environmental monitoring remains the basis for policy making, human biomonitoring strategies might be valuable complementary tools in this sense[25].

1.2.2 INTERNAL EXPOSURE AND HUMAN BIOMONITORING

Human biomonitoring (HBM) measures the concentration levels (internal dose) of chemicals, their metabolites or reaction products, commonly known as biomarkers, in human biological matrices, such as body fluids (blood, urine, saliva, etc.) and other specimens (feces, nails, hair, etc.)[25]. Biomonitoring data reflect the total body burden (internal exposure) which results from multiple exposure routes (inhalation, ingestion, or dermal contact) and all potential exposure media, such as food, air pollution, consumer products, water, industrial processes, etc[25,26].

According to the Spanish Ministry of Health and the WHO, HBM is a key component to support environment and health policymaking. As a result, in order to protect human health, the following tasks need to be addressed in HBM programs[25,27]:

a) To identify and prioritize substances of high concern.

b) To compare different subpopulation groups and identify the most vulnerable ones (those at higher risk of exposure).

c) To establish exposure reference values and health guideline values for the prioritized substances.

d) To identify geographical and time trends, as well as potential determinants of exposure (e.g., lifestyle factors).

e) To give recommendations to the population to decrease the risk of exposure.

f) To assess the effectiveness of policy actions.

Several factors must be carefully considered in the design of a HBM study and the interpretation of the resulting data. The selection of the most suitable combination of biomarker(s), which can be the parent compound or its metabolites, and the appropriate matrix, is the first issue to consider when designing a HBM study. Additionally, the sampling procedure should be robust and reproducible to obtain comparable data, and the matrix and the selected biomarkers must be stable during sampling, transportation, processing and analysis[28]. In some cases, normalization and correction procedures are applied to a better interpretation of HBM data, e.g., correction by creatinine and specific gravity in urine, or adjustment for lipid content in blood samples[29]. Equally important is the choice of an appropriate analytical method, which should be sufficiently sensitive and selective to produce reliable HBM data, since exogenous compounds are usually present in low concentrations in human samples[25,26]. These and other factors are discussed in depth in the following sections.

1.2.2.1 BIOLOGICAL MATRICES

Once exposure to an organic chemical occurs, it is absorbed and distributed in the human organism. Hydrophilic substances can be metabolized mostly in the liver by Phase I (redox or hydrolysis reactions) and Phase II (conjugation with a moiety) reactions, and then excreted as metabolites, or they can be excreted unchanged[30]. On the contrary, persistent chemicals are primarily accumulated in tissues, organs, or bones, either as biotransformation products or as the unchanged parent[25,30]. Thus, the physicochemical properties of a chemical influence its toxicokinetic, metabolism and excretion pathway, so they should be considered to choose the appropriate matrix for its analysis[25]. In addition, matrix availability is a relevant aspect to evaluate when designing a HBM study. This is the principal reason why urine is one of the preferred biological matrix in HBM[26]. Moreover, it is non-invasive and relatively easy to collect[15,31]. Urine analysis provides information about recent exposure to non-persistent and hydrophilic substances, such as bisphenols, parabens, and phthalates[32], which are rapidly excreted from the human body. However, the intra- and inter-individual variability of urine composition among/within individuals due to the influence of age, body weight, hydration status, among other factors, make urinary levels of chemicals difficult to interpret and compare[33].

For HBM of persistent organic pollutants (POPs), blood (plasma and serum) is the most common matrix, since it is in continuous contact with all organs and tissues where these chemicals are deposited[15,25]. Therefore, it is the most appropriate matrix to analyze long-term

exposure to some metals (lead, cadmium, selenium), per- and polyfluoroalkyl substances (PFAs), halogenated flame retardants (HFRs), dioxins, among others[26,34,35]. On one hand, blood sampling is a well-standardized procedure, and it is used in more than 65 % of national HBM programs[36]. Nevertheless, it is an invasive technique which may have a negative influence on the voluntary participation of individuals in HBM studies[15,25]. Other studies focusing on the evaluation of exposure to POPs in the prenatal and the 1st stage of life periods usually use umbilical cord blood, meconium, placenta, or human milk[25,37]. However, these matrices can be only sampled in certain subpopulation groups (women and newborns), and only at specific periods in their lives[38].

Nails and hair samples are becoming highly used matrices due to their non-invasive collection, easy sample shipping and storage[36], and have been employed to assess exposure to some heavy metals, such as mercury[36,39]. The biggest drawbacks of using these matrices are the possibility of external deposition of chemicals that can affect their analysis, as well as the required amount of sample, which can be a limiting factor, for example in small children[25,28].

Other non-invasive alternatives that have become important specimens in more recent years are saliva and exhaled breath. However, the applicability domain of saliva and breath specimens cover only specific types of compounds, such as low molecular weight compounds and volatile chemicals, respectively[25,40]. The utility of other types of samples, such as teeth or sweat, has begun to be explored, and they could provide added value for future HBM studies[25,40]. A diagram summarizing the biological matrices used in national HBM programs, the most measured compounds, and the main advantages and limitations of each matrix is presented in Figure 2. From an ethical and practical perspective, the use of non-invasive matrices in HBM studies should be promoted[38]. In addition, the collection of multiple sample types from each individual would allow us to have more information about human exposure to chemicals, facilitating the ‘exposome decoding’[40].

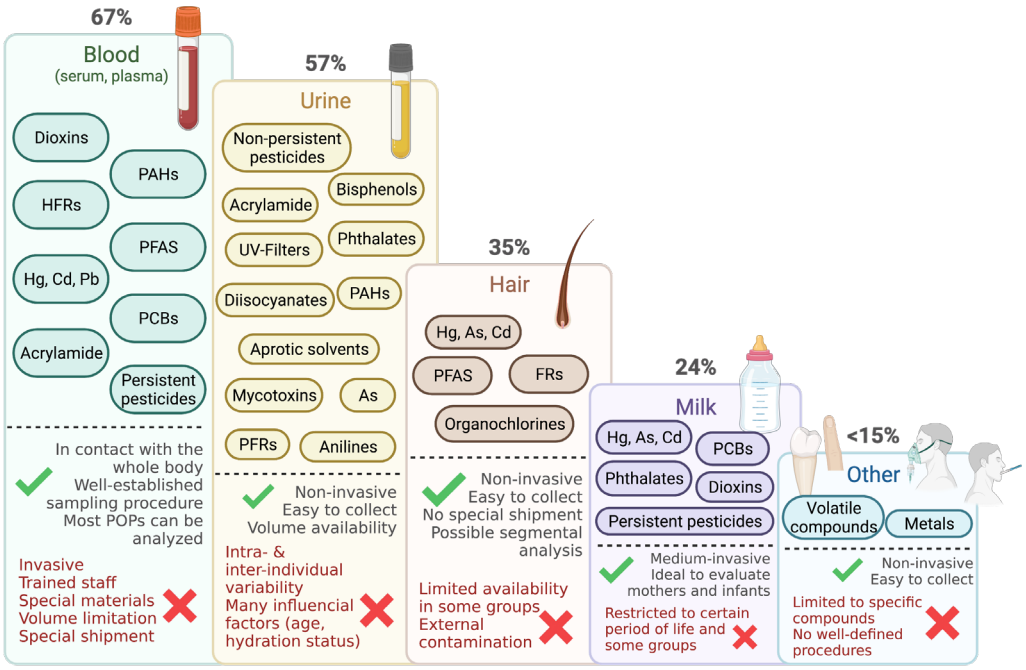


Figure 2. Human biological matrices most used in human biomonitoring (HBM) programs: percentage of HBM studies using each specific matrix, chemicals most frequently analyzed in each specimen, and their main advantages and disadvantages[25,26,34,36,41,42].

1.2.2.2 BIOMARKERS

Apart from practical issues, such as fieldwork considerations, availability of standards and the costs of analysis, the selection of the adequate biomarker(s) is based on: i) its toxicokinetic, metabolism and half-life in the human body, ii) in which human compartments can be found in order to choose the appropriate matrix, iii) stability in the selected matrix and individual susceptibility, iv) background of data, such as expected concentration ranges based on previous studies, v) laboratory equipment, specifically the availability of an analytical method with enough sensitivity to quantify the expected levels, and iii) possible external interfering factors affecting its determination[25,43].

Depending on the class of HBM study, three different categories of biomarkers have been defined:

i) *Biomarkers of exposure.* Exposure biomarkers reveal the concentration of a chemical (parent compound) or its metabolites in human biospecimens. The levels of a biomarker of exposure in the body give information about the degree of exposure to a substance, but not about associated potential health effects[25,34].

These biomarkers can be specific or non-specific of a certain parent compound. Non-specific biomarkers are common metabolites, or degradation products, of multiple parent chemicals within the same or different family[34]. Some non-specific metabolites are frequently used to evaluate the exposure of the population to a certain family of substances[42]. On the contrary, the measurement of a specific biomarker gives information about exposure to a specific substance from which it is derived[44]. Therefore, a comprehensive understanding of the specificity of biomarkers is also critical to not misinterpret HBM data.

ii) *Biomarkers of effect.* Effect biomarkers reflect measurable changes in biochemical, physiological, and behavioral parameters or other alterations in an organism due to an exogenous exposure[25]. They are used to establish associations between exposure (dose) and health disorders (response) in humans, and to identify predictors of risk[45]. A wide variety of chemical compounds have been identified as biomarkers of effect, mainly of genotoxic effects, from biomolecules (DNA and protein adducts) to physiological measurements, such as body weight or lung function tests[25,45].

iii) *Biomarkers of susceptibility.* Biomarkers of susceptibility are indicators of the elevated sensitivity of an individual to the adverse effects caused by the exposure to a xenobiotic that can be measured in a biological sample[25,46]. For example, polymorphism of some enzymes (cytochrome P450 and glutathione-S-transferase) and specific alleles in some genes are commonly used as susceptibility markers, since they have been linked to increased cancer risk and neurological disorders, respectively[25]. Therefore, these biomarkers are the key to identify individuals at higher risk of developing these diseases[46].

Generally, susceptibility and effect biomarkers are used in many epidemiological studies as a tool to link exposure to health disorders[25,40]. Therefore, these biomarkers complement exposure biomarkers and provide further evidence of the activity, toxicity, and in some cases, cumulative effects of chemicals and environmental contaminants[43,45]. Consequently, the monitoring of the three types of biomarkers is essential to better understand the human 'exposome' and the effect of these substances on health.

The present thesis focused on the analysis of biomarkers of exposure to organic chemicals, so only these ones were considered in the following sections.

1.2.2.3 ANALYTICAL METHODS

Analytical methods in HBM of organic chemicals differ among each other depending not only on the type of sample, but also on the concentration of their biomarkers of exposure in the biological sample. In addition, the chemical properties of the target analytes are decisive for

selecting the best separation and detection instrumental techniques. Sometimes, a reduction of the matrix complexity and/or a preconcentration of target chemicals are necessary to improve the selectivity and sensitivity of the method[31]. Concurrently, quality assurance and quality control (QA/QC) of the whole analytical process is needed to obtain precise and accurate analytical data. A general overview of the steps involved in the development of analytical methods for application in HBM studies is presented in Figure 3.

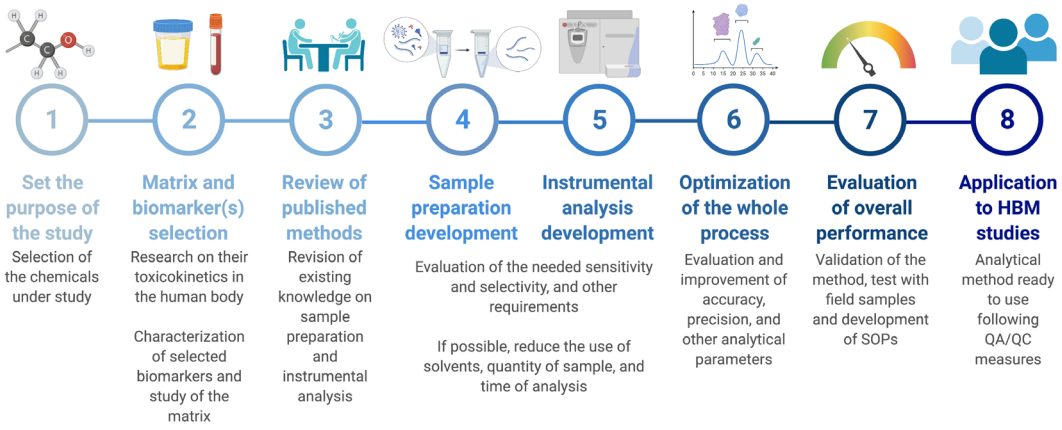


Figure 3. General workflow containing all needed steps to develop an analytical method to be applied in HBM studies: 1) establishment of the purpose of the study; 2) selection of the best matrix/biomarker(s); 3) review of existing knowledge; 4) and 5) development of the analytical method; 6) optimization of the methodology; 7) evaluation of the method performance and its applicability; and 8) application to HBM studies. *SOP: standard operational procedures; HBM: human biomonitoring; QA/QC: quality assurance/quality control*

1.2.2.3.1 SAMPLE PREPARATION

Generally, sample preparation procedures in analytical methods applied to HBM studies start with the thawing and homogenization of the sample[47]. The required volume of sample depends on the preconcentration factor of the sample treatment in each case.

In biological fluids, a preliminary enzymatic deconjugation step (or acid digestion/hydrolysis) is usually necessary for the hydrolysis of phase II metabolites (glucuronides, sulfates, etc.), for example urinary metabolites of PAHs, phthalates, parabens, or mycotoxins[41,42], due to the lack of conjugated standards in the market. In the case of nails and hair, a washing and drying step is required to avoid external contamination[48]. Then, to reduce matrix interferences and preserve instrument performance, as well as to enrich the target analytes to detectable concentrations, centrifugation (fluids)/pulverization (solid samples), extraction of the analytes from the matrix, a clean-up, and a preconcentration step are usually needed[25,31,48]. For example, in blood and breast milk samples, a prior precipitation and denaturation of proteins increases the efficiency of the subsequent extraction[48].

The most used extraction techniques applied to biological samples are solid-phase extraction (SPE) and liquid-liquid extraction (LLE)[26]. In SPE and LLE techniques, the selection of the most appropriate sorbents and solvents, respectively, depends on the chemical properties of the target compounds. In general, SPE methods based on C_{18} and silica cartridges are used for POPs, while mixed-mode cartridges are used for more polar compounds. The most common solvents in LLE methods are acetonitrile, methanol, ethyl acetate and hexane, and salts, such as $MgSO_4$ and NaCl, are usually added for enhancing the extraction recovery[47]. A generic approach named QuEChERS (Quick, Easy, Cheap, Effective, Rugged, Safe), which is based on the salting-out phenomenon, is also applied for extraction of organic chemicals in biological samples, for example of pesticides[49], bisphenols and parabens[50].

Routine analysis of biological samples by conventional extraction methods are time-consuming, solvent demanding and require large volumes of sample[48]. Instead, microextraction techniques, such as solid-phase microextraction and liquid-phase microextraction, which allow us to obtain a high enrichment together with a lower solvent consumption, are getting more popular[25,31]. Moreover, since HBM studies often comprise a large number of samples but in limited volume, 96-well plates-based methods have been developed in recent years[31,51]. Additionally, as the minimum sample treatment is gaining importance, the use of 'dilute-and-shoot' strategies are also increasing[47]. This alternative is applied when the concentration of the compounds in the matrix is high enough to be detected without any extraction step, while matrix interferences are diluted to minimum.

1.2.2.3.2 INSTRUMENTAL ANALYSIS

Due to the complexity of the matrix and the low concentrations of exposure biomarkers expected in human biological samples, selectivity and sensitivity are essential criteria to decide which is the most suitable analytical technique to analyze the target compounds[26].

In general, gas chromatography (GC) and liquid chromatography (LC) are the preferred separation techniques for the analysis of exposure biomarkers of organic chemicals in human samples. GC is commonly used to analyzed non-polar and volatile compounds, such as halogenated compounds, e.g., dioxins and PCBs[35]. Polar analytes can be also analyzed by GC but a prior derivatization step is needed[48]. By contrast, high performance and ultra-high performance liquid chromatography (HPLC and UHPLC, respectively) can be selected for the analysis of a wider range of polarities, although it is most extended in the determination of polar and semipolar organic analytes[47]. Phthalates, PAHs, PFAS and ultraviolet filters in urine samples are commonly determined by LC methods[41,42]. Other analytical techniques, such as enzyme-linked immunosorbent assay for mycotoxins in urine, milk or blood, have been used in HBM studies[41,42].

For quantitative (or target) analysis, LC and GC coupled to mass-based detectors, such as tandem mass spectrometry (MS/MS), are the most common methods used in HBM of organic chemicals due to their lower limits of quantification and the high selectivity compared to other detectors[25,52]. Mass spectrometer operation begins by converting analytes into molecular ions at the ionization source. Then, the resulting ions and their fragments (if any) that are produced in MS system are separated and detected according to their mass-to-charge ratio (m/z). Several different mass spectrometers and operational modes are available depending on the objective of the analysis[53]. Figure 4 shows the principle of operation of the analytical technique used in this thesis, a liquid chromatograph coupled to a tandem mass spectrometer (LC-MS/MS), which is explained in the following paragraphs.

In LC-MS/MS, once the sample is injected into the system, its chemical components are transported by the mobile phases and separated according to their affinity to the LC column. Subsequently, sample components arrive to the mass spectrometer at different times. Reverse-phased (RP) and hydrophilic interaction liquid chromatography (HILIC) columns are the most common ones in this field[47]. RP columns are based on non-polar interactions and are suitable for a wide range of chemicals. By contrast, HILIC columns are more efficient to separate mixtures of polar and very polar chemicals, since they are poorly retained in RP columns[47].

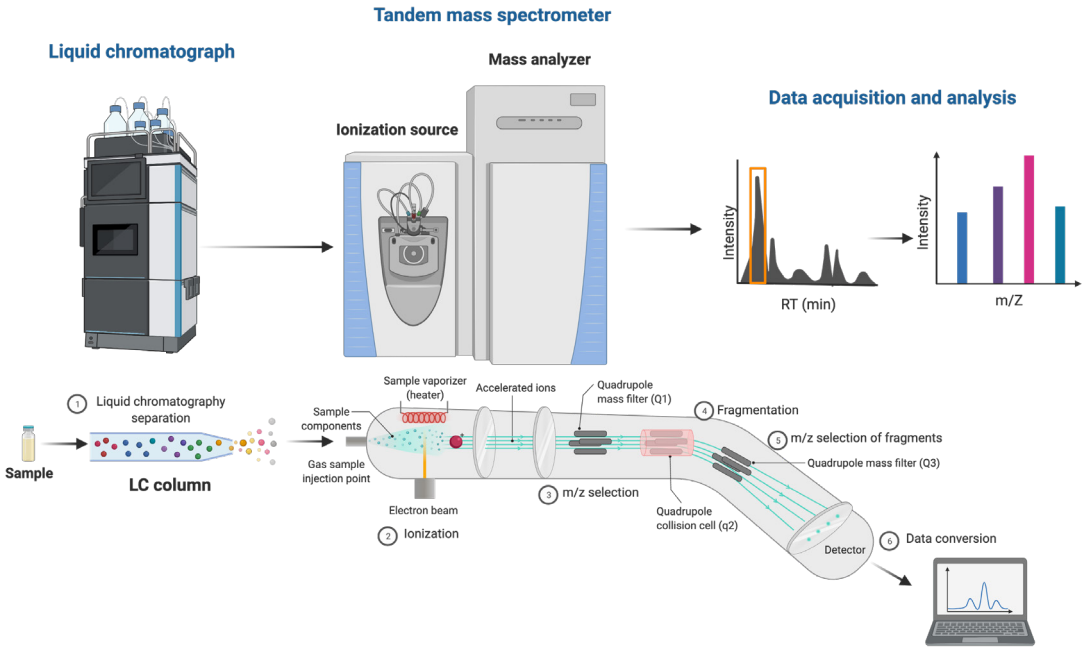


Figure 4. A flow scheme of a liquid chromatograph coupled to a triple quadrupole mass spectrometer (LC-MS/MS).

Before entering to the mass analyzer, the molecules are ionized in the ionization source normally by addition ($M+H^+$) or loss ($M-H^-$) of a proton, known as positive and negative ion modes, respectively. When salts are present in the mobile phases, ion adducts, such as ammonium or sodium adducts, can also occur. Due to the complexity of human samples, the ionization process of target analytes in LC-MS/MS methods can be influenced by the presence of other matrix components, leading to a reduction or an increase of their signal. These phenomena are known as ion suppression and ion enhancement, respectively, and should be considered and minimized in the optimization process[53]. The three following ionization methods are widely used in MS[52,53]:

i) Electrospray ionization (ESI) uses a high voltage to convert the sample to an aerosol which is dispersed into charged droplets. Then, the solvent evaporates, and the droplets "explode" creating a fine aerosol. This ionization mode works well with moderately polar analytes.

ii) Atmospheric pressure chemical ionization (APCI) converts the sample to an aerosol in a heated nebulizer. After that, the aerosol interacts with a corona discharge producing ions. This technique is effective for neutral and low polar molecules that are not well ionized in ESI sources.

iii) Atmospheric pressure photoionization is a variant of APCI which uses photons instead of a corona discharge to ionize the analyte molecules after nebulization. It is normally applied in the analysis of aromatic compounds.

After ionization, ions are driven to the tandem mass analyzer, which is composed by three quadrupoles. As it can be observed in Figure 4, a quadrupole consists of four parallel metal rods which are oppositely connected. The first (Q1) and third (Q3) quadrupoles act as mass filters. By applying a constant or variable voltage between the two pairs of rods, only ions of a certain m/z or m/z ranges can pass through them[52,53]. The second quadrupole (q2) is employed as a collision cell using an inert gas (Ar, N_2), which generates fragments of the ions coming from Q1 that may then be filtered (or not) in Q3[52,53]. Among the different scan modes available in MS/MS, selected reaction monitoring (SRM) is the preferred one for quantitation purposes due to its significantly increased sensitivity. In this mode of operation, a particular m/z is selected in Q1, which is fragmented into the collision cell (q2). Then, one or more specific fragment ions are selected by Q3 to achieve the mass detector[52]. In the development of a LC-MS/MS method, the ion source parameters and the collision energies (CEs) used for each m/z transition have to be optimized[53].

With the implementation of the 'exposome' concept, there is an increasing interest of developing suspect and non-target strategies as complementary tools to traditional target methods. To this end, the use of latest generation high-resolution mass spectrometry (HRMS) techniques is highly recommended, since they have a high mass accuracy, high mass-resolving power, high scan speed and a broad dynamic range. Due to these properties, HRMS allows the detection, annotation and identification of new substances and/or their metabolites in human

biological samples, and also enables the simultaneous determination of a larger number of compounds compared to target methods. Thus, it is a really useful technique to elucidate the chemical composition of the human 'exposome'. However, there is a lack of harmonization of suspect and non-target strategies that should be faced in future studies[54,55].

In the context of this thesis, the analytical technique applied to analyze biomarkers of exposure to organic chemicals was LC-MS/MS. For this reason, only LC-MS/MS is considered in the elaboration of the quality assurance/quality control section.

1.2.2.3.3 QUALITY ASSURANCE AND QUALITY CONTROL

In targeted studies, the selected analytical methodology should be robust and should provide acceptable precision and accuracy for the expected concentration range. Consequently, the validation of the method, and a frequent external and internal control of the reliability and comparability of generated data are crucial for any meaningful assessment and management of risks. Table 1 contains a compilation of basic QA/QC measures for each analytical stage of a HBM study that should be considered when validating and applying a LC-MS/MS method for target analysis of exposure biomarkers[28,55–57].

Sample collection is a crucial step in the whole analytical process. For this reason, it is advisable to train fieldworkers and provide them with a standard operating procedure (SOP). This SOP should give guidance on how to collect the sample to avoid contamination, which sampling materials are needed, required minimum number, volume, or mass of sample, and how to handle and store the samples correctly[58]. In addition, sampling blanks and field spiked blanks are also recommended to assess for any potential background contamination or to identify errors during collection and storage, respectively[55,58]. Prior to sample treatment, an efficient cleaning of labware might help us to avoid cross-contamination between batches. In addition, homogenization of the sample is essential to obtain a representative sample portion prior to analysis[55,58]. In the sample preparation process, matrix and reagent blanks should be incorporated to evaluate potential contamination from all analytical steps (solvents, labware and/or consumables)[55]. If the method includes an extraction and/or a clean-up step, matrix blanks spiked before and after these steps can be used to calculate recoveries and assess reproducibility of the process[56].

For QA/QC of the LC-MS/MS performance, a routine maintenance and internal calibration should be periodically checked. In order to detect instrumental contamination and carryover between injections, solvent blanks should be frequently injected within the same batch[56]. The evaluation of matrix effects is usually performed by comparing signals of reference standards prepared in solvent and signals of those prepared in matrix. In case of high matrix effects, the use of appropriate internal standards (IS) and a matrix-matched calibration curve should be implemented[55–57].

The identification of the analytes in field samples is based on the similarity of retention time (RT) and response ratio of selected fragment ions comparing to the corresponding peaks in the calibration curve[57]. For a proper quantification, a high linearity in the range of concentration levels expected for the analytes in the selected matrix should be ensured, as well as a limit of quantification (LoQ) low enough to be able to give quantifiable levels in most of the samples[28]. The analysis of potential interferences in matrix blank samples is used to evaluate the selectivity and specificity of the method[56].

One of the most important parts of the QA/QC strategy is the evaluation of the precision and accuracy of the reported results. Whenever possible, the analysis of certified reference materials (CRMs), which are controls or standards used to validate and check the quality and metrological traceability of analytical measurement methods, and the participation in laboratory intercomparison exercises, which consists of the organization, performance and evaluation of analysis results given by two or more laboratories in accordance with target values, providing independent verification of laboratory competence, should be encouraged for that purpose[59]. However, limited exposure biomarkers can be evaluated by the current available CRMs. Only the National Institute of Standards and Technology of the Department of Commerce (NIST) in the United States of America (U.S, USA) and the EC's Joint Research Centre (JRC) have been found to be suppliers of CRMs prepared from human biological samples[59]. These CRMs cover mainly the determination of some toxic metals and limited organic compounds, such as metabolites of PAHs, phthalates, phenols, and volatile organic compounds (VOCs) in blood and urine, respectively[60,61].

In the case of intercomparison exercises, the *Centre de Toxicologie du Québec at the Institut National de Santé Publique du Quebec* and the German External QUality Assessment Scheme (G-EQUAS) have been organizing permanent external quality assessment schemes (EQUAS) that enable participating laboratories to evaluate the accuracy and precision of their HBM analytical methods at least twice a year. These EQUAS comprise a wide range of chemicals and environmental pollutants and their metabolites in blood, urine, and hair samples[62,63]. In the frame of the human biomonitoring for Europe (HBM4EU) initiative, a QA/QC program organized in two schemes was conducted to create a network of European laboratories capable of providing comparable and consistent analytical HBM data of high quality[64]. In addition to this, major difficulties and needs, as well as the analytical capacities of European laboratories were identified. Consequently, more than 70 laboratories participated in the HBM4EU schemes with successful results, covering the analysis of nearly 100 biomarkers of exposure to different chemical groups, such as phthalates, bisphenols, PFAs, PAHs, some metals, amines, acrylamide, mycotoxins, pesticides, etc[64].

Table 1. Summary of the QA/QC measures normally used in each analytical stage of target LC-MS/MS methodologies to analyze biomarkers of exposure to contaminants in human biological samples[28,31,55–57].

Analytical stage	QA/QC measures
Sample collection	Selection of the adequate sampling material and storage conditions
	To set a minimum volume/weight of sample
	To use SOPs and training fieldworkers
	Field spiked samples and field blanks
Sample preparation	Cleaning of all labware, use of high-quality reagents
	Homogenization
	Procedural reagent and sample blanks
	Matrix blanks fortified before and after extraction
LC-MS/MS method	Periodical instrument maintenance and internal calibration
	Solvent blanks
	Injection of a reference standard solution or sample reinjection
	Comparison of the signals of reference standards dissolved in solvent vs in matrix
Identification and quantification process	Use of high-purity reference standards
	To check similarity of RT, peak shape, and response ratio of selected ions (normally, 2 product ions) between standards and samples
	Analysis of matrix blank samples
	Use of isotopically labelled ISs and a matrix-matched calibration curve
	Calibration curve with a concentration range and LoQs relevant for the analytes under study
	External QC materials: periodical use of CRM and or participation at publicly accessible international proficiency tests
	'In-house' QC samples: matrix blanks fortified at different levels of concentration in each batch (e.g., at low, mid and high concentration levels)

QA: quality assurance; QC: quality control; LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry; SOPs: standard operating procedures; RT: retention time; S/N: signal-to-noise ratio; ISs: Internal standards; LoQs: limits of quantification; CRM: certified reference materials

Evaluated parameters/purpose
To avoid external contamination, ensure a proper sample storage and the stability of target analytes
To confirm the viability of the analysis
To guarantee traceability and comparability of the results
Stability of the analytes and background contamination
To avoid cross-contamination
Representativeness of the sample
Potential external/procedural contamination (e.g., reagents, material, equipment)
Extraction efficiency (recovery) and reproducibility
LC: RT shift, resolution, and peak shape; MS/MS: mass accuracy, resolution and S/N ratio
Instrumental contamination and carry over
Signal measurement stability
Matrix effects
To ensure a proper identification and quantification
To ensure a proper identification and quantification, and avoid interferences
Selectivity and specificity
Compensation of matrix effects and losses during sample treatment
Good linearity and sensitivity (the LoQs are low enough to quantify the analyte in the selected matrix)
External evaluation of the precision and accuracy of the obtained data
Internal evaluation of the precision and accuracy of the data, intra- and inter-day variability

Simultaneously, many laboratories use 'in-house' QC as a cheaper alternative for daily evaluation of precision and accuracy of their HBM methods[55]. Thus, matrix blanks spiked with the target analytes at different concentration levels should be included in each sample batch to assess method suitability[55]. For the evaluation of internal QC results, previously defined acceptance criteria are necessary. In comparison to other fields, there are no official guidelines for the validation and QA/QC criteria of target HBM analytical methods. However, the *Bioanalytical Method Validation Guidance for Industry* of the U.S. Food and Drug Administration (FDA)[56], the SANTE/12682/2019 *on analytical quality control and method validation for pesticide residues analysis in food and feed*[57], and the QA/QC measures for suspect and non-target analysis of chemicals of emerging concern (CECs) in human studies proposed by Caballero-Casero et al. (2021)[55], could be used as starting points. In this sense, the new European Partnership for the Assessment of Risk from Chemicals (PARC) aims to develop the network of qualified laboratories for biomarkers analysis and some guidance documents to support the harmonized use of QA/QC principles in HBM and other less matured domains in the upcoming years[65,66].

Apart from all the aforementioned aspects, ease of use, cost, safety, and rapidity are also important characteristics to evaluate and optimize when developing a method, mainly for routine analysis purposes[58].

1.2.2.4 PROGRAMS AT NATIONAL, EUROPEAN, AND INTERNATIONAL LEVEL

National HBM programs are normally funded, coordinated and managed by their respective governmental centers, and aim to generate HBM data for a wide variety of chemicals which are representative of their populations (more than 100,000 participants in some cases) to control environmental exposure over time[36,67]. As it can be seen in Figure 5, a reduced number of countries among the world have currently long-term regular HBM programs at national level.

In America, the U.S. National Health and Nutrition Examination Survey (NHANES) started in 1971, being the first HBM program of the world. Since then, it has been continuously monitoring the internal exposure to environmental chemicals in the U.S. population at 2-year intervals[36]. In Canada, the Canadian Health Measures Survey (CHMS) was launched in 2007, comprising not only HBM data but also indoor air and tap water analysis, and now its 7th cycle is in progress (2022 – 2023)[68,69].

Under the Environmental Health Act in Korea, the Korean National Environmental Health Survey (KoNHES) was first conducted in 2009, which involved more than 6,000 Korean adults[70,71]. A year after, Japan started a nation-wide birth cohort study, the Japan Environment and Children's Study (JECS), which focused on children's health and development, recruiting and following-up around 100,000 families[72].

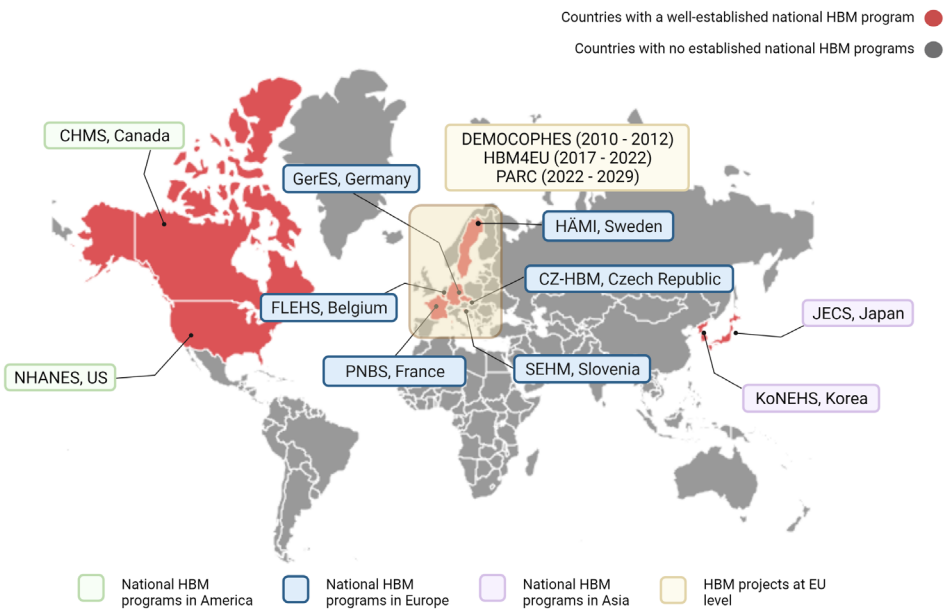


Figure 5. Global overview of HBM programs established at national level, and joint HBM initiatives conducted in Europe in the last decade. *HBM: human biomonitoring*

In the European Union, only six countries have well-implemented national HBM programs (Figure 5). For example, the German Environmental Surveys (GerES), which evaluate the exposure to pollutants of the population in Germany, are large-scale cross-sectional studies that have been conducted by the German Environment Agency (UBA) since 1985[73]. In the Czech Republic (CZ), the CZ-HBM project focused on their urban/suburban population was set up in 1994 as a part of its nationwide Environmental Health Monitoring System[74]. Approximately 30 years ago, the Swedish Environmental Protection Agency promoted the establishment of the Health-related environmental monitoring (HÄMI) within its environmental monitoring program area, which is currently managed by the Institute of Environmental Medicine at *Karolinska Institutet* (Sweden)[75]. In Belgium, the surveillance HBM program of the Flanders region, the Flemish Environment and Health Survey (FLEHS), has been integrated in its environmental health policy since 2002, using cross-sectional and prospective cohort studies with more than 5,500 participants. In the last two decades, the French national biomonitoring program (PNBS)[76] and the Slovenian national HBM program (SEHM)[36,77] were implemented by the Public Health France and the Ministry of Health of Slovenia, respectively, in accordance with their respective national legislations.

Most of these HBM programs are performed with general population, including children, adolescents, adults, and elderly, and some of them include also vulnerable groups, such as pregnant/lactating mothers and newborns. A broad range of chemicals have been covered, being metals, pesticides, PCBs, phenols, and phthalates the most studied ones. On the contrary,

perchlorate, acrylamide, parabens, nitrosamines, and mycotoxins have been measured in less than 30 % of the HBM programs. Regarding biological samples, urine and blood (serum and plasma) are by far the preferred specimens. However, hair has been used as a non-invasive sample to monitor children's exposure, and breast milk and cord blood are also collected when pregnant/lactating mothers are involved. Alternative human samples, such as teeth and dried blood spot have been only employed in the CZ-HBM and the JECS, respectively. In addition to this, the sampling process normally implies the collection of personal information through proper questionnaires containing questions about lifestyle, dietary habits, socio-demographic data, and other characteristics. By combining HBM data with the questionnaire information, potential determinants and patterns of exposure to chemicals and environmental contaminants might be identified.

Communication of HBM results varies by study/programs. HBM data of national programs are usually published as aggregated data or stratified data (by sex, age or subpopulation groups), either in public reports or in scientific articles while respecting ethical and data protection issues[36]. Other studies also provide individual results to participants together with an evaluation of their data. Since the performance of HBM studies depends on the willingness of the target population to provide samples for analysis, an effective dialogue between scientists and participants is crucial, starting from recruitment and ending with the dissemination of results and consequent actions taken by public health authorities[78]. In this sense, a clear and sensitive communication strategy for a proper interpretation of HBM data, including health and dietary recommendations, if necessary, is strongly recommended. An example of communication and follow-up of HBM data is described in the *Report of the official control plan of the food chain 2018* of the Food Safety Plan of the Valencian Region[79], where the results of Hg levels in hair of children in the BIOVAL study (Valencian Region, Spain) are evaluated. In this study, a well-established communication workflow was followed to inform parents of the participating children and the pediatricians of the Valencian Region about the obtained results. As an intervention measure, children whose hair Hg levels were above the threshold value (5 $\mu g \cdot g^{-1}$), fish consumption recommendations were given, showing a considerable reduction in Hg exposure after 4 months[79].

Due to the lack of national HBM programs, most countries have been collecting HBM data through specific research projects, generating heterogeneous and non-comparable data[67]. In this context, the EU's Environment and Health Action Plan (2004 – 2010) recognized the relevance of HBM and highlighted the need for further efforts to build a harmonized and coordinated HBM approach in Europe[80]. As a result, the EC conducted the COPHES/DEMOCOPHES (Consortium to Perform Human Biomonitoring on a European Scale/Demonstration of a Study to Coordinate and Perform Human biomonitoring on a European Scale) in 2009 – 2012, which was the first joint European HBM initiative developing aligned protocols for framework, fieldwork, chemical analysis, statistical analysis, and communication of HBM data[80]. This initiative was the first step

to implement a HBM strategy producing comparable and validated data on the exposure of the European population to chemicals[81].

Taking the COPHES/DEMOCOPHES as a starting point, the HBM4EU project (2017 – 2021) was launched by a joint effort of 26 countries and the EC under Horizon 2020 to coordinate and advance HBM in Europe[81]. The main objective of the HBM4EU initiative was to harmonize HBM strategies in order to obtain comparable HBM data among countries and population groups across the EU, and to create a sustainable European HBM platform to provide science-based evidence for chemical policies[67]. One of the first steps of the HBM4EU consortium was to develop a strategy for the prioritization of chemicals for action, considering the knowledge on chemical exposure and health outcomes. This work resulted in the publication of two lists of HBM4EU priority substances containing more than 15 chemical families of health concern[41,42]. In this context, many HBM studies focusing on the analysis of these substances have been conducted in different regions of the EU in an aligned way in order to generate comparable HBM data of European citizens[67,82]. The next steps for HBM in Europe will be implemented within the planned European PARC, which is promoted by 28 countries and includes ministries, national and EU agencies, as well as academia and research institutions[83]. The general objective of PARC is to consolidate and strengthen the research and innovation capacity for chemical risk assessment in Europe to protect human health and the environment[84]. In the HBM field, it aims to consolidate the work done in the HBM4EU by building harmonized and sustainable long-term HBM platforms at a national and a European level, and generating comparable HBM data across EU populations[65,66].

In Spain, the Ministry of Agriculture, Food and Environment promoted the BIOAMBIENT.ES project (2009 – 2010), which was the first nationwide cross-sectional study on a representative sample of the Spanish adult population. Under this project, the National Centre on Environmental Health of the *Carlos III* Institute of Health (ISCIII) was responsible for analyzing PCBs, PBDEs, metals, PAHs and pesticides in urine, blood, and hair samples from more than 1,800 participants[85]. Another relevant HBM study at a national level was the INMA project (Environment and Childhood project), a set of cohorts developed in seven different geographical areas in Spain, whose objective was to evaluate the health effects of exposure to environmental pollutants from gestation to birth and during childhood[86]. Additionally, other research HBM studies have been performed at a regional level in Madrid[87–89], Andalusia[90–92], Navarra[93], Catalonia[94–96], the Canary Islands[97,98] and the Valencian Region[39,99–101], among others. However, a well-established national HBM program does not currently exist in Spain. To cover this need, the Ministry of Health and the Ministry for Ecological Transition and Demographic Challenge have contemplated in *the Health and Environment Strategic Plan 2022 – 2026*, the urgent creation of a national hub for the implementation of periodic HBM campaigns in Spain[27].

1.3 INTERPRETATION OF HUMAN BIOMONITORING DATA

Due to the increasing number of HBM studies over the last two decades, a large volume of valuable HBM data is now available. These data can be compared over time within the same or among different populations to evaluate and support public health interventions. The geometric mean (GM) concentration of a given biomarker is generally chosen for the evaluation and comparison of HBM data, since it provides the best estimation of the central tendency of the observed internal dose, which is normally skewed to the left (more low exposures than high exposures). In addition to this, the 95th percentile (P95) concentration is used to estimate the upper bound of the internal dose, corresponding to highly exposed individuals[102]. In fact, the German HBM Commission proposed in the 1990s the use of the P95 of a representative population as a reference value (RV_{95}). RV_{95} is of no health relevance, but it is a relevant statistic to compare between similar populations or to identify which individuals within the same population group are exposed to a greater or lesser extent[103]. In order to accurately interpretate HBM data in a risk assessment context, several concepts and strategies based on toxicokinetic, toxicity and exposure information have been developed[102].

1.3.1 HEALTH-BASED GUIDANCE VALUES

Several regulatory authorities have derived different toxicity values for health effects, known as health-based guidance values (HBGVs), resulting from chronic exposure to chemical substances to evaluate the associated risk. For example, the U.S. EPA uses the concept of reference dose (RfD), defined as “an estimate of a daily oral exposure to the human population that is likely to be without an appreciable risk of deleterious effects during a lifetime”, and the reference concentration (RfC) for inhalation exposures[104]. Another frequently used term is the acceptable daily intake (ADI) proposed by the WHO, which is “an estimate of the amount of a substance in food (including drinking water) that can be consumed daily over a lifetime without presenting an appreciable risk to health”. Traditionally, ADI values were established for food additives and pesticide residues. By contrast, its equivalent for substances that have no intended function in these products was defined as a tolerable daily intake (TDI)[103]. These HBGVs consider only non-cancer effects and are generally expressed in units of milligrams per kilogram of bodyweight per day ($mg \cdot kg^{-1} \cdot day^{-1}$), as they represent chronic exposure to chemicals[104].

HBGVs can be derived from different points of departure (PODs), applying some uncertainty factors (UF), such as interspecies (UF_A) and intraspecies (UF_H) uncertainty factors, to consider limitations of the used data (equation 1). The most common PODs are: i) the no-observed-adverse-effect level (NOAEL), which is the highest experimentally determined dose without a significant adverse effect, ii) the lowest-observed-adverse-effect level (LOAEL), defined as the lowest experimental dose which implies a significant adverse effect, or iii) a benchmark dose

(BMD), such as the lower bound of the BMD confidence limit producing a 10 % change in the response rate of an adverse effect ($BMDL_{10}$)[105].

$$\text{HBGV (RfD,ADI)} = \text{POD (NOAEL, BMD)}/\text{UF} \quad (1)$$

For carcinogenic substances, no safe levels below which exposure does not pose a health risk can be established. In these cases, the U.S. EPA uses the term 'cancer slope factor', defined as "an upper-bound estimate of the increased human cancer risk from a lifetime exposure to an agent by ingestion or inhalation"[106].

1.3.2 EXPOSURE AND RISK ASSESSMENT OF HUMAN BIOMONITORING DATA

Figure 6 illustrates the major strategies that are currently employed to evaluate human exposure to chemicals in a risk assessment context[102,103]. As it was explained before, the external risk assessment is based on the occurrence of chemical substances in environmental samples (normally, foodstuffs) and the consumption data of the population, resulting in an estimated daily intake (EDI) which is compared to a HBGV. By contrast, the internal approach uses HBM data to evaluate the risk, which is commonly conducted in two different ways: forward and reverse dosimetry. The methodology used in the forward dosimetry approach is to convert a POD or a HBGV (if exists) of a given chemical compound into the corresponding concentration of its biomarker in the biological specimen of interest, such as the biomonitoring equivalent (BE) or the German control and action HBM values (HBM-I and HBM-II, respectively), using toxicokinetic information[107]. Then, the observed internal concentration (internal dose) in the target population is compared to the derived reference value for further evaluation. On the contrary, the reverse dosimetry uses toxicokinetic information to back-extrapolate from the observed biomarker concentration to an estimated external exposure dose (such as the EDI), which is then compared to the proper HBGV[107].

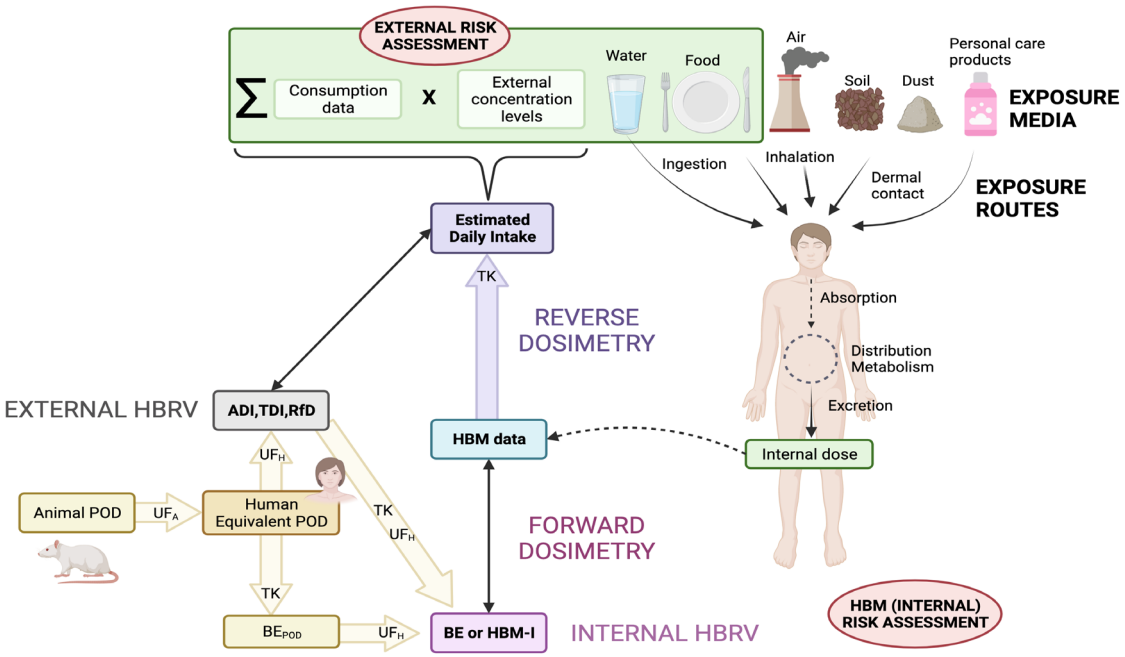


Figure 6. The different approaches to evaluate human exposure to chemicals and environmental pollutants: external risk assessment and HBM (internal risk assessment) using forward and reverse dosimetry. HBM: human biomonitoring; *HBGV: health-based guidance value; ADI: admissible daily intake; TDI: tolerable daily intake; RfD: reference dose; UF_A: interspecies uncertainty factors; UF_H: intraspecies uncertainty factors; TK: toxicokinetic data; BE: biomonitoring equivalent; HBM-I: German HBM control value; BE_{POD}: biomonitoring equivalent directly derived from a point of departure (POD) without considering intraspecies uncertainty factors.*

Next sections contain a detailed description of the forward and reverse dosimetry focusing on risk interpretation of urinary concentrations, since urine was the only matrix analyzed in the present thesis.

1.3.2.1 FORWARD DOSIMETRY

This method has been traditionally used to derive to biomarker guidance values for occupational health purposes. However, these values are inappropriate for evaluating the exposure of the general population[102,103]. In this context, two similar HBGVs, BE and HBM-I and -II, have been derived for many chemicals.

A BE is described as the concentration or a range of concentrations, for example from BE to BE_{POD} (biomonitoring equivalent directly derived from a POD without considering UF_H), of a chemical or its metabolites in a biological specimen (blood, urine, hair, etc.) that is consistent with an existing HBGV, such as RfD or TDI, or with a cancer-based exposure guidance value[107,108]. In order to identify if HBM data of a target population is below, near or above this BE, the hazard quotient (HQ) (equation 2), and the margin of safety (MOS) or margin of exposure (MOE) for genotoxic and carcinogenic chemicals (equation 3), have been established as follows[102,109]:

$$\text{HQ (dimensionless)} = \frac{[\text{Biomarker}]}{BE} \quad (2)$$

$$\text{MOE or MOS(dimensionless)} = \frac{BE}{[\text{Biomarker}]} \quad (3)$$

A HQ lower than 1 suggests that exposure to that substance is of a low concern. However, chemicals whose HQ values are above 1 indicate that further investigation and risk-assessment follow up are needed[102]. Generally, a MOE or MOS > 10,000 is considered of low concern from a public health point of view, but this cut-off value depends on the substance[109].

When there is a BE_{POD} available, the interpretation of the HBM data can be conducted through relative prioritization schemes (Figure 7). The resulting information is the priority level for further risk assessment follow-up of the exposure to a certain chemical compound. Biomarker concentrations below the BE, between the BE and the BE_{POD}, and above the BE_{POD}, are considered to be of low, medium and high priority, respectively[108]. Therefore, the use of BEs is a helpful tool to evaluate HBM data in terms of prioritization and identification of potential risks. In addition, it is a simple concept to communicate HBM data to the general population[102]. On the other hand, it cannot be used as a diagnostic tool to distinguish between 'safe' and 'unsafe' exposures, since it does not give information about the health impact associated to that exposure[103,107,108].

Another reference values widely used in HBM risk assessment are the German HBM values, HBM-I and HBM-II. The first one is practically identical to BE, and it is defined as "the concentration of a contaminant or its metabolites in a biological matrix below which no adverse health should be expected"[103]. By contrast, HBM-II is described as "the concentration of a contaminant or its metabolites in a biological specimen above which relevant adverse health effects may occur, and hence immediate action to reduce exposure must be taken and expert care in environmental medicine will be required"[103]. Thus, HBM-I values are called 'control values', and HBM-II are considered 'action levels'.

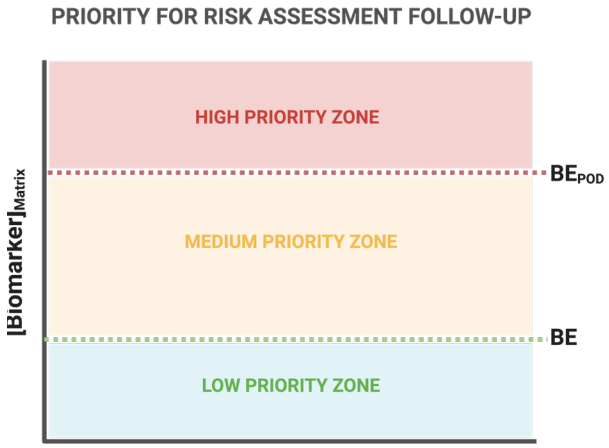


Figure 7. Prioritization scheme for the interpretation of biomonitoring data in a risk context using BEs. Biomarker concentrations lower than BE are considered of a low priority, while values between BE and BE_{POD} are of a medium priority, and above BE_{POD} of a high priority for risk assessment follow-up. *BE: biomonitoring equivalent; BE_{POD}: biomonitoring equivalent directly derived from a point of departure (POD) without considering intraspecies uncertainty factors.*

Nowadays, the limited availability of BE or HBM-I and -II values and the lack of official guidance to use HBM data in a risk assessment context are the principal obstacles for the implementation of HBM programs in Europe[110]. In this sense, the derivation of HBM reference values should be encouraged. As an alternative, for those chemicals with no defined HBM reference values, the reverse dosimetry approach can be applied instead.

1.3.2.2 REVERSE DOSIMETRY

In the reverse dosimetry approach, toxicokinetic data is used to transform HBM concentrations into estimated exposure doses. The simplest and most used method is the calculation of an EDI. In the case of urinary biomarker data, the generic expression to estimate the EDI is the following[102]:

$$\text{EDI}\left(\frac{\mu g}{kg\text{–}bw\cdot day}\right) = \frac{[Biomarker(s)]_U \; x \; AF \; x \; MX_P}{BW \; x \; F_{UE}} \quad (4)$$

where, $[Biomarker(s)]_U$ is the concentration (or sum of concentrations) of the biomarker(s) of exposure to the parent compound in urine (µmol/l or µmol/g creatinine); AF is an adjustment factor to minimize the effect of urine dilution, such as 24 h-urine volume ($l\cdot day^{-1}$) or creatinine excretion per day (g $Cre\cdot day^{-1}$), osmolality or specific gravity; MW_P is the molecular weight of the parent compound; BW is the body weight (kg) of the target population; and F_{UE} is the urinary

excretion factor, which is defined as the molar ratio of the amount of biomarker appearing in urine and the administered dose of the parent compound[102].

As in the reverse dosimetry approach, the HQ (equation 5), and MOE or MOS (equation 6) concepts can be applied to interpretate EDI values in a risk assessment context.

$$\text{HQ (dimensionless)} = \frac{\text{EDI}}{HBRV\ (ADI,RfD,TDI)} \quad (5)$$

$$\text{MOE or MOS (dimensionless)} = \frac{HBRV\ (ADI,RfD,TDI)}{\text{EDI}} \quad (6)$$

1.3.2.3 RISK ASSESSMENT OF EXPOSURE TO MIXTURES

Due to the fact that citizens are exposed to multiple chemical substances through several pathways, one of the key actions of the European *Chemicals Strategy for Sustainability - Towards a Toxic-Free Environment* is to consider the combination effect when assessing risk from chemicals[2]. In this context, EFSA have published the first reports assessing the cumulative risk to the combined exposure to pesticide residues by classifying them into cumulative assessment groups (CAGs) and assuming dose addition[111,112]. To this end, EFSA uses the combined (total) MOE (MOET) concept, as it is shown in equation 7, which can be also applied to HBM data. A MOET above 100 is generally considered protective of humans[111,112].

$$\text{MOET} = \frac{1}{\Sigma MOE_i} \quad (7)$$

Another method to calculate the combined exposure to chemicals is the hazard index (HI), which is defined as the sum of HQs for individual compounds with the same mode of action (MoA) occurring in the same specimen (equation 8). In the same way as HQ, a HI value >1 might indicate a potential risk[113].

$$\text{HI} = \Sigma \text{HQ}_i \quad (8)$$

For some structurally related substances, such as dioxins congeners or PAHs, a toxic equivalency factor (TEF) approach is used for evaluation of the cumulative risk, which assigns a potency factor to each chemical relative to the most toxic one[113].

Although some methodological tools are available for assessing mixture toxicity, there is a lack of knowledge on potential chemical interactions (additive, antagonistic, or synergistic) in exposure to mixtures. As a result, a systematic approach and health-based reference limits are still lacking, being evident the need of further research in this field[113].

1.4 BACKGROUND ON THE ORGANIC CHEMICALS STUDIED IN THIS THESIS

The selection of the organic chemical compounds studied in the present thesis was in line with the objectives of the HBM4EU initiative and the AESAN. The three selected groups were pesticides, PAHs and acrylamide, which are included in the two lists of priority substances resulting from the two HBM4EU prioritization rounds conducted in 2016 – 2017 due to the scarcity of available HBM data and general knowledge on them[114], and in the National Plan for Official Control of the Food Chain in Spain[115]. The following sections contain a comprehensive background and relevant HBM information of each substance group.

1.4.1 PESTICIDES

1.4.1.1 APPLICATIONS AND HEALTH EFFECTS RELATED TO PESTICIDES

Pesticides are chemical substances used to control harmful organisms, such as insects, rodents, fungi, and unwanted weeds, or vector-borne diseases (e.g., carried through mosquitoes), as well as to protect plants during production, storage, and transport, with the objective of achieving higher yields[116,117]. As a results, these substances allow agricultural industries to produce enough food to support the continuing growth of the global population, which is expected to have grown 30 % by 2050[118]. In addition to that, pesticides can occasionally be used for domestic purposes and in local public places, such as parks and gardens. In Europe, the major types of pesticides used in 2020 were[119]:

i) Insecticides, such as organophosphates (OPs), which contain a chemically reactive phosphate ester side chain, and pyrethroids (Pyr), which are classified by the absence (type I) or presence of an α-cyano group (type II),

ii) Herbicides (Herb), such as chloroacetanilides, phenoxies or triazines,

iii) Several groups of fungicides and bactericides.

Some of these pesticides have neurotoxic and endocrine disrupting properties and, due to their presence in the environment, wildlife and human health can be negatively affected by

exposure to them[120]. For example, chlorpyrifos, one of the most used OP insecticides in Europe in the last few years, has recently been banned [121] and classified as toxic for reproduction (category 1B) by EFSA[121]. Other OPs, such as malathion and parathion were also banned and classified as group 2A (Probably carcinogenic to humans) and 2B (Possibly carcinogenic to humans) by the International Agency for Research on Cancer (IARC)[122], respectively. For the moment, most Pyr have been considered as Group 3 (not classifiable as to its carcinogenicity to humans). On the contrary, the family of chlorophenoxy herbicides has been included in the group 2B since 1987[122].

In general, pesticides have been linked to the following health disorders: cancer (e.g. bladder and colon cancer), respiratory problems (e.g. asthma), Parkinson, Alzheimer, reproductive disorders (e.g. male infertility), among others[123]. In addition, several neurodevelopmental effects of pesticides on humans have been reported, concluding that exposure to these substances during pregnancy and childhood should be avoided[124]. In consequence, pregnant women and children are considered the most vulnerable population groups. On top of that, humans are exposed every day to combinations of different pesticides[125]. Thus, apart from the harmful effects that these substances can cause individually, interactions among pesticides, such as cumulative and synergistic effects, should be considered when evaluating exposure or their toxicological properties[112].

1.4.1.2 PESTICIDE POLICIES IN THE EUROPEAN FRAMEWORK

Due to the potential harmful effects of pesticides and their extensive use, existing policies and legislation on pesticides were first introduced at EU level in 1979[126]. In fact, the reduction of the use of pesticides and fertilizers in agriculture is one of the key actions included in the European Green Deal published in 2019[10]. Currently, there are several regulatory restrictions to control pesticide use, which are classified according to their application:

i) Regulation (EC) 1107/2009 for pesticides approved to be used as plant protection products (PPP)[127], including a “list of candidates for substitution”.

ii) Regulation (EU) 528/2012 for pesticides used as biocides (wood preservatives, insecticides, acaricides, and products to control other arthropods)[128].

iii) Regulation (EC) 726/2004 for those used in human and veterinary medicinal products[129].

In addition to that, public health authorities must monitor the presence of pesticides in final consumer products to ensure a reduced exposure of final consumers. To this end, the Regulation (EC) 396/2005 sets the maximum residue levels of pesticides in food and feed of plant and animal origin[130]. For infant formula and follow-on formula, the Regulation (EU) 2016/127 establishes a maximum of 0.01 or 0.003 mg of pesticide residues per kg of product, depending on the active substance[131]. However, their presence in breast milk has not been

covered yet by national control programs. In water, quality standards of 0.1 µg for individual active substances and 0.5 µg for the sum of all pesticides per liter of groundwater have been established by the Directive 2006/118/EC[132]. By contrast, there is not a European network for monitoring the presence of pesticides in ambient air.

1.4.1.3 EXPOSURE, METABOLISM, AND HUMAN BIOMONITORING OF PESTICIDES

Since pesticides are released not only into the food chain but also into the soil, water, and air, the general population is exposed to their residues through several pathways[133]. The principal route of exposure to pesticides is the ingestion, mainly of fruit and vegetable consumption, and water intake[134]. Nevertheless, non-dietary routes, such as inhalation and dermal contact via direct contact or dust, may also play an important role [134,135].

OPs, Pyr, and Herb are non-persistent pesticides, which means that these compounds do not bioaccumulate in the body and their metabolites are mainly excreted through urine in less than 72 hours after exposure[136]. A summary of the urinary metabolites of OPs, Pyr, and Herb analyzed in this thesis is presented in Table 2. Figure 8 represents the generic major metabolic pathways of OPs and the resulting metabolites[137,138]. In the phase I metabolism, the parent pesticide is usually transformed to a hydrolyzed or oxidized form by the cytochrome P450. In the second phase, glucuronyl-, sulfo- or glutathione-S-transferases catalyze the formation of the corresponding conjugates, which are more polar than the parent metabolites[137,138]. Thus, pesticide metabolites may be present in urine in a free or conjugated form[137,138]. Biotransformation products of pesticides can be non-specific or specific metabolites. Non-specific pesticide biomarkers are those metabolites that are common to a family of pesticides, for example dialkyl phosphates (DAPs) (Figure 8) and 3-phenoxybenzoic acid (PBA) are non-specific urinary metabolites of the OPs and Pyr families, respectively[136]. The analysis of non-specific metabolites offers insight into the general exposure to these compounds. On the contrary, the measurement of specific biomarkers (represented as 'X-OH' in figure 8), such as the specific metabolite of chlorpyrifos (3,5,6-trichloro-2-pyridinol, TCPY), only gives information about exposure to a specific parent.

As OPs, Pyr, and Herb are generally metabolized and excreted within 1-3 days, the analysis of their metabolites in urine (instead of blood or serum), normally by LC-MS/MS or GC-MS/MS, is a useful strategy to obtain valuable HBM information about recent exposure through all possible pathways[136]. However, hair and breast milk samples can be also used for the analysis of their parent compounds in unchanged form[114].

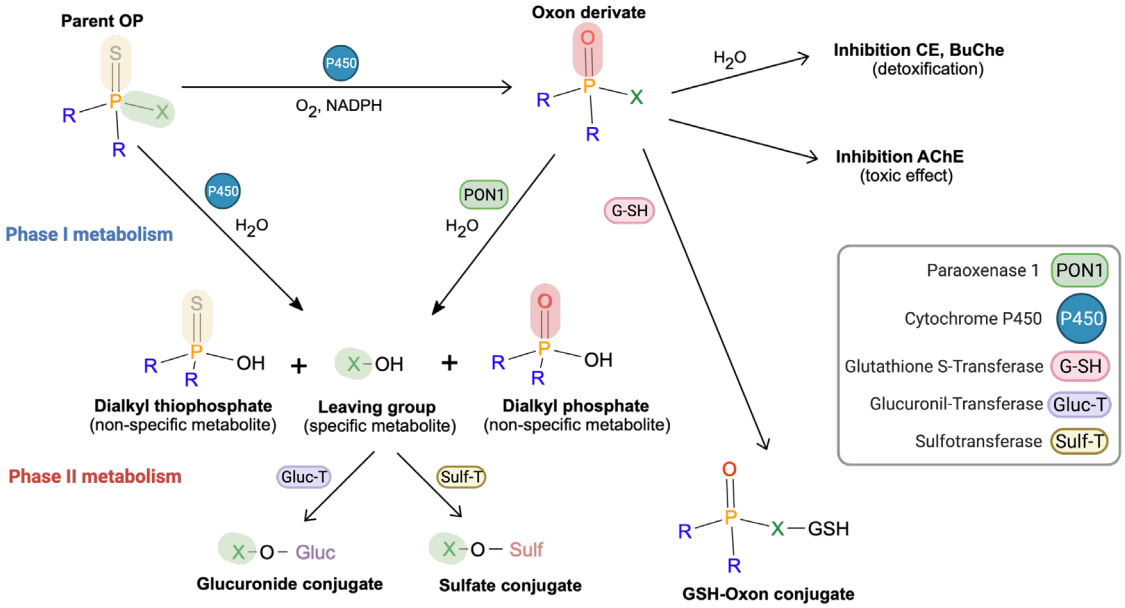
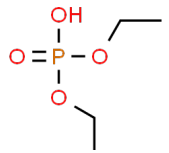
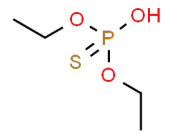
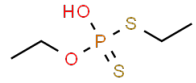
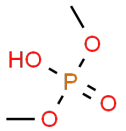
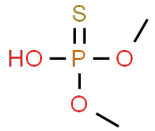
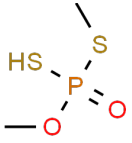


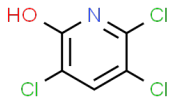
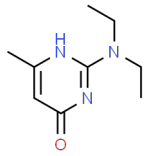
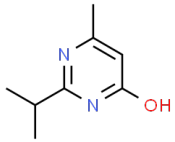
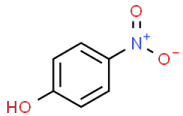
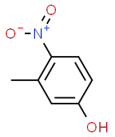
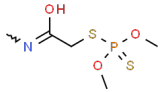
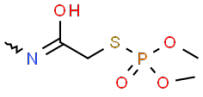
Figure 8. Generic biotransformation pathway of organophosphate pesticides (OPs) and resulting specific and non-specific metabolites. *NADPH: reduced nicotinamide adenine dinucleotide phosphate; R: radical chain; X: specific chemical group of each OP; CE: carboxylesterase; BuChE: butyrylcholinesterase; AChE: acetylcholinesterase; Gluc: glucuronide; Sulf: sulfate GSH: reduced glutathione.*

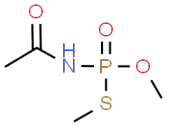
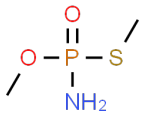
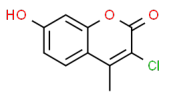
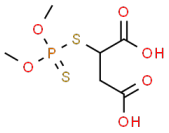
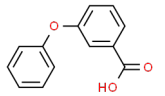
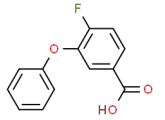
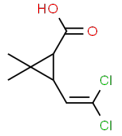
Many countries have included urinary pesticide metabolites in their HBM programs, such as the NHANES or the GerES, and there are many research projects focused on assessing internal exposure to pesticides in different population groups[114]. As a result, there are established RV_{95} for adults and children of some nationalities. However, HBM guidance values are not available for many pesticides, so an accurate risk assessment of HBM data is not possible yet[114]. No HBM-I or HBM-II have been established, and only BEs for 2,2-dibromovinyl-2,2-dimethylcyclopropane-1-carboxylic acid (DBCA), the specific metabolite of deltamethrin, the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D), and PBA are available[139–141]. However, some of these BEs are no longer applicable, since HBGVs have been updated or are based on weak data sets. Therefore, the reverse dosimetry approach is normally used when evaluating HBM data of pesticides in a risk context[142].

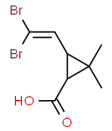
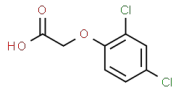
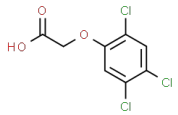
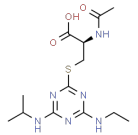
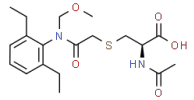
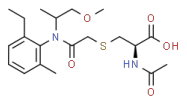
In Europe, Spain is one of the main agricultural producers, and it was the country with the second highest sales of pesticides, just before France, in 2018[143]. Although the Spanish population could be potentially highly exposed to these substances, only a few HBM projects focused on evaluating pesticide exposure have been conducted in lactating/pregnant women, adolescents, children, adults and occupationally exposed population living in different Spanish regions, such as Andalusia, the Murcia Region, Catalonia, the Valencian Region, and Galicia[136].

Table 2. Summary of the urinary metabolites of non-persistent pesticides analyzed in the present thesis: classification, applications, possible parent substances and their IARC classification, abbreviation, InChIKey, molecular formula, chemical structure, and type of biomarker (specific or non-specific).

Parent pesticide (IARC classification*)	Biomarker of exposure	Abbreviation	InChIKey	Molecular formula	Chemical structure	Type of biomarker
Organophosphate (OPs) pesticides: used in agriculture (specially fruits), gardens, veterinary practices, etc.						
OPs	Diethyl phosphate	DEP	UCQFCFPECQILOL-UHFFFAOYSA-N	$C_4H_{11}O_4P$		NS
	Diethyl thiophosphate	DETP	PKUWKAXTAVNIJR-UHFFFAOYSA-N	$C_4H_{11}O_3PS$		NS
	Diethyl dithiophosphate	DEDTP	OILHDBLLQZLFFI-UHFFFAOYSA-N	$C_4H_{11}O_2PS_2$		NS
	Dimethyl phosphate	DMP	KKUKTXOBAWVSHC-UHFFFAOYSA-N	$C_2H_7O_4P$		NS
	Dimethyl thiophosphate	DMTP	WWJJVKAEQGGYHJ-UHFFFAOYSA-N	$C_2H_7O_3PS$		NS
	Dimethyl dithiophosphate	DMDTP	PSRWXHIYWUDGMA-UHFFFAOYSA-N	$C_2H_7O_2PS_2$		NS

Parent pesticide (IARC classification*)	Biomarker of exposure	Abbreviation	InChIKey	Molecular formula	Chemical structure	Type of biomarker
Chlorpyrifos[b], chlorpyrifos-methyl[b], triclopyr[a] (not assessed)	3,5,6-Trichloro-2-pyridinol	TCPY	WCYYAQFQZQEUEN-UHFFFAOYSA-N	$C_5H_2Cl_3NO$		S
Pirimiphos-methyl[a] (not assessed)	2-Diethylamino-6-methyl-4-pyrimidinol	DEAMPY	NQCPECCCWDWTJJ-UHFFFAOYSA-N	$C_9H_{15}N_3O$		S
Diazinon[b] (Group 2A)	2-Isopropyl-4-methyl-6-hydroxypyrimidine	IMPY	AJPIUNPJBFBUKK-UHFFFAOYSA-N	$C_8H_{12}N_2O$		S
Fluorodifen[b] (not assessed), parathion-methyl[b] (Group 3), parathion[b] (Group 2B)	p-Nitrophenol	PNP	BTJIUGUIPKRLHP-UHFFFAOYSA-N	$C_6H_5NO_3$		S
Fenitrothion[b] (not assessed)	3-Methyl-4-nitrophenol	MNP	PIIZYNQECPTVEO-UHFFFAOYSA-N	$C_7H_7NO_3$		S
Dimethoate[b] (not assessed)	Dimethoate	DIMET	MCWXGJITAZMZEV-UHFFFAOYSA-N	$C_5H_{12}NO_3PS_2$		S
Dimethoate[b], omethoate[b] (not assessed)	Omethoate	OMET	PZXOQEXFMJCDPG-UHFFFAOYSA-N	$C_5H_{12}NO_4PS$		S

Parent pesticide (IARC classification*)	Biomarker of exposure	Abbreviation	InChIKey	Molecular formula	Chemical structure	Type of biomarker
Acephate[b] (not assessed)	Acephate	ACEP	YASYVMFAVPKPKE-UHFFFAOYSA-N	$C_4H_{10}NO_3PS$		S
Acephate[b], methamidophos[b] (not assessed)	Methamidophos	METHA	NNKVPIKMPCQWCG-UHFFFAOYSA-N	$C_2H_8NO_2PS$		S
Coumaphos[b] (not assessed)	3-Chloro-7-hydroxy-4-methylcoumarin	CMHC	ODZHLDRQCZXQFQ-UHFFFAOYSA-N	$C_{10}H_7ClO_3$		S
Malathion[a] (Group 2A)	Malathion dicarboxylic acid	MDA	NIUNKPWHNGMQRE-UHFFFAOYSA-N	$C_6H_{11}O_6PS_2$		S
Pyrethroid insecticides: used in crops protection, parks, gardens, household insecticides and mosquito control						
Pyrethroids	3-Phenoxybenzoic acid	PBA	NXTDJHZGHOFSQG-UHFFFAOYSA-N	$C_{13}H_{10}O_3$		NS
Cyfluthrin[b] (not assessed)	4-Fluoro-3-phenoxybenzoic acid	FPBA	VLXNXMTVRWIUJZ-UHFFFAOYSA-N	$C_{13}H_9FO_3$		S
Cyfluthrin[b] (not assessed), cypermethrin[c] (not assessed), permethrin[b] (Group 3)	2,2-Dichlorovinyl-2,2-dimethylcyclopropane-1-carboxylic acid	DCCA	LLMLSUSAKZVFOA-UHFFFAOYSA-N	$C_8H_{10}Cl_2O_2$		S

Parent pesticide (IARC classification*)	Biomarker of exposure	Abbreviation	InChIKey	Molecular formula	Chemical structure	Type of biomarker
Deltamethrin[a] (Group 3)	2,2-Dibromovinyl-2,2-dimethylcyclopropane-1-carboxylic acid	DBCA	MDIQXIJPQWLFSD-UHFFFAOYSA-N	$C_8H_{10}Br_2O_2$		S
Chlorophenoxy herbicides (Group 2B): used in weed control in monocotyledonous crops						
2,4-Dichlorophenoxyacetic acid[a]	2,4-Dichlorophenoxyacetic acid	2,4-D	OVSKIKFHRZPJSS-UHFFFAOYSA-N	$C_8H_6Cl_2O_3$		S
2,4,5-Trichlorophenoxyacetic acid[b] (not assessed)	2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	SMYMJHWAQXWPDB-UHFFFAOYSA-N	$C_8H_5Cl_3O_3$		S
Chloroacetanilide herbicides: used in grasses and broadleaved weed control						
Atrazine[b] (not assessed)	Atrazine mercapturate	ATZM	JYBXBVCDDNBLJW-VIFPVBQESA-N	$C_{13}H_{22}N_6O_3S$		S
Alachlor[b] (not assessed)	Alachlor mercapturate	ALAM	RFZSQTJRFQHGIJ-INIZCTEOSA-N	$C_{19}H_{28}N_2O_5S$		S
Metolachlor[c] (not assessed)	Metolachlor mercapturate	METM	HEFXMEPCHCUHDE-JRZJBTRGSA-N	$C_{20}H_{30}N_2O_5S$		S

IARC: International Agency for Research on Cancer; S: specific metabolite; NS: non-specific metabolite

[a]Approved in the EU (year 2022); [b]Not approved in the EU (year 2022); [c]Only some isomers are approved in the EU (year 2022)[144]

*Group 2A: Probably carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans[122]

1.4.2 POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

1.4.2.1 APPLICATIONS AND HEALTH EFFECTS RELATED TO PAHs

PAHs are a complex mixture of over 100 highly lipophilic organic compounds composed of two or more aromatic rings[145]. These substances are released into the environment during incomplete combustion of organic matter originating from natural sources (e.g., volcanic eruptions, forest fires), and anthropogenic and industrial activities, such as tobacco smoke, vehicle exhausts, processing of fuels, as well as from some culinary practices (drying, grilling, roasting, frying, and smoking food)[145–147]. Additionally, they are used in the manufacture of medicines, plasticizers, pigments, drying agents, and pesticides[146].

Numerous epidemiological studies have identified some PAHs as genotoxic, carcinogenic, mutagenic, and teratogenic substances[148–150] since they can cause damage to DNA, proteins, and lipids via oxidative stress[151]. Consequently, they may have adverse immunologic, developmental, and reproductive effects on humans[152–154]. More specifically, human exposure to PAHs has been linked to many chronic diseases including cancer, diabetes, cardiovascular diseases, and hypertension[114,155].

In recent years, PAHs have become a public health concern, since the IARC classified benzo(a)pyrene as Group 1 (carcinogenic to humans) in 2012[122]. Other PAHs have been identified as Group 2B, such as chrysene and naphthalene, while other PAH compounds (e.g., fluorene, phenanthrene, pyrene) have been considered as Group 3, because of limited or inadequate experimental evidence[122]. In addition, the US EPA has developed a list of 16 PAHs, including benzo(a)pyrene, naphthalene, fluorene, pyrene and phenanthrene, which should be prioritized for monitoring because of their potential human health and/or ecological toxicity[156]. Similarly, the Agency for Toxic Substances and Disease Registry (ATSDR) has also included PAHs in its list of priority substances, as they are potentially considered to be among the most health-threatening substances due to their potential toxicity and the high frequency of human exposure to them, being priority candidates for further research[157].

In this context, children are considered to be among the most sensitive subpopulation groups, since they have a less efficient detoxification system, lower body weight and higher inhalation and intake rates compared to adults, resulting in higher exposure to PAHs[155], which may disrupt their growth and development process[158]. Another group of special concern is lactating mothers, not only because exposure to PAHs can influence their health but also the health of their children through breastfeeding[159].

1.4.2.2 PAH POLICIES IN THE EUROPEAN FRAMEWORK

To control and mitigate PAH exposure in the general population, the EU has implemented several regulatory measures for PAHs in food and the environment. In 2006, MLs for certain PAHs in foods, such as oils, fats, smoked products, baby foods, among others, were established by the Regulation No 1881/2006. This regulatory measure emphasizes the obligation of EU national authorities to withdraw from the market those foods that do not comply with the defined MLs[16]. In addition to monitoring compliance with the MLs, the *Code of Practice for the Reduction of Contamination of Food with PAHs from Smoking and Direct Drying Processes* was adopted at international level in 2009. This Code of Practice of the *Codex Alimentarius* is a guidance document that provides information and recommendations to national authorities, food industries and consumers to prevent and reduce PAH contamination in foodstuffs[160]. Likewise, several PAHs are deemed as priority hazardous substances under the Directives 2000/60 and 2016/2284/EC, which aim to improve the quality of water and ambient air, respectively [161,162].

Moreover, Entry 50 of Annex XVII of the REACH regulation together with Regulation No 1272/2013, restrict PAH content in rubbers and plastic components of consumer articles, defined as those products that "may come into direct and prolonged contact with the human skin or oral cavity under normal conditions of use", such as sport equipment, household tools or clothing, by a limit of 1 $mg \cdot kg^{-1}$[1163,164]. This restriction also includes toys and childcare articles, to which this limit has been lowered to 0.5 $mg \cdot kg^{-1}$, due to the high vulnerability of children to PAH exposure[163,164].

1.4.2.3 EXPOSURE, METABOLISM, AND HUMAN BIOMONITORING OF PAHs

In general, the main pathway of human exposure to PAHs is the ingestion of contaminated foodstuffs. According to EFSA, the two highest contributors to the PAH dietary exposure are cereals and seafood[145], although they have been also detected in meat, eggs and dairy products, among others[165,166]. However, inhalation of tobacco smoke, including passive smoking, and dermal contact are also significant contributors to PAH exposure in (passive) smokers and occupationally exposed individuals (e.g., firefighters, asphalt workers, etc.), respectively[145,167]. The inhalation of polluted air is a marginal contributor to total PAH exposure (less than 10 %), except for people using indoor biomass or living in areas close to intense combustion sources, such as high-density traffic roads, refineries, certain industries or waste incinerators[114].

Once exposure to PAHs occurs, they are rapidly absorbed from the gastrointestinal tract, distributed in many tissues and metabolized in the human body[168] (Figure 9). Metabolism of PAHs predominantly occurs in the liver, where they are transformed into reactive epoxides by the cytochrome P450 and then reduced or hydrolyzed to di- and hydroxylated (OHPAHs)[114,169].

OHPAHs are then conjugated to glucuronic acid or sulphate compounds to increase their polarity and, finally, excreted in urine (low molecular weight PAHs) or feces (high molecular weight PAHs) [41]. In the kidney, the conjugation of PAHs with glutathione generates mercapturic acids derivatives, that can also be found in urine[114]. In general, urine is the most analyzed matrix to evaluate recent exposure to low molecular weight PAHs, as free or conjugated hydroxylated metabolites, due to their relative short elimination half-lives[114,168]. However, due to their lipophilic characteristics, many PAHs can also be found in blood and, therefore, bioaccumulate in body fat, such as breast milk or adipose tissue[114,170] (Figure 9).

The wide exposure of the population to PAHs and their toxicity has led to a growing interest in evaluating the body burden of PAHs in the general population. Due to the carcinogenic activity of some high molecular weight PAHs, there is no 'safe' threshold, such as TDI, for PAH exposure. In consequence, the US EPA recommends the use of TEFs considering benzo(a) pyrene as the most toxic PAH (TEF = 1)[171]. However, EFSA claimed that the MOE concept, using the $BMDL_{10}$ as a HBGV, is a more reliable approach to evaluate the risk associated to dietary PAH exposure[145]. In Europe, the HBM4EU initiative aimed to generate more knowledge on exposure trends of PAHs and associated risks by using OHPAHs as internal indicators of exposure in urine samples[41]. However, BE values for PAHs have not been derived, so reverse dosimetry is the most common method to evaluate the risk associated to urinary levels of PAHs[147,172].

Among all OHPAHs, the urinary metabolite of pyrene, 1-hydroxypyrene (1OHPYR), is the most common biomarker used as indicator of exposure to the PAH family[155,173]. Nevertheless, given that humans are exposed to a mixture of PAHs and not only to a single compound, the HBM of other OHPAHs along with 1OHPYR would provide an efficient overall assessment of PAH exposure[167,174]. In fact, urinary metabolites of naphthalene, fluorene, phenanthrene, chrysene, and benzo(a)pyrene are also commonly used as biomarkers of exposure to PAHs in many national HBM programs and research studies[69,169,175–177]. A summary of the urinary OHPAHs analyzed in the present thesis is presented in Table 3.

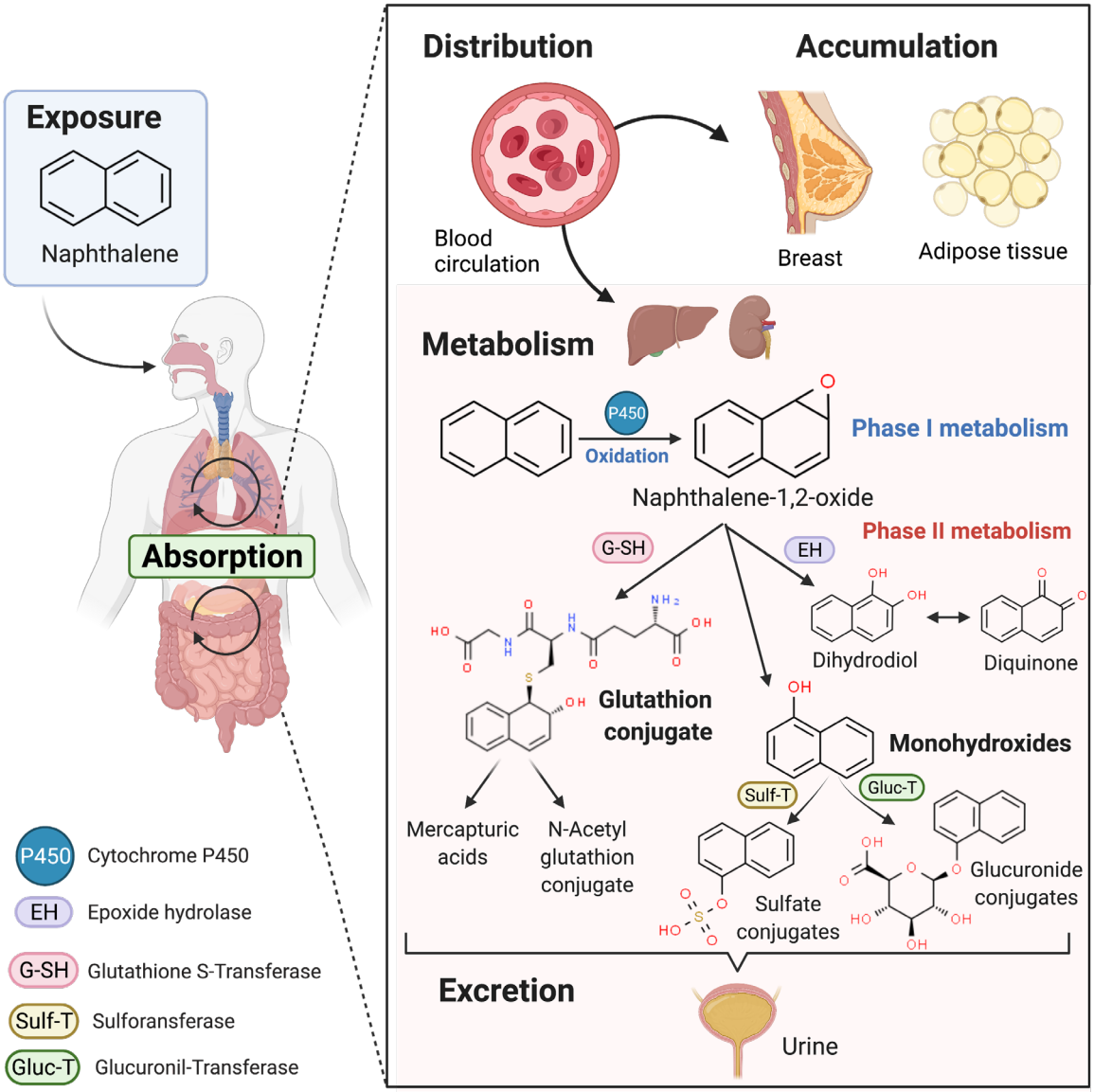
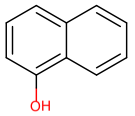
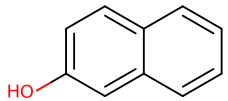
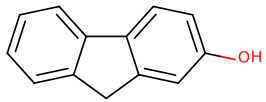
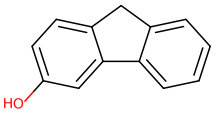
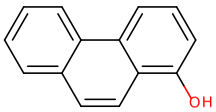
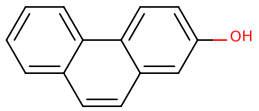
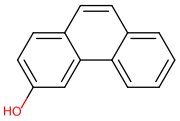
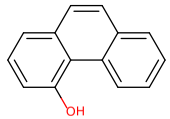
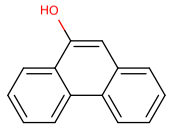
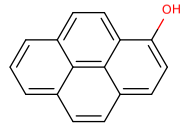
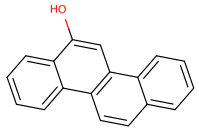


Figure 9. Metabolism of PAHs in the human body and resulting urinary metabolites (naphthalene as an example).

In Spain, the BIOAMBIENT.ES project was the first national-level HBM survey to estimate urinary levels of 1OHPYR and several hydroxy phenanthrenes in a representative sample of adults in 2010[178]. Similarly, the researchers of the INMA project analyzed 1OHPYR in urine of pregnant women living in different Spanish locations[179]. In the case of children, only one HBM study on PAHs has been conducted so far in Granada (Spain)[180]. In conclusion, there are limited HBM data on PAH exposure in the Spanish population.

Table 3. Summary of the urinary metabolites of PAHs analyzed in the present thesis: parent compounds, IARC classification, abbreviation, InChIKey, molecular formula and chemical structure.

Parent compound	IARC classification*	Biomarker of exposure	Abbreviation	InChIKey	Molecular formula	Chemical structure
Naphthalene	Group 2B	1-Hydroxynaphthalene	1OHNAP	KJCVRFUGPWSIIH-UHFFFAOYSA-N	$C_{10}H_8O$	
		2-Hydroxynaphthalene	2OHNAP	JWAZRIHNYRIHIV-UHFFFAOYSA-N	$C_{10}H_8O$	
Fluorene	Group 3	2-Hydroxyfluorene	2OHFLU	ZDOIAPGLORMKTR-UHFFFAOYSA-N	$C_{13}H_{10}O$	
		3-Hydroxyfluorene	3OHFLU	PVUBSZGNXLNTLX-UHFFFAOYSA-N	$C_{13}H_{10}O$	
Phenanthrene	Group 3	1-Hydroxyphenanthrene	1OHPHEN	GTBXZWADMKOZQJ-UHFFFAOYSA-N	$C_{14}H_{10}O$	
		2-Hydroxyphenanthrene	2OHPHEN	YPWLZGITFNGGKW-UHFFFAOYSA-N	$C_{14}H_{10}O$	

Parent compound	IARC classification*	Biomarker of exposure	Abbreviation	InChIKey	Molecular formula	Chemical structure
Phenanthrene	Group 3	3-Hydroxyphenanthrene	3OHPHEN	NGPOABOEXMDQBT-UHFFFAOYSA-N	$C_{14}H_{10}O$	
		4-Hydroxyphenanthrene	4OHPHEN	SIMYIUXARJLHEA-UHFFFAOYSA-N	$C_{14}H_{10}O$	
		9-Hydroxyphenanthrene	9OHPHEN	DZKIUEHLEXLYKM-UHFFFAOYSA-N	$C_{14}H_{10}O$	
Pyrene	Group 3	1-Hydroxypyrene	1OHPYR	BIJNHUAPTJVVNQ-UHFFFAOYSA-N	$C_{16}H_{10}O$	
Benzo(a)pyrene	Group 1	3-Hydroxybenzo(a) pyrene	3OHBAP	SPUUWWRWIAEPDB-UHFFFAOYSA-N	$C_{20}H_{12}O$	

IARC: International Agency for Research on Cancer

*Group 1: Carcinogenic to humans; Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans[122]

1.4.3 ACRYLAMIDE

1.4.3.1 APPLICATIONS AND HEALTH EFFECTS RELATED TO ACRYLAMIDE

Acrylamide (AA) or 2-propenamide is a low molecular weight, highly water-soluble and volatile organic compound, classified in group 2A by the IARC[122]. It is used worldwide in the chemical industry as a monomer in the synthesis of polyacrylamide, which is employed as thickener and flocculating agent in the paper and textile industries, in water treatment, for building and construction purposes, in cosmetics, and in food packaging[181,182]. Moreover, AA is also present in tobacco smoke[183].

Several *in vivo* and *in vitro* studies have shown that AA produces neurotoxic, carcinogenic, mutagenic, genotoxic, and immunotoxic effects[184]. In humans, it has been found to have negative effects on children's development[185,186], since AA is able to be transferred to the fetus through the placenta and blood-brain barriers, and through breast milk consumption in breastfed infants[187,188], concluding that pregnant and nursing women should avoid foods containing this substance. Other human health conditions, such as cardiovascular diseases[189] and oxidative stress damage[190,191], have also been associated with exposure to AA. By contrast, although the last EFSA's scientific report *Assessment of the genotoxicity of acrylamide* shows additional evidence of its genotoxic and non-genotoxic effects which may contribute to the development of human cancers[192], several epidemiological studies claim that there are no consistent evidence about the association of AA exposure and cancer risk[193].

1.4.3.2 ACRYLAMIDE POLICIES IN THE EUROPEAN FRAMEWORK

In 2002, the discovery of the formation of AA in carbohydrate-rich foods at temperatures above 120 ºC and under low-moisture conditions (for example, during frying, roasting, and baking) via the Maillard reaction attracted significant attention of the scientific society and public health authorities[194]. This chemical reaction takes place between the amino group of certain amino acids (e.g. asparagine) and the carbonyl group of reducing sugars (e.g. glucose), naturally present in these foods[195,196]. Consequently, different regulatory measures have been taken in Europe to mitigate the human exposure to AA by food intake. Since 2007, national food authorities in the EU countries have been continuously monitoring the presence of AA in certain food commodities[197]. In 2015, the EFSA's CONTAM panel published a scientific opinion, concluding that AA levels in food products were not consistently decreased in previous years, and dietary exposure to AA in the European citizens indicated a concern with respect to its carcinogenicity[181]. As a result, the EC established the Regulation (EU) 2017/2158 containing "mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food"[198]. In addition to this, some uses of AA are restricted under Annex XVII of REACH and it is included in the "candidate list of Substances of Very High Concern for Authorization"[199]. Moreover, the use of AA is banned in cosmetic products (Regulation (EC) 1223/2009)[23] and in food-contact materials (Regulation (EU) 10/2011)[200], and a maximum level of 0.10 $\mu g \cdot l^{-1}$ is established for AA residues in drinking water by the Council Directive 98/83/EC[201].

1.4.3.3 EXPOSURE, METABOLISM AND HUMAN BIOMONITORING OF ACRYLAMIDE

Diet is the main contributor to AA exposure in the general (non-smoking) population[181,202]. In fact, the general population is exposed to AA mainly through the consumption of potato crisps, French fries, bakery products (bread and biscuits), and coffee because of their composition, processing conditions, or high consumption frequency[181,202]. However, non-dietary exposure routes have been also identified, such as inhalation in (passive) smokers or dermal uptake in exposed workers.

After exposure, AA is rapidly absorbed and distributed to all body tissues. Figure 10 shows an overview of AA metabolism in humans. In phase I of its metabolism, AA is partly metabolized by cytochrome P450 2E1 to glycidamide (GA)[203]. In addition, AA and GA can form hemoglobin adducts (AAVal and GAVal, respectively), normally used as biomarkers of long-term exposure to AA by blood analysis[204,205]. In phase II, AA and GA are conjugated with glutathione and converted to their corresponding mercapturic acids, N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA) and N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA3), respectively, which are excreted in urine[203]. About 50 % of the AA is eliminated in the urine during the first 24 hours after the ingestion. In humans, AAMA can be also oxidized to its corresponding sulfoxide, acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine (AAMA-Sul)[206], and GA can be further hydrolyzed to glyceramide (2,3-dihydroxypropionamide) by an epoxide hydrolase[207]. In general, urine is the preferred specimen for HBM of AA not only because it is a non-invasive matrix which is easy to obtain in large volumes, but also due to the fact that urinary AA metabolites, i.e., AAMA, GAMA3 and AAMA-Sul, are stable biomarkers of recent exposure that can be quantified with high specificity and sensitivity[114]. The three urinary mercapturic acid metabolites of AA analyzed in the present thesis are listed in Table 4.

Risk assessment of AA exposure in humans is commonly carried out by estimating its daily intake per kg of body weight based on its occurrence in food commodities and comparing it with the $BMDL_{10}$ derived for animals[181]. However, this approach only considers the exposure derived from its dietary ingestion, which may not reflect the actual uptake of AA by the human body, since it depends not only on individual eating habits in terms of cooking methods, portion size and frequency of consumption[208], but also on the protein content of each individual diet[209]. In order to evaluate the total exposure dose to AA from all possible pathways of exposure, the analysis of its metabolites in urine or blood by HBM studies could be a reliable alternative[110]. In this sense, Hays and Aylward, (2008) derived BEs for the interpretation of the concentration of AA metabolites in urine (13 µg AAMA·l^{-1}) and blood (8 and 13 fmol·mg $globin^{-1}$ for AAVal and GAVal, respectively) in a risk assessment context[210].

Most of the recent HBM studies on AA exposure focused on smokers[211–214] or occupationally exposed population[215–219]. Generally, very few studies have been reported on internal AA exposure in the general population and especially in vulnerable groups[220–223], such as infants and young children[224,225]. To date, among the European countries, only Germany has reported quantifiable data on the urinary levels of AAMA and GAMA3 in children and adolescents[223,224,226].

Therefore, considering the potential carcinogenic activity of AA and the scarcity of studies focused on studying exposure across general and vulnerable population groups, obtaining HBM data on the internal AA exposure is of substantial interest.

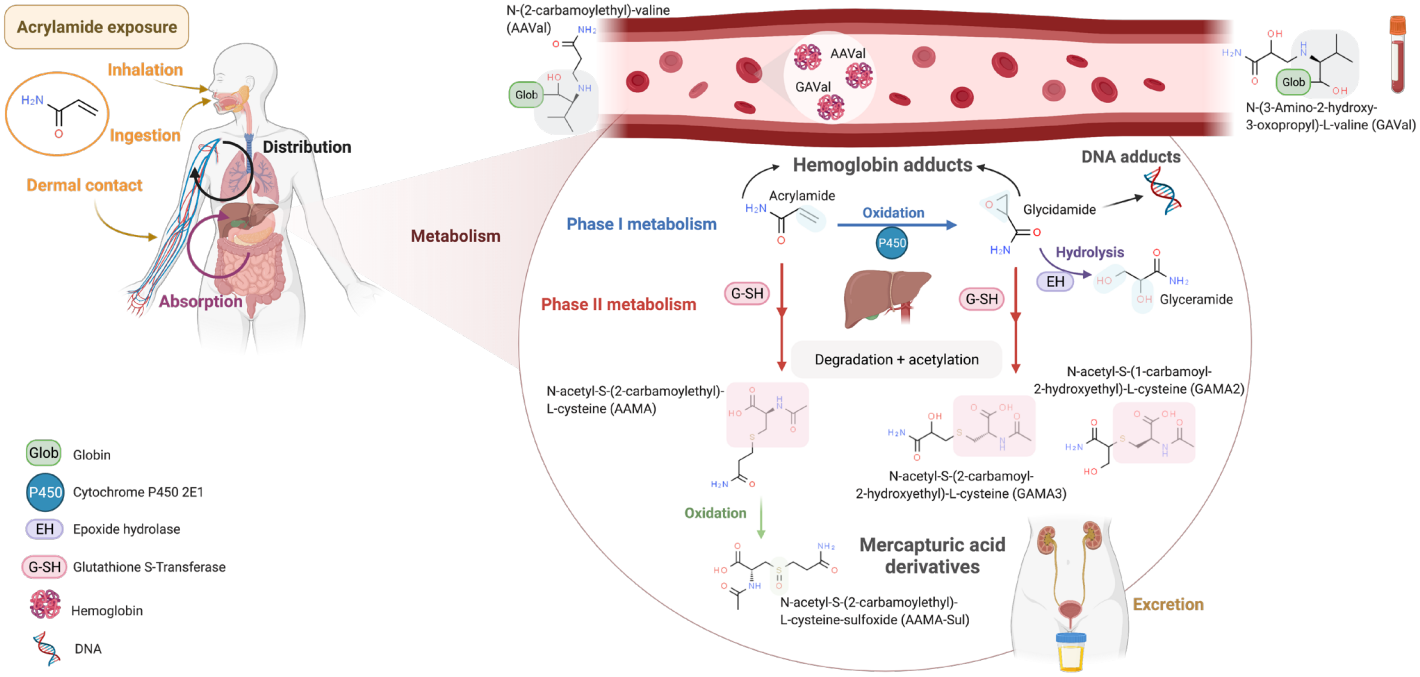
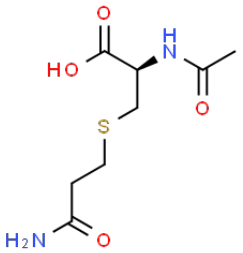
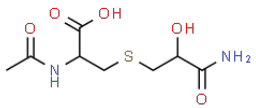
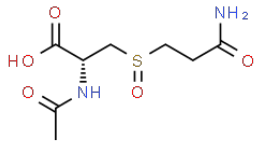
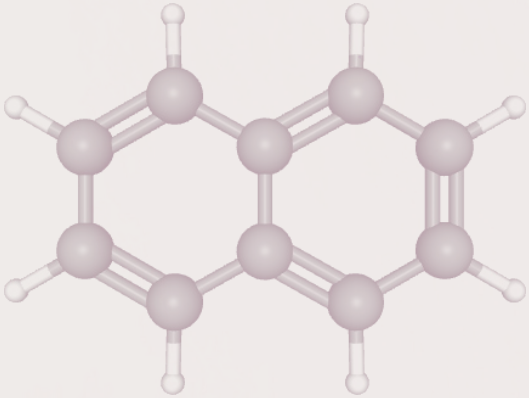
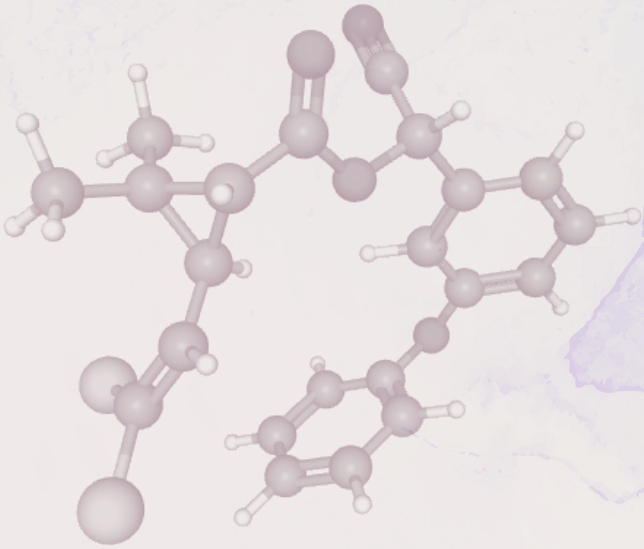
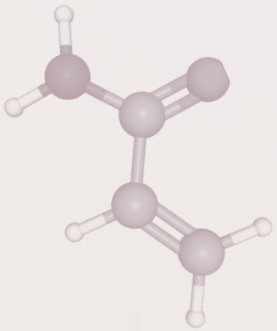
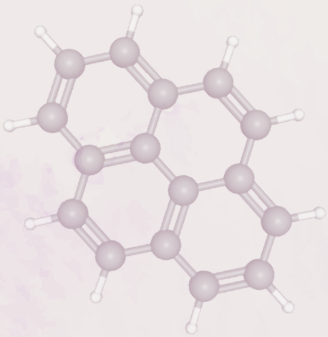
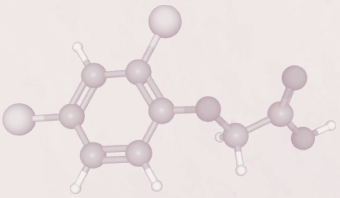
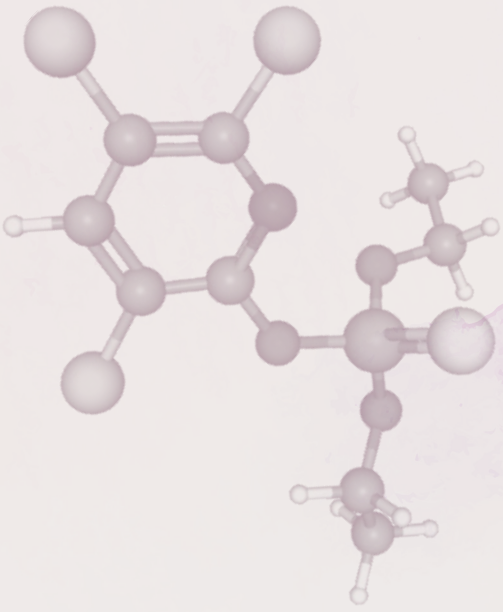
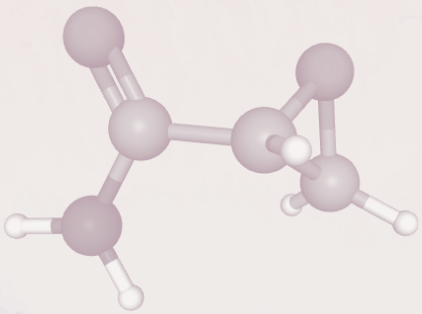


Figure 10. Metabolism of acrylamide in the human body and resulting urinary and blood metabolites.

Table 4. Summary of the urinary metabolites of acrylamide analyzed in the present thesis: parent compound, IARC classification, abbreviation, InChIKey, molecular formula and chemical structure.

Parent compound	IARC classification	Biomarker of exposure	Abbreviation	InChIKey	Molecular formula	Chemical structure
Acrylamide	Group 2A: Probably carcinogenic to humans[122]	N-acetyl-S-(2-carbamoylethyl)-L-cysteine	AAMA	GGBCHNJZQQEQRX-LURJT-MIESA-N	$C_8H_{14}N_2O_4S$	
		N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine	GAMA3	GFVUOIIZUCFXSF-LWOQYN-TDSA-M	$C_8H_{14}N_2O_5S$	
		Acetyl-3-[(3-amino-3-oxopropyl)-sulfinyl]-L-alanine	AAMA-Sul	MIZMPWFZMWDOPJ-AIUX-ZDRISA-N	$C_8H_{14}N_2O_5S$	

IARC: International Agency for Research on Cancer

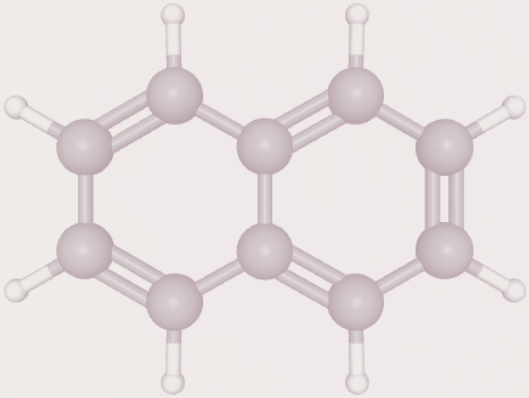
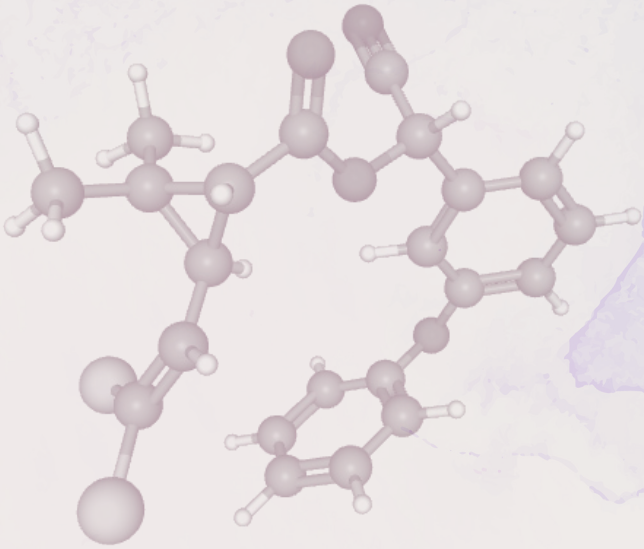
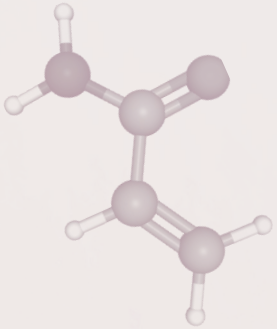
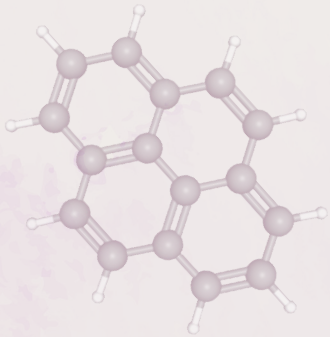
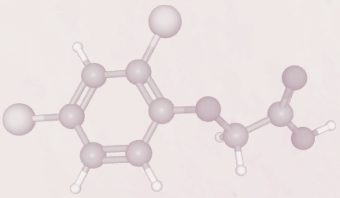
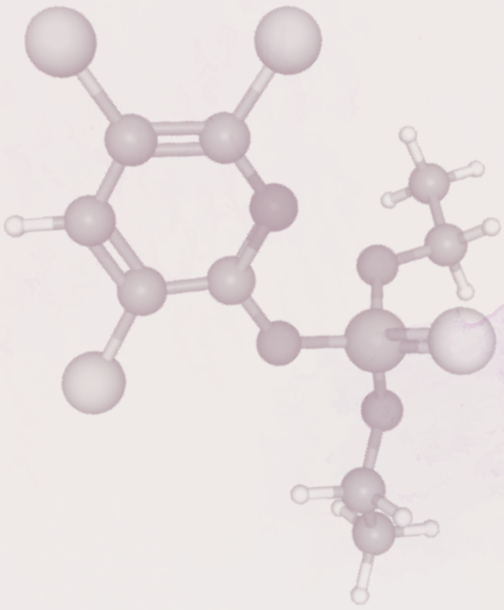
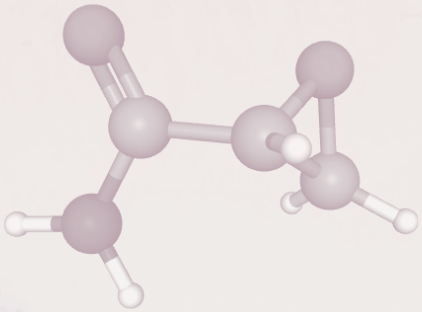
2

RESEARCH AIMS

2 RESEARCH AIMS

The aim of this doctoral thesis was to develop different analytical tools for their application to HBM projects, and to design and carry out some specific HBM studies on children and lactating mothers focusing on non-persistent pesticides, low molecular weight PAHs and acrylamide. Specifically, the objectives were:

1. To develop, optimize and validate a new methodology to analyze six non-specific metabolites of organophosphate pesticides in urine using LC-MS/MS (Article I.1).
2. Development of a HBM study on exposure to non-persistent pesticides in lactating mothers (Article I.2) and children (Article I.3): reporting of urinary levels, risk assessment estimations and statistical analysis.
3. Execution of a HBM study on exposure to low molecular weight PAHs in lactating mothers (Article II.1) and children (Article II.2): urinary levels descriptors, evaluation of the associated risks, and identification of potential determinants of exposure.
4. Conducting a HBM study on exposure to acrylamide in lactating mothers (Article III.1) and children (Article III.2): description of urinary levels, assessment of exposure in a risk context, and associations with exposure media.

3

MATERIALS AND METHODS

3 MATERIALS AND METHODS

This section summarizes and contextualizes the design of the two HBM projects that are the basis of this thesis, and includes a detailed description of the analytical methods developed and validated to analyze metabolites of pesticides, PAHs and acrylamide in urine, as well as the statistical and risk evaluation procedures used in each case.

3.1 STUDY DESIGN: TARGET POPULATION, SAMPLE AND DATA COLLECTION

The two population groups studied in the present thesis were lactating mothers and children, which were recruited within the BETTERMILK project and the BIOVAL program of the Valencian Region, respectively. Both studies aimed to generate knowledge on the exposure of vulnerable groups living in this region to chemicals and environmental contaminants by HBM.

An overview of the design of both HBM studies is illustrated in Figure 11. In the BETTERMILK project, the recruitment of lactating women took place between June and November 2015 at the University and Polytechnic Hospital *La Fe* (Valencia, Spain), period in which these women came to the hospital for giving birth. A total of 120 women aged 20 – 45 years matched the selection criteria and volunteered to participate in the study. The BIOVAL program was a cross-sectional HBM program carried out by the Public Health Directorate of the Regional Government of Valencia (Spain) in 2016. In total, a representative sample of 666 children (5 – 12 years old) were randomly recruited from 25 primary schools located in 16 municipalities of the entire Valencian territory (see geographical distribution in Figure 11).

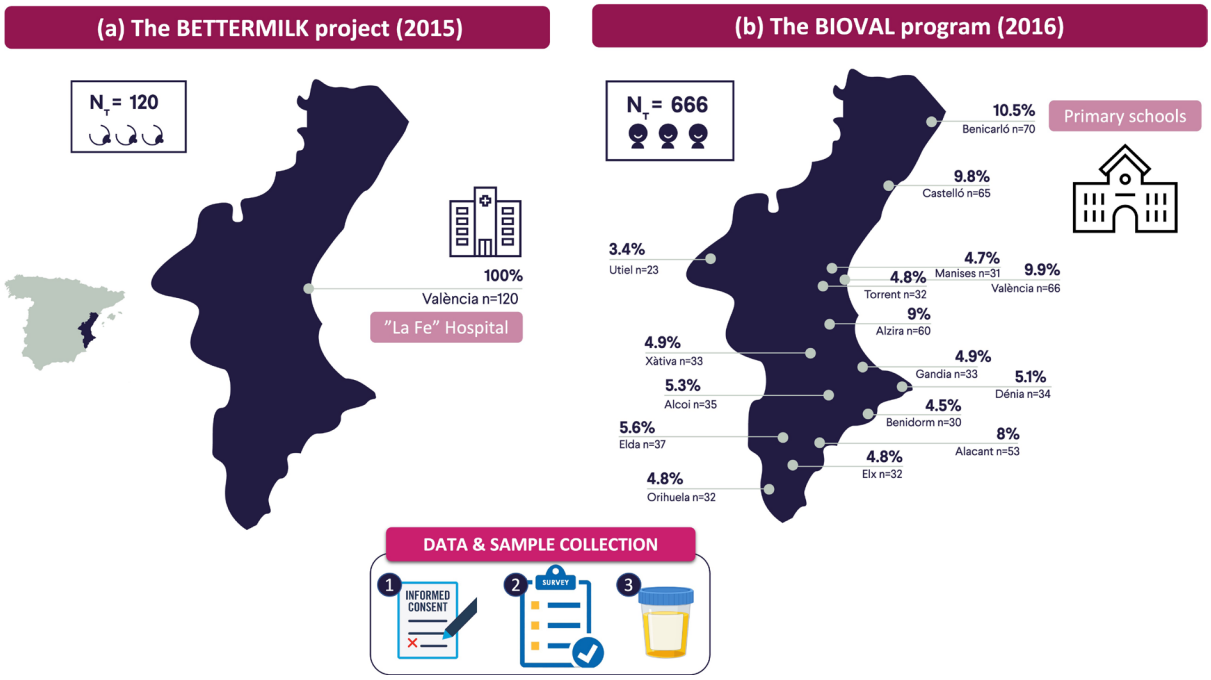


Figure 11. General overview of each human biomonitoring study design included in this thesis: (a) the BETTERMILK project, in which 120 lactating mothers living in Valencia were recruited in *La Fe* Hospital (2015), and (b) the BIOVAL program (2016), showing the geographical distribution of children recruited in primary schools along the three provinces of the Valencian Region. Adapted from Yusà et al. (2021)[227].

All participants provided a written informed consent prior to the data and sample collection. Each donor was provided with a kit containing instructions, disposable gloves and a sterile polypropylene bottle for the urine collection. First-morning urine samples were collected by themselves at home, which were kept at 4 ºC, and then transferred to the Biobank for Biomedical and Public Health Research of the Valencian region (IBSP-CV Biobank, Spain, PT13/0010/0064) where they were aliquoted in 10-ml tubes and stored at -80 ºC until analysis. Other biological samples (breast milk and hair) were collected but not analyzed in the present thesis. Additionally, detailed information on dietary data (food consumption frequency and a 72-h dietary recall), lifestyle, and general socio-demographic characteristics of the volunteers was obtained by means of a self-administered questionnaire.

The BETTERMILK project and the BIOVAL program, including their study design and sampling protocols, were approved by the Ethics Committee for Clinical Research of the Directorate General of Public Health and the Centre for Advanced Research in Public Health of the Valencian Government. The Biomedical Scientific Ethics Committee of the University and Polytechnic Hospital *La Fe* also gave its approval for conducting the BETTERMILK study. Data processing was carried out in compliance with ethics and legal aspects at the national and European level, particularly the General Data Protection Regulation.

3.2 CHEMICAL ANALYSIS

3.2.1 CHEMICALS, REAGENTS, STANDARDS AND SOLUTIONS

All solvents used here were specific for pesticide residue analysis (PAR) or of analytical grade (HPLC or LC coupled to MS, LC-MS, grade). Acetonitrile (PAR and LC-MS), water (HPLC and LC-MS grade), hexane (PAR), ethyl acetate (PAR), methanol (LC-MS grade), hydrochloric acid (HCl) 37 % (v/v), and ethyl acetate (PAR) were supplied by VWR Prolabo (Barcelona, Spain). Diethyl ether (reagent grade, stabilized with 7 ppm of butylated hydroxytoluene) and ammonium acetate (HPLC grade) were purchased from Scharlau (Barcelona, Spain). Acetic acid (purity 98 – 100 %), β-glucuronidase/arylsulfatase (from *Helix pomatia*; β-glucuronidase activity 30 $U \cdot ml^{-1}$, and sulphatase activity 60 $U \cdot ml^{-1}$), silanization solution I (~5 % dimethyldichlorosilane in heptane), Supel™ QuE Z-Sep (300 mg Z-Sep, 900 mg $MgSO_4$, 12 ml), and sodium acetate (> 99 %) were supplied by Merck/Sigma-Aldrich (Madrid, Spain). QuEChERS Original extraction packets (4 g $MgSO_4$ and 1 g NaCl) and ceramic homogenizers for QuEChERS extraction were obtained from Agilent Technologies (Madrid, Spain). Certified reference materials (CRM) 3672 and 3673 were purchased from the NIST (USA).

All certified standards and their IS used here were at least 97 % of analytical purity. Specifically, DMP, DMDTP and DEP were purchased from Chromlab (Barcelona, Spain); DMTP was from Chiron AS (Trondheim, Norway); DETP was supplied by Cambridge Isotope Laboratories (Massachusetts, USA); and DEDTP was purchased from Merck/Sigma-Aldrich. Dibutyl phosphate (DBP) was supplied by Dr. Ehrenstorfer (Augsburg, Germany) and was used as IS for DAP analysis, since it has similar properties as the six selected DAPs. Although standard addition has been described as the best method to correct the potential matrix effects[57], it was not chosen because the amount of urine sample was limited and it requires a longer analytical procedure. For OHPAH analysis, certified standards of 1OHNAP (100 $mg \cdot ml^{-1}$ in acetonitrile) and 2OHNAP were obtained from Neochema (Bodenheim, Germany) and Dr. Ehrenstorfer, respectively. 2OHFLU, 1OHPHEN, 2OHPHEN, 3OHPHEN, 4OHPHEN, and 3OHBAP (50 $mg \cdot ml^{-1}$ in toluene) were supplied by Chiron SA. 3OHFLU, 9OHPHEN, and 1OHPYR were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada), Merck/Sigma-Aldrich, and AccuStandard (Connecticut, USA), respectively. Isotopically labeled analogues used as IS for OHPAH determination, specifically $^{13}C_6$-1OHNAP (50 $mg \cdot ml^{-1}$ in toluene), $^{13}C_6$-2OHFLU (50 $mg \cdot ml^{-1}$ in toluene), $^{13}C_6$-2OHPHEN (50 $mg \cdot ml^{-1}$ in toluene), and $^{13}C_6$-1OHPYR (50 $mg \cdot ml^{-1}$ in toluene) were supplied by Cambridge Isotope Laboratories, Inc., $^{2}H_7$-2OHNAP by Merck/Sigma-Aldrich, and $^{2}H_8$-9OHPHEN and $^{2}H_{11}$-3OHBAP by Toronto Research Chemicals, Inc. Finally, for analysis of AA metabolites, certified standards of AAMA, GAMA3 dicyclohexylammonium salt, AAMA-Sul sodium salt, and their isotopically labelled analogues, specifically AAMA-d_3, GAMA3-d_3 dicyclohexylammonium salt, and AAMA-Sul-d_3 sodium salt, were purchased from Toronto Research Chemicals, Inc.

Individual commercial standards delivered as solids were weighted and dissolved in acetonitrile (DAPs and OHPAHs) or water (AA metabolites) to obtain concentrations between 200 and 500 $\mu g \cdot ml^{-1}$, approximately. These individual stock solutions were stored in the dark at -20 ºC for at least 6 months. Multi-analyte standard solutions (working solutions) for DAPs, OHPAHs and AA metabolites, containing concentrations for each compound between 100 and 1000 $ng \cdot ml^{-1}$, 1 and 1000 $ng \cdot ml^{-1}$, and 0.25 and 2000 $ng \cdot ml^{-1}$, respectively, were prepared by diluting the individual stock solutions with the corresponding solvent. IS working solutions were also prepared containing all isotopic labelled standards in the appropriate solvent at concentrations between 150 and 500 $ng \cdot ml^{-1}$. Working solutions were stored in the dark at -20 ºC for at least 3 months.

3.2.2 ANALYTICAL METHODS

3.2.2.1 DETERMINATION OF PESTICIDE METABOLITES IN URINE

The analysis of specific urinary metabolites of pesticides was carried out by the staff of the Public Health Laboratory of Valencia. Therefore, although the urinary concentrations of these metabolites were used for further evaluation in Chapter I, the development, optimization and validation of this methodology, which consisted of a QuEChERS extraction and LC-MS/MS determination, is not part of this thesis and is not included here. Only the determination of DAPs in urine is described in this section.

A novel method for the determination of six DAPs in urine was developed and fully optimized in the present thesis. To this end, three sample preparation methods, 'dilute-and-shoot', QuEChERS based on LLE with different salts and clean-up sorbents, and SPE with STRATA X-AW (weak anion exchange) cartridges at different conditions were tested. The final method is shortly explained hereunder (see Figure 12) and is described in a SOP under the quality system of the Public Health Laboratory of Valencia. Briefly, an amount of 4 ml of human urine sample is spiked with 5 $\mu g \cdot ml^{-1}$ of the IS working solution and HCl 6M. Then, the mixture is centrifuged and transferred into a polypropylene tube, where ethyl acetate and diethyl ether are used for extraction. After cooling down in an ice bath, a QuEChERS Original extraction packet and one ceramic homogenizer are added and shaken vigorously. The total organic layer is separated by centrifugation and collected in a clean tube, where then is evaporated under a N_2 stream. The dry residue is dissolved in mobile phase, centrifuged, transferred into an injection vial and analyzed on the corresponding LC-MS/MS system.

For DAP chromatographic separation, a KINETEX HILIC and a Hypersil GOLD HILIC columns of the same dimensions (2.1 × 100 mm, 3 µm), were evaluated in a Finnigan Surveyor HPLC system from Thermo Fisher Scientific (Bremen, Germany). Various pHs and modifiers were also tested in water (mobile phase A) and in acetonitrile (mobile phase B). The final chromatographic

conditions are presented in Figure 12. Detection of DAPs was performed on a Finnigan TSQ Quantum Ultra detector (San José, CA, USA) equipped with an ESI source in a negative mode. SRM mode was used and CEs for the most abundant transitions were optimized for each compound separately. Tube lens (TLs) were set at values which were automatically optimized by the mass spectrometer. Quantification (Q) and confirmation (q) ions observed for each DAP correspond to the products resulted from the loss of one or both alkyl chains (methyl or ethyl groups) bonded to the phosphate group. Moreover, the ion source parameters, named spray voltage (SV), capillary temperature (CT), sheath gas (SG), and auxiliary gas (AG), were optimized by design of experiments (DoE) consisting of a two-level Plackett-Burman design (P-B) followed by a central composite design (CCD), using the statistical package MINITAB for Windows, Release 14.1 (Minitab Inc., Birmingham, UK). Data acquisition was performed using the Thermo Scientific Trace Finder™ 3.2 software. The procedural LoQ was 0.5 $ng \cdot ml^{-1}$ for all DAPs.

The whole analytical procedure and experimental conditions for sample preparation and DAP analysis is fully described in Figure 12.

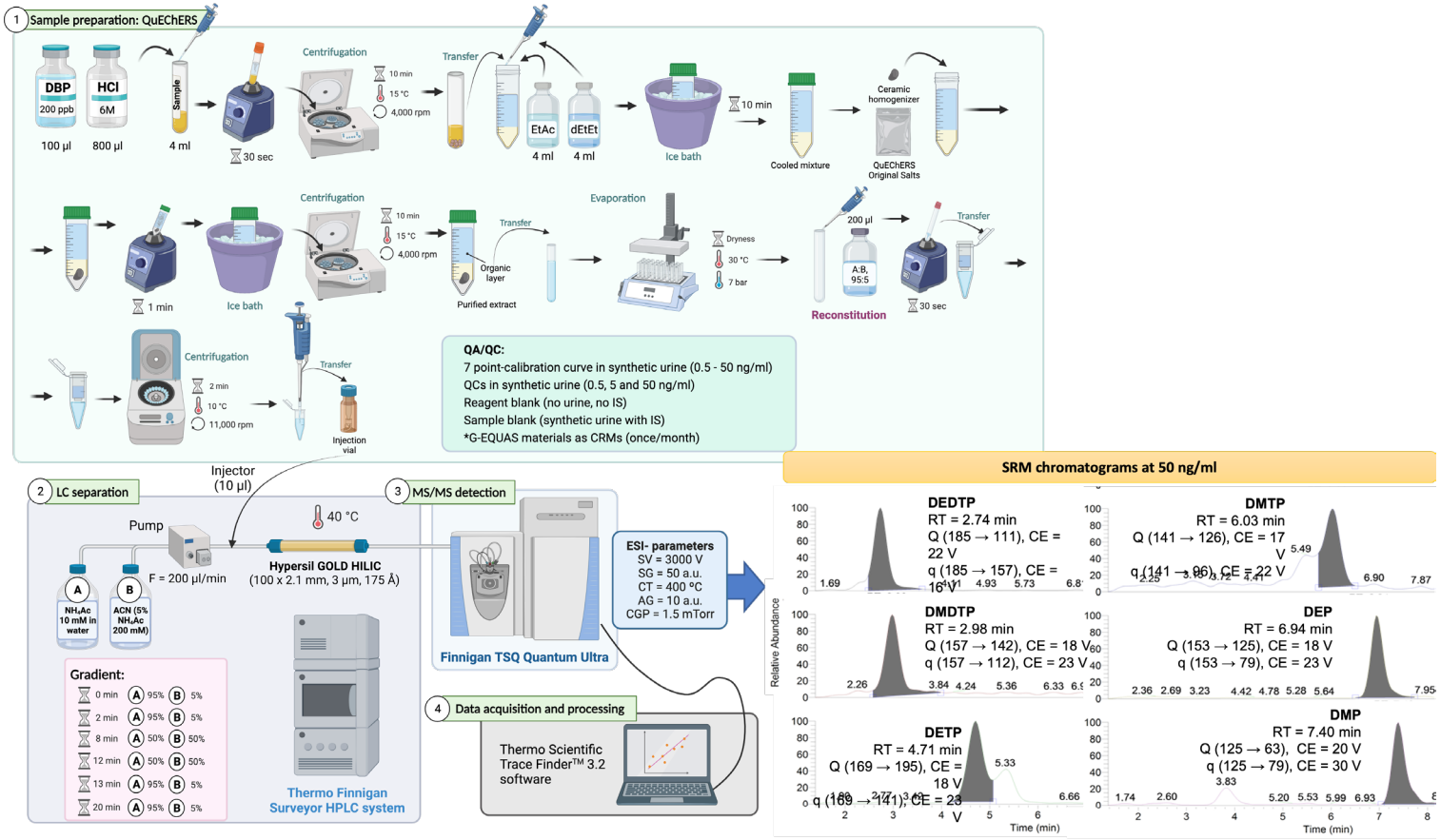


Figure 12. Diagram of the steps involved in the analysis of six DAPs in urine. 1) Sample preparation by QuEChERS using DBP as IS, and ethyl acetate and diethyl ether as extraction solvents, together with the QA/QC samples included in each sequence; 2) LC separation: experimental conditions of the Thermo Finnigan Surveyor HPLC system equipped with a Hypersil Gold HILIC column, gradient and flow rate of mobile phases A (containing ammonium acetate in water) and B (containing ammonium acetate in acetonitrile; 3) MS/MS detection: conditions of the ESI source working in negative mode, and SRM chromatograms for each DAP at 50 ng·ml^{-1} showing their corresponding Q and q ions, together with their RT and CEs. Tube lens were set at values which were automatically optimized by the mass spectrometer; 4) Data acquisition and processing using Trace Finder™ 3.2 software. *QuEChERS: Quick, Easy, Cheap, Effective, Rugged & Safe; DBP: dibutyl phosphate; IS: internal standard; EtAc: ethyl acetate; dEtEt: diethyl ether; QA/QC: quality assurance/quality control; G-EQUAS: German External QUality Assessment Schemes; CRM: certified reference material; LC: liquid chromatography; HPLC: high-performance liquid chromatograph; HILIC: hydrophilic interaction chromatography; F: flow rate; NH_4Ac: ammonium acetate; ACN: acetonitrile; MS/MS: tandem mass spectrometry; ESI: electrospray ionization; SV: ion spray voltage; SG: sheath gas; CT: capillary temperature; AG: auxiliary gas; CGP: collision gas pressure; SRM: selected reaction monitoring; Q: quantification ion; q: confirmation ion; RT: retention time; CE: collision energy.*

3.2.2.2 DETERMINATION OF PAH METABOLITES IN URINE

The analysis of the 11 PAH urinary metabolites was performed applying the procedure developed by Lankova et al. (2016)[228] but with a few modifications. The complete analytical method is described in a SOP under the quality system of the Public Health Laboratory of Valencia (see Figure 13). The deconjugation of glucuronide and sulfate derivatives of OHPAHs by enzymatic hydrolysis was optimized applying two different enzymes, β-Glucuronidase/Arylsulfatase from *Helix pomatia* and β-Glucuronidase from *Escherichia coli*, at different incubation times (30 min, 2 h, 6 h and 17 h) to field urine samples and the CRM 3672. In addition, the use of Supel™ QuE Z-Sep and β-Gone β-Glucuronidase Removal (Phenomenex) as clean-up steps were evaluated. Those experimental conditions showing better recoveries, accuracies and higher areas for most analytes were chosen (data not shown). In brief, the final method uses an amount of 2.5 ml of the homogenized sample spiked with the IS working solution at concentrations between 1.5 and 3 ng·ml^{-1}. This mixture is subjected to an enzymatic deconjugation step using β-Glucuronidase/Arylsulfatase from *Helix pomatia* (37 ºC, 17 h), followed by LLE with ethyl acetate and a clean-up with Supel™ QuE Z-Sep. Then, the clean extract is evaporated and reconstituted in mobile phase.

OHPAHs in the extract were determined using a Vanquish HPLC system from Thermo Fisher Scientific Inc. equipped with a KINETEX Core-shell technology F5 (Phenomenex, USA) column (2.1 x 150 mm, 2.6 µm). Their detection was performed on a Finnigan TSQ Quantum Ultra detector equipped with an ESI source in the negative ion mode. The OHPAHs were determined by using SRM, and data acquisition was carried out using the Thermo Scientific Trace Finder™ 3.2 software. Only one m/z transition for each OHPAH was observed. For 2OHFLU, the ion at m/z 180 was attributable to its deprotonated molecule. Whereas fragmentation of the other OHPAHs occurred by means of the loss of the carboxyl group (M–28). In addition, the ESI parameters mentioned above were optimized by DoE considering all isomers but also the maximization of the signal of the least sensitive metabolite (3OHBAP). The procedural LoQ was 0.05 ng·ml^{-1} for 3OHFLU and 3OHBAP, and 0.01 ng·ml^{-1} for the rest of OHPAHs. Figure 13 shows a schematic diagram of the whole analytical process and experimental conditions for determination of the 11 OHPAHs in urine.

3.2.2.3 DETERMINATION OF ACRYLAMIDE METABOLITES IN URINE

A 'dilute-and-shoot' method based on Brisson et al. (2014)[229] was used to analyze AAMA, GAMA3 and AAMA-Sul in the urine samples. The whole analytical process is fully described in a SOP under the quality system of the Public Health Laboratory of Valencia (see Figure 13). Before applying the method to field samples, two different dilution factors (1:2 and 1:5 with mobile phase) and two strategies for interferences reduction (centrifugation and filtration) were tested to select the methodology giving the best sensitivity, least matrix interferences and the

lowest procedural losses for the three analytes. Briefly, the final method consists of spiking 200 µl of the homogenized urine sample with the IS working solution at 25 ng·ml^{-1}, and diluted them in 0.08 % acetic acid in water to 1 ml. Then, the mixture was shaken and injected into the HPLC-MS/MS system.

The chromatographic separation of the three AA metabolites was performed in a Vanquish HPLC system, equipped with an Atlantis dC18 column (150 x 2.1 mm, 2.6 µm, 100 Å) from Waters (Milford, MA, USA). Three different modifiers (acetic acid, formic acid and ammonium formate) of the mobile phase were evaluated. Finally, 0.08 % acetic acid was selected, since higher areas and better peak shapes were observed (data not shown). The detection was performed in a TSQ Altis™ Triple Quadrupole Mass Spectrometer from Thermo Fisher Scientific Inc. The instrument operated with a heated electrospray ionization source (HESI) in negative ion mode and SRM. The positive ionization mode was also studied but it gave lower areas for the three AA metabolites (data not shown). Data acquisition and processing was carried out using the Thermo Scientific Trace Finder™ 5.1 software. Q and q transitions for AAMA correspond to the mercapturic acid (m/z = 162) and the thiolated acrylamide (AA-SH, m/z = 104) moieties, respectively. Similarly, GAMA3 showed the thiolated glycidamide moiety (GA-SH, m/z = 120) and the remaining chemical structure at m/z = 128 (loss of GA-SH). In AAMA-Sul, the oxidated mercapturic acid moiety (m/z = 178) and a loss of a m/z = 116, which could not be assigned to only one chemical structure, were observed. As for DAPs and OHPAHs, the optimization of the HESI source parameters, including SV, SG, AG, ion transfer tube temperature (ITT), sweep gas (SwG) and vaporizer temperature (VT), as well as the radiofrequency (RF) lens, was conducted using a DoE considering the three biomarkers but maximizing the signal of AAMA-Sul, which was the least sensitive compound. The procedural LoQ was 0.25 ng·ml^{-1} for all AA metabolites. A flow diagram of the whole analytical procedure and experimental conditions for analysis of urinary AA metabolites is presented in Figure 14.

3.2.2.4 CREATININE ANALYSIS

The University and Polytechnic Hospital *La Fe* (Valencia, Spain) and the University Hospital *Doctor Peset* (Valencia, Spain) were responsible for measuring creatinine (Cre) concentration in the urine samples of the BETTERMILK and BIOVAL studies, respectively, using the *Jaffé* reaction colorimetric method. The aim of this determination was to to correct possible differences in dilutions of urine samples and to determine whether a urine is valid for assessing chemical exposures. In accordance with the WHO, those adult urine specimens with a creatinine concentration outside the range of 30 – 300 mg·dl^{-1} were excluded from the present research[230]. As a “normal” urinary creatinine range for children has not been described yet in the literature, the same criterion was applied for both study populations (lactating mothers and children).

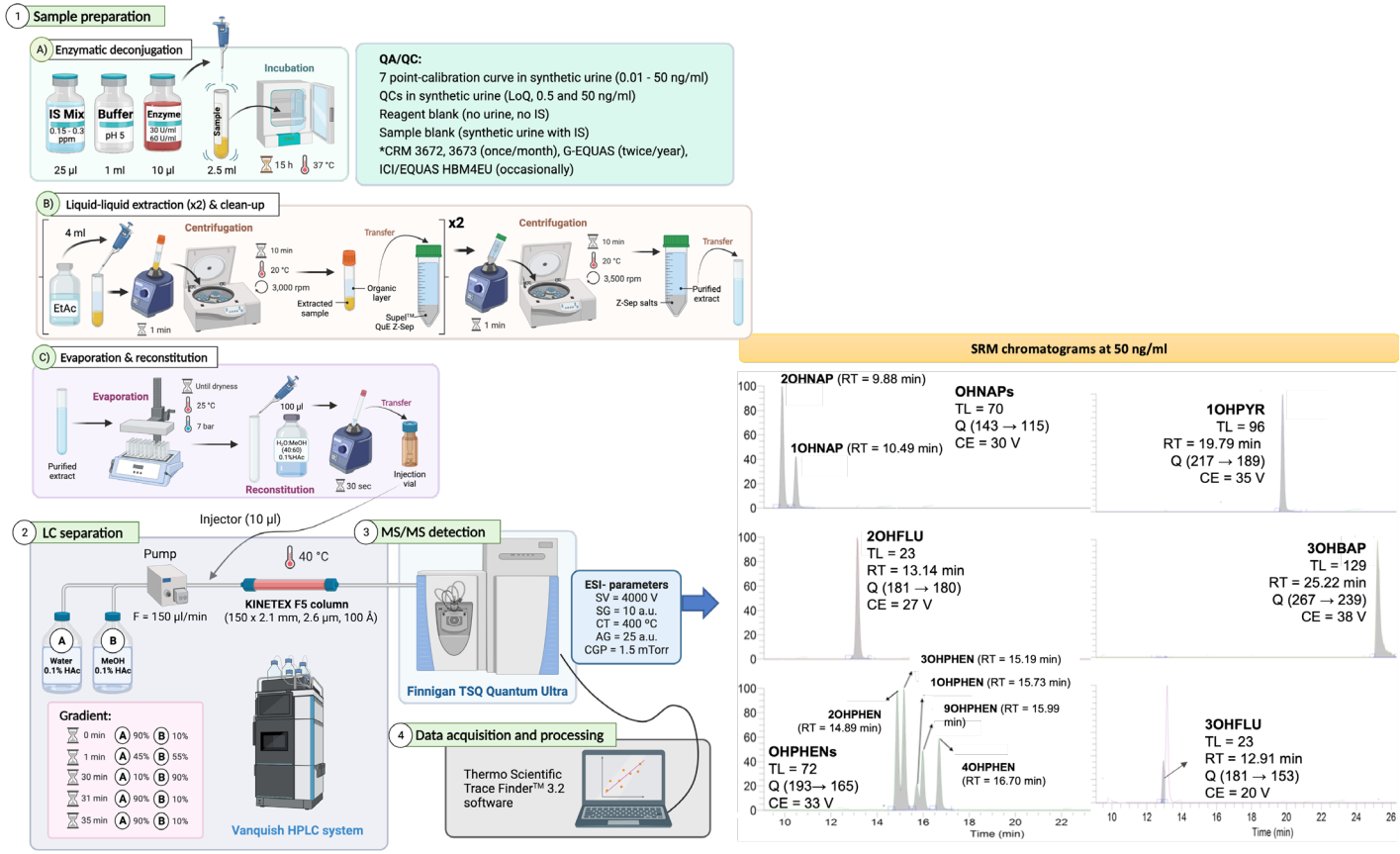


Figure 13. Diagram of the steps involved in the analysis of 11 OHPAHs in urine. 1) Sample preparation by (A) an enzymatic deconjugation using an IS Mix (working solution containing all IS at concentrations between 150 and 300 ng·ml^{-1}), an acetate buffer (pH = 5), and the enzyme β-Glucuronidase/Arylsulfatase (30 units·ml^{-1}/60 units·ml^{-1}), together with the QA/QC samples included in each sequence; (B) liquid-liquid extraction using ethyl acetate and a clean-up with Z-Sep; and, (C) evaporation and reconstitution in mobile phase containing water, methanol and acetic acid. 2) LC separation: experimental conditions of the Vanquish HPLC system equipped with a KINETEX F5 column. 3) MS/MS detection: conditions of the ESI source working in negative mode, and SRM chromatograms at 50 ng·ml^{-1} for each group of OHPAH isomers showing their mass transitions used for quantification, together with their corresponding TL, RT and CEs. 4) Data acquisition and processing using Trace Finder™ 3.2 software. *IS: internal standard; QA/QC: quality assurance/quality control; G-EQUAS: German External QUality Assessment Schemes; CRM: certified reference material; EtAc: ethyl acetate; MeOH: methanol; HAc: acetic acid; LC: liquid chromatography; HPLC: high-performance liquid chromatograph; F: flow rate; MS/MS: tandem mass spectrometry; ESI: electrospray ionization; SV: ion spray voltage; SG: sheath gas; CT: capillary temperature; AG: auxiliary gas; CGP: collision gas pressure; SRM: selected reaction monitoring; Q: quantification ion; TL: tube lens; RT: retention time; CE: collision energy.*

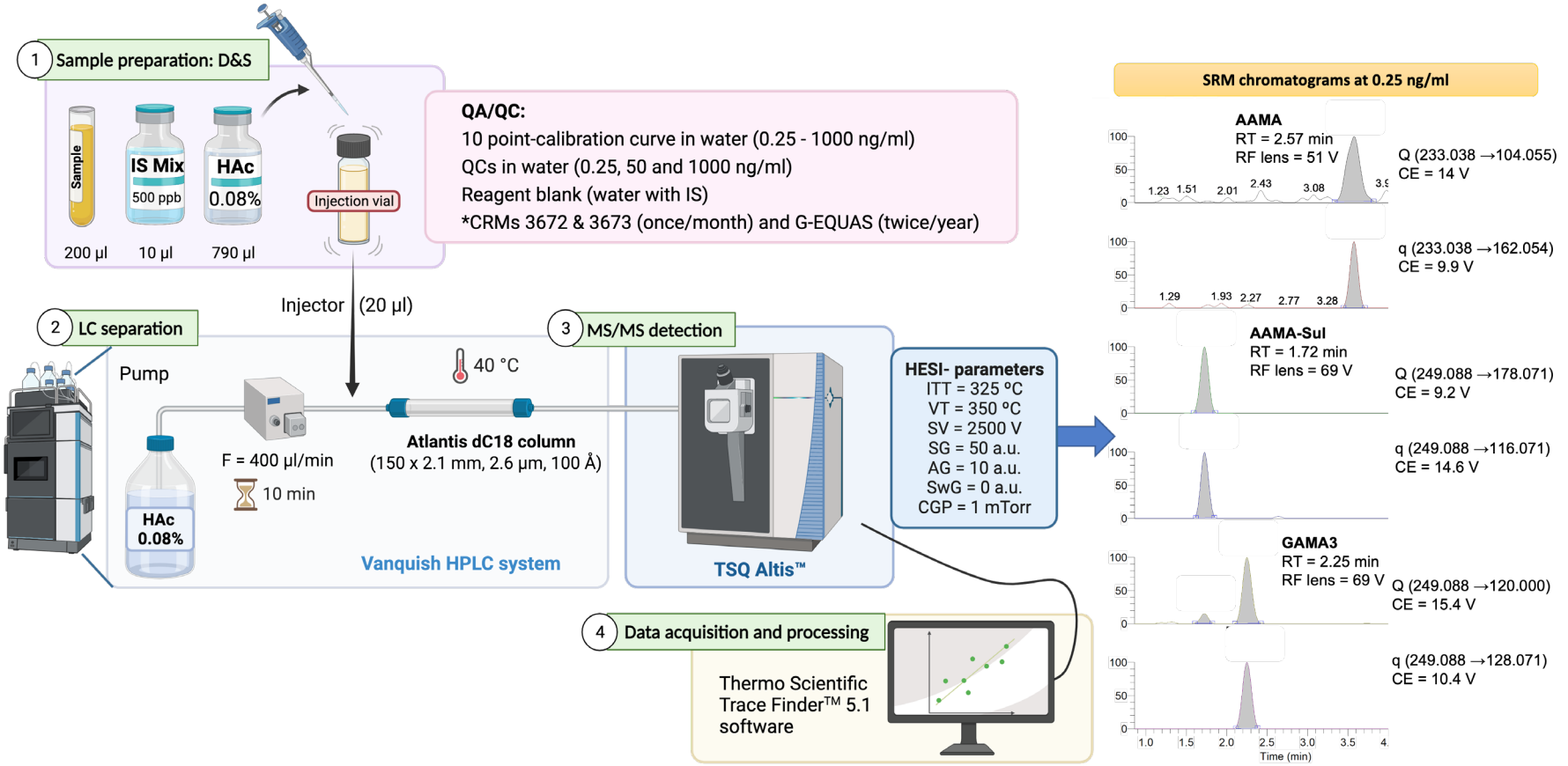


Figure 14. Diagram of the steps involved in the analysis of AAMA, AAMA-Sul and GAMA3 in urine: 1) Sample preparation by *dilute-and-shoot* (D&S) using an IS Mix (working solution containing all IS at 500 ng·ml^{-1}) and a solution of acetic acid, together with the QA/QC samples included in each sequence; 2) LC separation: experimental conditions of the Vanquish HPLC system equipped with an Atlantis dC18 column; 3) MS/MS detection: conditions of the HESI source working in negative mode, and SRM chromatograms at the limit of quantification (0.25 ng·ml^{-1}) showing Q and q ions for each analyte, together with their corresponding RT, RF lens, and CEs; and, 4) Data acquisition and processing using Trace Finder™ 5.1 software. *IS: internal standard; HAc: acetic acid; QA/QC: quality assurance/quality control; G-EQUAS: German External QUality Assessment Schemes; CRM: certified reference material; LC: liquid chromatography; HPLC: high-performance liquid chromatograph; HILIC: hydrophilic interaction chromatography; F: flow rate; MS/MS: tandem mass spectrometry; HESI: heated electrospray ionization; ITT: ion transfer tube temperature; VT: vaporizer temperature; SV: ion spray voltage; SG: sheath gas; AG: auxiliary gas; SwG: sweep gas; CGP: collision gas pressure; SRM: selected reaction monitoring; Q: quantification ion; q: confirmation ion; RT: retention time; RF lens: radiofrequency lens; CE: collision energy.*

3.2.3 METHOD VALIDATION, QUALITY ASSURANCE AND QUALITY CONTROL

The analytical methodologies used in the present thesis were developed and applied under a highly consolidated quality system (UNE-EN ISO/IEC 17025:2017 standard) in the Public Health Laboratory of Valencia. The validation and QA/QC methodologies for quantifying DAPs, OHPAHs and AA metabolites in urine were based on the *Bioanalytical Method Validation Guidance for Industry* of the FDA[56], and the *SANTE/11813 guide for pesticide residues analysis in food and feed*[57]. A SOP containing a detailed and written description of the methodology, as well as specific procedures to control critical quality parameters during analysis, was established for each analytical method before initiating validation and analysis of field samples. A general acquisition sequence including all QA/QC samples used in each analytical batch for validation and routine analysis is presented in Figure 15.

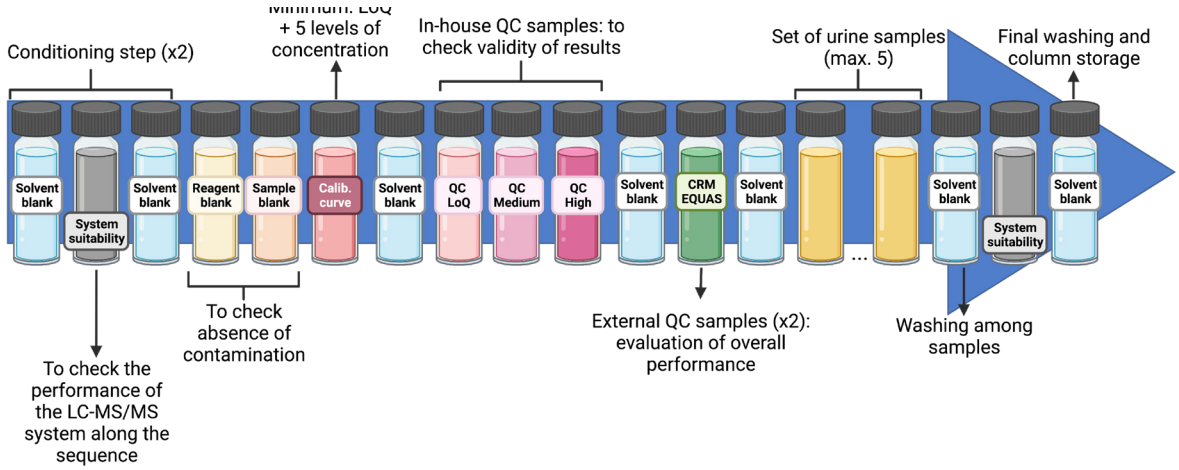


Figure 15. General acquisition sequence order employed in each batch of LC-MS/MS analysis: solvent blanks, system suitability solution, reagent and sample blanks, calibration curve (calib. curve), 'in-house' QCs at LoQ, medium and high concentration levels, external QC samples, and field urines. *LC-MS/MS: liquid chromatography coupled to tandem mass spectrometry; QC: quality control; LoQ: limit of quantification; CRM: certified reference materials; G-EQUAS: German External QUality Assessment Schemes; (x2): minimum number of injections equal to 2; (max. 5): maximum number of consecutive samples equal to 5.*

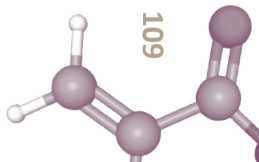
The validation of each method was conducted for the following parameters: linearity, accuracy, precision, sensitivity and specificity. It was performed in five runs in five different days considering elements and acceptance criteria presented in Table 5. Due to the lack of blank field urines, the following matrices for preparing the matrix-matched calibration curves were evaluated in the validation process: water, an 'in-house' synthetic urine as proposed by the Centers for Disease Control and Prevention (CDC)[231], and Surine™ Negative Urine Control Cerilliant® (Merck/Sigma-Aldrich). To this end, QA/QC parameters (Table 5) were assessed in

each case to select the matrix which compensated as much as possible the potential matrix effects, showing the best QA/QC results. Finally, a calibration curve using the 'in-house' synthetic urine for DAPs and OHPAHs, and water for AA metabolites were employed in each sequence. The quantification and confirmation criteria for LC-MS/MS determinations were based on the SANTE/11813 guideline[57], and are listed in Table 6.

After confirming that the developed method worked as intended, routine analysis of the urines from the BETTERMILK and BIOVAL studies could be started in batches of 25 samples, following the same acceptance criteria as in the method validation (Table 5). The quantification was carried out using the analyte to IS peak area ratio to compensate for the influence of the matrix and/or potential experimental losses of the analytes during the analytical procedure. Additionally, three 'in-house' QCs (LoQ, medium and high levels of concentration) were prepared and processed in the same way as the calibration curve. These QCs were used to check the efficiency of the extraction procedure and to ensure the proper quantification of analytes in the field urine samples in terms of accuracy and precision, covering all concentration levels of the calibration curve. Reagent and sample blanks were introduced in each batch to confirm the absence of any background contamination during the whole experimental procedure. In addition, solvent blanks were injected to avoid carryover among samples during the analytical sequence. A system suitability solution containing all analytes in a certain concentration was used to conditionate the LC-MS/MS system and check its performance before and during the injection of a complete analytical sequence. As an evaluation of the overall performance, the laboratory participated in the G-EQUAS intercomparison programs, which included the evaluation of the quantification of some pesticide biomarkers (DAPs, TCPY, PNP, DBCA, PBA, FPBA), three OHPAHs (1OHNAP, 2OHNAP and 1OHPYR), and two AA metabolites (AAMA and GAMA3) in urine. In addition, the proper quantification of 10 OHPAHs (all except for 3OHBAP) and the same two AA metabolites were also internally assessed by analyzing the CRM 3672 (urine of smokers, which generally contains these metabolites at high concentrations) or 3673 (urine of non-smokers, which generally contains these metabolites at low concentrations) once a month to better ensure the validity of the results. Furthermore, the laboratory participated in the five rounds of the interlaboratory comparison investigation (ICI)/EQUAS program organized by the HBM4EU initiative, resulting as "HBM4EU laboratory for the analysis of OHPAHs in human urine". The rest of pesticide metabolites, 3OHBAP and AAMA-Sul were not externally evaluated due to the lack of CRMs and/or interlaboratory assays.

Table 5. Summary of analytical parameters, elements, tools and acceptance criteria used for the method validation and QA/QC in each routine batch of this thesis.

Parameters	Elements & tools		Acceptance criteria
Linearity	Calibration curve (at least six different concentration points covering the concentration range expected in each study)	• Analysis of variance (ANOVA) of the regression residuals (Lack-of-fit test): $$Critical\ F\ value = \frac{SS_{LOF}/df(SS_{LOF})}{SS_{PE}/df(SS_{PE})}$$ where SS_{LOF} is the sum of squares due to lack-of-fit, SS_{PE} is the sum of squares due to pure error, and 'df' their corresponding degrees of freedom • Coefficient of determination (R^2)* • Percentage of residual standard deviations (%RSD)*	• ANOVA: F-statistic > critical F value • $R^2 > 0.98$ • %RSD ± 20 %
Accuracy and precision	Replicates at three different concentration levels of the calibration curve ('in-house' QCs)	• Percentage of relative recovery (%R): $$\%R = \frac{Concentration_{Observed}}{Concentration_{Theorical}}\ x100$$ • Percentage of coefficient of variation (%CV): $$\%CV = \frac{S}{\bar{x}}\ x100$$ where $\bar{x}$ is the mean of the observed concentrations, and s its standard deviation	• %R = 85 – 115 % for medium and high concentration levels • %R = 80 – 120 % for LoQ level • %CV ≤ 20 %
Sensitivity	Replicates at LoQ level of the calibration curve	• %R • %CV • Signal-to-noise ratio (S/N)	• %R = 80 – 120 % • %CV ≤ 20 % • S/N ≥ 10
Specificity	Reagent (no analyte, no IS) and sample (no analyte) blanks	• Percentage of specificity (%S), calculated as the response ratio of each analyte in the blank and at the LoQ: $$\%S = \frac{Area_{Blank}}{Area_{LoQ}}\ x100$$	• %S < 30%



Parameters	Elements & tools		Acceptance criteria
Overall performance	CRM 3672 (urine of non-smokers) & 3673 (urine of smokers) for OHPAHs and acrylamide, G-EQUAS, and HBM4EU intercomparison programs (only for OHPAHs)	• Z-Score (absolute value), $\|Z\| = \frac{\|x - C\|}{\sigma} \times 100$ where x is the obtained result, C is the consensus value and σ is the standard deviation of all results	• $\|Z\| \leq 2$

QA: quality assurance; QC: quality control; LoQ: limit of quantification; IS: internal standard; CRM: certified reference material; G-EQUAS: German External QUality Assessment Scheme; HBM4EU: Human biomonitoring for Europe project.

*Automatically calculated by the Thermo Scientific Trace Finder™ software (3.2 and 5.1 versions)

Table 6. Summary of the identification requirements for the LC-MS/MS determinations applied in the present thesis.

Detectors used in this thesis	Evaluated parameters	Requirements for identification
MS/MS triple quadrupole detectors from Thermo Fisher Scientific Inc.: • DAPs and OHPAHs: Finnigan TSQ Quantum Ultra • Acrylamide: TSQ Altis™ Acquisition mode: SRM Ionization: ESI (DAPs and OHPAHs) and HESI (Acrylamide) in negative mode	Number of product ions	If possible ≥ 2, one for quantification (Q ion) and another for identification (q ion)
	Overlapping	Q and q ion peaks must fully overlap
	Signal-to-noise ratio (S/N)	S/N ≥ 3
	Choice of the Q ion	The most abundant transition for each analyte, which should be sufficient selective
	Percentage of relative ion ratio (%RR) $\%RR = \frac{Area_{q\ ion}}{Area_{Q\ ion}} \text{ x}100$	Reference values of %RR are set based on the average of calibration standards from a same sequence %RR from unknown urine samples should be within ±30 % of the reference value
	Retention time (RT)	A tolerance of ± 0.1 min in the RT of the same analytical set is permitted between the Q ion in unknown urine samples and the calibration curve Larger deviations are acceptable when both RTs and peak shapes of the analytes match with those of their respective IS

LC: liquid chromatography; MS/MS: tandem mass spectrometry; DAPs: dialkyl phosphates; OHPAHs: hydroxylated polycyclic aromatic hydrocarbons; SRM: selected reaction monitoring; ESI: electrospray ionization; HESI: heated electrospray ionization.

3.3 DATA AND STATISTICAL ANALYSIS

To perform the statistical analysis, IBM SPSS Statistics (versions 23.0 and 26.0) and R software (version 3.5.2) were used. First, detection frequency (DF, % samples > LoQ), arithmetic mean (AM), geometric mean (GM), 25th, 50th, 75th and 95th percentiles (P25, P50, P75, and P95), maximum observed concentration and standard deviation (SD) were calculated to summarize analyte levels in ng·ml^{-1} and µg·g creatinine^{-1}. In addition, the maximum likelihood estimation method proposed by EFSA[232] was used to statistically impute missing analyte concentrations (if any), those not detected or detected below the LoQ, but only in those analytes with a DF ≥ 40 %.

For studying correlations among the main metabolites, the Spearman's test was used. Those metabolites with the same parent compound were converted to nmol·l^{-1} and added together, as in the case of DEPs (ΣDEPs = DEP + DETP + DEDTP), DMPs (ΣDMPs = DMP + DMTP + DMDTP), some OHPAHs (ΣOHNAPs = 1OHNAP + 2OHNAP; ΣOHFLUs = 2OHFLU + 3OHFLU; and ΣOHPHENs = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN), and urinary AA metabolites (ΣUAAM = AAMA + GAMA3 + AAMA-Sul). For the construction of the prediction models, the sum of DAPs (ΣDAPs = ΣDEPs + ΣDMPs), ΣOHNAPs, ΣOHFLUs, ΣOHPHENs, 1OHPYR, and ΣUAAM were used. Moreover, as the data were log-normally distributed, the natural logarithm of the observed concentrations was applied to build the corresponding exposure prediction models, including

information from the questionnaires as independent variables. Qualitative variables having categories with frequencies higher than 90 % were excluded from the models. To ensure that creatinine levels did not affect the statistical significance of other variables, urinary concentrations without adjustment for creatinine were used (ng·ml^{-1} or nmol·l^{-1}), adding creatinine concentration as a separate quantitative variable[230]. As a result, linear regression models for log-transformed concentrations of ΣDAPs, ΣOHNAPs, ΣOHFLUs, ΣOHPHENs, 1OHPYR, and ΣUAAM were performed using Forward Stepwise Regression based on the corrected Akaike information criterion[233] or Backward Stepwise Regression based on the Robust Final Prediction Error criterion when some outlier values were observed in the data set[234]. Additionally, numerical explanatory variables were divided by two times their SD to obtain directly-comparable regression coefficient magnitudes[235]. Only data from those participants with a completed questionnaire were used in the construction of the prediction models. A p-value less than or equal to 0.05 was considered as statistically significant.

3.4 EXPOSURE AND RISK ASSESSMENT

With the aim to estimate the exposure of both population groups to pesticides, PAHs and AA, the reverse dosimetry approach was used. For this purpose, EDIs (µg·kg-bw^{-1}·day^{-1}) were estimated based on GM and P95 urinary levels of their respective metabolites according to formula 4. Given that the non-occupational population and children are exposed to these substances mainly through dietary ingestion, EDIs were calculated considering only this exposure route. Furthermore, EDIs were calculated only for biomarkers with a DF ≥ 40 %, considering that concentration of metabolites which come from the same parent compound were aggregated and then assessed jointly, such as ΣDAPs, ΣOHNAPs, ΣOHFLUs, ΣOHPHENs, and ΣUAAM. It was assumed that the presence of their biomarkers in urine was mainly due to metabolism to their respective parents, and not to exposure to the metabolites themselves. Toxicokinetic parameters used to calculate EDIs are presented in Table 7.

To evaluate the non-cancer risk associated to the calculated EDIs, the reverse dosimetry approach was applied using the concept of HQ (formula 5) for the three organic compound groups (pesticides, PAHs and acrylamide), and MOEs (formula 6) for pesticides and acrylamide. Additionally, for cumulative risk assessment, the HI (formula 8) for substances with the same MoA (OPs and PAHs, separately), and MOET (formula 7) for pesticides included in the same CAG, those causing alterations of the motor system ($MOET_{motor}$) and those affecting the acetylcholinesterase (AChE) activity ($MOET_{AChE}$), were also estimated by dose addition. For acrylamide, the forward dosimetry approach was also used to interpret urinary AAMA levels in two different ways: i) a direct comparison with the BE and BE_{POD} by calculating HQs (formula 2); and ii) construction of prioritization schemes (see Figure 7). A compilation of the parameters and considerations used for risk assessment estimations in the BETTERMILK and BIOVAL studies is presented in Table 8. Due to the lack of epidemiological studies demonstrating that the assessed substances are carcinogenic for humans, the cancer risk was not calculated in the present study for any of them.

Table 7. Parameters used for calculation of EDIs for GM and P95 of urinary pesticides, PAHs and acrylamide in the BETTERMILK and BIOVAL studies. Stratification performed in each study population (if any) is also presented.

Compound class	Parent	MWP ($g \cdot mol^{-1}$)	Biomarker	F_{UE}	BETTERMILK project			BIOVAL study		
					Strata	BW	AF	Strata	BW	AF
*Pesticides	Chlorpyrifos -ethyl	350.59	TCPY	0.50[236]	None	60 kg	[d]V_{24h} (1.6 $l \cdot day^{-1}$)	None	33 kg	[g]V_{24h} (0.66 $l \cdot day^{-1}$)
			ΣDEPs	0.50[236]						
	Dimethoate	229.26	ΣDMPs	0.90[142]						
	Parathion	291.30	PNP	0.37[237]						
	Deltamethrin[a]	505.21	PBA	0.25[140]						
	λ-Cyhalothrin[b]	449.50	PBA	0.30[238]						
PAHs	Naphthalene	128.17	ΣOHNAPs	1[c]	Smoking habit (smokers, non-smokers, former smokers)	60 kg	[d]V_{24h} (1.6 $l \cdot day^{-1}$) [e]Cre_{24h} (18 $mg \cdot kg\text{-}bw^{-1} \cdot day^{-1}$)	Age range		[g]V_{24h} (0.66 $l \cdot day^{-1}$)
	Fluorene	166.22	ΣOHFLUs	0.78[c]				5 – 8 years	27 kg	
	Phenanthrene	178.23	ΣOHPHENs	0.09[c]				9 – 12 years	39 kg	
	Pyrene	202.26	1OHPYR	0.08[c]						
Acrylamide	Acrylamide	71.08	ΣUAAM	0.50[203]	Smoking habit (smokers, non-smokers, former smokers)	60 kg	[d]V_{24h} (1.6 $l \cdot day^{-1}$) [f]Cre_{24h} (1.03 $g \cdot day^{-1}$)	None	33 kg	[g]V_{24h} (0.66 $l \cdot day^{-1}$) [h]Cre_{24h} (0.5 $g \cdot day^{-1}$)

EDI: estimated daily intake; GM: geometric mean; P95: 95th percentile; MW_P: molecular weight of the parent compound; F_{UE}: excretion factor; BW: body weight; AF: adjustment factor

*The selection of the parent compound for pesticide metabolites which have various parent pesticides was based on the following criteria: i) if possible, an active substance approved by the Regulation (EC) No 1107/200; and ii) if there was more than one possible parent, the pesticide with the lowest health-based guidance value (HBGV) was chosen.

[a]Only evaluated in the BETTERMILK project, since the lowest admissible daily intake (ADI) was found for this pyrethroid at the time the risk assessment was conducted

[b]Only assessed in the BIOVAL program

[c]Mean values of two studies[168,239], except for naphthalene in which the maximum possible F_{UE} value was selected

[d]Default value of the total urinary volume excreted within 24h for adults[140]

[e]Total urinary creatinine excreted within 24h standardized by BW[240]

[f]Total urinary creatinine excreted within 24h normalized by BW, age (34 years old) and height (164 cm)[241]

[g] Default value of the total urinary volume excreted within 24h for children[140]

[h]Default value of the total urinary creatinine excreted within 24h for children[140]

Table 8. Parameters used for risk assessment of exposure to pesticides, PAHs and acrylamide in the BETTERMILK and the BIOVAL studies[a].

Parent	HBGV	Biomarker	Risk assessment	
			Individual	Cumulative
Pesticides	ADI (µg·kg-bw^{-1}·day^{-1})[144] $NOAEL_{Motor}$ (µg·kg-bw^{-1})[242] $NOAEL_{AChE}$ (µg·kg-bw^{-1})[242]		$HQ_i = EDI_i/ADI_i$[d] $MOE_{i,motor} = NOAEL_{i,Motor}/EDI_i$[e] $MOE_{i,AChE} = NOAEL_{i,AChE}/EDI_i$[e]	$HI = \Sigma HQ_i$[d] $1/MOET_{Motor} = \Sigma 1/MOE_{i,Motor}$[e] $1/MOET_{AChE} = \Sigma 1/MOE_{i,AChE}$[e]
Chlorpyrifos-ethyl	10 10000 500	TCPY	HQ_{TCPY} $MOE_{TCPY\text{-}Motor}$ $MOE_{TCPY\text{-}AChE}$	$HI_{OPs} = HQ_{\Sigma DEPs} + HQ_{\Sigma DMPs}$ $1/MOET_{AChE} = 1/MOE_{\Sigma DEPs\text{-}Motor} + 1/MOE_{\Sigma DMPs\text{-}Motor}$ $1/MOET_{Motor} = 1/MOE_{\Sigma DEPs\text{-}AChE} + 1/MOE_{\Sigma DMPs\text{-}AChE} + 1/MOE_{PBA}$
		ΣDEPs	$HQ_{\Sigma DEPs}$ $MOE_{\Sigma DEPs\text{-}Motor}$ $MOE_{\Sigma DEPs\text{-}AChE}$	
Dimethoate	2 20000 1000	ΣDMPs	$HQ_{\Sigma DMPs}$ $MOE_{\Sigma DMPs\text{-}Motor}$ $MOE_{\Sigma DMPs\text{-}AChE}$	
Parathion	4 1750 250	PNP	HQ_{PNP} $MOE_{PNP\text{-}Motor}$ $MOE_{PNP\text{-}AChE}$	
Deltamethrin[b]	10 - -	PBA	HQ_{PBA} - -	
λ-Cyhalothrin[c]	2.5 - 500	PBA	HQ_{PBA} - MOE_{PBA}	

Parent	HBGV	Biomarker	Risk assessment	
			Individual	Cumulative
PAHs[d]	RfD (μg·kg-bw^{-1}·day^{-1})[243–246]		$HQ_i = EDI_i/RfD_i$[d]	$HI = \Sigma HQ_i$[d]
Naphthalene	20	ΣOHNAPs	HQ_{NAP}	$HI_{PAHs} = HQ_{NAP} + HQ_{FLU} + HQ_{PHEN} + HQ_{PYR}$
Fluorene	40	ΣOHFLUs	HQ_{FLU}	
Phenanthrene	30	ΣOHPHENs	HQ_{PHEN}	
Pyrene	30	1OHPYR	HQ_{PYR}	
Acrylamide	$BMDL_{10}$ (430 μg AA·kg-bw^{-1}·day^{-1})[181]	ΣUAAM		$MOE_{AA} = BMDL_{10}/EDI_{AA}$[f]
	BE (13 μg·l^{-1}; 16 μg·g Cre^{-1})[210] BE_{POD} (130 μg·l^{-1}; 160 μg·g Cre^{-1})[210]	AAMA		$HQ_{BE} = C_{AAMA}/BE$[d] $HQ_{BE\text{-}POD} = C_{AAMA}/BE_{POD}$[d] Prioritization diagrams[g]

HBGV: health-based guidance value; ADI: admissible daily intake; NOAEL: no observed adverse effect level in mammals; AChE: affects the acetylcholinesterase activity; Motor: causes alterations of the motor system; HQ: hazard quotient; HI: hazard index; EDI: estimated daily intake; MOE: margin of exposure; MOET: total margin of exposure; RfD: reference dose; $BMDL_{10}$: Benchmark Dose Lower Confidence Limit; BE: biomonitoring equivalent; BE_{POD}: biomonitoring equivalent directly derived from a point of departure (POD) without considering intraspecies uncertainty factors.

[a]MOEs and MOETs for pesticides were only calculated in the BIOVAL program

[b]Only evaluated in the BETTERMILK project, since the lowest ADI was found for this pyrethroid at the time the risk assessment was conducted

[c]Only assessed in the BIOVAL program; it does not affect acetylcholinesterase activity, so it does not have an associated $NOAEL_{AChE}$ and MOE_{AChE} was not calculated for this compound[111,112]

[d]HQs and HIs below 1 are considered a low risk of exposure to individual substances and mixture of compounds, respectively

[e]MOEs and MOETs above 100 are considered a low risk due to pesticide exposure[111,112]

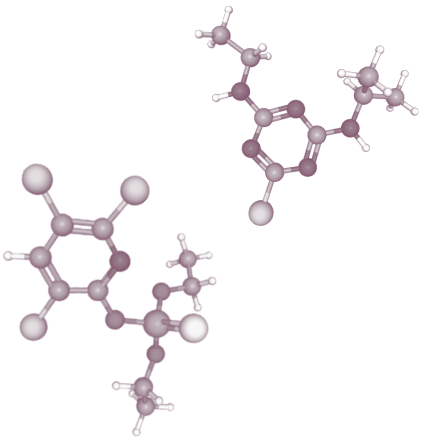
[f]A MOE above 125 are considered sufficient to conclude that there is no concern for non-genotoxic health effects of AA[181]

[g]A concentration of AAMA in urine below BE is considered of a low priority, between BE and BE_{POD} is of a medium priority, and above BE_{POD} is of a high priority for risk assessment follow-up[108]

4

CHAPTER I

BIOMONITORING OF NON-PERSISTENT PESTICIDES IN CHILDREN AND MOTHERS: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT



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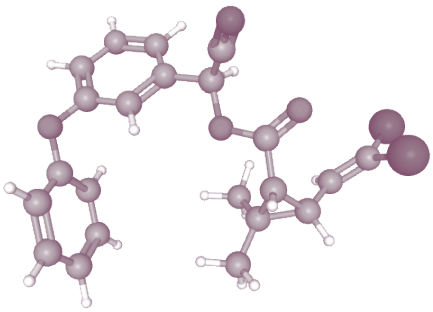


CHAPTER I

4.1 DEVELOPMENT OF A NOVEL METHODOLOGY FOR DETERMINATION OF DIALKYL PHOSPHATES IN HUMAN URINE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

4.1.1 RESULTS AND DISCUSSION

4.1.1.1 SAMPLE PREPARATION

'Dilute-and-shoot', QuEChERs and different SPE methods were tested for the urine extraction of DAPs. The results obtained by 'dilute-and-shoot' were unsatisfactory due to the signal suppression presented for all analytes (low responses), leading to high LoQs. Based on a previous publication[247], a total number of 12 different automatic SPE extraction methods with Strata X-AW (weak anion exchange) cartridges with various extraction solvents (acetonitrile, acetone, methanol and water) and pH modifiers (formic acid, ammonium and sodium acetate, ammonia and triethylamine) were tested. However, these SPE methods were not adequate to measure DAPs, specifically at low levels, due to unsatisfactory recoveries (%R < 70 %) and a high presence of interfering substances, mainly for DMP and DEP. QuEChERs-based methods using two different extraction solvents (acetonitrile, and a mixture of ethyl acetate:diethyl ether, 1:1), several salts (sodium chloride, magnesium sulfate, sodium citrate and citric acid disodium salt) and clean-up sorbents, i.e., Primary Secondary Amine and activated carbon, were tested to choose the sample preparation methodology showing the best performance[49,248]. Finally, original QuEChERS pouches, containing 1 g sodium chloride and 4 g magnesium sulfate, and a mixture of diethyl ether:ethyl acetate (1:1) were selected with any additional clean-up step (no sorbent), since it did not significantly improve the relative recoveries. This extraction

procedure provided higher recoveries for all analytes (82 – 117 %), higher responses and enough interference removal throughout the concentration range compared to 'dilute-and-shoot' and the SPE methods, achieving LoQs of 0.5 ng·ml^{-1} for all DAPs.

4.1.1.2 CHROMATOGRAPHIC SEPARATION

For DAP separation, two HILIC columns (2.1 × 100 mm, 3 µm) with a different stationary phase surface were tested: a KINETEX HILIC column (unbounded silica) and a Hypersil GOLD HILIC (surface modified with a tertiary amine) column. A chromatogram showing the separation of DAPs in both columns under the same conditions is presented in Figure 16. As can be observed, the KINETEX HILIC column showed wide peaks, mainly for DEP and DMP, and the retention time of DBP was too high (31.05 min), resulting in a long chromatogram (Figure 16a). Thus, the Hypersil GOLD HILIC column was selected since it showed optimal chromatographic resolution in the shortest time (Figure 16b). To improve the ionization process of DAPs, various pHs and modifiers (as ammonium acetate, formic acid and ammonium formate) were also tested in water (mobile phase A) and in acetonitrile (mobile phase B)[247–249]. Finally, an aqueous solution of ammonium acetate 200 mM (pH = 6.3) was chosen for both mobile phases as the modifier giving the most optimal resolution. An example of the quantification and confirmation of some DAPs in a field urine sample using the selected chromatographic conditions is presented in Figure 16c.

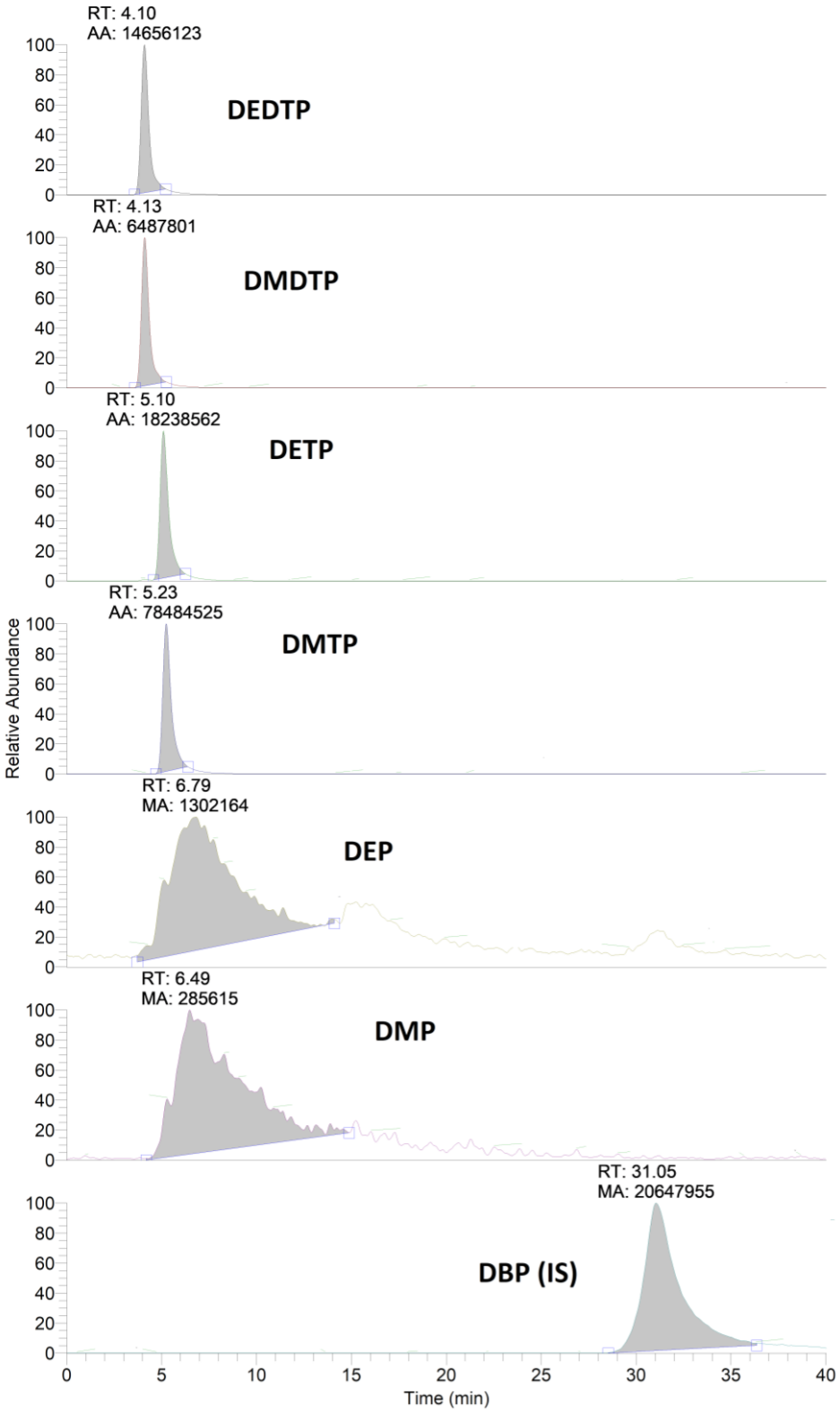


Figure 16a. SRM chromatograms of the six dialkyl phosphates (DEDTP, DMDTP, DETP, DMTP, DEP and DMP) and DBP (internal standard, IS) obtained with a KINETEX HILIC column (2.1 × 100 mm, 3 µm) from a standard solution of 100 ng·ml^{-1}. *SRM: selected reaction monitoring; HILIC: hydrophilic interaction chromatography*

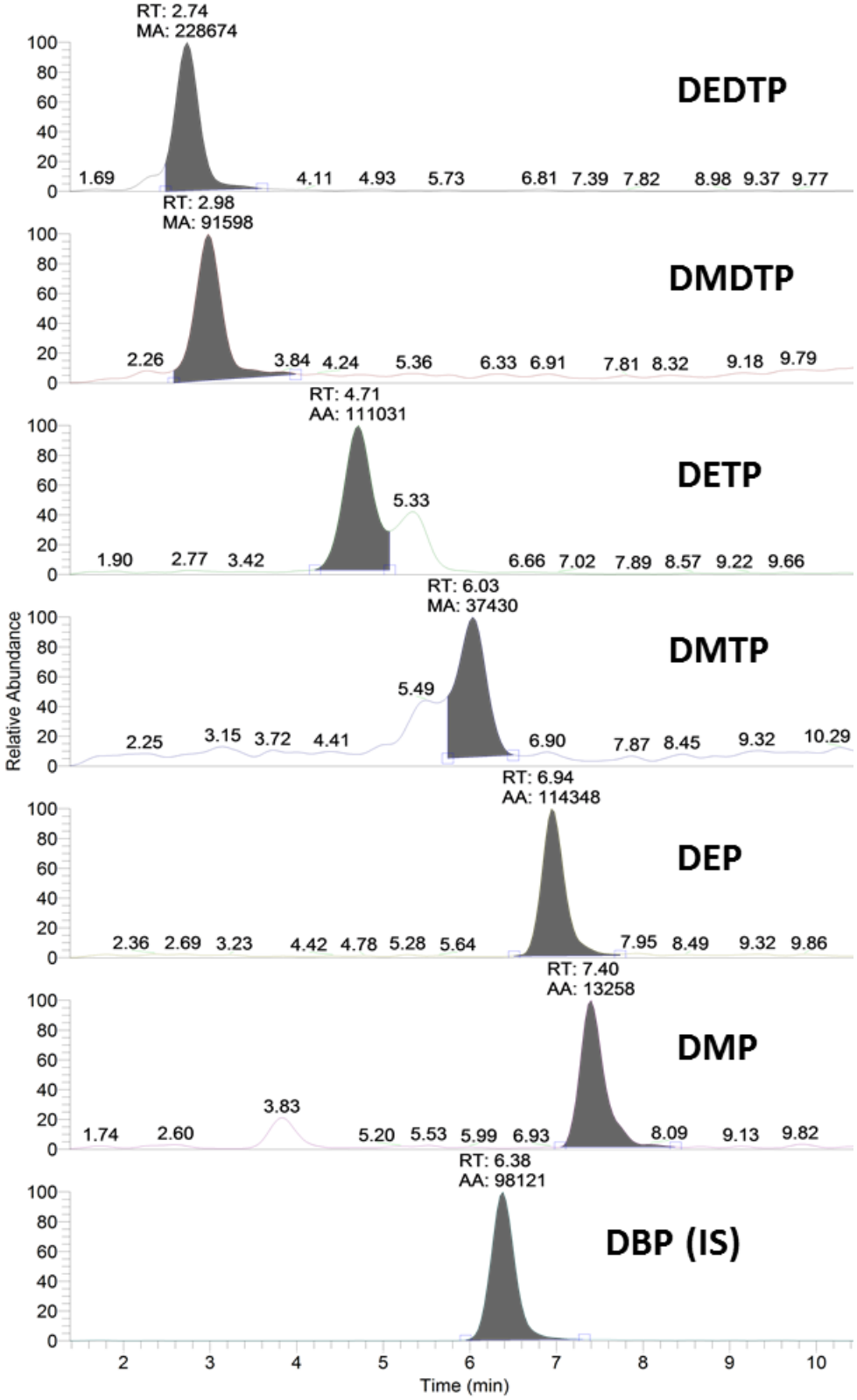


Figure 16b. SRM chromatograms of the six dialkyl phosphates (DEDTP, DMDTP, DETP, DMTP, DEP and DMP) and DBP (internal standard, IS) obtained with a Hypersil GOLD HILIC column (2.1 × 100 mm, 3 µm) from a standard solution of 50 ng·ml^{-1}. *SRM: selected reaction monitoring; HILIC: hydrophilic interaction chromatography.*

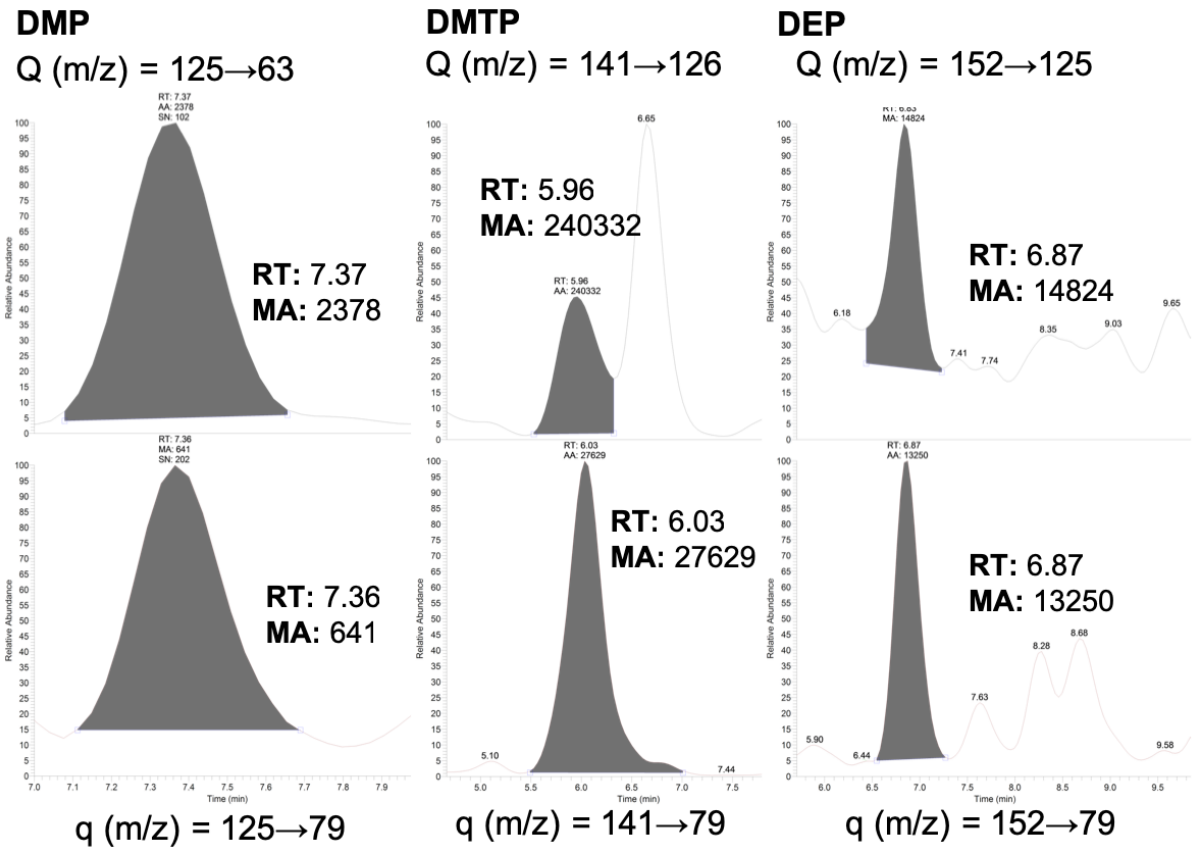


Figure 16c. SRM chromatograms of the six dialkyl phosphates (DEDTP, DMDTP, DETP, DMTP, DEP and DMP) and DBP (internal standard, IS) obtained with a Hypersil GOLD HILIC column (2.1 × 100 mm, 3 µm) from a field urine sample, in which DMP, DMTP and DEP were detected and confirmed with a concentration > LoQ (0.5 ng·ml^{-1}). *SRM: selected reaction monitoring; HILIC: hydrophilic interaction chromatography; LoQ: limit of quantification.*

4.1.1.3 MASS SPECTROMETRY DETECTION

A preliminary optimization of MS/MS parameters was necessary to maximize the analytical response for each compound using syringe infusion mode. For this purpose, full scan spectrum of each DAP was obtained by direct injection of 1 µg·ml^{-1} of individual standard solutions to the mass spectrometry detector with a flow rate of 20 µl·min^{-1}. After that, the chosen precursor ion was fragmented and the two most abundant product ions, used as quantification ion (Q ion) and confirmation ion (q ion), were selected. CEs were optimized using the automated optimization procedure for each pair precursor/product ion. Precursor ions, product ions and their optimized CEs were previously shown in Figure 12. Tube lens were automatically optimized by the mass spectrometer.

According to prior experiments carried out in our laboratory and the manual provided by the manufacturer, four factors are potentially affecting the ESI efficiency: spray voltage (SV), capillary temperature (CT), sheath gas (SG), and auxiliary gas (AG). To optimize these parameters, i.e., making the analytical response of DAPs as high as possible, a DoE consisting of a P-B and a CCD was applied using a standard software named MINITAB. The DoE is a reliable tool to generate an ordered and optimal sequence of experiments, facilitating the analysis of results and extraction of conclusions in an organized way. Since doing a few more experiments was not a limitation in this study, it was preferred to introduce duplicates and points to the center in the experimental designs to consider the variability between runs due to the instrument performance, exceeding what would be strictly necessary in ordinary DoE designs.

Firstly, a P-B design of 15 experiments (12 runs, the minimum number of experiments for four factors, and one center point in triplicate to estimate variability between runs) was selected to determine the relative influence of the four factors on the six DAP responses in the ESI source. The ranges analyzed in this screening were: SV (2000 – 5000 V), CT (200 – 350 ºC), SG (5 – 40 psi) and AG (5 – 20 arbitrary units, a.u.). The conditions and results of each P-B experiment (estimated effects and statistical significance at 95 % confidence level) are presented in Table 9. A p-value < 0.05 was only observed for CT and SG in all DAPs, except from DMDTP and DMTP, respectively. Therefore, these parameters were chosen to be more accurately optimized by a CCD. Additionally, estimated effects for AG were negative for all analytes, and variable for SV, i.e., negative for DMP, DMDTP, DETP, and DEDTP; and, positive for DMTP and DEP. For this reason, these factors were fixed at low (10 psi) and intermediate (3000 V) levels, respectively.

Table 9. Placket-Burman design and the resulting estimated effects and p-values used for optimization of the electrospray ionization source settings for the analysis of dialkyl phosphates by liquid chromatography coupled to tandem mass spectrometry.

Run	Capillary Temperature (ºC)	Auxiliary Gas (a.u.)	Sheath Gas (psi)	Spray Voltage (V)
1	275	13	23	3500
2	200	5	5	5000
3	350	5	5	2000
4	350	5	40	2000
5	200	20	40	2000
6	200	20	40	5000
7	200	20	5	2000
8	350	20	5	5000
9	350	20	40	2000
10	350	5	40	5000
11	275	13	23	3500
12	350	20	5	5000
13	200	5	5	2000
14	200	5	40	5000
15	275	13	23	3500

Analyte	Estimated effects (p-value)			
DMP	291745 (0.004)[a]	-50632 (0.520)	346388 (0.001)[a]	-6500 (0.933)
DMTP	4996436 (0.005)[a]	-1781499 (0.220)	10898384 (0.000)	533002 (0.703)
DMDTP	1476188 (0.128)	-576941 (0.529)	6872825 (0.000)[a]	-1452620 (0.134)
DEP	841588 (0.007)[a]	-176220 (0.483)	1215725 (0.001)[a]	68646 (0.782)
DETP	2393253 (0.003)[a]	-557461 (0.368)	5528047 (0.000)[a]	-337217 (0.581)
DEDTP	3677305 (0.022)[a]	-930662 (0.502)	12594782 (0.000)[a]	-1242960 (0.375)

[a]p-value < 0.05

After the P-B, a CCD was used for a further optimization of CT and SG. This kind of experimental design can build the response surface for each analyte and find the factor settings that maximize the analytical response for all compounds simultaneously. The designed CCD model included 26 experiments (13 runs including 5 center points, 4 cube points and 4 axial points and 2 replicates) with an Alpha value of 1.41421. The ranges analyzed in this step were: 150 – 400 ºC for the CT, and 5 – 50 psi for the SG, extending the range outside the initial settings due to the selected alpha value (> 1). These axial points outside the initial ranges remained at reasonable levels of CT and SG for the MS/MS system. A multiple regression equation was used to fit the responses observed, including second order and interaction terms. The obtained response surfaces displayed the effect of the two independent variables (CT and SG) on the DAPs responses. The six response surfaces obtained by MINITAB for each DAP were different and are presented in Figure 17. Finally, the 'response optimizer' in the MINITAB software aided the selection of the factor settings that concurrently optimized the responses of the six DAPs. In this way, a composite desirability that combines the desirability of each analyte into a single value could be maximized, considering that all responses had the same importance. A desirability value of 0.0 is assigned to the lowest response, and a value of 1.0 corresponds to the highest response. The composite desirability obtained was 0.97, and the final established factor settings were 400 ºC for the CT and 50 psi for the SG.

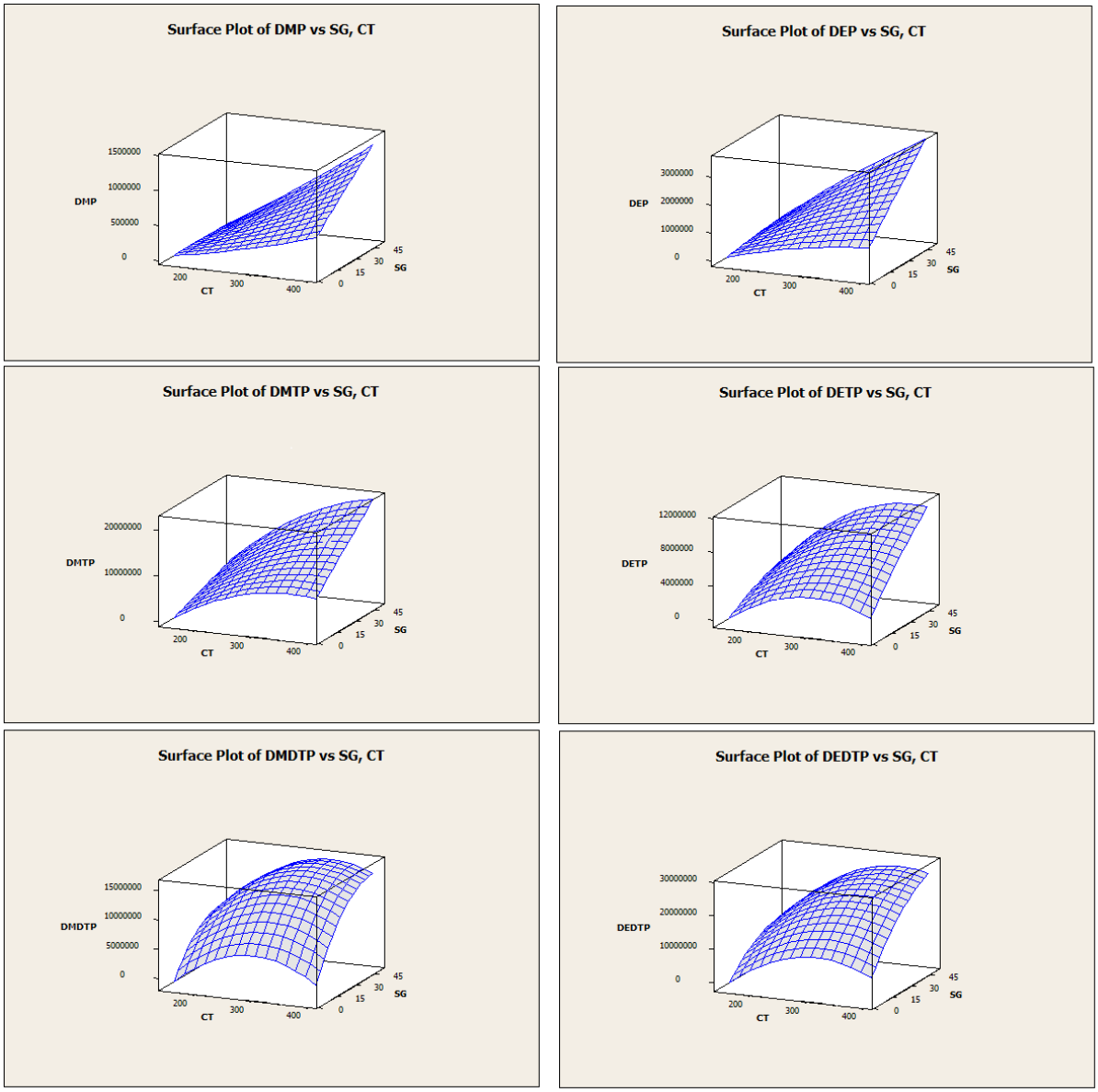


Figure 17. Results of the center composite design experiments: surfaces plots for DMP, DEP, DMTP, DETP, DMDTP and DEDTP vs SG (sheath gas) and CT (capillary temperature).

4.1.1.4 METHOD VALIDATION

Results of the method validation (linearity, accuracy, precision and selectivity) at three levels of concentration (0.5, 5 and 50 ng·ml^{-1}), and of the G-EQUAS 60/2017 analysis are presented in Table 10. The lineal dynamic range was 0.5 – 50 ng·ml^{-1} and the procedural LoQ was 0.5 ng·ml^{-1} for all DAPs. A good linearity was achieved, since all R^2 values were above 0.98 and residuals were below 20 %. The obtained %R and %CVs ranged from 82 % (DMP, at 5 ng·ml^{-1}) to 117 % (DMP and DEDTP, at LoQ level), and from 3% (DMP, at LoQ level) to 20 % (DMDTP, at 5 ng·ml^{-1}), respectively. No relevant contamination or interferences were found in either the reagent or sample blanks (%S < 30 %). Furthermore, $|Z| \leq 2$ were obtained in analysis of the G-EQUAS/60-2017 samples, used here as CRMs. Thus, the proposed analytical method fulfilled all performance criteria that were previously defined. The developed method was tested first with a set of 20 field urines, and then applied to all samples collected in the BETTERMILK and BIOVAL studies, obtaining concentrations within the range reported in the literature (see Chapters 4.2 and 4.3, respectively).

4.1.1.5 COMPARISON WITH PREVIOUS METHODOLOGIES

Figure 18 makes a comparison between the analytical characteristics of the current study and other reported methods. In general, LoQs obtained here are lower than those found in previous studies based on LC. Only one of those studies reported LoQs, ranging from 0.03 to 0.49 ng·ml^{-1}, below ours (0.50 ng·ml^{-1}), but it requires a large amount of sample (10 ml), a sample pre-treatment (lyophilization) and a long run-time analysis (35 min)[250]. In addition, the developed method shows considerably better %R (between 82 and 117 %) than those reported in most LC-MS methods (13 % – 122 %), mainly for DEDTP and DMP. Moreover, the maximum %CV obtained for DEDTP (11 %), DMP (12 %), DMTP (19 %) and DMDTP (20 %) are much lower than those observed in other studies, which are from up to 24, 70, 34 and 30 %, respectively. Therefore, the developed strategy gives the most precise and accurate results of all LC-MS methodologies reported so far. The simultaneous analysis of DAPs is also carried out by GC because this technique allows the achievement of lower LoQs than LC methods[251–254]. However, GC methodologies need a complex derivation step, leading to higher times and costs of analysis[251–256].

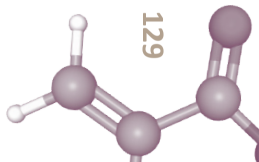
Regarding the sample treatment, according to the acquired knowledge in our laboratory, QuEChERs, in contrast to SPE, speeds up the sample preparation workflow due to the absence of conditioning and equilibration steps, simplifying the extraction procedure. In view of these facts, it is concluded that the suggested LC method allows a quicker and easier analysis of urinary DAPs than previously reported methods based on SPE. In addition, it has adequate LoQs and it was successfully validated with a rigorous and strict QA/QC procedure to guarantee reliable results, being a useful strategy to be applied in routine analysis.

Table 10. Results of the parameters evaluated in the validation of the method (n = 5) for the analysis of six dialkyl phosphates in urine: linearity, accuracy, precision, and specificity.

Analyte	LoQ (ng·ml^{-1})	Linearity		Accuracy (%R)			Precision (%CV)			%S	G-EQUAS 60/2017 results (ng·ml^{-1})					
		R^2	Residuals	LoQ level	Medium level	High level	LoQ level	Medium level	High level		Sample	Our result	Reference value	SD (number of participating laboratories)	|Z|	Evaluation*
DMP	0.5	0.997 – 0.999	< 20 %	117	82	97	3	12	8	15.5	A	14.17	17.02	5.74 (14)	0.5	+
											B	97.34	117.51	15.09 (14)	1.3	
DMTP		0.990 – 0.997		109	94	111	12	17	19	17.5	A	13.57	14.08	4.01 (14)	0.1	+
											B	79.40	90.98	27.07 (14)	0.4	
DMDTP		0.989 – 0.992		115	98	98	4	20	9	18.7	A	4.65	3.45	0.59 (14)	2.0	+
											B	7.92	8.45	1.16 (14)	0.5	
DEP		0.993 – 0.995		116	95	104	4	16	5	10.5	A	13.85	11.25	3.56 (14)	0.7	+
											B	39.75	38.35	6.01 (14)	0.2	
DETP		0.992 – 0.998		109	89	98	12	17	7	21.0	A	78.57	74.89	15.42 (14)	0.2	+
											B	26.12	22.97	3.72 (14)	0.8	
DEDTP		0.989 – 0.997		117	107	107	3	11	11	21.0	A	1.14	0.86	0.20 (13)	1.4	+
											B	3.34	2.92	0.68 (13)	0.6	

*All |Z| were below or equal to 2, and all the validation criteria were met.

LoQ = limit of quantification; %R = percentage of relative recovery (%); %CV: percentage of coefficient of variation (%); %S: percentage of selectivity (%); SD: standard deviation; Medium level = 5 ng·ml^{-1} in urine; High level = 50 ng·ml^{-1} in urine; |Z|: Z-score absolute value



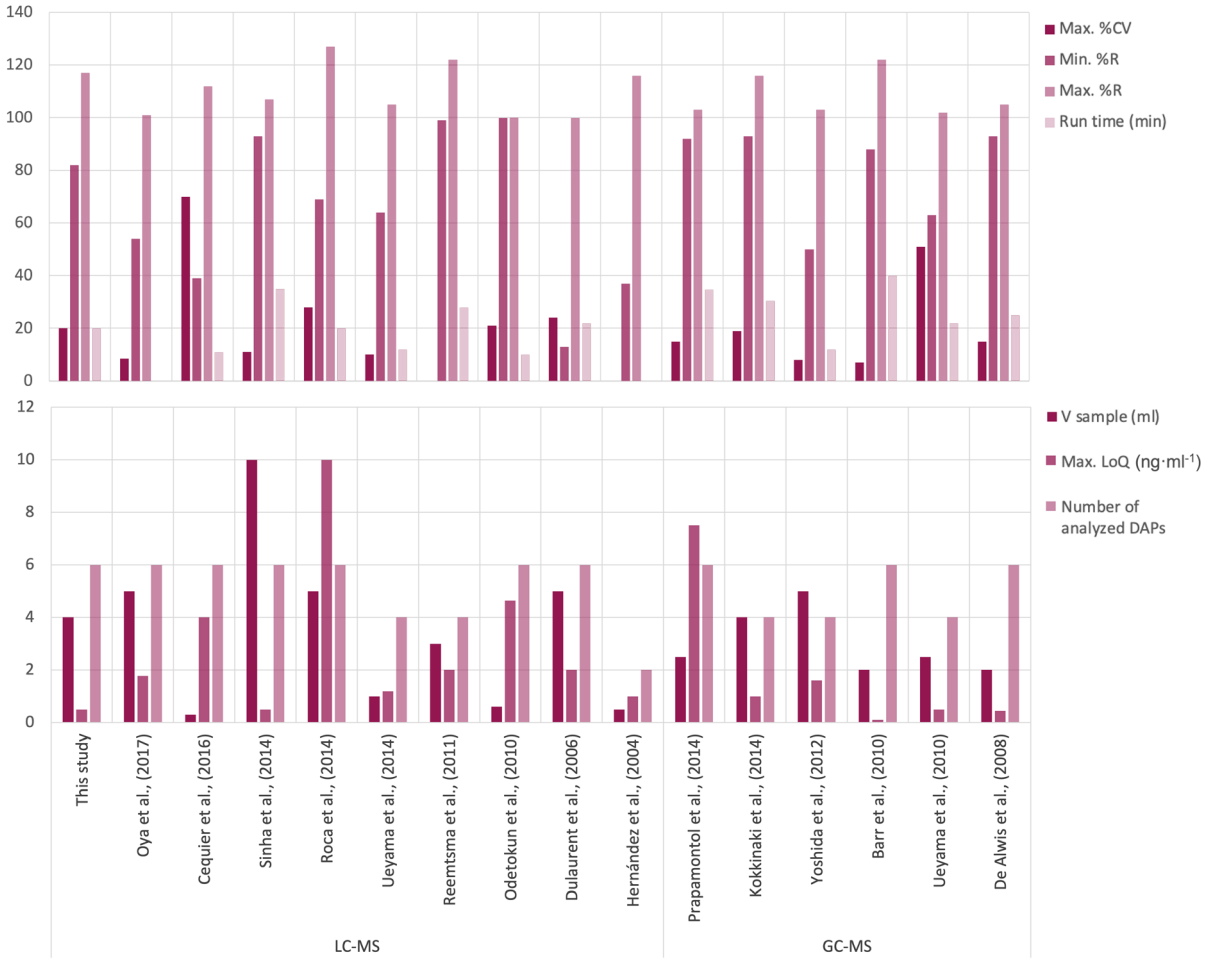


Figure 18. Comparison of existing analytical methodologies based on LC[49,247–250,257–260] and GC[251–256] coupled to MS to analyze urinary DAPs in terms of %CV, %R range (min. and max.), run time of chromatographic analysis (min), necessary volume of sample (ml), max. reported LoQ (ng·ml^{-1}), and total number of analyzed DAPs (max. number = 6). *LC-MS: liquid chromatography coupled to mass spectrometry; GC-MS: gas chromatography coupled to mass spectrometry; DAPs: dialkyl phosphates; %CV: percentage of coefficient of variation; %R: percentage of relative recovery; LoQ: limit of quantification; Max.: maximum; Min.: minimum; V: volume.*

4.1.2 CONCLUSIONS

The developed method is a new and straightforward analytical strategy for simultaneous analysis of six DAPs in human urine: DEP, DETP, DEDTP, DMP, DMTP and DMDTP. DAPs were extracted from urine by QuECHERs, a salting-out method to enhance the extraction process. A procedural LoQ of 0.5 ng·ml^{-1} was achieved for all analytes using LC-MS/MS. No clean-up or derivatization was necessary. This new methodology for detecting and quantifying DAPs is faster, easier and shows better analytical performance compared to previous methodologies. In addition, it was successfully validated and applied to a set of field urines showing its satisfactory applicability as a HBM tool in risk assessment studies.

4.1.3 ARTICLE I.1: *DEVELOPMENT OF A NOVEL METHODOLOGY FOR DETERMINATION OF DIALKYL PHOSPHATES IN HUMAN URINE USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY*

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Development of a novel methodology for determination of dialkyl phosphates in human urine using liquid chromatography-tandem mass spectrometry

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ABSTRACT

In this study a fast and sensitive method was developed for the quantitative determination of six dialkyl phosphates (DAPs) in human urine using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The proposed methodology was based on liquid-liquid extraction (LLE) with diethyl ether and ethyl acetate using $MgSO_4$ and NaCl, followed by liquid chromatography and mass spectrometry detection in the selected reaction mode (SRM) with negative electrospray ionization (ESI-). Detection settings were optimized by design of experiments (DoE). Dibutyl phosphate (DBP) was used as internal standard. Several criteria established in the SANTE/11813/2017 guidance document for pesticide residues and analysis in food and feed were used to validate the suggested method: recoveries (R %) between 70 and 120% and coefficients of variation (CV %) ≤ 20%. The procedural limit of quantification (LoQ) was 0.50 ng mL^{-1} for the six DAPs. The R% and CV% ranges were 82–117% and 3–20%, respectively. In total, 20 spot urine samples from lactating mothers were satisfactorily analyzed by the proposed analytical strategy.

1. Introduction

Nowadays, pesticides are widely used in agriculture, in the household (on plants/gardens and against common insects) and for domestic animals [1]. In the 1970s, organophosphate pesticides (OPs) replaced organochlorine pesticides (OCs) because they are less persistent [2]. OPs such as chlorpyrifos and malathion are the most globally-used synthetic compounds in agriculture. These compounds are also used in private industries, hospitals, schools, private houses and sports stadiums, so the general population is exposed to OPs [2,3]. These pesticides have a greater toxicity than OCs considering that they poison insects and mammals by inhibiting the acetylcholinesterase (AChE) enzyme and disrupting their nervous system [4,5]. OPs are reported to be endocrine disruptors and to produce neurotoxic effects [1]. Thus, considering OPs can contaminate food, air, water and soil, not only the general population but also vulnerable populations such as infants, children, and pregnant and lactating women can be exposed to these environmental chemicals in three ways: dermal absorption (occupational exposure), inhalation (outdoor and indoor air) and ingestion (fruits, vegetables and drinks) [1,2,6–9].

Once human exposure to OPs occurs, they do not accumulate in the human body since they are swiftly metabolized to specific and non-specific metabolites [2]. These dialkyl phosphates (DAPs) metabolites, including dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP), are non-specific metabolites of OPs. At least one of the aforementioned six biomarkers is produced as a result of the metabolism of the OPs, with some exceptions (such as acephate and metamidophos) [10,11]. Considering that DAPs appear at low concentrations in blood, they are usually determined in human urine due to their highly polar characteristic [10,12–16]. The measurement of DAP concentrations provides the possibility to have valuable information about recent cumulative exposure to OPs (1–3 days) [2,4,9,17,18].

In 2016, the European Commission created the European Joint Programme, called HBM4EU (2017–2022), whose objective was "to provide better evidence of the actual exposure of citizens to chemicals and a robust interpretation of human biomonitoring data and the possible impact of chemical exposure on human health" [19]. The HBM4EU project expects to generate comparable data on human

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exposure to chemicals in Europe and identify exposure pathways. The results of this EU project will contribute to the improvement of human health, designing policy measures to reduce exposure to contaminants [20]. Pesticides are included on the second list of priority substances in the HBM4EU project. Therefore, the development of novel and sensitive methods to identify internal exposure to pesticides is necessary.

Several recent studies about biomonitoring of OPs using urinary DAPs as biomarkers in pregnant and lactating women have been described [12,13,17,21–24]. The majority of the exposure studies are based on methods that determine DAPs in urine by gas chromatography (GC) due to its low limits of quantification (LOQs), ranging from 0.01 to 7.5 ng mL^{-1} [3,21,25–31]. However, the sample treatment used in GC methods is complex because it requires a derivation step, leading to long analysis times and not being able to apply it in routine analyses. On the other hand, most of the latest methods based on liquid chromatography (LC) have high LoQs (up to 10 ng mL^{-1}) [9,32–39]. Moreover, previous reported LC methods have bad recoveries (R % < 70% or > 120%)[9,32,33,35,36,38,39] and high coefficients of variation (CV% > 20%) [9,37,38]. Some of these LC methods do not analyze all six DAPs [35,36,39]. A detailed comparison between current methods to analyze DAPs in urine is shown in Table S1.

The aim of this study was to develop a high throughput method to analyze six DAPs (DMP, DMTP, DMDTP, DEP, DETP and DEDTP) in human urine using liquid chromatography coupled to mass spectrometry (LC-MS/MS) determination. The liquid-liquid extraction (LLE) method with a salting-out process was selected and optimized to extract DAPs from urine samples. No pre-treatment or purification steps were necessary, leading to a quick sample treatment. High recoveries and accurate results were achieved. The developed strategy was applied in 20 urine samples from Spanish lactating mothers.

2. Experimental section

2.1. Reagents and chemicals

Acetonitrile (for pesticide residue analysis), water (HPLC grade), hydrochloric acid (HCl) 37% (v/v) and ethyl acetate (for pesticide residue analysis) were supplied by VWR Prolabo (Barcelona, Spain). Diethyl ether (reagent grade, stabilized with 7 ppm of butylated hydroxytoluene, BHT) and ammonium acetate (HPLC grade) were supplied by Scharlau (Barcelona, Spain) and acetic acid was supplied by Merck (Barcelona, Spain). QuEChERS Extract Pouches-Original method (salt packet containing 4 g $MgSO_4$ and 1 g NaCl) and ceramic homogenizers for QuEChERS extraction were obtained from Agilent Technologies (Madrid, Spain).

2.2. Standards and solutions

The six selected DAPs are non-specific metabolites from OPs: DMP, DMDTP and DEP were purchased from Chromlab (Barcelona, Spain); DMTP was from Chiron AS (Trondheim, Norway); DETP was supplied by Cambridge Isotope Laboratories (Massachusetts, USA); and DEDTP was purchased from Sigma-Aldrich (Barcelona, Spain). Dibutyl phosphate (DBP) was supplied by Dr. Ehrenstorfer (Ausburg, Germany) and was used as internal standard (IS). The elemental composition, structure and transitions of DAP metabolites together with the IS are presented in Table 1. Each commercial standard was weighted and dissolved in acetonitrile to obtain a concentration of approximately 200 µg mL^{-1}. The stock solutions were stored in the dark at −20 °C for at least 6 months. Multi-analyte standard solutions containing 100 and 1000 ng mL^{-1} were prepared by diluting the individual stock solutions with acetonitrile. IS intermediate solution of 1000 ng mL^{-1} and a IS working solution of 200 ng mL^{-1} were also prepared containing DBP. Intermediate and working solutions were stored in the dark at −20 °C for at least 3 months. The matrix-matched calibration curve and quality control samples were prepared by spiking synthetic urine at concentrations between 0.5 and 50 ng mL^{-1} with the multi-analyte solutions and IS working solution before the extraction procedure.

2.3. Sample collection and storage

The sample analyses were performed in the Public Health Laboratory of Valencia (Spain). The 20 samples of human urine analyzed in this study are part of a biomonitoring project called BETTERMILK. Urine samples were obtained during 2015 from lactating women living in the Valencian region (Spain) aged between 20 and 45 years. A spot, first- morning urine sample was collected from each participant and kept cool at 4 °C. Samples were transported to the laboratory and properly stored at −80 °C, until analysis, in the

Biomedical and Public Health Research Biobank of the Valencian Community (IBSP-CV Biobank) (PT13/0010/0064), integrated in the Spanish National Biobanks Network and in the Valencian Biobanking Network.

2.4. Sample preparation

After homogenization by shaking the whole sample, an amount of 4 mL of human urine sample was transferred into a 10 mL amber glass centrifuge tube. The sample was spiked with 100 µL of the IS working solution (200 ng ml^{-1}) and homogenized. After that, a volume of 800 µL of HCl 6 M was added to the spiked sample. To decrease urinary turbidity, the mixture was centrifuged at 3724*g* and 15 °C for 10 min. Then, it was transferred into a 50 mL polypropylene tube for extraction. Ethyl acetate (4 mL) and diethyl ether (4 mL) were added, and the mixture was introduced in an ice bath for approximately 10 min to avoid further overheating when salts were added. After the mixture was cooled down, 4 g anhydrous $MgSO_4$ and 1 g NaCl were added in order to increase ionic strength and improve analyte partitioning to organic phase. One ceramic homogenizer was added to avoid salt agglomeration and the tube was mixed by vortex for 1 min. The tube was introduced in the ice bath again and once cold, the mixture was centrifuged at 3724*g* and 15 °C for 10 min. The total organic layer was taken and transferred to a 10 mL glass tube to evaporate under a N_2 stream (30 °C, 7 bar) until dry. The dry residue was then dissolved in 200 µL of ammonium acetate 10 mM:acetonitrile containing 5% of ammonium acetate 200 mM (95:5), placed into a Millipore 0.2 µm Eppendorf and ultra-centrifuged (11,363 g, 2 min and 10 °C). The final extract was transferred into an injection vial and analyzed on the HPLC–MS/MS system. In Fig. 1, the analytical scheme for urinary DAPs determination is shown.

2.5. HPLC-MS/MS analysis

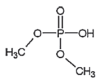
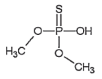
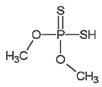
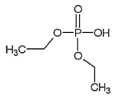
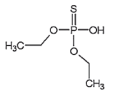
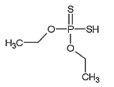
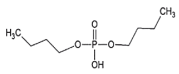
2.5.1. Chromatographic conditions

Chromatographic separation was carried out with the Finnigan Surveyor high-performance liquid chromatography (HPLC) system from Thermo Fisher Scientific (Bremen, Germany) equipped with a Hypersil GOLD HILIC column (2.1 × 100 mm, 3 µm) from Thermo Fisher Scientific (Bremen, Germany). The mobile phases were (A) an aqueous solution of ammonium acetate 10 mM and (B) acetonitrile containing 5% of an aqueous solution of ammonium acetate 200 mM. The gradient was as follows: 0 min 95% A, 2 min 95% A, 8 min 50% A, 12 min 50% A, 13 min 95% A, 20 min 95% A. The flow rate used was 200 µL min^{-1} and the injection volume was 10 µL. Data acquisition was performed by the Thermo Scientific Trace Finder™ 3.2 software.

2.5.2. Mass spectrometry detector settings

Detection of DAPs was performed on the Finnigan TSQ Quantum Ultra detector (San José, CA, USA) equipped with an electrospray ionization source (ESI). Negative ESI mode and selected reaction monitoring (SRM) mode were used for all the analytes. Collision gas pressure was set at 1.5 mTorr. Collision Energies (CEs) for the most

Table 1
Name, acronym, CAS number, structure, elemental composition, transitions and collision energy of dialkyl phosphates (DAPs).

Analyte	Acronym	CAS Number	Structure	Elemental composition	Transitions			CE (V)
					Precursor	Q[a] (m/z)	q[b] (m/z)	
Dimethyl phosphate	DMP	813-78-5		$C_2H_7O_4P$	125.0	63.0	79.0	20[a] (30)[b]
Dimethyl thiophosphate	DMTP	23754-87-2		$C_2H_7O_3PS$	141.0	126.0	96.0	17[a] (22)[b]
Dimethyl dithiophosphate	DMDTP	756-80-9		$C_2H_7O_2PS_2$	157.0	142.0	112.0	18[a] (23)[b]
Diethyl phosphate	DEP	598-02-7		$C_4H_{11}O_4P$	153.0	125.0	79.0	15[a] (30)[b]
Diethyl thiophosphate	DETP	5871-17-0		$C_4H_{11}O_3PS$	169.0	95.0	141.0	18[a] (23)[b]
Diethyl dithiophosphate	DEDTP	298-06-6		$C_4H_{11}O_2PS_2$	185.0	111.0	157.0	22[a] (16)[b]
Dibutyl phosphate (Internal Standard)	DBP	107-66-4		$C_8H_{18}O_4P$	209.0	79.0	153.0	37[a] (15)[b]

[a]Quantification ion.
[b]Confirmation ion.

abundant transitions were optimized for each compound separately using syringe infusion mode. For this purpose, full scan spectrum of each DAP was obtained by direct injection of 1 μg/mL of individual standard solutions to the mass spectrometry detector with a flow rate of 20 μL/min. After that, the chosen precursor ion was fragmented and the two most abundant product ions, used as quantification ion and

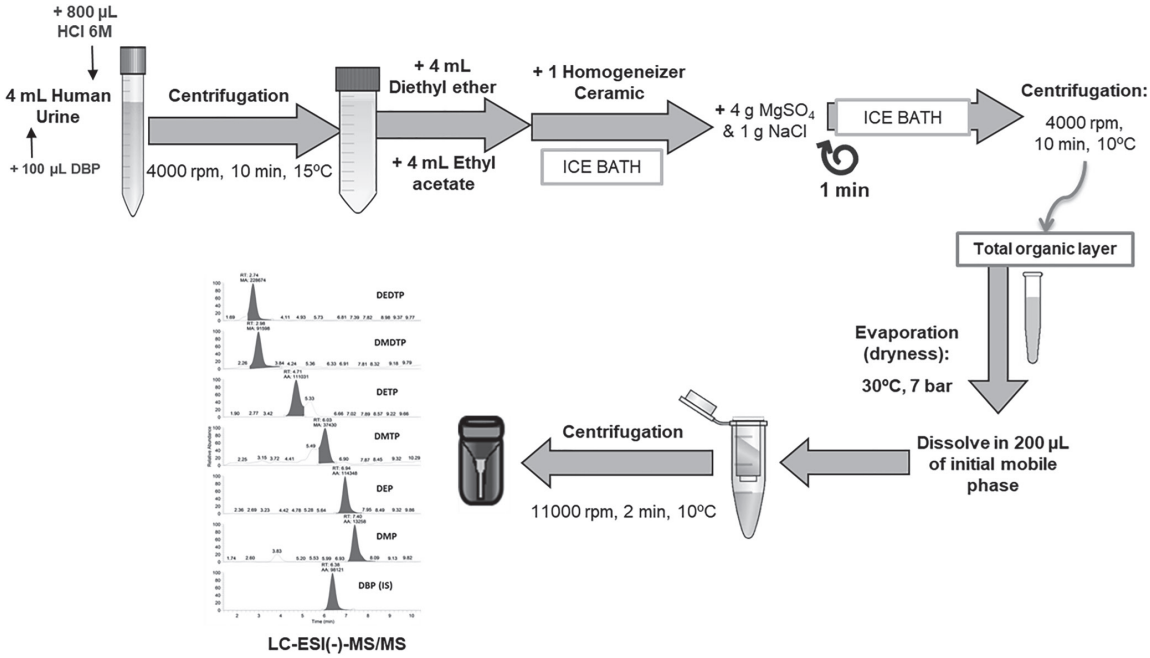


Fig. 1. Analytical scheme for urinary dialkyl phosphates determination by LLE and LC-MS/MS.

confirmation ion, were selected. The CE was optimized using the automated optimization procedure for each pair precursor/product ion. Precursor ions, product ions and the corresponding CEs are shown in Table 1.

2.5.2.1. Optimization of the ESI source parameters. The ion source parameters were optimized by design of experiments (DoE) using the statistical package MINITAB for Windows, Release 14.1 (Minitab Inc., Birmingham, UK). According to prior experiments carried out in our laboratory and the ESI manual provided by the manufacturer, four factors were potentially affecting the ESI efficiency: spray voltage (SV), capillary temperature (CT), sheath gas (SG), and auxiliary gas (AG). Due to the fact that doing a few more experiments was not a limitation in this study, it was preferred to introduce duplicates and points to the center in the experimental designs in order to take into account the variability between runs due to the instrument performance, exceeding what would be strictly necessary in ordinary DoE designs. The final objective was ensuring the validity of the conclusions. In this sense, the DoE consisting of Plackett-Burman and central composite design is a good tool to generate an ordered and optimal sequence of experiments, facilitating the analysis of results and extraction of conclusions in an organized way using standard software (MINITAB).

Firstly, a two-level Plackett-Burman (P-B) design for the four factors was used as a screening step to select those that were more relevant for ESI source optimization. The ranges analyzed in this screening were: SV (2000–5000 V), CT (200–350 °C), SG (5–40 psi) and AG (5–20 a.u.). The selected P-B design was composed of 15 experiments: 12 runs (minimum number of experiments for four factors using MINITAB 14.1) and one center point in triplicate in order to estimate variability between runs.

After evaluating the results of the P-B (Section 3.2.2.2), a central composite design (CCD) was used for a further optimization of CT and SG. This kind of experimental design can build the response surface for each analyte and find the factor settings that maximize the analytical response for all compounds. For this purpose, a CCD with 26 experiments was carried out: 13 runs including 5 center points, 4 cube points and 4 axial points and 2 replicates with an alpha value of 1.414 (default in MINITAB). The ranges analyzed in this step were: CT (150–400 °C) and SG (5–50 psi), extending the range outside the initial settings due to the selected alpha value (> 1). These axial points outside the initial ranges remained at reasonable levels of CT and SG for the MS/MS system.

A multiple regression equation was used to fit the responses observed, including second order and interaction terms. The obtained response surfaces displayed the effect of the two independent variables (CT and SG) on the DAPs responses. Finally, the 'response optimizer' in the MINITAB 14.1 software aided the selection of the factor settings that concurrently optimized the responses of the six DAPs. In this way, a composite desirability that combines the desirability of each analyte into a single value could be maximized, considering that all the responses have the same importance. The optimized settings were: spray voltage, 3000 V; capillary temperature, 400 °C; sheath gas, 50 psi; and auxiliary gas, 10 psi.

2.5.3. Quantification and confirmation criteria

The quantification was performed with the area corresponding to the most abundant transition for each analyte. (Table 1: Quantification ion). The signal-to-noise ratio (S/N) of the peaks should be ≥ 3. According to the SANTE/11813/2017 guidance document, compound identification and confirmation in the urine samples were based on three criteria [40]:

i. No fewer than two product ions for each analyte: one for quantification and one for confirmation (Table 1: Confirmation ion).
ii. The retention time (RT) of the DAPs in the unknown samples should coincide with the RT in the matrix-matched standards with a maximum tolerance of ± 0.1 min in the same batch. A difference larger than ± 0.1 min is allowed when the same deviation occurs with the IS peak.
iii. The relative ion ratio from both product ions in the samples should be within ± 30% of the average of the matrix-matched standards from a same working sequence.

2.6. Matrix effect

The comparison between the response from standard solutions and matrix-matched standards was not carried out in this study because previously reported methodologies based on LC have affirmed that there is an important matrix effect (ME) in urinary DAP determination [9,33,36,38,39]. Therefore, several authors have confirmed that the ESI source is likely due to signal suppression [9,41]. In accordance with the SANTE/11813/2017 guidance document, for multi-analyte methods, it is better to use more than one IS to compensate ME, such as individual isotopically labeled internal standards from each DAP [33]. However, individual ISs are too costly and it is difficult for most laboratories to obtain them. So, DBP was selected as IS due to the fact that it has similar properties as the six selected DAPs and, together with matrix-matched calibration, it compensates ME as much as possible [31,35,38]. Although internal standard addition has been described as the best method to correct the ME [40], it was not chosen because the amount of urine sample was limited and it requires a longer analytical procedure. Furthermore, due to the difficulty finding DAP-free urine, the calibration curve was elaborated using synthetic urine prepared in-house according to the procedure proposed by the CDC [42].

2.7. Method validation

The analytical method validation and the performance criteria were carried out following some points from the SANTE/11813/2017 guide for pesticide residues analysis in food and feed [40]. Linearity, accuracy, precision and specificity were evaluated as follows:

- *Linearity*. Linearity was checked using seven-point calibration curves, spiking the synthetic urine at different concentration levels (from 0.5 to 50 ng mL^{-1}). Analysis of Variance (ANOVA) and $R^2 > 0.95$ were used for assessing the validity of the linear model. Also, the deviation of calculated concentrations of the different calibration standards in the curve from the real concentrations should not differ more than ± 20%.
- *Accuracy (R%) and precision (CV%)*. The accuracy was calculated in terms of relative recovery in each batch by spiking the synthetic urine at three different concentration levels: LoQ, and intermediate and high level of the calibration curve. Recoveries and CVs should be between 70 and 120% and $\leq$20%respectively. Mean recoveries with an associated precision of the method were also evaluated by measuring replicates of each concentration for six different days.
- *Quantification limits*. The procedural LoQ for each compound was established at the lowest validated spiked level meeting the aforementioned criteria for accuracy and precision. Furthermore, for LoQ levels, the S/N ratio must be ≥ 10.
- *Specificity*. The analysis of a reagent blank and a sample blank was also performed in each batch to test contamination and interferences. The response in reagent blank and blank control samples must be < 30% of LoQ level.

2.8. Quality assurance

The Public Health Laboratory of Valencia works following the ISO/IEC 17025 standard, so this new methodology was developed under a highly consolidated quality system. In each analytical batch, quality control (QC) samples were used to check the efficiency of the extraction procedure and to ensure the proper quantification of DAPs in the field

samples. The QC samples were prepared by spiking the synthetic urine at LoQ, and at the intermediate and the highest level of the calibration curve, and were subjected to the same extraction and analytical procedures as the field samples and the calibration curve. Furthermore, proficiency testing materials supplied by the German External Quality Assessment Scheme (G-EQUAS) were analyzed as standard reference materials to support the quality assurance of the method.

2.9. Scientific ethics committee

The study and sampling protocol were approved by the Scientific Ethics Committee of the Valencian Research Centre for Public Health (FISABIO) of the Valencian Government (Dirección General de Salud Pública, DGSP) and the Biomedical Scientific Ethics Committee of the University and Polytechnic Hospital La Fe. Informed Consent, approved by the Ethic Committee, was signed by all participants.

3. Results

3.1. Sample preparation

'Dilute and shoot', LLE and solid phase extraction (SPE) methods were tested for the urine treatment. The results obtained by 'dilute and shoot' were unsatisfactory due to high matrix effects and the signal suppression obtained for all analytes (low responses). Based on a previous publication [37], automatic SPE extractions with Strata X-AW (weak anion exchange) cartridges with various extraction solvents (acetonitrile, acetone, methanol and water) and pH modifiers (formic acid, ammonium and sodium acetate, ammonia and triethylamine) were tested (Table S2). However, these SPE methods were not adequate to measure DAPs, specifically at low levels, because the recoveries were unsatisfactory (R% < 70%) mainly for DMP and DEP. The salting-out based method showed better responses and higher recoveries than SPE extraction throughout the concentration range. According to two previously reported methods [9,38], several extraction solvents (acetonitrile, ethyl acetate, diethyl ether, etc.), QuEChERS based salts (sodium chloride, magnesium sulfate, sodium citrate and citric acid disodium salt) and clean-up sorbents, such as Primary Secondary Amine (PSA) and activated carbon (C*), were tested to choose the best sample preparation (Table S3). Finally, original QuEChERS pouches (1 g NaCl and 4 g $MgSO_4$) and a mixture of diethyl ether:ethyl acetate (1:1) were selected without an additional clean-up step because this extraction procedure provided the highest recoveries for all DAPs and enough interference removal.

3.2. HPLC-MS/MS determination

3.2.1. Chromatographic separation

For DAP separation, two HILIC columns (2.1 × 100 mm, 3 µm) with a different stationary phase surface were checked: a KINETEX HILIC column (unbounded silica) and a Hypersil GOLD HILIC (surface modified with a tertiary amine) column. The KINETEX HILIC column showed wide peaks, mainly for DEP and DMP, and DBP retention time was too high (31.05 min), leading to a long chromatogram (Fig. S1). Thus, the Hypersil GOLD HILIC column was selected since it showed optimal chromatographic resolution in a shorter time. To improve the DAP ionization process, various pHs and modifiers (as ammonium acetate, formic acid and ammonium formate) were also tested in water (mobile phase A) and in acetonitrile (mobile phase B) [35,37,38]. Finally, an aqueous solution of ammonium acetate 200 mM (pH = 6.3) was chosen as the best modifier for both mobile phases. Fig. 2 shows the SRM chromatograms from a standard solution (50 ng mL^{-1}) and a field sample obtained with the optimized chromatographic conditions.

3.2.2. Mass spectrometry detection

3.2.2.1. Preliminary optimization of MS/MS parameters. A preliminary optimization of MS/MS parameters was necessary to maximize the analytical response for each compound. With this aim, syringe infusion mode was used in order to obtain the most abundant precursor ion and product ions, and their corresponding CEs as it is described in Section 2.5.2 (Table 1).

3.2.2.2. Optimization of the ion source settings. The parameters SV, CT, SG and AG of the ESI source were optimized by DoE (P-B and CCD) in order to make the analytical response of DAPs as high as possible. Firstly, a P-B design of 15 experiments was selected to determine the relative influence of these factors on the DAP responses. The conditions of each P-B experiment and the P-B final results (estimated effects and statistical significance at 95% confidence level) are presented in Tables S4 and S5, respectively. In Table S5, two factors with a p-value < 0.05 for almost all DAPs can be observed: CT and SG. Therefore, these parameters were chosen to obtain a more accurate optimization by a CCD. Additionally, estimated effects for AG were negative for all analytes and variable for SV. For this reason, these factors were fixed at low (10 psi) and intermediate (3000 V) levels, respectively.

After the P-B, a CCD was carried out to further optimization of CT and SG. The CCD model created included 26 experiments with an Alpha value of 1.41421 and the results are shown in Table S6. The six response surfaces obtained by MINITAB for each DAP were different (Fig. S2). Thus, it was needed to get a factor setting that simultaneously maximized the desirability for each DAP by using the 'response optimizer' in the MINITAB 14.1 software. The desirability value is 0.0 for the lowest response and 1.0 for the highest response obtained in the CCD experiments. The composite desirability obtained was 0.97 and the factor settings established were a capillary temperature of 400 °C and a sheath gas of 50 psi.

3.3. Validation

For all analytes, the lineal dynamic range was 0.5–50 ng mL^{-1} and the procedural LoQ was 0.5 ng mL^{-1}. Good linearity was achieved for all DAPs, in all cases $R^2 > 0.95$. The obtained recoveries and CVs ranged from 82% (DMP, at medium level) to 117% (DMP and DEDTP, at low level), and from 3% (DMP, at low level) to 20% (DMDTP, at medium level), respectively. No relevant contamination or interferences were found in the reagent or sample blanks. Furthermore, the laboratory used the G-EQUAS/60-2017 quality assurance process as a standard reference material with successful results (Calculated |Z scores| ≤ 2 in all cases). Thus, the proposed analytical method fulfilled all requisites of quality assurance. An intra-laboratory validation of the developed method was performed. Linearity, accuracy and precision at three levels (LoQ, medium and high), and G-EQUAS 60/2017 results are presented in Table 2.

3.4. Comparison with previous methodologies

Table S1 makes a comparison between the characteristics of the current study and other reported methods. In general, the obtained LoQs are lower than those found in previous studies based on liquid chromatography (LC). Only one LC method reported LoQs, ranging from 0.0287 to 0.49 ng mL^{-1}, below ours (0.50 ng mL^{-1}) [34], but it requires a large amount of sample (10 mL), a sample pre-treatment (lyophilization) and a long run-time analysis (35 min). Comparing with other LC-MS methodologies, the developed method shows R% considerably better (between 82% and 117%) than the vast majority, mainly for DEDTP and DMP, whose reported R% are between 13% and 122%. Moreover, the maximum CV% obtained for DEDTP (11%), DMP (12%), DMTP (19%) and DMDTP (20%) are much lower than those observed in other LC methods, which are from up to 24%, 70%, 34% and 30%, respectively. So far, the developed strategy gives the most precise and accurate results of all LC-MS methodologies. On the other hand, the simultaneous analysis of DAPs is usually carried out by GC

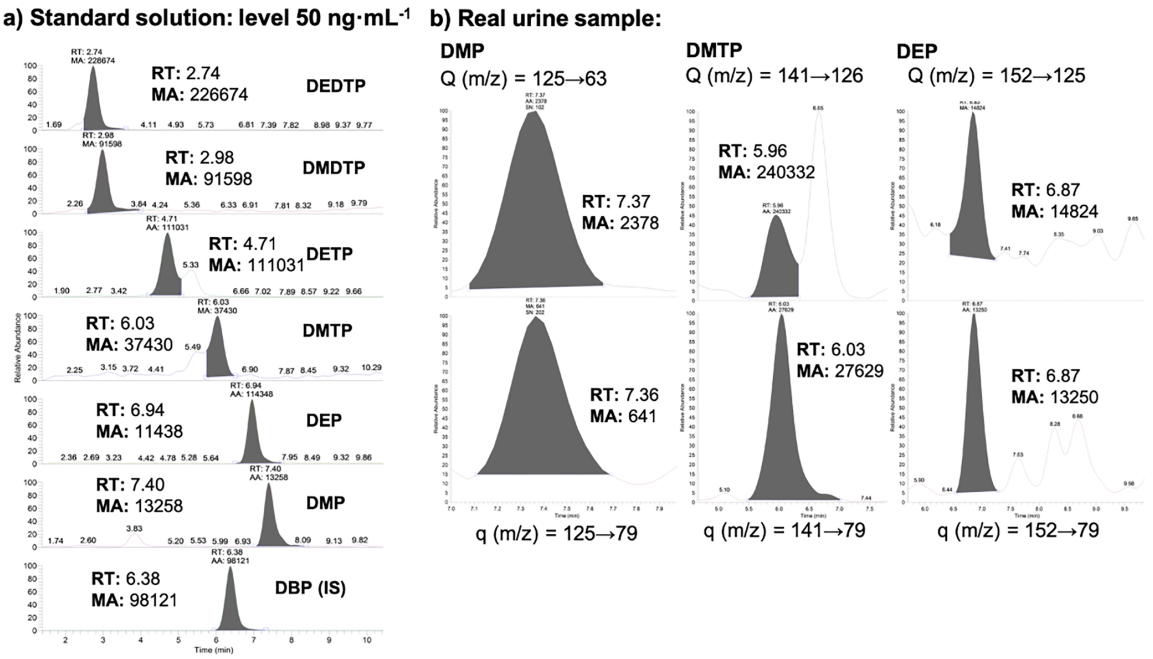


Fig. 2. SRM chromatograms of dialkyl phosphates obtained with the optimized chromatographic conditions from (a) a standard solution (level of 50 ng mL^{-1}) and (b) a field sample. The detected and confirmed DAPs with a concentration > LoQ in the urine sample were DMP, DMTP and DEP.

because this technique allows the achievement of lower LoQs than LC methods [3,27,30,31]. However, GC methods need a complex derivation step and high analysis times [3,27–31]. According to the acquired knowledge in our laboratory, LLE, in contrast to SPE, speeds up the sample preparation workflow due to the absence of conditioning and equilibration steps, simplifying the extraction procedure. So, it is clear that the suggested LC method allows quicker and easier analysis of six DAPs in urine than previously reported methods. In addition, it has adequate LoQs and it has been validated with a rigorous and strict quality assurance procedure to guarantee reliable results. Thus, it could

Table 2
Method validation results for DAPs: linearity, recovery (R%) precision (CV%) at three levels (LoQ, medium and high), and evaluation of G-EQUAS 60/2017. [a], [b]

Compound	Linearity (R^2) (Min–Max)	Concentration level[c] (ng·mL^{-1})	R (%)	CV (%)	G-EQUAS 60/2017 Results (ng·mL^{-1})			Calculated |Z-Score|	Internal Evaluation
					Our result	Ref. value (Tolerance range)	Standard Deviation (SD) (Number of participants)		
DMP	0.997–0.999	0.5	117	3	A 14.17	17.02 (10.27–23.77)	5.74 (14)	0.5	+
		5	82	12	B 97.34	117.51 (84.48–150.54)	15.09 (14)	1.3	+
		50	97	8	**FINAL EVALUATION:** Satisfactory[d]				
DMTP	0.990–0.997	0.5	109	12	A 13.57	14.08 (8.08–20.08)	4.01 (14)	0.1	+
		5	94	17	B 79.40	90.98 (57.41–124.55)	27.07 (14)	0.4	+
		50	111	19	**FINAL EVALUATION:** Satisfactory[d]				
DMDTP	0.989–0.992	0.5	115	4	A 4.65	3.45 (1.83–5.07)	0.59 (14)	2.0	+
		5	98	20	B 7.92	8.45 (5.91–11.07)	1.16 (14)	0.5	+
		50	98	9	**FINAL EVALUATION:** Satisfactory[d]				
DEP	0.993–0.995	0.5	116	4	A 13.85	11.25 (6.87–15.63)	3.56 (14)	0.7	+
		5	95	16	B 39.75	38.35 (28.69–48.01)	6.01 (14)	0.2	+
		50	104	5	**FINAL EVALUATION:** Satisfactory[d]				
DETP	0.992–0.998	0.5	109	12	A 78.57	74.89 (52.90–96.88)	15.42 (14)	0.2	+
		5	89	17	B 26.12	22.97 (17.27–28.67)	3.72 (14)	0.8	+
		50	98	7	**FINAL EVALUATION:** Satisfactory[d]				
DEDTP	0.989–0.997	0.5	117	3	A 1.14	0.86 (0.56–1.16)	0.20 (13)	1.4	+
		5	107	11	B 3.34	2.92 (2.23–3.61)	0.68 (13)	0.6	+
		50	107	11	**FINAL EVALUATION:** Satisfactory[d]				

[a]Limit of quantification (LOQ) = 0.5 ng·mL^{-1} for all analytes.
[b]Lineal dinamic range (LDR) = 0.5–50 ng·mL^{-1} for all analytes.
[c]Concentration in urine.
[d]|Z-Score| ≤ 2.

Table 3
Uncorrected (ng·mL^{-1}) and creatinine-corrected (μg·g^{-1}Cre, in parentheses) levels of DAPs in urine samples fromValencian mothers (n = 20).[a]

Metabolites	DF% (≥LoQ)	GM	Median	P95	Maximum
DMP	65	1.75 (1.59)	1.44 (1.41)	6.50 (6.40)	7.87 (11.49)
DMTP	45	2.83 (2.08)	2.94 (1.51)	6.85 (7.76)	8.27 (8.34)
DMDTP	10	–	–	–	1.34 (1.24)
DEP	100	2.39 (2.15)	2.50 (1.91)	10.97 (10.86)	13.15 (12.06)
DETP	55	2.25 (2.24)	2.74 (1.98)	5.13 (7.40)	5.28 (7.54)
DEDTP	0	–	–	–	–

LoQ = Quantification Limit.
DF = Detection Frequency.
GM = Geometric mean.
P95 = 95 Percentile.
[a]GM, median and P95 calculated only when DF% > 40%.

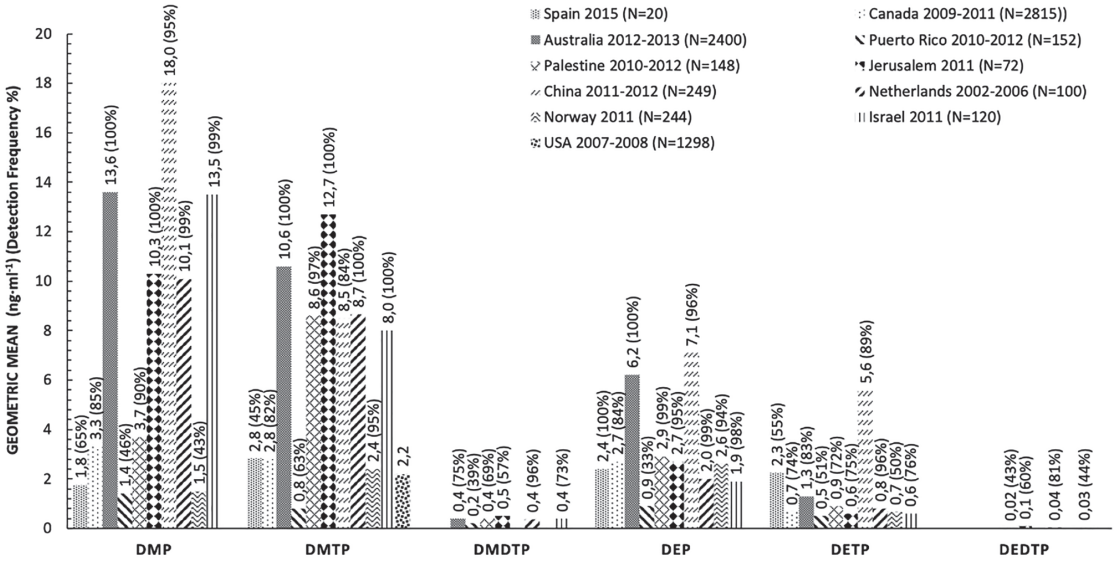


Fig. 3. Urinary levels (geometric means in ng·mL^{-1}) and detection frequencies of dialkyl phosphates reported by several studies from pregnant/lactating women and female population. Comparison between different countries.

be a useful strategy to be applied in routine analysis.

3.5. Analysis of urine samples from lactating mothers

A total of 20 urine samples from lactating mothers were analyzed by the proposed analytical method. The descriptive statistics of unadjusted and adjusted DAP levels found in the urine samples are shown in Table 3. According to previous studies regarding DAP levels in the general population (general women and women of childbearing age) and pregnant/lactating women (Fig. 3), the obtained results were within the reported ranges. The geometric mean was not calculated when the detection frequency was lower than 40%. However, it has to be taken into account that our results were not representative because only 20 samples were analyzed with the purpose to test the developed methodology. For this reason, our reported results could not be comparable with data from large biomonitoring studies. In addition, it should be noted that each study was performed with a different analytical technique/method with different LoQs. On the other hand, geometric means in nmol L^{-1} were transformed to ng mL^{-1} using the conversion factor proposed in the Report on Human Biomonitoring of Environmental Chemicals in Canada to compare them [43]. In a way, DMP and DMTP geometric means are different among populations, being able to distinguish two groups: Australia [44], Jerusalem [13], China [23], the Netherlands [24] and Israel [15] with higher levels (ranges between 10.1 and 18.0 ng·ml^{-1} for DMP and 8.0–12.7 ng ml^{-1} for DMTP), and Spain (this study), Canada [11], Puerto Rico [22], USA [45] and Norway [33] with lower levels (ranges between < 0.47–3.7 ng ml^{-1} for DMP and 0.8–2.8 ng ml^{-1} for DMTP). The Palestine population [13] has intermediate levels of DMP and DMTP. This division of populations could reflect differences and similarities in food consumption habits and pesticide regulation between countries. Similar concentrations were found for the rest of DAPs. Geometric means for DMDTP and DEDTP in other populations are below our LoQs. In general, Chinese and Australian women stand out for their high DAP levels. Regarding detection frequencies, DEP and DMP are the DAPs with higher detection frequencies in nearly all assessed countries, followed by DETP and DMTP. In general, DMDTP and DEDTP are the least detected metabolites.

4. Conclusions

The developed method is a new and straightforward analytical strategy for simultaneous analysis of six DAPs in human urine: DEP, DETP, DEDTP, DMP, DMTP and DMDTP. DAPs were extracted from urine by LLE using $MgSO_4$ and NaCl to enhance the extraction process. A procedural LoQ of 0.5 ng mL^{-1} was achieved for all analytes using

liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS). No clean-up or derivatization was necessary. This new methodology for detecting and quantifying DAPs was successfully validated and it could be applied in routine analysis.

In total, 20 urine samples from lactating mothers were satisfactorily analyzed. The obtained DAP levels agree with those previously reported in studies regarding the general population and pregnant/lactating women. Thus, it was demonstrated that this analytical strategy could be employed as a biomonitoring tool in future risk assessment studies.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2019.121810.

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4.2 BIOMONITORING OF NON-PERSISTENT PESTICIDES IN MOTHERS: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT

4.2.1 RESULTS AND DISCUSSION

A total of 120 women participated in the BETTERMILK study and 119 of them provided a urine sample. Three samples had an abnormal urinary concentration of creatinine (out of the range 30 – 300 mg·dl^{-1}). For this reason, only 116 urine samples with creatinine concentrations between 30 and 240 mg·dl^{-1} (mean of 100 mg·dl^{-1}) were finally included in the present study. Table 11 describes the lifestyle, socio-demographic characteristics and food consumption habits of the target population. The participants had a median age of 34 years, and most of them were employed (84 %), lived in an urban area (80 %), and had university studies (68 %).

Table 11. Socio-demographic characteristics, lifestyle and food consumption habits of the lactating women participating in the BETTERMILK project and included in the analysis of non-persistent pesticide metabolites in urine (n = 116).

Socio-demographic characteristics	n (%)	Mean (minimum – maximum)
Primipara	-	-
Yes	68 (59 %)	-
No	48 (41 %)	-
Age (years)	-	34 (20 – 45)
Missing data	1 (1 %)	
BMI before pregnancy (kg·m^{-2})	-	22 (17 – 35)
Country of birth	-	-
Spain	100 (88 %)	-
Foreign	14 (12 %)	-
Missing data	2 (2 %)	
Place of residence	-	-
Urban	82 (80 %)	-
Rural	21 (20 %)	-
Missing data	13 (11 %)	

Socio-demographic characteristics	n (%)	Mean (minimum – maximum)
Educational level	-	-
Without studies or primary studies	13 (11 %)	-
Secondary studies	24 (21 %)	-
University studies	79 (68 %)	-
Occupational Status	-	-
Employed	96 (84 %)	-
Unemployed	18 (16 %)	-
Missing data	2 (2 %)	
Physical activity	-	-
Frequently (3 or more days/week)	37 (33 %)	-
Occasionally (1 or 2 days/week)	49 (43 %)	-
Never	27 (24 %)	-
Missing data	3 (3 %)	
Smoking habit	-	-
Currently smokes	9 (8 %)	-
Former smoker	48 (41 %)	-
Never have smoked	59 (51 %)	-
Time with the windows open during the day (hours)	-	6.5 (1.0 – 24.0)
Garden or plants at home	-	-
Yes	68 (59 %)	-
No	48 (41 %)	-
Animal deworming in the last month	-	-
Yes	19 (16 %)	-
No	97 (84 %)	-
Closeness to farming activity	-	-
Yes (< 200 m)	35 (30 %)	-
No (> 200 m)	80 (70 %)	-
Missing data	1 (1 %)	-
Use of pesticides at home in the last month	-	-
Yes	60 (53 %)	-
No	56 (47 %)	-

Food consumption frequency (g·month^{-1})	n (%)	Mean (minimum – maximum)
Dairy products	-	13084 (1404 – 43446)
Eggs	-	720 (30 – 1800)
Meat products	-	55689 (738 – 13710)
Fishery products	-	4283 (1040 – 15640)
Vegetables	-	13264 (1181 – 38906)
Fruits	-	16607 (3721 – 84484)
Legumes and cereals	-	4999 (2048 – 26960)
Oils and fats	-	801 (153 – 1541)
Bakery products	-	1244 (172 – 7303)
Miscellany (snacks and sauces)	-	1356 (343 – 29706)
Drinks (ml·month^{-1})	-	44111 (1728 – 76563)
Missing data	6 (5 %)	-

4.2.1.1 URINARY LEVELS OF PESTICIDE METABOLITES

The descriptive analysis of metabolite concentrations is summarized in Table 12. In 99 % of the urine samples, at least one of the analyzed DAPs was detected. In general, DFs of DAPs ranged from 3 % (DEDTP) to 91 % (DEP). The highest GM was obtained for DEP (1.9 ng·ml^{-1}). However, the highest maximum urinary concentration was detected for DMP (14.7 ng·ml^{-1}). DMDTP and DEDTP were the DAPs with the lowest DFs and urinary levels, probably because they are metabolites of a reduced number of OPs[261,262].

Moreover, 98 % of the analyzed urine samples had at least one pesticide-specific metabolite. The metabolite of chlorpyrifos-ethyl, chlorpyrifos-methyl and triclopyr, known as TCPY, was the specific biomarker detected in most samples, with a DF of 85 %, followed by PNP, which has been identified previously as a specific metabolite of parathion and methyl-parathion (banned in the EU), with a DF of 84 %. The metabolite PBA was the only frequently detected Pyr biomarker, with a DF of 65 %, which presented the highest maximum detected urinary level (72.7 ng·ml^{-1}) of all analyzed metabolites. Overall, four non-specific metabolites of OPs (DEP, DETP, DMP, DMTP), two specific metabolites of OPs (TCPY and PNP), and one non-specific metabolite of Pyr (PBA) were the most frequently detected biomarkers in the analyzed urine samples.

As expected, the metabolite of a currently banned herbicide, 2,4,5-T, was not detected in any sample. Conversely, other specific biomarkers of prohibited pesticides were found in some urines, such as ACEP and METHA, IMPY, ATZM, MNP and METM, which are specific metabolites of acephate, diazinon, atrazine, fenitrothion and metolachlor, respectively. The presence of these metabolites in urine may be justified because residues of their parent pesticides, despite being banned, have been detected in several environmental samples in the Valencian region, such as fruit, vegetables and ambient air[135,263].

Table 12. Descriptive statistics of the urinary pesticide metabolite concentrations in ng·ml^{-1} (µg·g Cre^{-1}) for the lactating mothers included in the BETTERMILK study (n =116).[a]

Class of parent pesticide	Metabolite	LoQ (ng·ml^{-1})	DF (%)	P25
Herbicides	2,4-D	0.50	22	< LoQ
	ALAM	0.25	18	-
	ATZM	0.25	4	-
	METM	0.25	3	-
	2,4,5-T	0.50	0	-
Pyrethroids	PBA	0.25	65	< LoQ
	DBCA	2.50	13	-
	DCCA	1.25	4	-
	FPBA	0.50	3	-
OPs (specific biomarkers)	TCPY	0.25	85	0.8 (0.7)
	PNP	0.25	84	0.4 (0.4)
	METHA	0.25	28	< LoQ
	IMPY	0.25	23	< LoQ
	MNP	0.25	22	< LoQ
	MDA	0.50	15	-
	DEAMPY	0.25	13	-
	OMET	0.25	9	-
	DIMET	0.25	8	-
	CMHC	0.25	7	-
	ACEP	0.25	2	-
OPs (non-specific biomarkers)	DEP	0.50	91	1.2 (1.9)
	DMP	0.50	65	< LoQ
	DMTP	0.50	47	< LoQ
	DETP	0.50	51	< LoQ
	DMDTP	0.50	22	< LoQ
	DEDTP	0.50	3	-
	∑DMPs[b,c]	-	-	6 (7)
	∑DEPs[b,d]	-	-	13 (15)
	∑DAPs[b,e]	-	-	24 (27)

LoQ = Quantification Limit; DF = Detection Frequency (% samples > LoQ); P25 = 25th percentile; AM = Arithmetic mean; GM= Geometric mean; P75 = 75th percentile; P95 = 95th percentile; Min. = minimum; Max. = maximum; SD = Standard deviation

[a]Means and percentiles were only calculated in analytes with DF > 40 % and > 20 %, respectively

[b]Units: nmol·l^{-1} (nmol·g Cre^{-1})

[c]∑DMPs = DMP + DMTP + DMDTP

[d]∑DEPs = DEP + DETP + DEDTP

[e]∑DAPs = ∑DEPs + ∑DMPs

P50	AM	GM	P75	P95	Range (Min. – Max.)	SD
-	-	-	< LoQ	0.9 (1.0)	< LoQ – 2.0 (< LoQ – 1.4)	-
-	-	-	-	-	< LoQ – 1.1 (< LoQ – 0.8)	-
-	-	-	-	-	< LoQ – 0.5 (< LoQ – 0.5)	-
-	-	-	-	-	< LoQ – 9.0 (< LoQ – 6.1)	-
-	-	-	-	-	-	-
3.9 (1.5)	5.4 (4.7)	1.4 (1.4)	5.7 (4.6)	18.8 (22.8)	< LoQ – 72.7 (< LoQ – 51.8)	11.1 (8.4)
-	-	-	-	-	< LoQ – 3 (< LoQ – 10)	-
-	-	-	-	-	< LoQ – 9 (< LoQ – 7)	-
-	-	-	-	-	< LoQ – 3.0 (< LoQ – 2.3)	-
2.3 (2.0)	2.6 (2.6)	1.5 (1.5)	3.3 (3.2)	7.9 (7.5)	< LoQ – 16.8 (< LoQ – 18.8)	2.8 (2.9)
1.1 (0.9)	1.6 (1.4)	0.8 (0.8)	1.9 (1.6)	5.4 (3.4)	< LoQ – 23.9 (< LoQ – 22.6)	2.6 (2.3)
-	-	-	0.3 (0.3)	0.9 (1.2)	< LoQ – 5.1 (< LoQ – 3.3)	-
-	-	-	0.3 (0.3)	1.5 (1.4)	< LoQ – 4.7 (< LoQ – 5.8)	-
-	-	-	< LoQ	2.2 (2.1)	< LoQ – 11.6 (< LoQ – 8.3)	-
-	-	-	-	-	< LoQ – 12 (< LoQ – 6)	-
-	-	-	-	-	< LoQ – 3 (< LoQ – 4)	-
-	-	-	-	-	< LoQ – 1.6 (< LoQ – 1.5)	-
-	-	-	-	-	< LoQ – 6 (< LoQ – 4)	-
-	-	-	-	-	< LoQ – 1.8 (< LoQ – 2.6)	-
-	-	-	-	-	< LoQ – 20 (< LoQ – 15)	-
1.9 (2.1)	2.6 (2.7)	1.9 (1.9)	3.5 (3.3)	6.7 (7.1)	< LoQ – 13.2 (< LoQ – 15.7)	2.2 (2.3)
1.2 (1.0)	1.4 (1.4)	0.9 (0.9)	1.7 (1.8)	3.3 (3.7)	< LoQ – 14.7 (< LoQ – 6.3)	1.8 (1.2)
< LoQ	1.2 (1.1)	< LoQ	1.5 (1.3)	4.4 (4.3)	< LoQ – 10.4 (< LoQ – 8.7)	1.8 (1.6)
0.5 (0.5)	1.7 (1.9)	0.6 (0.6)	3.0 (2.9)	5.2 (6.0)	< LoQ – 12.6 (< LoQ – 24.1)	2.1 (3.0)
-	-	-	< LoQ	1.8 (2.0)	< LoQ – 4.2 (< LoQ – 7.2)	-
-	-	-	-	-	< LoQ – 0.5 (< LoQ – 0.5)	-
12 (13)	19 (19)	12 (12)	22 (23)	65 (49)	2 – 119 (1 – 103)	21 (18)
23 (23)	27 (29)	21 (21)	33 (36)	61 (62)	4 – 105 (2 – 244)	19 (27)
38 (40)	46 (47)	38 (38)	56 (57)	112 (98)	7 – 173 (4 – 258)	31 (35)

In Figure 19a to 19c, PNP, TCPY, ∑DAPs and PBA urinary levels found in this research are compared to those levels reported in other studies with adults, pregnant and breastfeeding women from different countries. The concentrations of ∑DAPs presented in this study were lower than those reported in other countries, mainly in The Netherlands[264] with a GM almost five times higher than in the present study (Figure 19a). In general, TCPY and PNP concentrations in this study fell within the range of values observed in the rest of the populations around the world (see Figures 19b and 19c, respectively). However, it should be noted that Spain reported the highest GM in the case of urinary PBA, indicating a higher exposure to Pyr than in other countries (Figure 19d). Comparing the results of the present research with those from two studies carried out in Spain, one on pregnant women (2003 – 2006)[265] and the other one on general population[266], several differences could be observed. On one hand, levels of ∑DAPs were higher in the urine of women collected during 2003 – 2006 than those collected in 2015 for this study (Figure 19a). On the contrary, urinary TCPY and PNP concentrations seemed to increase from 2003 – 2006 to 2015. On the other hand, it should be noted that the study performed on the Spanish general population[266] reported higher levels of TCPY, PNP and PBA than the target population under study (see Figures 19b to 19d). Changes and differences in the type of pesticides used over time, targeted pests in the study areas, season of sample collection, place of residence and lifestyles of the participants are some of the exposure determinants that could lead to these variations among studies.

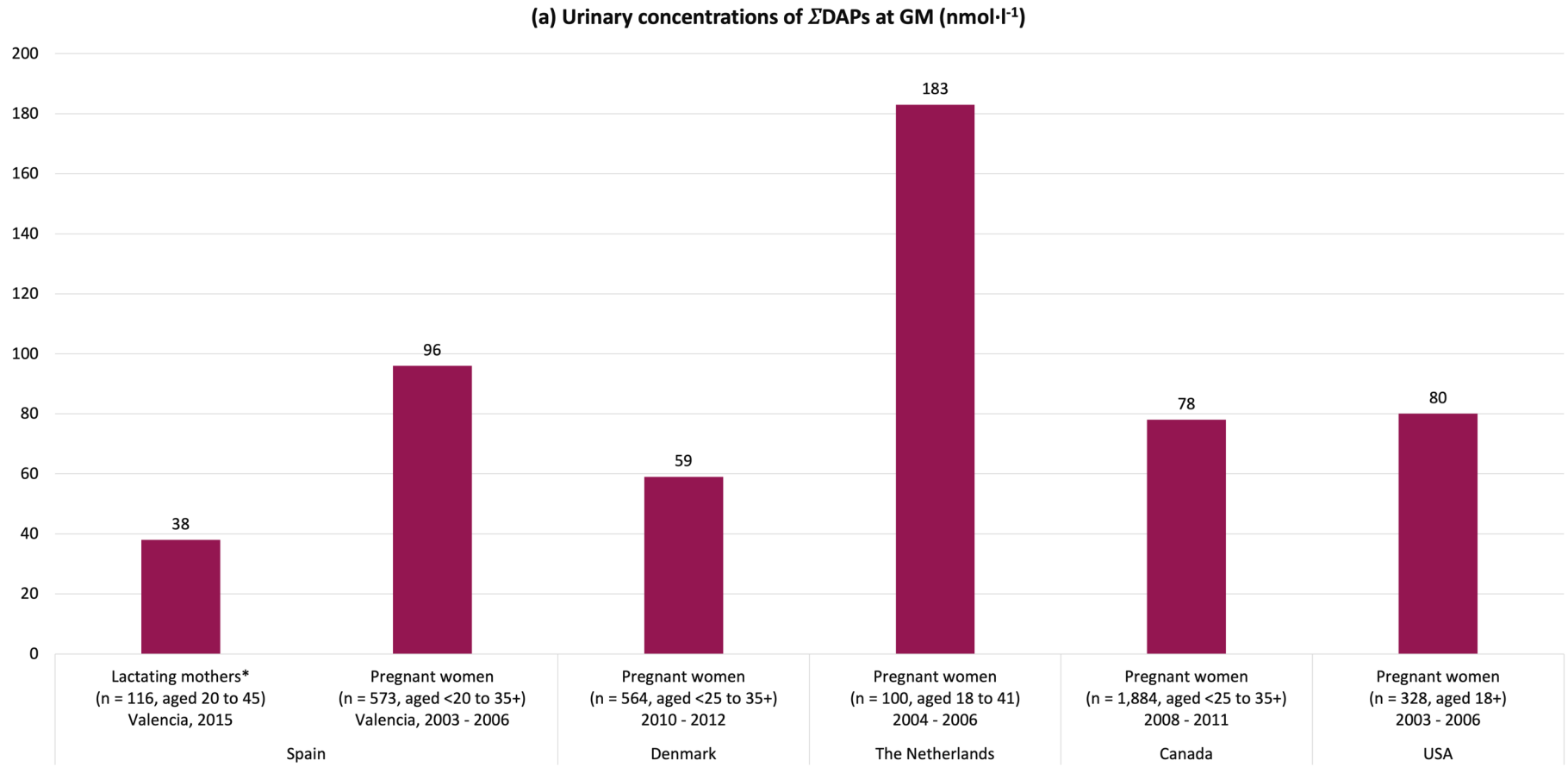

Figure 19a. Comparison of existing HBM data of urinary ∑DAPs (nmol·l^{-1}) in mothers and pregnant women at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain[179], Denmark[267], The Netherlands[264], Canada[268] and USA[269]. **Present study; GM: geometric mean; '+' means 'older than'.*

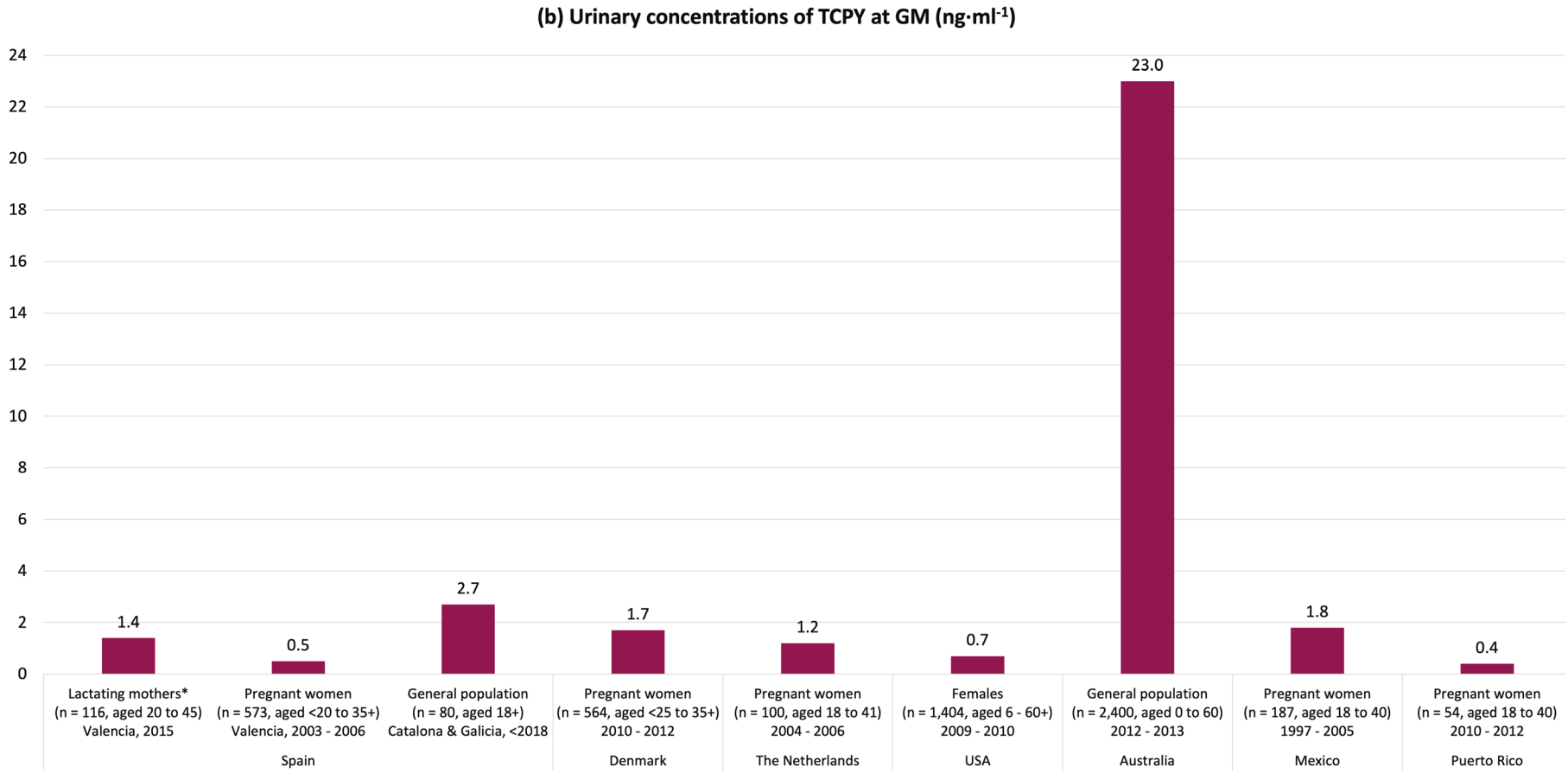

Figure 19b. Comparison of existing HBM data of urinary TCPY (ng·ml^{-1}) in mothers, pregnant women, general population and females at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain[265,266], Denmark[267], The Netherlands[264], USA[261], Australia[270], Mexico[271], and Puerto Rico[272]. **Present study; GM: geometric mean; '+' means 'older than'.*

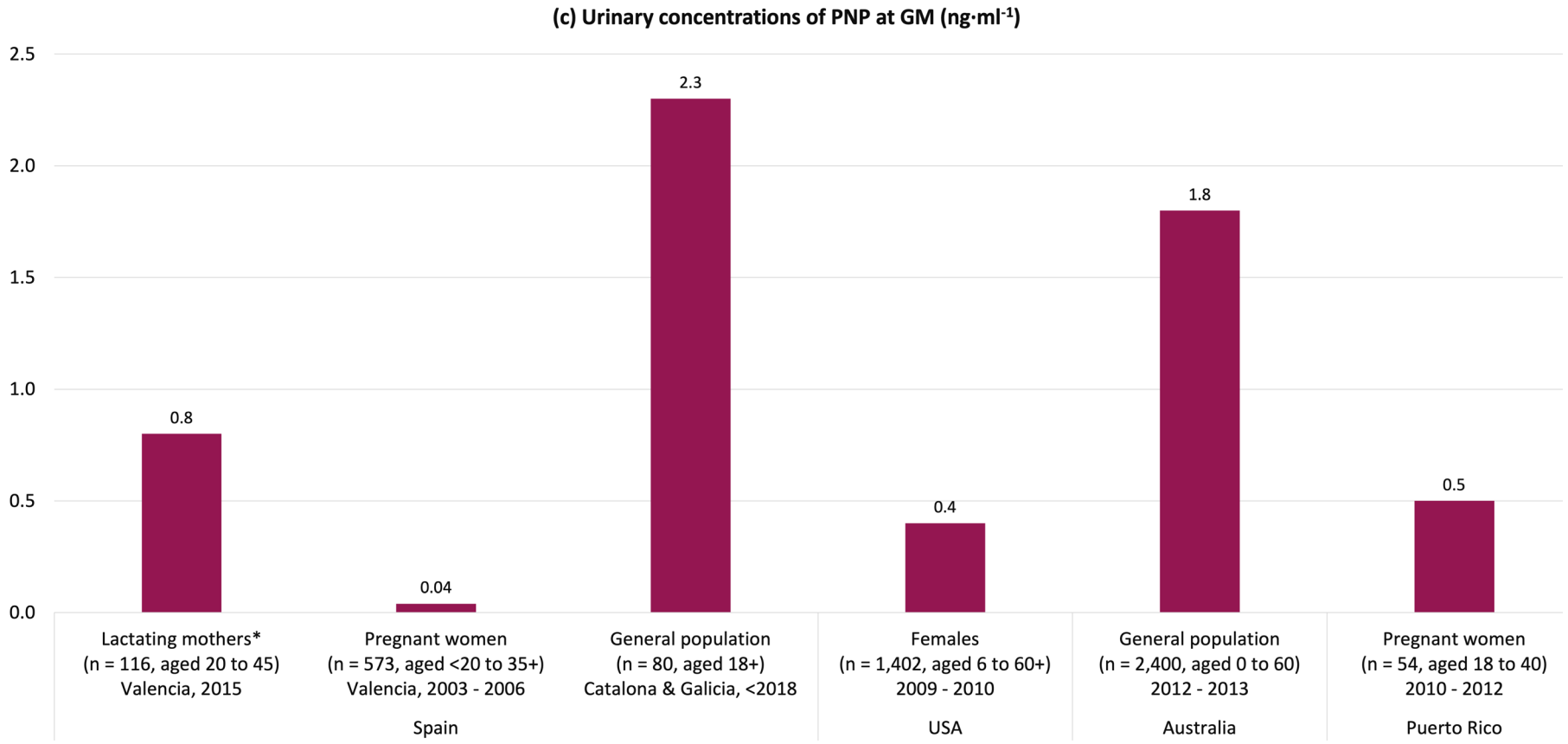

Figure 19c. Comparison of existing HBM data of urinary PNP (ng·ml^{-1}) in mothers, pregnant women, general population and females at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain[265,266], USA[261], Australia[270] and Puerto Rico[272]. **Present study; GM: geometric mean; '+' means 'older than'.*

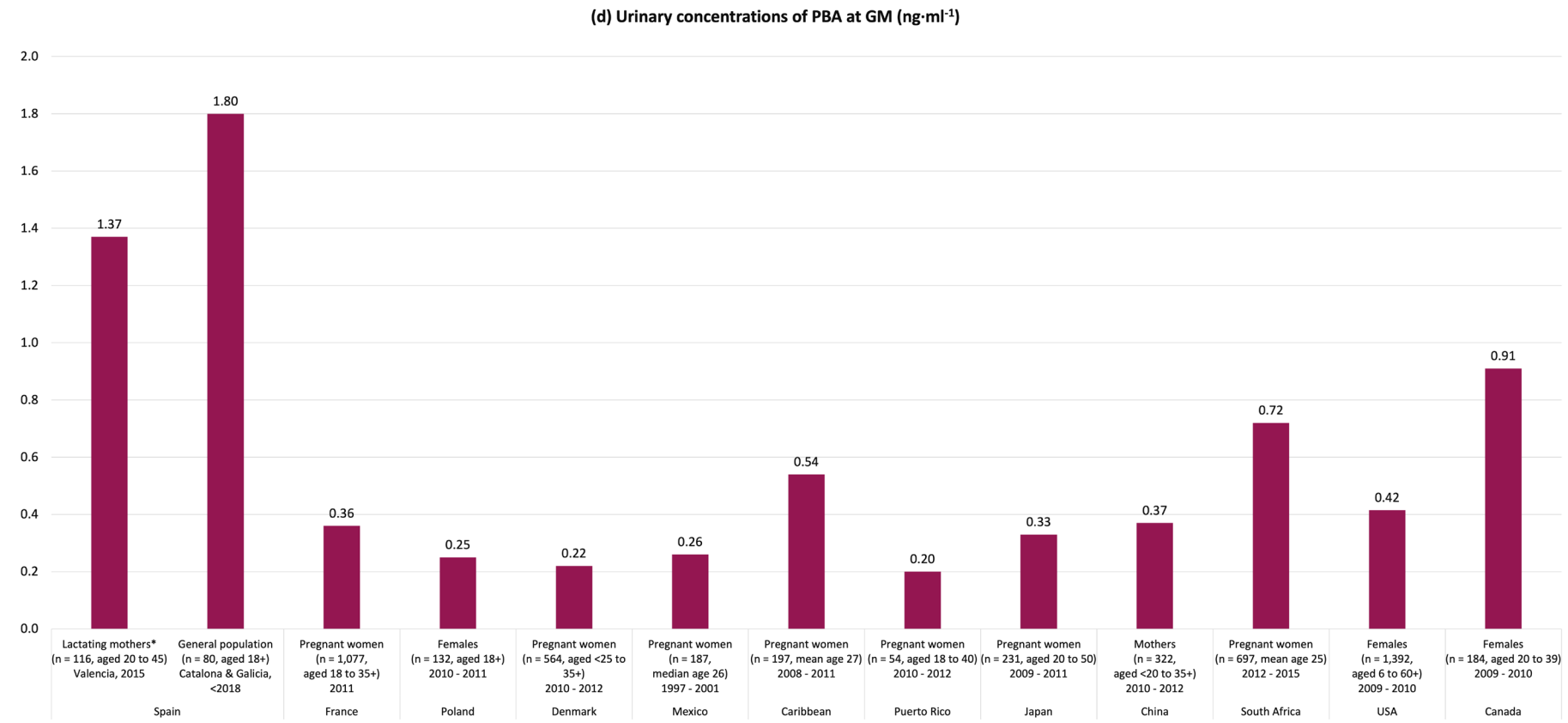

Figure 19d. Comparison of existing HBM data of urinary PBA (ng·ml^{-1}) in mothers, general population, pregnant women and females at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain[266], France[273], Poland[274], Denmark[267], Mexico[275], Caribbean countries[276], Puerto Rico[272], Japan[277], China[278], South Africa[279], USA[261], and Canada[262]. **Present study; GM: geometric mean; '+' means 'older than'.*

4.2.1.2 STATISTICAL ANALYSIS

A Spearman correlation matrix was obtained to explore the relationship between the most detected pesticide biomarkers (∑DAPs, PNP, TCPY and PBA). As a result, a strong correlation was found between TCPY and ∑DAPs (correlation coefficient 0.253, $p < 0.01$), and TCPY and ∑DEPs (correlation coefficient 0.238, $p < 0.05$), showing that their presence in the analyzed urine samples could be due principally to chlorpyrifos-ethyl exposure. Moreover, the positive correlation of PBA with TCPY (correlation coefficient 0.275, $p < 0.01$) might be related to the fact that Pyr and chlorpyrifos are usually applied to the same type of crops[280]. These pesticides are some of the most widely used insecticides in Spain, especially in fruits, cereals and vegetables[280]. Furthermore, residues of chlorpyrifos-ethyl and some Pyr, such as cypermethrin and deltamethrin, were found in some fruit and vegetables samples from Spain in levels above the Maximum Residue Levels in 2017[281]. In addition, chlorpyrifos-ethyl was the third most detected pesticide residue in food in the Valencian region in 2015, and more than 3.5 % of the analyzed food samples tested positive for cypermethrin and deltamethrin in 2015[263]. Moreover, chlorpyrifos-ethyl was detected in 29 % of air samples analyzed in the Valencian region from 2008 to 2014[135]. Thus, the wide use of this combination of pesticides and their presence in similar environmental samples justify the correlation between internal exposure levels of TCPY and PBA in the participants under study. For PNP, significant correlations were found with ∑DMPs (correlation coefficient 0.201, $p < 0.05$) and PBA (correlation coefficient 0.197, $p < 0.05$), but there is no explanation for these findings.

Associations of urinary levels of ∑DAPs, PNP, TCPY and PBA with possible influencing factors (socio-demographic variables, food consumption frequencies and parameters related to the exposure to pesticides) were also statistically explored. Figure 20 shows the standardized coefficients of the regression models built to assess the relationship among metabolite levels and potential predictors of exposure, with their corresponding 95 % confidence intervals (CIs). Overall, the fitted regression models were weak, accounting for 4 – 42 % of the variance (R^2). Surprisingly, no associations were found between fruit or vegetable consumption and urinary levels of pesticide biomarkers. This fact could be explained because differences in the preparation/treatment of these products in the participants' meals could influence the final pesticide concentration due to degradation, volatilization or extractions of the pesticide residues[282].

According to Figure 20, mothers with higher BMI had lower urinary concentrations of TCPY. Similar results were previously reported by Cequier et al. (2017), who found higher levels of DAPs in woman with lower BMI due to a high fruit consumption[282]. In contrast to these findings, was no association between healthy dietary patterns (high consumption of fruits or vegetables) and BMI was observed here, so no conclusions can be obtained with this information alone. In addition, participants living near farming activities (< 200 m) had significantly higher levels of TCPY in urine

(11.1 nmol·l^{-1}) compared with those living far from these areas (8.3 nmol·l^{-1}). Similarly, women who had a terrace with plants, garden or similar in their houses presented higher urinary levels of DAPs (48.4 nmol·l^{-1}) than women living in houses without garden/plants (36.9 nmol·l^{-1}). These findings are most likely due to the use of OPs in these areas or nearby. For PBA, no significant associations were observed.

Until now, PNP has been considered as a biomarker of parathion and methyl-parathion insecticides, but both substances were banned in the EU in the early 21st century and have not been detected recently in any food or air sample in the Valencian region[135,263]. Moreover, according to the National Institute of Safety, Health and Welfare at Work from Spain, urinary PNP can also be found after exposure to nitrobenzene[283], a residue from motor vehicle fuels and smoking processes, which is also used as a raw material to produce several commercial items, such as aniline, solvents, lubricant oils, colorants, and medicines[284,285]. According to the regression model of PNP, women who lived in urban areas and/or kept the windows open more hours during the day had higher levels of this metabolite in their urines. Therefore, its presence in urine could be due principally to inhalation as route of exposure. Taking all this into account, although many studies consider PNP as a specific metabolite of parathion and methyl-parathion, the origin of this metabolite in urine is still unclear, possibly owing to exposure to other compounds. However, no further conclusions can be drawn from the obtained results.

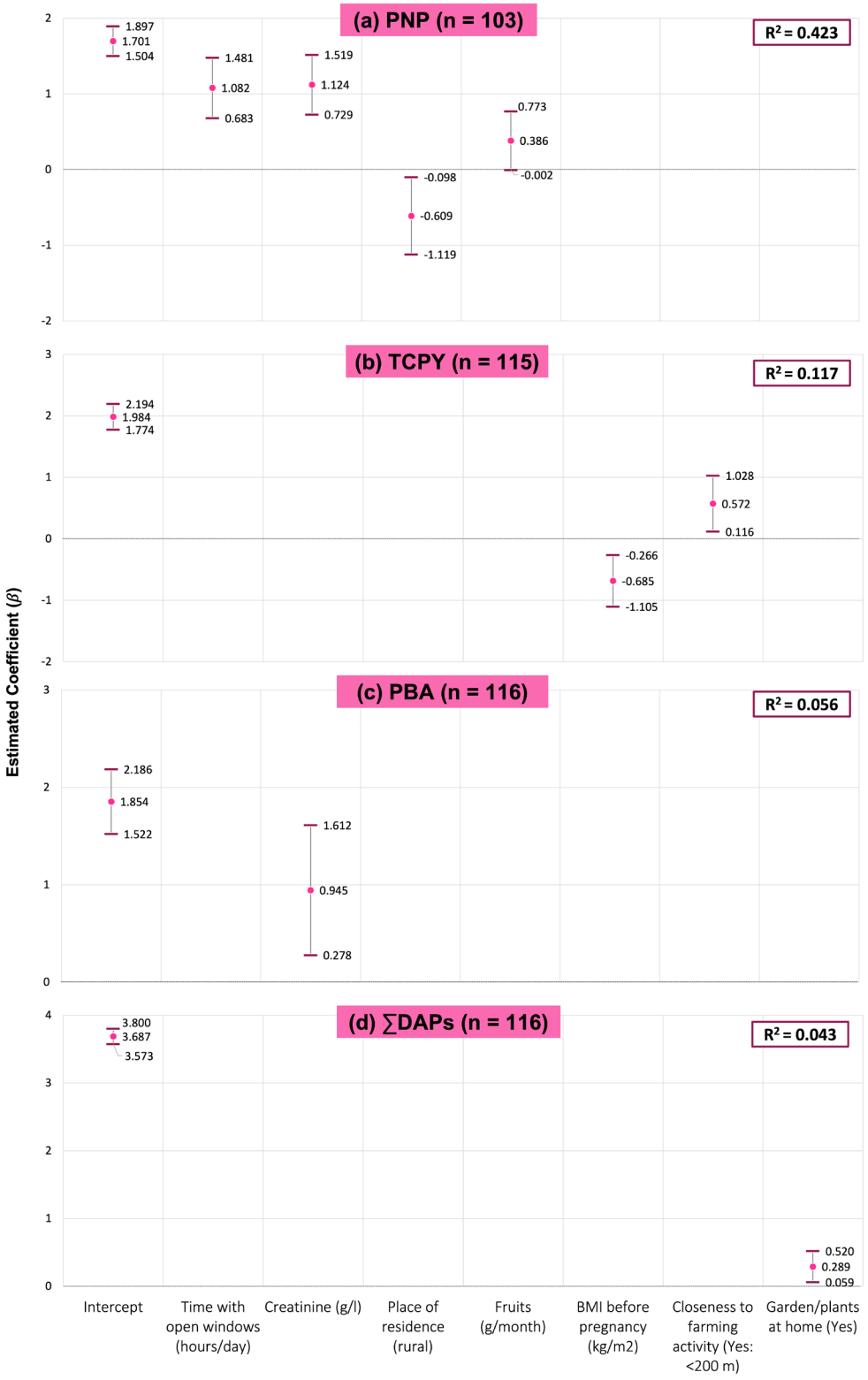

Figure 20. Linear regression models fitted for the log-transformed (a) PNP, (b) TCPY, (c) PBA, and (d) ∑DAPs concentrations (only participants with a completed questionnaire were considered, total number shown within parenthesis), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2) for the mothers of the BETTERMILK project.

4.2.1.3 RISK ASSESSMENT

For the interpretation of the obtained levels in a risk assessment context, EDIs for parathion, chlorpyrifos, dimethoate and deltamethrin were calculated. However, considering that PNP is not only a metabolite of parathion, but that it could come from other substances, the EDI and HQ for parathion should be interpreted with caution. EDI at GM level for dimethoate showed the lowest value of all evaluated pesticides (0.09 μg·kg bw^{-1}·day^{-1}). The highest EDI_{GM} was obtained for chlorpyrifos-ethyl (0.40 μg·kg bw^{-1}·day^{-1}) using ∑DEPs as non-specific biomarker. Deltamethrin resulted in the highest EDI_{P95} (4.73 μg·kg bw^{-1}·day^{-1}), since it had the lowest F_{UE} and its metabolite (PBA) had the highest maximum level found in urine samples. For this reason, deltamethrin is by far the pesticide to which the Spanish mothers seem to be more exposed.

In general, EDI_{P95} for chlorpyrifos (1.14 μg·kg bw^{-1}·day^{-1}) and deltamethrin observed here are higher than those reported in a study regarding external exposure to pesticides through fruit and vegetable consumption in Spanish adults[286], especially for deltamethrin. This fact can be explained because the risk assessment performed in the present study considered not only (dietary) ingestion, but also other routes of exposure. Furthermore, calculated EDIs for parathion (ranging from 0.12 to 0.81 μg·kg bw^{-1}·day^{-1}) and dimethoate (ranging from 0.09 to 0.44 μg·kg bw^{-1}·day^{-1}) are similar to those obtained for pregnant women in a recent study concerning internal exposure to OPs[142]. However, it should considered that humans could be exposed directly to pesticide metabolites due to the degradation of the parent compound in environmental media (food, air, water, etc.). Consequently, this fact may lead to an overestimation of the exposure to the parent pesticide in the present study[44].

Figure 21 shows the HQ for each pesticide and the HI for OPs using GM and P95 data. To calculate the HI for OPs, individual HQs obtained for chlorpyrifos based on ∑DEPs levels and for dimethoate based on ∑DMPs concentrations were added, representing the overall exposure to this pesticide family since DAPs are common metabolites of most OPs. Deltamethrin was not included in the calculation of this HI because it does not have the same MoA as OPs. As it can be seen in Figure 21, similar HQs were found for all pesticides when the GM is considered, ranging from 0.01 (chlorpyrifos-ethyl based on TCPY levels) to 0.05 (dimethoate). Higher differences were found at P95, in which HQs are between 0.07 (chlorpyrifos-ethyl based on TCPY levels) and 0.47 (deltamethrin).

Overall, the highest HQ at P95 was found for exposure to deltamethrin, as it has already been seen in a previous publication[286], followed by dimethoate, whose ADI is the lowest. Compared to the HQ results from the Centers for Disease Control and Prevention (CDC)[287], the Spanish population under study seems to be more exposed to deltamethrin than the American population. However, it should be considered that pesticides applied in Spain can be different from those applied in other parts of the world. Regarding chlorpyrifos, despite being one of

the most used pesticides in Spain[280] and its residues being found in some Spanish fruit and vegetables above the Maximum Residue Levels[281], this pesticide showed very low HQ values in this study. Likewise, the HI for OPs observed here are in the range of HI values reported in other populations of pregnant women from different countries[142].

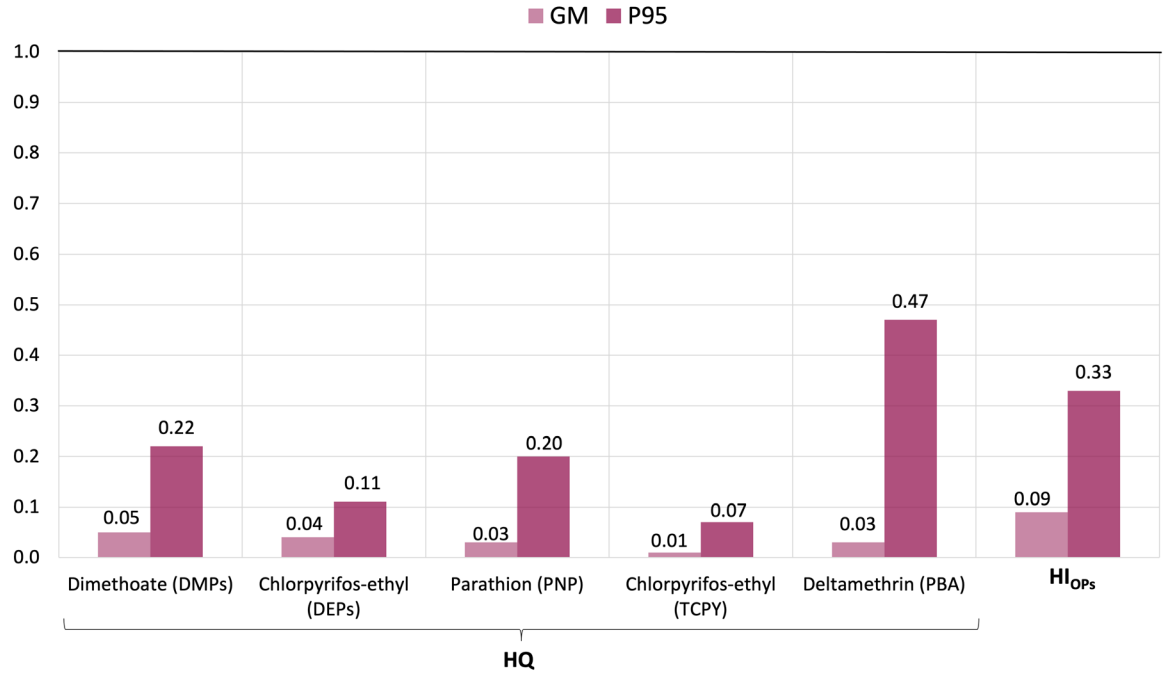

Figure 21. Results of the risk assessment at GM and P95 distribution levels for dimethoate, chlorpyrifos-ethyl, parathion and deltamethrin in the mothers of the BETTERMILK study: HQ for individual pesticides and HI for OPs (sum of HQ for DMPs and DEPs). *GM: geometric mean; P95: 95th percentile; HQ: hazard quotient (dimensionless); HI: hazard index (dimensionless); OPs: organophosphates.*

All calculated HQs and HI were below one. Thus, results of the risk assessment suggested that there is a relative low health risk due to the exposure to these pesticides in the Spanish mothers under study. However, it must be considered that synergies between OPs and Pyr were not considered in this study, leading to the underestimation of the real risk of exposure to the Pyr-OPs mixture. In addition, active substances are periodically reassessed to renew their approval, so new potential health impacts, which can lead to new HBGVs, can be described afterwards. For example, new evidence of neurodevelopmental and reproductive toxicity have been attributed to exposure to chlorpyrifos in humans. Therefore, in view of the extensive use of this pesticide, EFSA considered that the approval criteria which are applicable to human health, as laid down in Article 4 of Regulation (EC) No 1107/2009, were not met and consequently, and the use of chlorpyrifos has been recently banned in Europe (2019)[121,288].

In conclusion, due to the extensive use of pesticides in Spain, the biomonitoring of these substances should continue for a better assessment of population exposure over time, mainly the most sensitive populations groups, such as children and pregnant women.

4.2.2 CONCLUSIONS

In the present study, six non-specific metabolites of OPs and 20 specific metabolites of OPs, Herb and Pyr insecticides were analyzed in urine samples from 116 Spanish lactating mothers. The pesticide metabolites with the highest DFs were DEP, TCPY, PNP and PBA, with GMs of 1.9 $ng \cdot ml^{-1}$, 0.8 $ng \cdot ml^{-1}$, 1.5 $ng \cdot ml^{-1}$ and 1.4 $ng \cdot ml^{-1}$, respectively. Based on the obtained concentrations, the study population has similar levels of OP metabolites to other populations around the world, but higher levels of PBA. The closeness to farming activities, the place of residence and the presence of garden or plants at home seemed to be important contributors to the exposure to these substances. EDI values show that the mothers under study are potentially more exposed to deltamethrin and chlorpyrifos compared to other pesticides. The resulting biomonitoring data was interpreted in a risk assessment context comparing calculated EDIs with the corresponding ADI values. In view of the resulting HQs and HI within a conservative scenario (P95), none of these pesticides exceeded the safety limit, and therefore a relatively low health risk for these lactating women living in Valencia is considered.

4.2.3 ARTICLE I.2: *BIOMONITORING OF NON-PERSISTENT PESTICIDES IN URINE FROM LACTATING MOTHERS: EXPOSURE AND RISK ASSESSMENT*

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Biomonitoring of non-persistent pesticides in urine from lactating mothers: Exposure and risk assessment

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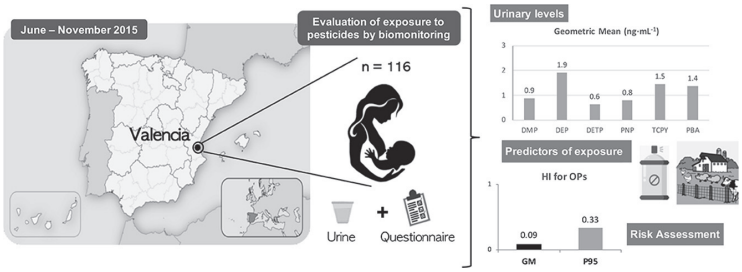
HIGHLIGHTS

- 26 pesticide biomarkers were analyzed in 116 urine samples from lactating women.
- The highest detection frequency (91%) and GM (1.9 μg/L) were obtained for DEP.
- Place of residence and the use of pesticides are major predictors of OP exposure.
- Spanish mothers were more exposed to pyrethroids than those from other countries.
- Risk assessment for OPs and pyrethroids showed hazard quotients lower than 1.

GRAPHICAL ABSTRACT



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ABSTRACT

The aim of the present study was to assess the exposure to pesticides in urine from Spanish lactating mothers (n = 116). Six nonspecific (dialkyl phosphates) and 20 specific metabolites of organophosphate pesticides (OPs), herbicides and pyrethroids were analyzed. The most frequently detected biomarkers were diethyl phosphate, p-nitrophenol, 3,5,6-trichloro-2-pyridinol and 3-phenoxybenzoic acid, whose geometric means were 1.9 ng·mL^{-1}, 0.8 ng·mL^{-1}, 1.5 ng·mL^{-1} and 1.4 ng·mL^{-1}, respectively. Herbicide metabolites were the least frequently detected biomarkers with detection frequencies between 0% (2,4,5-Trichlorophenoxyacetic acid) and 22% (2,4-Dichlorophenoxyacetic acid). Multiple regression analyses showed that the closeness to a farming activity, the place of residence and the presence of garden/plants at home were some of the most important contributors to urinary levels of pesticide metabolites. Estimated daily intake (EDI), hazard quotient (HQ) and hazard index (HI) were obtained in order to interpret urinary levels of the most frequently detected pesticide metabolites in a risk assessment context. The highest EDIs were obtained for chlorpyrifos (0.40–1.14 μg·kg bw^{-1}·day^{-1}) and deltamethrin

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(0.34–4.73 μg·kg bw^{-1}·day^{-1}). The calculated HQ for chlorpyrifos, dimethoate, parathion and deltamethrin ranged from 0.01 to 0.47, and HI for OPs ranged from 0.09 to 0.33 showing that apparently there were low health risks due to the exposure to these pesticides in this group of Spanish breastfeeding women.

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1. Introduction

Organophosphate insecticides (OPs), synthetic pyrethroids and some herbicides are the most widely used pesticides in the world (Lewis et al., 2015; Zhang et al., 2013). These chemical products are commonly used in agricultural fields, residential areas (e.g. lawns, gardens and parks) and also indoors (Dalsager et al., 2018; Heffernan et al., 2016).

Pesticides are toxic not only to target organisms, but also to other aquatic and terrestrial species, such as humans. The majority of pesticides have genetic, epigenetic, oxidative stress and endocrine-disrupting effects in humans (Sabarwal et al., 2018). Human exposure to pesticides is associated with a broad range of health disorders: different types of cancer (breast, colorectal, prostate, etc.), neurodegenerative Parkinson's and Alzheimer's disease, and reproductive and respiratory disorders (Sabarwal et al., 2018).

Three pathways of human exposure to these pesticides are well-known: inhalation of air, dermal contact and ingestion of food and water (Li and Kannan, 2018; Silver et al., 2019). Human risk assessment of exposure to pesticides is traditionally carried out by monitoring environmental samples, such as air, water, food or other sources of potential exposure (Quijano et al., 2016; Yusa et al., 2015). In this way, an estimated external exposure to pesticides can be obtained. However, the analysis of biological matrices (biomonitoring), such as urine, blood or milk, is becoming more relevant in this field because it allows one to determine a human's internal exposure to pollutants from all routes of exposure, offering the possibility to obtain more precise risk assessments (Yusa et al., 2015). OPs, pyrethroids and herbicides, among others, are the most frequently evaluated pesticides in human biomonitoring (HBM) studies. These non-persistent substances tend not to accumulate in the human body and are consequently quickly metabolized and excreted via urine within 4–72 h after exposure (Roca et al., 2014). Therefore, the analysis of non-persistent pesticide metabolites in urine is a useful strategy in order to obtain valuable information about recent exposure to pesticides (1–3 days) through all possible sources (Roca et al., 2014). Biomarkers of exposure to these chemical products can be non-specific or specific metabolites of the parent compound. Non-specific pesticide biomarkers are those metabolites that are common to a family of pesticides. The analysis of non-specific metabolites offers insight into the general exposure to these compounds. On the contrary, the measurement of specific pesticide biomarkers gives information about exposure to the specific active substances from which they are derived (Ryan et al., 2007). These metabolites may be present in urine in a free or conjugated form (Yusa et al., 2015).

Apart from being useful for generating biomonitoring reference values for different target populations (adults, pregnant women, children, etc.), the pesticide exposure levels obtained by biomonitoring studies can be compared to health-based guideline values, allowing for the interpretation of biomonitoring data in a risk assessment context (Katsikantami et al., 2019). Several biomonitoring studies use Biomonitoring Equivalents (BEs) or HBM I and HBM II values as biological reference values (Apel et al., 2017; Aylward et al., 2011), which are calculated using the toxicokinetic properties of the chemical and several uncertainty factors (Steckling et al., 2018). However, there are no BEs or HBM values described for a great number of pesticides, and thus another methodology should be employed in these cases. Consequently, a recent publication suggests using the estimated daily intake (EDI) based

Table 1
Lifestyle, sociodemographic characteristics and exposure to pesticides information of the target population.

	n total[a]	n (%)
Mother		
Creatinine concentration (g·L^{-1})[c]	116	1.0 (0.3–2.4)[b]
Number of children	116	–
Primiparous	–	68 (59)
Multiparous	–	48 (41)
Age (years)	115	34 (20–45)[b]
Weight before pregnancy (kg)	114	60 (42–92)[b]
Height (cm)	114	164 (150–184)[b]
BMI before pregnancy (kg·m^{-2})[c]	116	22 (17–35)[b]
Diet during pregnancy	114	–
Yes	–	16 (14)
No	–	98 (86)
Country of birth	114	–
Spain	–	100 (88)
Foreign	–	14 (12)
Place of residence[c]	103	–
Urban	–	82 (80)
Rural	–	21 (20)
Education level	116	–
Only primary school	–	13 (11)
Secondary school	–	24 (21)
University degree	–	79 (68)
Occupational status[c]	114	–
Employed	–	96 (84)
Unemployed	–	18 (16)
Physical exercise[c]	113	–
Frequently	–	37 (33)
Occasionally	–	49 (43)
Never	–	27 (24)
Smoker[c]	116	–
Smoker	–	9 (8)
Exsmoker	–	48 (41)
Non-smoker	–	59 (51)
Time with the windows open during the day (hours)[c]	116	6.5 (1.0–24.0)[b]
Garden or plants at home[c]	116	–
Yes	–	68 (59)
No	–	48 (41)
Animal deworming in the last month[c]	116	–
Yes	–	19 (16)
No	–	97 (84)
Closeness to farming activity[c]	115	–
Yes (<200 m)	–	35 (30)
No (>200 m)	–	80 (70)
Use of pesticides at home in the last month[c]	116	–
Yes	–	60 (53)
No	–	56 (47)
Children		
Gestational age (weeks)	88	40 (35–41)[b]
Weight (at birth) (g)	113	3360 (2160–4350)[b]
Height (at birth) (cm)	74	51 (46–55)[b]
Cranial Perimeter (cm)	42	34 (33–37)[b]

[a] Number of participants who answered this question.
[b] Median (minimum–maximum).
[c] Variables included in the linear regression models.

Table 2
Food consumption frequency by groups in g·month^{-1}.

Food consumption[a]	n total	Median (minimum–maximum) (g·month^{-1})
Dairy products	110	13,084 (1404–43,446)
Eggs	110	720 (30–1800)
Meat products	110	55,689 (738–13,710)
Fishery products	110	4283 (1040–15,640)
Vegetables	110	13,264 (1181–38,906)
Fruits	110	16,607 (3721–84,484)
Legumes and cereals	110	4999 (2048–26,960)
Oils and fats	110	801 (153–1541)
Bakery products	110	1244 (172–7303)
Miscellany[b]	110	1356 (343–29,706)
Drinks (mL·month^{-1})	110	44,111 (1728–76,563)

[a] All these variables were included in the regression models.
[b] Miscellany group is composed of snacks and sauces.

on internal concentrations of pesticide metabolites and characteristics of the target population under evaluation in order to tackle this issue (Katsikantami et al., 2019).

According to Eurostat and the Food and Agriculture Organization of the United Nations (FAO), Spain is the European country with the highest sale of pesticides (almost 80 million kg in 2016) and is situated in 12th position in the European Union (EU) in the use of pesticides per area, with 3.63 million kg of pesticides applied for hectare of cropland in 2016 (EUROSTAT, 2018; FAO, 2019). Moreover, it is one of the main agricultural producers in the EU (EUROSTAT, 2018). Approximately 72,000 tons of pesticides were used during 2017 in Spain (EUROSTAT, 2018; Ministerio de Agricultura y Pesca, 2017). Therefore, it is assumed that the Spanish population could be highly exposed to these pesticides.

The objectives of this study were: i) to determine the urinary levels of currently used pesticide metabolites in Spanish breastfeeding mothers; ii) to identify correlations between urinary levels of pesticide metabolites and sociodemographic characteristics, dietary habits and use of pesticides of the target population; and, iii) to perform a risk assessment for the most frequently detected pesticides.

2. Materials and methods

2.1. Study design and sample collection

The study design and population recruitment were previously described in detail (Yusà et al., 2017). Briefly, women who had given birth from June to November 2015 in the University and Polytechnic Hospital "La Fe" (Valencia, Spain) were asked to give a urine sample. Selection criteria were based on four characteristics: i) good health of mother and baby, ii) full gestation period without incident, iii) minimum residence in Valencia of 10 years, and iv) decision to breastfeed the newborn for at least 2 months.

Finally, a total of 120 women of 20–45 years of age agreed to participate in the study. The participants completed a questionnaire after pregnancy with detailed physical and sociodemographic characteristics, lifestyles, food consumption frequency, household characteristics and use of pesticides information. These variables are shown in Tables 1 and 2.

Urine collection was carried out by the participants themselves, who were provided with a 125 mL polypropylene urine container and the urine collection instructions. A first-morning urine sample was collected from each volunteer between 2 and 8 weeks after birth and kept at 4 °C. Samples were transported to the laboratory and stored at −80 °C, until analysis, in the Biobank for Biomedical Research and Public Health of the Valencian region (IBSP-CV Biobank, Spain). In the end, 119 urine samples were gathered and analyzed in the Public Health Laboratory of Valencia.

The study and sampling protocol were approved by the Scientific Ethics Committee of the Foundation for the Promotion of Health and Biomedical Research in the Valencia Region (FISABIO) of the Valencian Government (Dirección General de Salud Pública, DGSP) and the Biomedical Scientific Ethics Committee of the University and Polytechnic Hospital La Fe.

2.2. Chemical analysis

2.2.1. Determination of pesticide metabolites

Specific and non-specific (dialkyl phosphates) urinary biomarkers of pesticides selected in this study are shown in Table S1. The analytical methods used to analyze these substances in urine are shown in the Supplementary material (SM1 and SM2). Dialkyl phosphates (DAPs) and specific metabolites were extracted from the urine using liquid-liquid and QuEChERS extraction (Roca et al., 2014), respectively, and they were analyzed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). The procedural limit of quantification (LoQ) for each metabolite is presented in Table 3. Creatinine concentration was also measured using the Jaffé's reaction to correct possible differences in dilutions of spot urine samples and to determine whether a spot urine sample is valid for assessing chemical exposures. A valid urine sample should have a creatinine concentration within the range 30–300 mg·dL^{-1} (Barr et al., 2005).

2.2.2. Quality assurance/quality control

The laboratory works following the ISO/IEC 17025 standard, hence the analyses were carried out under a highly consolidated quality system. The quality assurance/quality control was performed following some aspects of the SANTE/11813/2017 guide for the analysis of pesticide residues in food and feed (European Commission, 2017). In each set of samples, quality control (QC) samples and a matrix-matched calibration curve (from the LoQ to 50 ng·mL^{-1}) were used to check the extraction efficiency and ensure a proper quantification of real samples. The QC samples were prepared by spiking blank samples at three different concentration levels (LoQ, intermediate and high levels of the corresponding calibration curve) and they were subjected to the same extraction and analysis procedures than the real samples and calibration curve. Before extraction, dibutyl phosphate for DAPs and analogous isotopic labelled internal standards for specific metabolites (Roca et al., 2014) were added in order to compensate matrix effects and possible losses occurred during the experimental procedure. Reagent and sample blanks were also used in order to confirm the absence of contamination. The acceptance criteria were: recoveries between 70 and 120% and coefficients of variation $\leq$20%. The quantification and confirmation criteria for the LC-MS/MS were based on the SANTE/11813/2017 guide and is described in Dualde et al. (2019). The laboratory participates in two German External Quality Assessment Scheme (G-EQUAS) intercomparison programs per year in order to ensure the validity of the results.

2.3. Statistical analysis

Statistical analysis was performed using R and SPSS software (versions 3.5.2 and 23.0, respectively). Metabolite levels below the LoQ were estimated using the maximum likelihood estimation method described by the European Food Safety Authority (EFSA, 2010). This method assumes that the data are distributed according to a parametric distribution and estimates the parameters of this distribution, maximizing the probability of obtaining the observed sample. A log-normal distribution was assumed for urinary metabolite levels. In order to summarize information on food consumption, the questionnaire variables that reflect the frequency of individual food consumption were converted to semi-

Table 3
Urinary levels (ng·mL^{-1}) of the most frequently detected (DF > 40%) pesticide metabolites in Spanish mothers (n = 116).[a]

Metabolites	LoQ	DF (%)	GM (95% CI)	Median (95% CI)	Percentiles			Maximum
					25th	75th	95th	
Non-specific metabolites of OPs								
DEP	0.50	91	1.9 (1.5–2.3)	1.9 (1.5–2.3)	1.2	3.5	6.7	13.2
DMP	0.50	65	0.9 (0.6–1.2)	1.2 (0.8–1.5)	<LoQ	1.7	3.3	14.7
DETP	0.50	51	0.6 (<LoQ–1.0)	0.5 (<LoQ–0.9)	<LoQ	3.0	5.2	12.6
DMTP	0.50	47	<LoQ	<LoQ	<LoQ	1.5	4.4	10.4
[b]∑DEPs	–	–	21.5 (18.0–25.0)	22.9 (19.4–26.4)	13.0	33.1	61.0	105.4
[c]∑DMPs	–	–	13.6 (9.4–17.7)	13.0 (8.8–17.1)	6.9	27.3	65.3	131.1
[d]∑DAPs	–	–	39.9 (33.9–45.9)	38.8 (32.8–44.8)	24.6	57.6	111.8	179.9
Specific metabolites of OPs								
TCPY	0.25	85	1.5 (0.9–2.0)	2.0 (0.5–2.5)	0.8	3.3	7.9	16.8
PNP	0.25	84	0.8 (0.2–1.3)	0.9 (0.4–1.4)	0.4	1.9	5.4	23.9
Non-specific metabolites of pyrethroids								
PBA	0.50	65	1.4 (<LoQ–3.4)	1.7 (<LoQ–3.8)	<LoQ	5.7	18.8	72.7

LoQ = quantification limit.
DF = detection frequency (>LoQ).
GM = geometric mean.
CI = confidence intervals.
[a] Data from the rest of pesticide metabolites can be found in Table S3.
[b] ∑DEPs (nmol·L^{-1}) = DEP + DETP + DEDTP.
[c] ∑DMPs (nmol·L^{-1}) = DMP + DMTP + DMDTP.
[d] ∑DAPs (nmol·L^{-1}) = ∑DMPs + ∑DEPs.

quantitative intakes (g·month^{-1}, or mL·month^{-1} in drinks), using estimations from Table S2, and grouped by the kind of food. Monthly intake of individual food items was estimated according to the formula (1). Food consumption by groups was achieved by adding the monthly intake of individual foods of the same group. In total, 11 groups were created: dairy products, eggs, meat products, fishery products, vegetables, fruits, legumes and cereals, oils and fats, bakery products, drinks and miscellany.

$$Food\ intake\left(g\ or\ ml\cdot month^{-1}\right) = Portions\ size\ (g\ or\ mL) \times Intake\ frequency\left(portions\cdot month^{-1}\right) \quad (1)$$

First, a descriptive analysis of the studied variables was performed. Categorical variables, such as educational level (primary school, secondary school or university degree), were described with absolute frequencies and percentages. Quantitative variables were summarized by their median and range. Additionally, specific and non-specific metabolite concentrations were summarized by calculating the most relevant descriptors: detection frequency (DF), geometric mean (GM), median, 25th, 75th and 95th percentiles (P25, P75 and P95) and maximum, with their corresponding 95% confidence intervals (CI).

The urinary concentrations of pesticide metabolites were not normally distributed, so non-parametric methods were applied to study their relationship with variables. Mann-Whitney and Kruskal-Wallis tests were used to compare metabolite levels according to the categories of categorical variables. The Spearman correlation test was used to study correlations between urinary biomarker levels and quantitative variables included in the study. A statistically significant relationship was considered when p-value was less than or equal to 0.05. These analysis were performed considering unadjusted concentrations by creatinine, as recommended by the HBM4EU initiative (Vrijheid et al., 2017), and only for the most frequently detected metabolites (those with DF ≥ 40%). Creatinine concentration was considered as a separate quantitative variable in the statistical analysis. Dialkyl phosphates were considered as a sum (∑DAPs = DMP + DMTP + DMDTP + DEP + DETP + DEDTP).

Finally, multiple linear regression models were built to evaluate the association of sociodemographic, lifestyle and dietary variables with metabolite levels. Metabolite levels were log-transformed in order to achieve a normal distribution of the response variables. Regression models were constructed following a forward variable selection procedure based on the Bayesian Information Criterion (BIC), considering all variables that could explain or show a relationship with urinary concentrations of pesticide metabolites (see Tables 1 and 2). In order to obtain directly-comparable regression coefficient magnitudes, the numerical explanatory variables were centered and divided by two times their standard deviation (Gelman, 2008). The 95% CI were calculated for the regression coefficients to assess their precision and uncertainty. Lastly, regression assumptions were verified: normality of the residuals was checked using the Shapiro-Wilk test, non-autocorrelation using Durbin-Watson statistic, homoscedasticity by scatter plots and non-multicollinearity of explanatory variables in the final models with tolerance and condition indexes.

2.4. Risk assessment

EDI was calculated in this study to interpret urinary levels of pesticide biomarkers in a risk assessment context. For this purpose, a toxicokinetic model was used to transform urinary levels of metabolites into an EDI of parent pesticides in the population under review, using Eq. (2) (Katsikantami et al., 2019).

$$EDI\left(\frac{g}{kg\ bw\cdot day}\right) = \frac{C_U\left(\frac{mol}{L}\right)\times V_{24h}\times MW_P\left(\frac{g}{mol}\right)}{F_{UE}\times BW\ (kg)} \quad (2)$$

EDI: estimated daily intake
C_U: molar concentration of the metabolite in urine
V_{24h}: total urinary volume excreted within 24 h (1.6 L·day^{-1})
MW_P: molecular weight of the parent pesticide
F_{UE}: urinary excretion factor of the parent pesticide (ranged from 0.25 to 0.90)
BW: mean body weight of the participants (60 kg)

Parent pesticides were selected following these two criteria:

a) If possible, an approved pesticide was chosen.
b) If there was more than one possibility, the parent pesticide with the lowest health-based guidance value (conservative scenario) was selected.

In this study, risk assessment was evaluated including only the most frequently detected biomarkers (DF > 40%). Therefore, EDI was calculated for dimethoate, chlorpyrifos-ethyl, parathion and deltamethrin, assuming their metabolites came only from these parental compounds. The exposure to chlorpyrifos-ethyl was calculated twice: based on its specific biomarker (TCPY) and its non-specific biomarkers (ΣDEPs). ΣDMPs and PNP were used to calculate the exposure to dimethoate and parathion, respectively. PBA is a common metabolite of several pyrethroids, and thus in order to perform the risk assessment from a conservative approach, only deltamethrin was considered as the PBA parent because it has the lowest acceptable daily intake (ADI) value.

Hazard quotient (HQ) and hazard index (HI) were also calculated. The HQ allows one to evaluate the risk of exposure to pesticides individually. It can be obtained using Eq. (3) with the ADI as a reference guideline, which ranges between 2 (for dimethoate) and 10 μg·kg bw^{-1}·day^{-1} (for chlorpyrifos-ethyl and deltamethrin). If the HQ was less than one, it was considered a low risk for health. Aggregate exposure to OPs was estimated by calculating the HI as the sum of individual HQs for chlorpyrifos-ethyl and dimethoate, which have a common mechanism of action. Risk assessment of exposure to pyrethroid-OPs mixture was not calculated because the currently existing models used to evaluate the risk of exposure to this pesticide mixture are not accurate enough to obtain consistent results (Hernández et al., 2013).

$$HQ_i = \frac{EDI_i}{ADI_i} \quad (3)$$

3. Results

A total of 120 women participated in the study and 119 provided a urine sample. Three samples had a urinary concentration of creatinine out of the range 30–300 mg·dL^{-1}. The WHO recommendation is that if a sample is too dilute or too concentrated, another urine void should be collected. However, in this study it was not possible to collect another sample from the participants, thus these three urine samples were excluded from the current study (Barr et al., 2005). Finally, 116 urine samples were included in the statistical analysis in order to explore correlations between the obtained urinary biomarker levels and lifestyle, sociodemographic characteristics and food consumption habits of the participants. Tables 1 and 2 summarize the descriptive analysis of the target population characteristics. The participants had a median age of 34 years, and most of them were employed (84%), lived in an urban area (80%), and had university studies (68%).

3.1. Urinary concentrations of pesticide metabolites

The descriptive analysis of metabolite concentrations is summarized in Table 3 (biomarkers with DF > 40%) and Table S3 (biomarkers with DF < 40%), and adjusted urinary levels (μg·g^{-1}Cre) are presented in Table S4. In 99% of the samples, at least one of the urinary DAPs was detected. Detection frequencies of DAPs ranged from 3% (DEDTP) to 91% (DEP). The highest GM was obtained for DEP (1.9 ng·mL^{-1}). However, the highest maximum urinary concentration was detected for DMP (14.7 ng·mL^{-1}). DMDTP and DEDTP were the DAPs with the lowest detection frequencies and urinary levels. Moreover, 98% of the analyzed urine samples had at least one pesticide-specific metabolite. 3,5,6-Trichloro-2-pyridinol (TCPY), a metabolite of chlorpyrifos-ethyl, chlorpyrifos-methyl and triclopyr, was the specific biomarker detected in most samples, with a DF of 85%, followed by p-nitrophenol (PNP), which has been identified previously as a specific metabolite of parathion and methyl-parathion (banned in the EU), with a DF of 84%. Only one pyrethroid insecticide biomarker was frequently detected: 3-phenoxybenzoic acid (PBA), with a DF of 65%. The highest GM within these pesticide metabolites was obtained for TCPY (1.5 ng·mL^{-1}), while PBA had the highest maximum detected urinary level (72.7 ng·mL^{-1}).

2,4,5-Trichlorophenoxyacetic acid (2,4,5-T), a metabolite of a currently banned herbicide, was not detected in any sample. Conversely, other specific biomarkers of prohibited pesticides were found in some samples such as acephate (ACEP) and methamidophos (METHA), 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMPY), atrazine mercapturate (ATZM), 3-methyl-4-nitrophenol (MNP) and metolachlor mercapturate (METM), which are specific metabolites of acephate, diazinon, atrazine, fenitrotion and metolachlor, respectively.

3.2. Factors influencing pesticide biomarker levels

Correlations between concentrations of the most detected biomarkers (ΣDAPs, PNP, TCPY and PBA) were evaluated. In addition, correlations among these metabolite levels and possible influencing factors (sociodemographic variables, food consumption frequencies and parameters related to the exposure to pesticides) were also statistically explored. Obtained results are described in the following sections.

3.2.1. Correlation between biomarkers

A Spearman correlation matrix (Table S5) was obtained to explore the relationships between the different biomarkers. The sum of DMPs (ΣDMPs) and the sum of DEPs (ΣDEPs) were only included in this part of the statistical analysis. As presented in Table S5, TCPY was positively correlated with ΣDAPs, ΣDEPs and PBA. In the case of PNP, significant correlations were found for ΣDMPs and PBA.

3.2.2. Predictors of exposure

A bivariate analysis with the Spearman correlation test was used to evaluate the relationships between biomarker levels and quantitative parameters, such as those related to sociodemographic characteristics (Table S6) and food consumption frequency (Table S7). Median biomarker concentrations and their distribution by categories for variables related to lifestyle and sociodemographic characteristics are shown in Table S6.

Fig. 1 shows the standardized coefficients of the regression models built to assess the relationship among metabolite levels and sociodemographic and dietary parameters, with their corresponding 95% CI. Further statistical information about these models is presented in Table S8. Variables related to the children's characteristics (gestational age, weight and length at birth, and cranial perimeter) were not investigated in the multivariate model because they were not true predictors but dependent variables.

According to Fig. 1, TCPY was negatively associated with BMI before pregnancy. In addition, participants living near farming activities (<200 m) had higher levels of TCPY in urine (11.1 nmol·L^{-1}) compared with those living far from these areas (8.3 nmol·L^{-1}). Moreover, it can be observed that women who had a terrace with plants, garden or similar in their houses had higher urinary levels of DAPs (48.4 nmol·L^{-1}) than women living in houses without garden/plants (36.9 nmol·L^{-1}). PNP was the only metabolite weakly associated with fruit intake

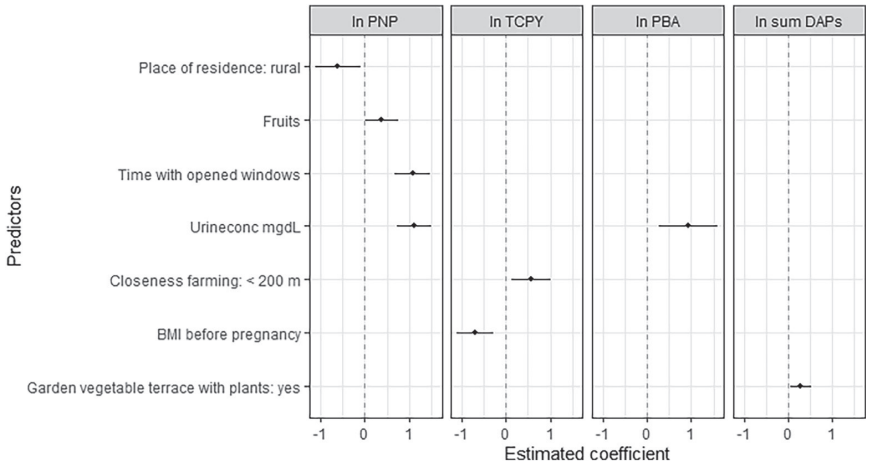

Fig. 1. Standardized coefficients of the linear regression models obtained for lnPNP, lnTCPY, lnPBA and lnΣDAPs, with their corresponding 95% confidence intervals.

Table 4
Parameters selected for the calculation of EDI for dimethoate, chlorpyrifos-ethyl, parathion and deltamethrin based on GM and P95.

Biomarker	Selected parent compound	Selection reason	MW_P ($g \cdot mol^{-1}$)	F_{UE}	ADI[e] ($\mu g \cdot kg\ bw^{-1} \cdot day^{-1}$)	EDI ($\mu g \cdot kg\ bw^{-1} \cdot day^{-1}$)		V_{24h} ($L \cdot day^{-1}$)	BW (kg)
						EDI_{GM}	EDI_{P95}		
∑DMPs	Dimethoate	Approved parent pesticide with the lowest ADI	229.26	0.90[a]	2	0.09	0.44	1.6[d]	60
∑DEPs	Chlorpyrifos-ethyl	Only possible parent pesticide approved in the UE	350.59	0.50[b]	10	0.40	1.14		
PNP	Parathion	Parent pesticide with the lowest ADI	291.30	0.37[c]	4	0.12	0.81		
TCPY	Chlorpyrifos-ethyl	Approved parent pesticide with the lowest ADI	350.59	0.50[b]	10	0.14	0.75		
PBA	Deltamethrin	Approved parent pesticide with the lowest ADI	505.21	0.25[d]	10	0.34	4.73		

MW_P: molecular weight of the parent pesticide.
F_{UE}: urinary excretion factor of the parent pesticide.
ADI: acceptable daily intake.
EDI: estimated daily intake.
V_{24h}: total urinary volume excreted within 24 h.
BW: mean body weight of the target population before pregnancy.
[a] (Katsikantami et al., 2019).
[b] (Bouchard et al., 2005).
[c] (Gosselin et al., 2004).
[d] (Aylward et al., 2011).
[e] (European Commission, 2019).

Table 5
Hazard quotient for dimethoate, chlorpyrifos-ethyl, parathion and deltamethrin, and hazard index for OPs based on GM and P95.

Biomarker	Selected parent compound	HQ_{GM}	HQ_{P95}	HI_{GM} (OPs)	HI_{P95} (OPs)
∑DMPs	Dimethoate	0.05	0.22	0.09	0.33
∑DEPs	Chlorpyrifos-ethyl	0.04	0.11		
PNP	Parathion	0.03	0.20		
TCPY	Chlorpyrifos-ethyl	0.01	0.07		
PBA	Deltamethrin	0.03	0.47		

(regression coefficient = 0.39), although not statistically significant. Furthermore, higher PNP levels were found in urine from mothers who lived in urban areas and kept the windows open more hours during the day. A significant relationship was also found between the creatinine concentration from the mothers' urine and levels of urinary PNP and PBA. Overall, the fitted regression models were weak but accounted for 4–42% of the variance (R^2), and these findings agree with the correlations obtained in the bivariate analysis.

3.3. Risk assessment

EDI values calculated for dimethoate, chlorpyrifos-ethyl, parathion and deltamethrin ranged between 0.09 and 4.73 $\mu g \cdot kg\ bw^{-1} \cdot day^{-1}$ and are shown in Table 4. The highest EDI_{GM} was obtained for chlorpyrifos-ethyl (0.40 $\mu g \cdot kg\ bw^{-1} \cdot day^{-1}$), using ΣDEPs as non-specific biomarker. Deltamethrin resulted in the highest EDI_{P95} (4.73 $\mu g \cdot kg\ bw^{-1} \cdot day^{-1}$), since it had the lowest F_{ue} and the highest maximum level of PBA found in urine samples.

Table 5 shows the HQ for each pesticide and the HI for OPs (sum of chlorpyrifos-ethyl and dimethoate) using GM and P95 data. Deltamethrin was not included in the calculation of the HI because it does not have the same mechanism of action as the OPs and therefore cannot be combined in one common HI value. Thus, in order to obtain a HI for OPs, the HQs for chlorpyrifos based on ΣDEPs (0.04–0.11), and for dimethoate based on ΣDMPs (0.05–0.22) were added since DAPs are common metabolites of the majority of OPs. Regarding the HQs from Table 5, similar results were found for all pesticides when the GM was used. All calculated HQs (0.01–0.47) and HI (0.09–0.33) were less than one.

4. Discussion

Four non-specific metabolites of OPs (DEP, DETP, DMP, DMTP), two specific metabolites of OPs (TCPY and PNP), and one non-specific metabolite of pyrethroids (PBA) were the most frequently detected biomarkers in the analyzed urine samples. The two analyzed dithiophosphates (DMDTP and DEDTP) were the least frequently detected DAPs, probably due to the fact that they are metabolites of a reduced number of OPs (CDC, 2018; Health Canada, 2013). The highest GM was obtained for DEP, a non-specific metabolite of OPs such as chlorpyrifos; and the highest maximum urinary level obtained was for PBA, a common metabolite of several pyrethroids such as deltamethrin. Regarding correlations between biomarkers, the strong correlation between TCPY and DAPs showed that their presence in the analyzed urine samples could be due principally to chlorpyrifos-ethyl exposure. Moreover, the positive correlation of PBA with TCPY might be related to the fact that pyrethroids and chlorpyrifos are usually applied to the same type of crops (Ministerio de Agricultura y Pesca, 2013). These pesticides are some of the most widely used insecticides in Spain, especially on fruits, cereals and vegetables (Ministerio de Agricultura y Pesca, 2013). Furthermore, residues of chlorpyrifos-ethyl and some pyrethroids (cypermethrin and deltamethrin) were found in some fruit and vegetables samples from Spain in levels above the Maximum Residue Level in 2017 (AECOSAN, 2017). In addition, chlorpyrifos-ethyl was the third most detected pesticide residue in food from the Valencian region in 2015 and >3.5% of the analyzed food samples tested positive for cypermethrin and deltamethrin in 2015 (Dirección General de Salud Pública, 2019). Moreover, chlorpyrifos-ethyl was detected in 29% of air samples analyzed in the Valencian region from 2008 to 2014 (López et al., 2017). Thus, the wide use of these pesticides and their presence in environmental samples justify the internal exposure levels of TCPY and PBA in the participants of our study. However, no associations were found between fruit or vegetable consumption and urinary levels of these pesticide biomarkers. This fact could be explained because, as previously described, differences in the preparation/treatment of these products in the participants' meals could influence the final pesticide concentration due to degradation, volatilization or extractions of the pesticide residues (Cequier et al., 2017). Furthermore, it was observed that mothers with higher BMI had lower urinary concentrations of TCPY. Similar results were previously reported by Cequier et al., 2017, who found higher levels of DAPs in woman with lower BMI due to a high fruit consumption. In contrast of these findings, in our study no associations were found between healthy dietary patterns (high consumption of fruits or vegetables) and BMI, so no conclusions about this association could be obtained with this information alone. In addition, participants who lived <200 m from a farming activity (30%) and had a house with a garden or similar (59%) presented significantly higher urinary levels of TCPY and ΣDAPs in urine, respectively, most likely due to the use of OPs in these areas or nearby.

Up until now, PNP has been considered as a biomarker of parathion and methyl-parathion insecticides, but these substances have been banned in the EU since the 20th century and have not been detected in any food or air sample in the Valencian region (Dirección General de Salud Pública, 2019; López et al., 2017). Moreover, according to the National Institute of Safety, Health and Welfare at Work from Spain, urinary PNP can also be found after exposure to nitrobenzene (Instituto Nacional de Seguridad e Higiene en el Trabajo, 2010), a raw material for the production of several commercial items (aniline, solvents, lubricant oils, colorants, medicines, etc.) and a residue from motor vehicle fuels and smoking processes (Chapman et al., 2018; World Health Organization, 2003). According to our PNP regression model, people who lived in urban areas and kept the windows open during the day had higher levels of urinary PNP. In view of these results, its presence in urine could be due principally to inhalation as route of exposure. Taking all this into account, although many studies consider PNP as a specific metabolite of parathion and methyl-parathion, the origin of this metabolite in urine is still unclear, possibly owing to exposure to other compounds. However, no further conclusions could be drawn from the obtained results.

In the case of ACEP, METHA, IMPY, ATZM, MNP and METM, their presence in urine was justified because residues of their parent pesticides, in spite of them being banned, were found in several environmental samples in the Valencian region, such as fruit, vegetables and ambient air (Dirección General de Salud Pública, 2019; López et al., 2017).

PNP, TCPY, DAPs and PBA urinary levels found in this research were compared to those levels reported in other studies with adults, pregnant and breastfeeding women from different countries (Fig. 2). The concentrations of DAPs presented in this study are lower than those reported in other countries, mainly in The Netherlands (Ye et al., 2008) with a GM almost five times higher than in our study. In general, TCPY and PNP concentrations in this study fall within the range of values observed in the rest of the populations around the world. However, it should be noted that Spain reports the highest GM in the case of urinary PBA, indicating a higher exposure to pyrethroids than in other countries. Comparing our results with those from two other studies carried out in Spain, one with pregnant women between 2003 and 2006 (Llop et al., 2017) and another one in the general population (Garí et al., 2018), several differences can be observed. On the one hand, levels of DAPs are higher in the urine of women collected during 2003–2006 than those collected in 2015 for this study. On the contrary, urinary TCPY and PNP concentrations seem to increase from 2003 to 2006 to 2015. On the other hand, it should be noted that the Spanish general population (Garí et al., 2018) has higher levels of TCPY, PNP and PBA than our target population. However, changes and differences in the type of pesticides used over time, targeted pests in the study areas, season of sample collection, and place of residence and lifestyles of the participants are some of the possible determinants that could lead to these variations among studies.

In order to interpret the obtained levels in a risk assessment context, EDIs for parathion, chlorpyrifos, dimethoate and deltamethrin were calculated (Table 4). However, taking into account that PNP is not only a metabolite of parathion, but that it could come from other sources of exposure, the EDI and HQ for parathion should be interpreted with caution.

EDI_{P95} for chlorpyrifos and deltamethrin are higher than those reported in a study regarding external exposure to pesticides through fruit and vegetable consumption in Spanish adults (Quijano et al., 2016), especially for deltamethrin. This fact can be explained because the risk assessment performed in the present study considers not only diet exposure, but also other sources of exposure. Furthermore, calculated EDIs and HQs for parathion

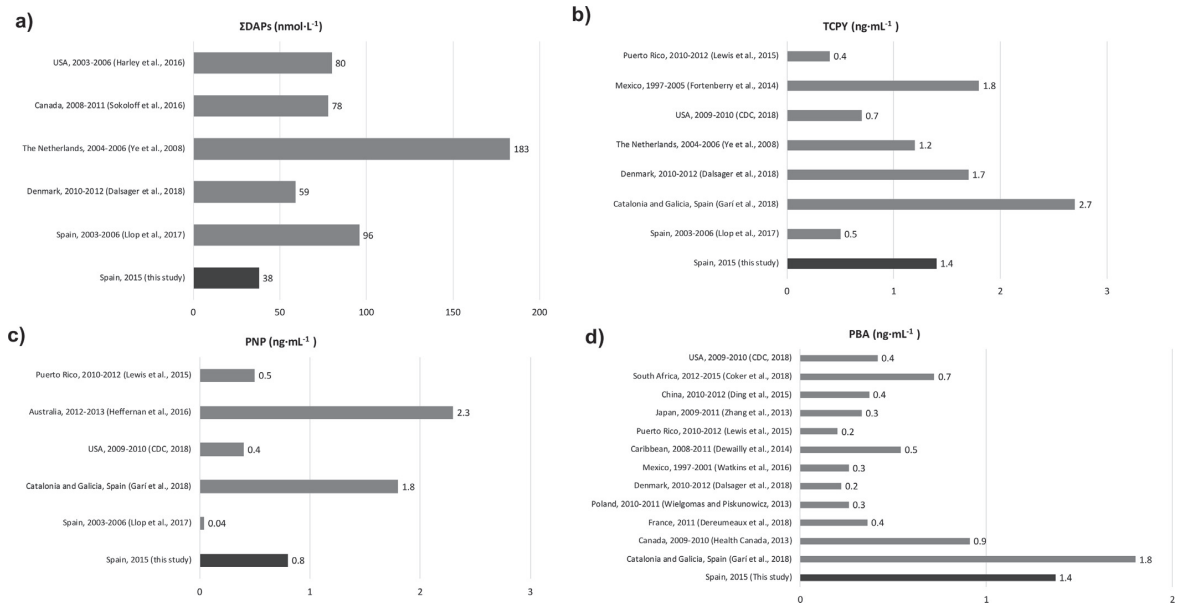

Fig. 2. Geometric mean in different countries of the world for a) ΣDAPs, b) TCPY, c) PNP and d) PBA. (See above-mentioned references for further information.)

and dimethoate are similar to those obtained for pregnant women in a recent study concerning internal exposure to OPs (Katsikantami et al., 2019). Comparing EDI_{P95} values, deltamethrin was by far the pesticide to which the Spanish mothers seemed to be more exposed. However, the target population could be exposed directly to pesticide metabolites due to the degradation of the parent compound in environmental media (food, air, water, etc.). Consequently, this fact may lead to the overestimation of the exposure to the parent pesticide (Ryan et al., 2007).

Regarding HQ_{P95} values for OPs and deltamethrin (Table 5), all the obtained values were below one, and therefore, no risk existed for the Spanish mothers. However, the highest risk was associated with the exposure to deltamethrin, as it has already been seen in a previous publication (Quijano et al., 2016), followed by dimethoate, whose ADI was the lowest. Compared to the HQ results from the Centers for Disease Control and Prevention (CDC) (Aylward et al., 2013), our study group in Spain seemed to be at a higher risk than the American population due to the exposure to deltamethrin. However, it has to be considered that pesticides applied in Spain can be different from those applied in other parts of the world. In addition, in spite of chlorpyrifos being one of the most used pesticides in Spain (Ministerio de Agricultura y Pesca, 2013) and its residues being found in some Spanish fruit and vegetables above the Maximum Residue Level (AECOSAN, 2017), very low HQ values were found for this pesticide in this study. Likewise, the HI for OPs was far from one (Table 5), in the range of that reported for other populations of pregnant women in 13 countries (Katsikantami et al., 2019).

Thus, considering the obtained HQs and HI, there was a relative low health risk due to the exposure to these pesticides in the Spanish mothers from our study, and they were considered to be safe. However, it must be taken into account that synergies between OPs and pyrethroids were not considered in this study, leading to the underestimation of the real risk of exposure to the pyrethroid-OPs mixture. In addition, the active substances are periodically reassessed in order to renew their approval so that new health impacts can be described, such as the genotoxic potential, and the neurodevelopmental and reproductive toxicity recently described for chlorpyrifos (EFSA, 2019).

In conclusion, due to the extensive use of pesticides in Spain, the biomonitoring of these substances should continue for a better assessment of population exposure, mainly the most sensitive populations groups, such as children and pregnant women.

5. Study limitations

The participants were recruited from just one hospital that covers a limited geographic location. Likewise, the sample size was small and the educational level of the participants was skewed toward university studies. Moreover, the collection of urine samples was carried out in different months (from June to November) and seasons (summer and autumn), leading to possible differences among the urinary biomarker levels of the participants, since this variability was not taken into account in the statistical analysis and they may not be comparable to each other. In addition, the questionnaire did not include questions regarding what type of food the participants consumed (such as organic food), and if they peeled, washed or treated fruits and vegetables before eating them. Consequently, this study can be considered as a pilot study, and the conclusions should not be extrapolated to the entire population.

6. Conclusions

In the present study, six non-specific metabolites of OPs and 20 specific metabolites of OPs, herbicides and pyrethroid insecticides were analyzed in urine samples from Spanish lactating mothers. The pesticide metabolites with the highest DF were DEP, TCPY, PNP and PBA, with GM of 1.9 $ng{\cdot}mL^{-1}$, 0.8 $ng{\cdot}mL^{-1}$, 1.5 $ng{\cdot}mL^{-1}$ and 1.4 $ng{\cdot}mL^{-1}$, respectively. Based on the obtained concentrations, the study population had similar levels of OP metabolites as other populations around the world, but higher levels of PBA. The closeness to farming activities, the place of residence and the presence of garden or plants at home seemed to be important con-

tributors to the exposure to these substances. The resulting biomonitoring data was interpreted in a risk assessment context comparing the calculated EDI with ADI values. EDIs showed that these mothers were mostly exposed to deltamethrin and chlorpyrifos. However, in view of the calculated HQs and HI within a conservative scenario (P95), none of these pesticides exceeded the safety limit, and therefore a relatively low health risk for these lactating women living in Valencia (Spain) was obtained.

Declaration of competing interest

All authors report no conflict of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.134385.

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4.3 BIOMONITORING OF NON-PERSISTENT PESTICIDES IN CHILDREN: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT

4.3.1 RESULTS AND DISCUSSION

Out of the 666 recruited children in the BIOVAL program, only 572 urine samples were analyzed because 94 of them did not reach the minimum volume required for this study. In addition, four urine samples were removed from the current research because they had a creatinine level below 30 mg·dl^{-1}. As a result, 568 urine samples were considered in the descriptive and statistical analyses. Creatinine concentration in these samples ranged from 32.6 to 265.9 mg·dl^{-1}. The socio-demographic characteristics and information about the food consumption of the participants in the 72 hours before collecting the urine sample are presented in Table 13. Most of the children (93 %) had at least one parent employed, 78 % of them were living in urban areas, and 60 % had at least one parent with university studies.

Table 13. Socio-demographic characteristics, lifestyle and food consumption habits of the children of the BIOVAL study included in the analysis of non-persistent pesticide metabolites in urine (n = 568).

Parents' characteristics	n (%)
Country of birth	-
Both parents were Spanish	492 (87 %)
At least one of the parents was foreign	60 (10 %)
Missing data	16 (3 %)
Maximum level of education	-
Without studies or primary studies	58 (10 %)
Secondary studies	169 (30 %)
University studies	341 (60 %)
Employment situation	-
Both unemployed	38 (7 %)
At least one parent employed	530 (93 %)

Children's characteristics	n (%)	Mean (minimum – maximum)
BMI (kg·m^{-2})	-	
Missing data	17 (3 %)	17 (12 – 45)
Age (years)	-	**8 (5 – 12)**
Province	-	-
Alicante	204 (36 %)	-
Castellón	126 (22 %)	-
Valencia	238 (42 %)	-
Place of residence	-	-
Urban area	444 (78 %)	-
Rural area	22 (4 %)	-
Other	101 (17.8 %)	-
Missing data	1 (0.2 %)	-
Sex	-	-
Male	287 (51 %)	-
Female	281 (49 %)	-
Country of birth	-	-
Spain	557 (98 %)	-
Foreign	8 (2 %)	-
Missing data	3 (0 %)	-
Physical activity	-	-
Frequently (3 or more days/week)	299 (53 %)	-
Occasionally (1 or 2 days/week)	209 (37 %)	-
Never	60 (10 %)	-

Food consumption in the last 72 h (grams)	Mean (minimum – maximum)
Eggs	60 (0 – 360)
Meat products	470 (0 – 2295)
Fishery products	220 (0 – 1730)
Dairy products	1008 (0 – 2820)
Vegetables	337 (0 – 1798)
Fruits	560 (0 – 2625)
Nuts	0 (0 – 210)
Legumes and cereals	524 (0 – 2248)
Canned vegetables, fruits and legumes	0 (0 – 1465)
Oils and fats	10 (0 – 120)
Miscellany (snacks, pre-cooked meals, pastries and sauces)	85 (0 – 420)

4.3.1.1 URINARY LEVELS OF PESTICIDE METABOLITES

The descriptive analysis for each metabolite is summarized in Table 14. In general, DFs ranged from < 1 % (2,4,5-T) to 92 % (DEP). The highest GM was obtained for DEP (1.5 $ng \cdot ml^{-1}$), one of the non-specific metabolites of OPs. As can be seen in Table 14, the specific metabolites of parathion and chlorpyrifos (PNP and TCPY, respectively) and the non-specific metabolite of Pyr (PBA) were found in more than 74 % of the samples with GMs higher than 1 $ng \cdot ml^{-1}$. Another frequently detected non-specific biomarker of OPs was DMP, with a DF of 65 %. The highest maximum detected urinary level (256 $ng \cdot ml^{-1}$) was observed for DCCA, a metabolite of cyfluthrin, cypermethrin, and permethrin. The rest of metabolites were detected in less than 37 % of the urine samples. It is worth noting that 85 % of the children had five or more pesticide metabolites in their urine samples. Indeed, at least one metabolite of OPs and Pyr was detected in 97 and 83 % of the children, respectively. By contrast, biomarkers of Herb were only detected in 41 % of the children.

Table 14. Descriptive statistics of the urinary pesticide metabolite concentrations in ng·ml^{-1} (µg·g Cre^{-1}) for children of the BIOVAL program (n = 568).[a]

Class of parent pesticide	Metabolite	LoQ (ng·ml^{-1})	DF (%)	P25	P50
Herbicides	2,4-D	0.25	35	< LoQ	< LoQ
	ATZM	0.25	4	-	-
	ALAM	0.125	4	-	-
	METM	0.125	3	-	-
	2,4,5-T	0.125	0.2	-	-
Pyrethroids	PBA	0.50	79	0.6 (0.7)	1.6 (1.6)
	DCCA	5.00	20	< LoQ	< LoQ
	DBCA	1.25	14	< LoQ	< LoQ
	FPBA	0.125	4	-	-
OPs (Specific biomarkers)	PNP	0.25	85	0.5 (0.6)	1.4 (1.3)
	TCPY	0.50	74	< LoQ	1.1 (1.2)
	DEAMPY	0.25	37	< LoQ	< LoQ
	METHA	0.125	27	< LoQ	< LoQ
	IMPY	0.25	9	-	-
	MDA	1.25	7	-	-
	MNP	0.25	7	-	-
	DIMET	0.25	3	-	-
	OMET	0.25	2	-	-
	CHMC	0.50	1	-	-
	ACEP	0.50	1	-	-
OPs (Non-specific biomarkers)	DEP	0.50	92	0.9 (0.9)	1.5 (1.5)
	DMP	0.50	65	< LoQ	0.9 (1.0)
	DMTP	0.50	33	< LoQ	< LoQ
	DMDTP	0.50	27	< LoQ	< LoQ
	DETP	0.50	21	< LoQ	< LoQ
	DEDTP	0.50	3	-	-
	∑DMPs[b,c]	-	-	6.3 (6.2)	14.1 (14.8)
	∑DEPs[b,d]	-	-	8 (8.2)	12.4 (13.1)
	∑DAPs[b,e]	-	-	18 (18)	31 (32)

LoQ = Quantification Limit; DF = Detection Frequency (% samples > LoQ); P25 = 25th percentile; AM = Arithmetic mean; GM = Geometric mean; P75 = 75th percentile; P95 = 95th percentile; Min. = minimum; Max. = maximum; SD = Standard deviation

[a]Means and percentiles were only calculated in analytes with DF > 40 % and > 20 %, respectively

[b]Units: nmol·l^{-1} (nmol·g Cre^{-1})

[c]∑DMPs = DMP + DMTP + DMDTP

[d]∑DEPs = DEP + DETP + DEDTP

[e]∑DAPs = ∑DEPs + ∑DMPs

AM	GM	P75	P95	Range (Min. – Max.)	SD
< LoQ	< LoQ	0.4 (0.4)	1.3 (1.3)	< LoQ – 12.4 (< LoQ – 9.5)	-
-	-	-	-	< LoQ – 0.8 (< LoQ – 1.5)	-
-	-	-	-	< LoQ – 1.1 (< LoQ – 1.0)	-
-	-	-	-	< LoQ – 1.4 (< LoQ – 2.0)	-
-	-	-	-	< LoQ – 0.3 (< LoQ – 0.6)	-
4.0 (3.9)	1.5 (1.6)	3.6 (3.7)	11.6 (11.7)	< LoQ – 130.3 (< LoQ – 127.7)	11.2 (10.0)
< LoQ	< LoQ	< LoQ	47 (46)	< LoQ – 256 (< LoQ – 339)	-
< LoQ	< LoQ	< LoQ	6 (7)	< LoQ – 219 (< LoQ – 242)	-
-	-	-	-	< LoQ – 2.5 (< LoQ – 2.8)	-
1.7 (1.7)	1.0 (1.1)	2.3 (2.2)	5.2 (5.0)	< LoQ – 11.8 (< LoQ – 15.9)	1.7 (1.8)
3.1 (3.2)	1.2 (1.2)	3.2 (3.5)	11.1 (13.0)	< LoQ – 102.2 (< LoQ – 91.7)	6.2 (6.3)
< LoQ	< LoQ	0.7 (0.7)	4.0 (3.8)	< LoQ – 49.8 (< LoQ – 58.7)	-
< LoQ	< LoQ	0.14 (0.16)	0.79 (0.84)	< LoQ – 3.60 (< LoQ – 2.86)	-
-	-	-	0.7 (0.8)	< LoQ – 7.0 (< LoQ – 11.7)	-
-	-	-	1.4 (1.6)	< LoQ – 12.4 (< LoQ – 10.5)	-
-	-	-	0.5 (0.6)	< LoQ – 11.6 (< LoQ – 28.7)	-
-	-	-	-	< LoQ – 3 (< LoQ – 4)	-
-	-	-	-	< LoQ – 3 (< LoQ – 3)	-
-	-	-	-	< LoQ – 1.5 (< LoQ – 1.2)	-
-	-	-	-	< LoQ – 1.6 (< LoQ – 1.7)	-
2.2 (2.5)	1.5 (1.6)	2.4 (2.8)	5.9 (7.8)	< LoQ – 21.4 (< LoQ – 55.1)	2.4 (3.3)
3.1 (3.4)	0.9 (1.0)	3.0 (3.2)	12.9 (14.5)	< LoQ – 81.8 (< LoQ – 74.1)	7.0 (7.4)
< LoQ	< LoQ	0.9 (0.9)	14.7 (16.1)	< LoQ – 218.8 (< LoQ – 179.4)	-
< LoQ	< LoQ	0.6 (0.6)	2.5 (3.2)	< LoQ – 180.6 (< LoQ – 257.5)	-
< LoQ	< LoQ	< LoQ	4 (3)	< LoQ – 114 (< LoQ – 122)	-
-	-	-	-	< LoQ – 1.5 (< LoQ – 1.4)	-
59.6 (62.1)	16.4 (17.0)	35.9 (38)	238.4 (251.6)	0.3 – 2774.1 (0.3 – 2346.9)	199.5 (190.9)
20.3 (22.2)	13.4 (13.8)	19.9 (23.7)	61.4 (67.3)	1.0 – 759.1 (1.1 – 814.5)	38.2 (42.3)
80 (84)	37 (38)	61 (70)	285 (302)	5 – 2784 (3 – 2395)	206 (197)

The comparison of the observed GM urinary levels of the most frequently detected metabolites (∑DEPs, ∑DMPs, TCPY, PNP, and PBA) with other HBM studies on children across different countries is presented in Figures 22a-d.

In the present research, lower concentrations of urinary TCPY were found compared to Australia[289] and Thailand[290], but higher than those reported in other countries, such as Italy[291], Slovenia[292], and China[293] (Figure 22b). It is important to highlight that, due to the recent prohibition of chlorpyrifos in the EU, exposure to chlorpyrifos should be closely monitored in children since, as mentioned before, it has been related to the development of neurodevelopmental outcomes in this population group[121,288].

In the case of PBA, the present study reported the highest concentration in comparison with other countries in the world (Figure 22d). This finding is consistent with the data provided by Eurostat, which affirms that Spain, a leading agricultural producer in the EU, was also one of the greatest consumers of pesticides in 2016[294]. By contrast, urinary ∑DEPs and ∑DMPs levels seemed to be lower in children in Spain than in other parts of the world (Figure 22a). Finally, the urinary levels of PNP obtained in this research were similar to those reported in other countries around the world, but much higher levels of PNP were observed in children from Thailand[290] (Figure 22c), probably because the sampling period of this specific survey was conducted before 2009, close to the date of the final prohibition of parathion. However, bearing in mind that both parathion and methyl-parathion are currently banned in the EU, and that exposure to nitrobenzene could be also a cause of the presence of PNP in urine, no more conclusions can be drawn.

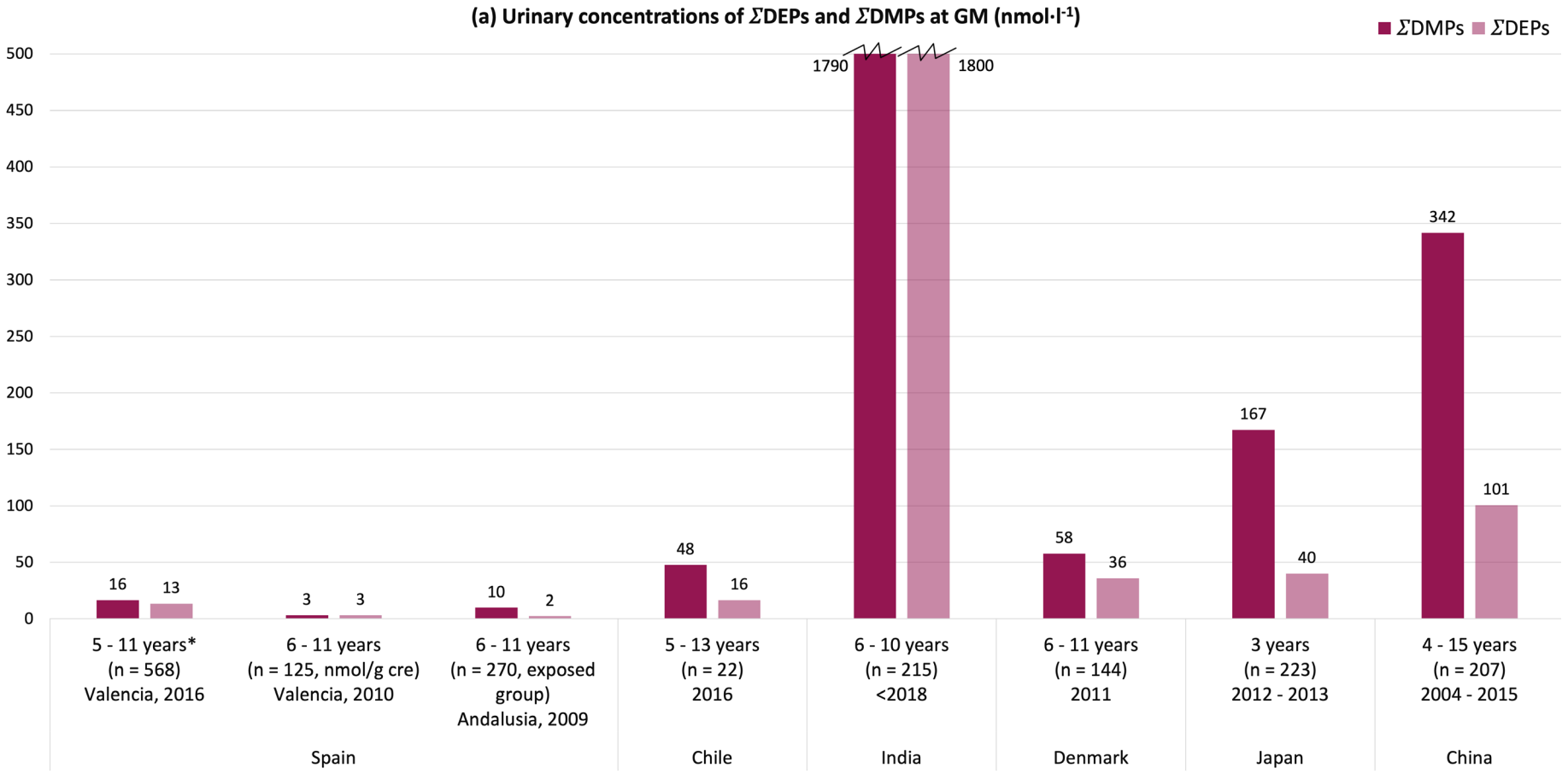

Figure 22a. Comparison of existing HBM data of urinary ∑DMPs and ∑DEPs (nmol·l-1) in children of different ages at different sample collection years (number of samples within parenthesis) across the following countries: Spain[99,295], Chile[296], India[297], Denmark[298], Japan[299], and China[300]. **Present study; GM: geometric mean; cre: creatinine.*

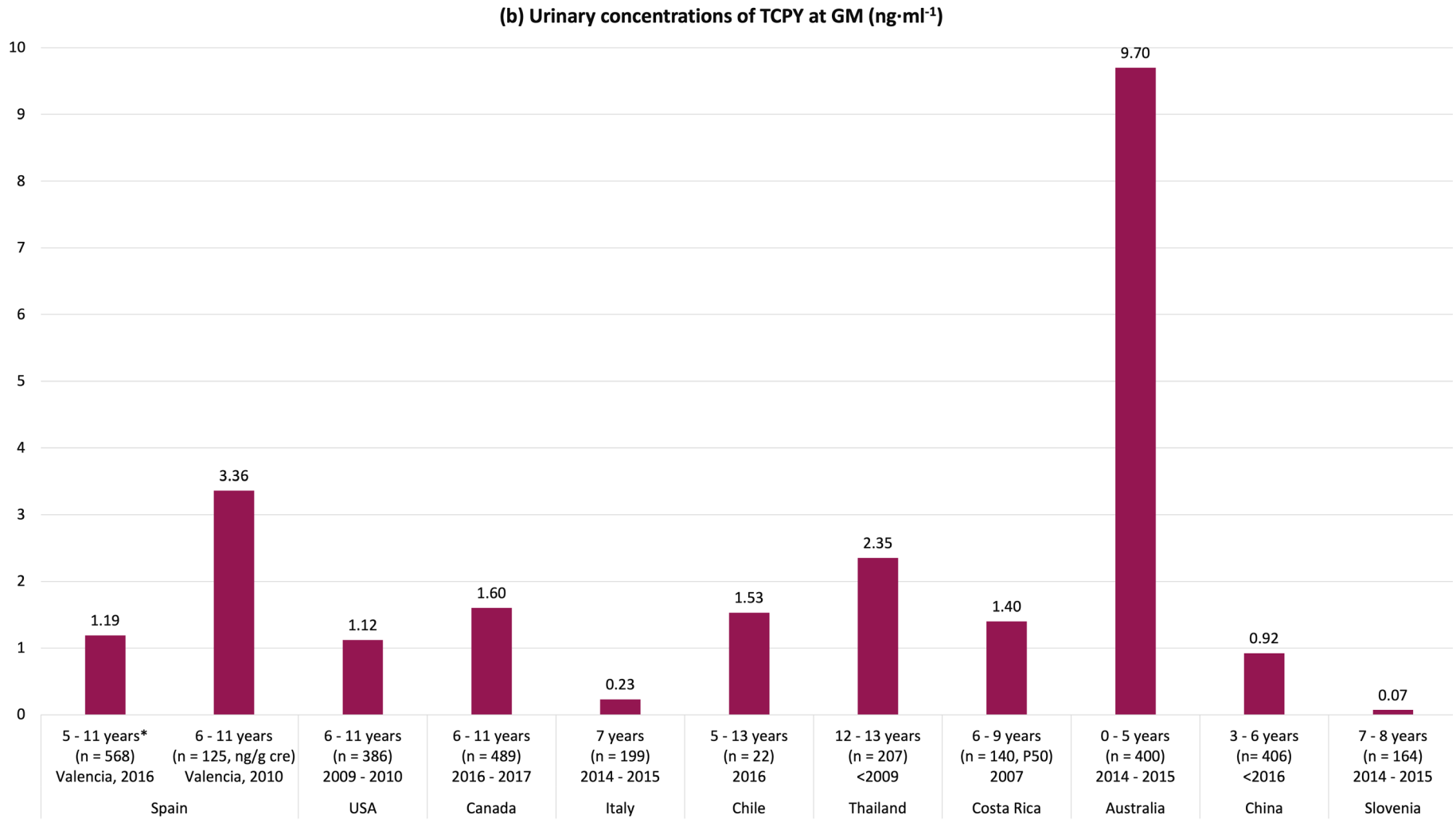

Figure 22b. Comparison of existing HBM data of urinary TCPY (ng·ml^{-1}) in children of different ages at different sample collection years (number of samples within parenthesis) across the following countries: Spain[99], USA[301], Canada[302], Italy[291], Chile[296], Thailand[290], Costa Rica[303], Australia[289], China[304], and Slovenia[292]. **Present study; GM: geometric mean; P50: 50th percentile; cre: creatinine.*

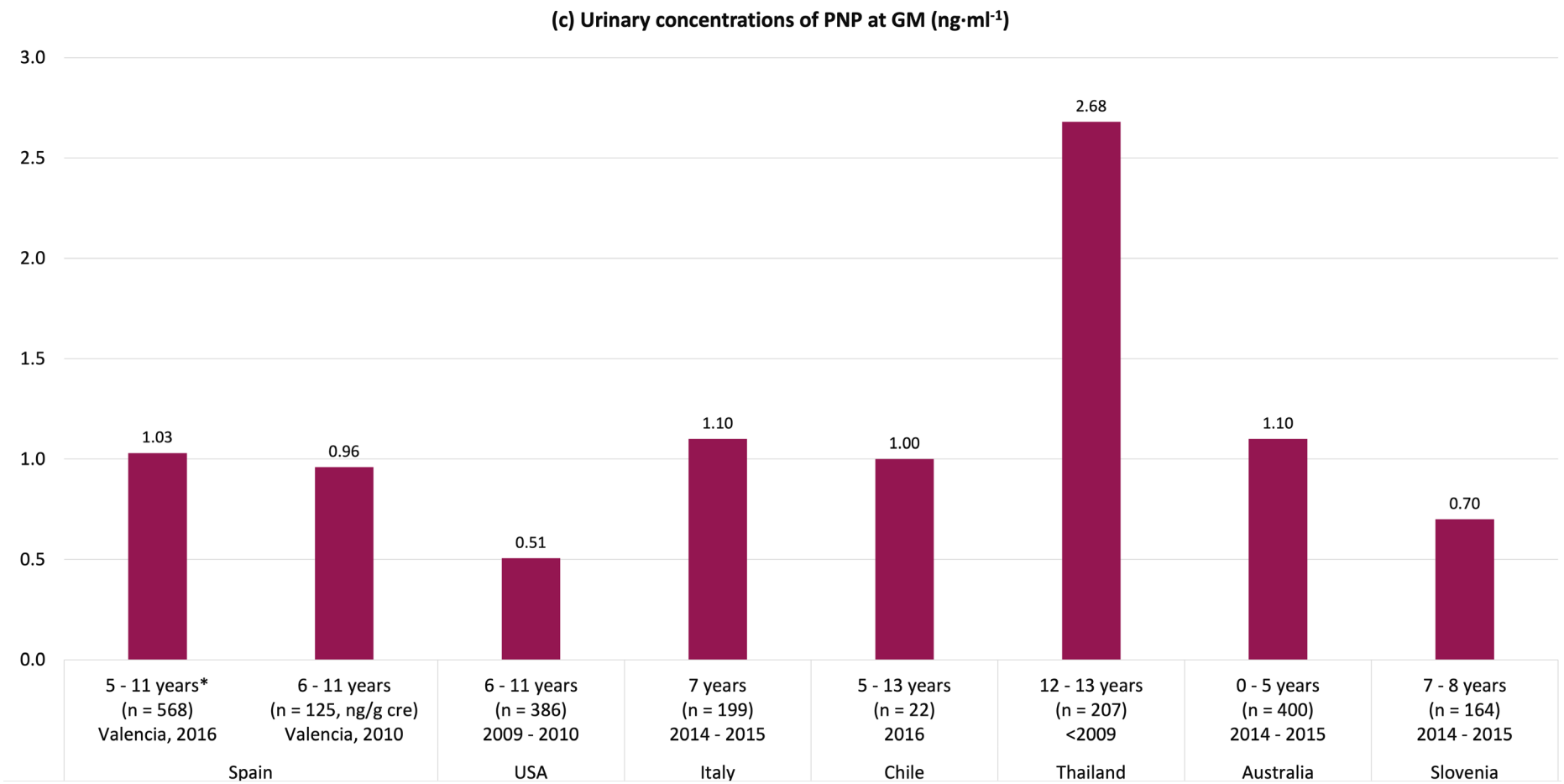

Figure 22c. Comparison of existing HBM data of urinary PNP (ng·ml^{-1}) in children of different ages at different sample collection years (number of samples within parenthesis) across the following countries: Spain[99], USA[301], Italy[291] , Chile[296], Thailand[290], Australia[289], and Slovenia[292]. **Present study; GM: geometric mean; cre: creatinine.*

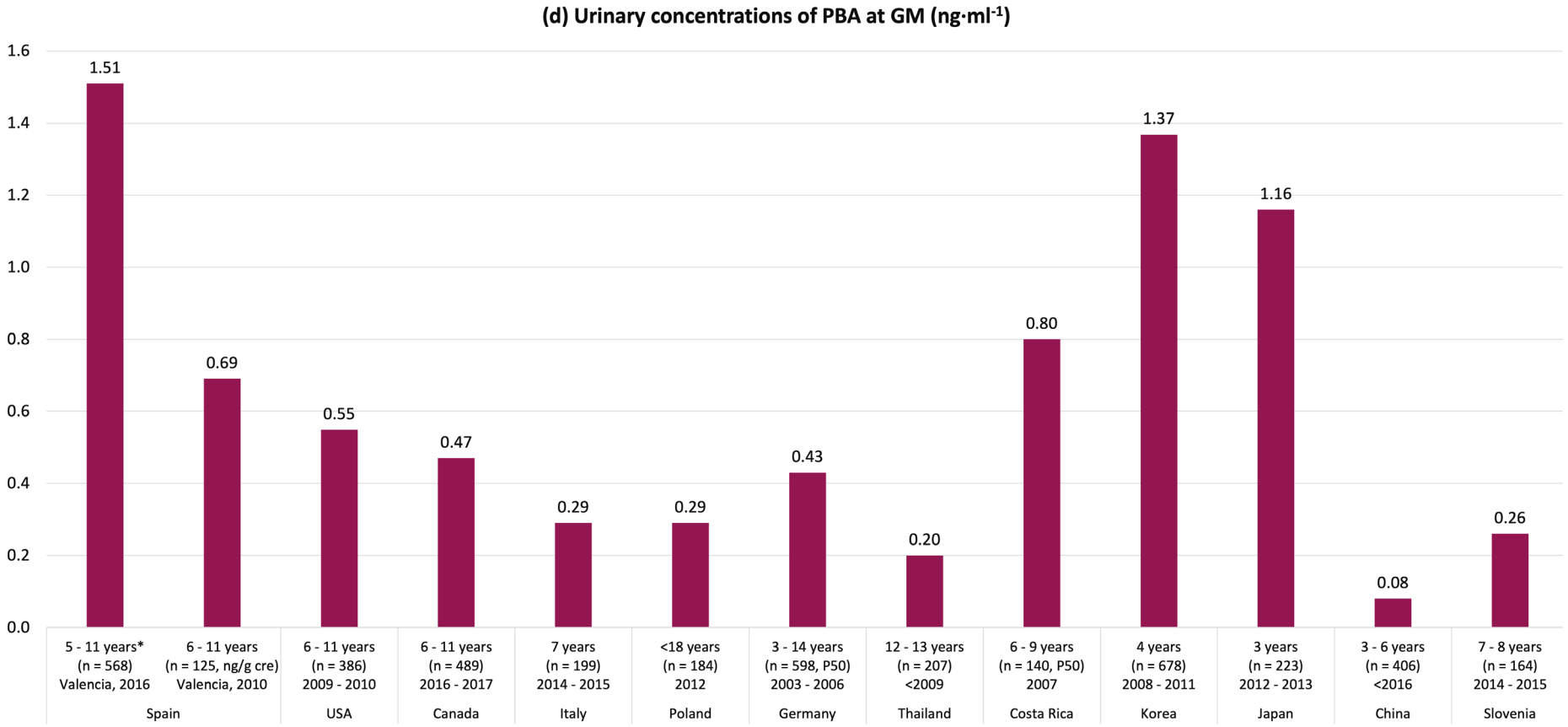

Figure 22d. Comparison of existing HBM data of urinary PBA (ng·ml^{-1}) in children of different ages at different sample collection years (number of samples within parenthesis) across the following countries: Spain[99], USA[301], Canada[302], Italy[291], Poland[274], Germany[305], Thailand[290], Costa Rica[303], Korea[306], Japan[299], China[304], and Slovenia[292]. **Present study; GM: geometric mean; P50: 50th percentile; cre: creatinine.*

Comparing the results obtained here with two previous HBM studies conducted with children from Spain in 2009[295] and 2010[99], exposure to chlorpyrifos appears to have decreased from 2010 to 2016 (present study), since the levels of urinary TCPY were lower in the present study (see Figure 22b). This finding is consistent with the decrease of 6.5 % in the number of food samples with chlorpyrifos and chlorpyrifos-methyl residues detected in the Valencian Community from 2010 to 2015[307,308]. On the other hand, the opposite trend was observed for Pyr exposure, since levels of urinary PBA seem to have increased over the years in Spain (see Figure 22d). This fact might be related to the increase of 46 % observed in sales of Pyr (in tons) between 2011 and 2016 in Spain[309,310]. In the case of DAPs levels (Figure 22a), the reason for their increase along years is not clear because data to support these observations, such as the total amount of OPs used in those time periods in Spain, are not available.

4.3.1.2 STATISTICAL ANALYSIS

A Spearman correlation matrix was performed to explore relationships among TCPY, PNP, PBA, the sum of dimethyl phosphates (∑DMPs) and the sum of diethyl phosphates (∑DEPs). As a result, PBA was observed to be positively correlated with TCPY and ∑DMPs ($p < 0.01$ and < 0.05, respectively), probably because Pyr and chlorpyrifos, together with other OPs, can be used together to control different insect pests in the same type of crops. The relationship between ∑DMPs and ∑DEPs (correlation coefficient of 0.213, $p < 0.01$) may be explained not only by the same cause mentioned above, but also by the drift of application of different OPs in contiguous cultivation areas[307,308]. In the case of PNP, no explanation has been found for its correlation with all metabolites ($p < 0.05$ for TCPY, ∑DEPs and ∑DMPs, and $p < 0.01$ for PBA), as the use of its parent pesticides are banned in Europe.

Associations among the most frequently detected biomarkers (PBA, TCPY, PNP, and ∑DAPs) and all parameters related to the characteristics of the target population (see Table 13) were statistically studied. The resulting regression models with their standardized coefficients and their corresponding 95 % CI are represented in Figure 23. Given that the robust regression models for TCPY, PNP, PBA and ∑DAPs accounted for only between 5 and 12 % of the variance (R^2), the results cannot be conclusive.

Regarding predictors of exposure related to dietary habits, a significant positive association was observed between PBA and recent vegetable intake. This finding is consistent with the results of the Food Safety monitoring program carried out in the Valencian Community in 2015, in which residues of deltamethrin, cypermethrin and λ-cyhalothrin (parent compounds of PBA) were detected in 15 %, 12 %, and 6 % of the food samples analyzed, respectively[308]. In the case of ∑DAPs, the ingestion of legumes and cereals seemed to increase their concentration in urine, as described previously (Figure 20). On the other hand, while ∑DAPs presented a negative correlation with the consumption of canned vegetables, fruits, legumes, and cereals, PNP presented

a positive one. However, no explanation can be attributed to these findings. By contrast, no association was found between TCPY urinary levels and fruit or vegetable consumption, which is not consistent with the results reported by the Valencian Government, given that chlorpyrifos-ethyl was detected in more than 40 % of food samples from the Valencian Community in 2015[308]. This fact is probably related to the fast elimination of TCPY in urine (half-live about 27 hours)[311], whereas questionnaires of the BIOVAL study only covered the food consumption 72 hours before sampling. Therefore, these different time windows may be a limitation to find better correlations.

Regarding the association with socio-demographic characteristics, TCPY and PNP concentrations were lower in those children and those parents born in countries other than Spain, respectively, probably because these families have different dietary habits from people born in Spain, but since less than 10 % of the participants were foreign, this finding was not considered relevant. For PNP, TCPY and ∑DAPs, a significant association with the province of residence was observed but in different directions, so no firm conclusions can be drawn. In the case of ∑DAPs, young children were observed to have increased concentrations of these metabolites in urine, probably due to their increased exposure, e.g., higher dietary intake, compared to their BW.

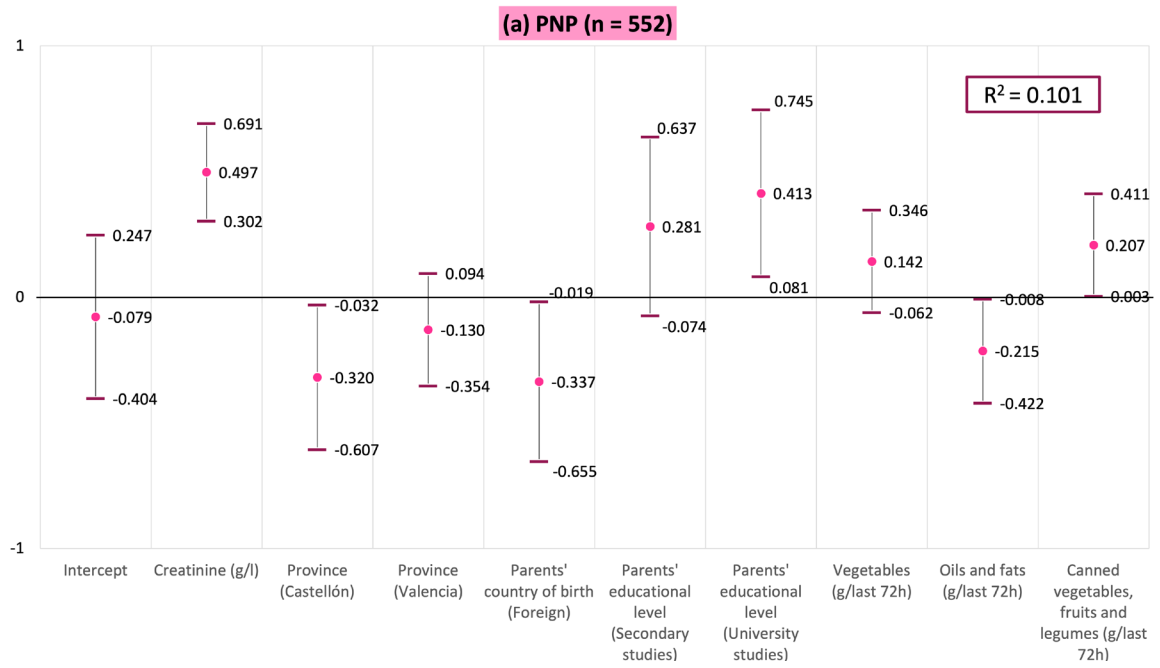

Figure 23a. Linear regression model fitted for the log-transformed PNP concentrations of the children participating in the BIOVAL study (only donors with a completed questionnaire were considered, total number within parenthesis), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2).

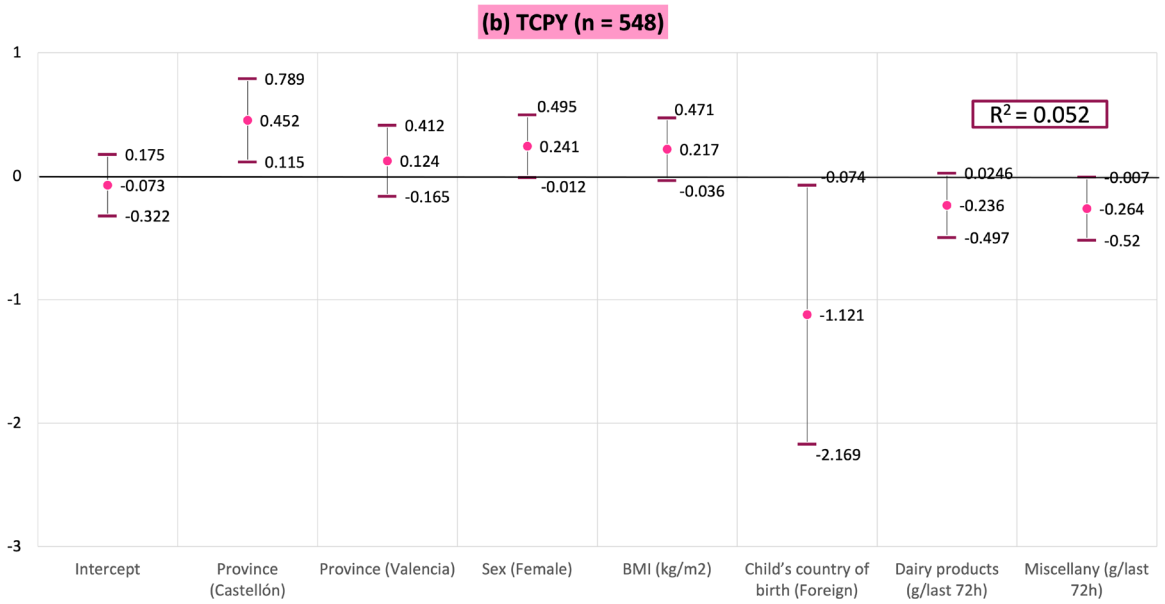

Figure 23b. Linear regression model fitted for the log-transformed TCPY concentrations of the children participating in the BIOVAL study (only donors with a completed questionnaire were considered, total number within parenthesis), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2).

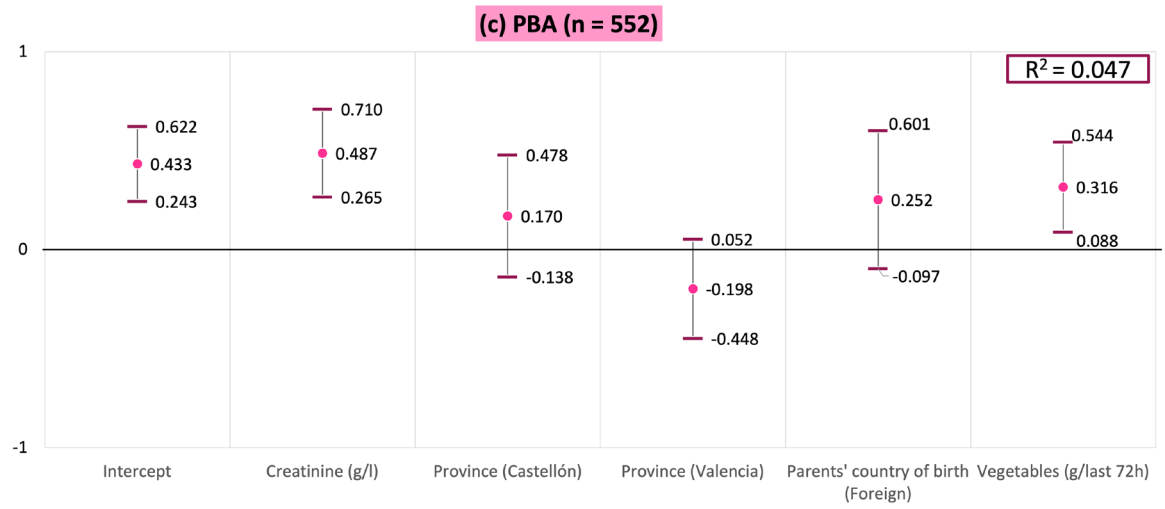

Figure 23c. Linear regression model fitted for the log-transformed PBA concentrations of the children participating in the BIOVAL study (only donors with a completed questionnaire were considered, total number within parenthesis), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2).

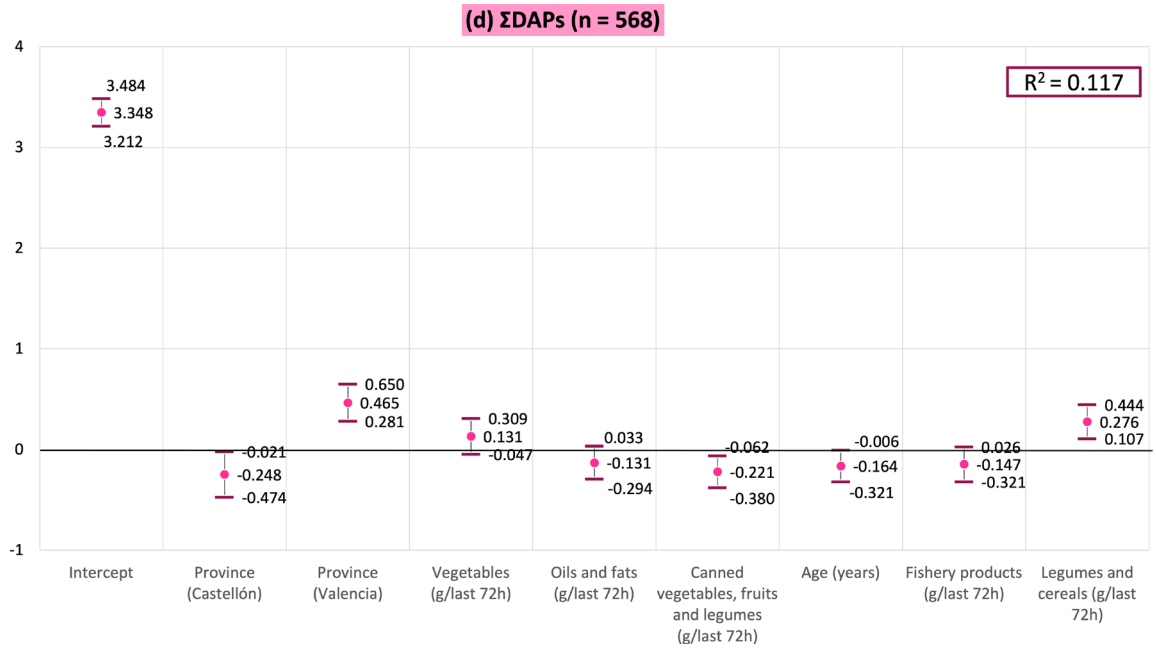

Figure 23d. Linear regression model fitted for the log-transformed ∑DAPs concentrations of the children participating in the BIOVAL study (only donors with a completed questionnaire were considered, total number within parenthesis), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2).

4.3.1.3 RISK ASSESSMENT

EDI for chlorpyrifos-ethyl, dimethoate, parathion, and λ-cyhalothrin were calculated based on their urinary metabolite concentrations at GM (EDI_{GM}) and P95 (EDI_{P95}) levels. In the case of chlorpyrifos-ethyl, EDI was calculated twice: using the concentrations of its specific metabolite (TCPY) and its non-specific metabolite (ΣDEPs), because it was the only approved pesticide that had been recognized as a precursor of both biomarkers when the BIOVAL study took place.

In general, the highest EDI_{GM} was observed for λ-cyhalothrin (0.21 μg·kg bw^{-1}·day^{-1}) since its non-specific metabolite PBA has the lowest F_{UE} (0.30) and one of the highest urinary concentration levels. Similar tendencies have been found in the lactating mothers of the BETTERMILK study (see Section 4.2.1.3), concluding that the Spanish population might be more exposed to Pyr than to OPs. On the other hand, the lowest EDI_{GM} was found for dimethoate (0.08 μg·kg bw^{-1}·day^{-1}) and chlorpyrifos-ethyl based on TCPY levels (0.08 μg·kg bw^{-1}·day^{-1}). Regarding the EDI_{P95}, values ranged from 0.56 μg·kg bw^{-1}·day^{-1} (parathion) to 1.62 μg·kg bw^{-1}·day^{-1} (λ-cyhalothrin).

Results of the risk assessment based on MoA, HQs and HI, are shown in Figure 24a. Regarding HQs, a range from 0.01 (chlorpyrifos-ethyl) to 0.08 (λ-cyhalothrin) was observed based on the GM level, and between 0.08 (chlorpyrifos-ethyl) and 0.65 (λ-cyhalothrin) for the 95th percentile. The cumulative risk was only assessed for OPs because Pyr have not the same MoA as OPs, so they cannot be added together. The HI for OPs was calculated adding the HQs

of chlorpyrifos-ethyl (∑DEPs) and dimethoate (∑DMPs) since their EDIs were calculated from the DAP concentrations, i.e., the representative metabolites of almost the entire OPs family. HIs for the GM and P95 levels of OPs were 0.06 and 0.69, respectively.

As it can be observed, all HQs and HIs were below 1. However, higher HQs and HIs were observed in the children under study compared to women from the same region of Spain (see Figure 21), most likely because of the differences in body burden. In agreement with other studies from other countries[142], dimethoate was observed to have the highest HQ among OPs, although lower HQs and HIs are observed in the children under study compared to other nationalities. In fact, in 2019, EFSA announced the non-renewal of the approval of dimethoate as an active substance in plant protection products in Europe since the endocrine disrupting potential of this substance could not be excluded[312].

Figures 24b and 24c present the results of the cumulative exposure calculations based on CAGs, CAG-motor (CAG of pesticides causing alterations of the motor system) and CAG-AChE (CAG of pesticides affecting the acetylcholinesterase activity), at GM and P95 distribution levels, respectively. MOEs for CAG-motor ranged from 309 (at P95 for λ-cyhalothrin) to 238686 (at GM for dimethoate), and MOEs for CAG-AChE were between 445 (P95 for parathion) and 11934 (at GM for dimethoate). In addition, MOETs for CAG-motor were calculated considering MOEs for chlorpyrifos-ethyl (∑DEPs), dimethoate (∑DMPs), and λ-cyhalothrin, which acted as representatives of the OPs and Pyr families, respectively; while the MOETs for CAG-AChE were estimated considering only MOEs for chlorpyrifos-ethyl (∑DEPs) and dimethoate (∑DMPs), as in the cumulative risk assessment based on MoA, since Pyr pesticides are not AChE inhibitors. As a result, calculated MOETs ranged from 296 (P95) to 2241 (GM) for CAG-motor, and from 341 (GM) to 2177 (P95) for CAG-AChE. Regarding CAG-AChE, chlorpyrifos-ethyl was the most significant contributor (82 %) to the MOET at the GM level, since it has the lowest $NOAEL_{AChE}$, due to its biomarkers (∑DEPs) presented the highest urinary levels among the evaluated OP metabolites. In the case of CAG-motor, λ-cyhalothrin made the greatest contribution (> 95 %) to both levels of distribution.

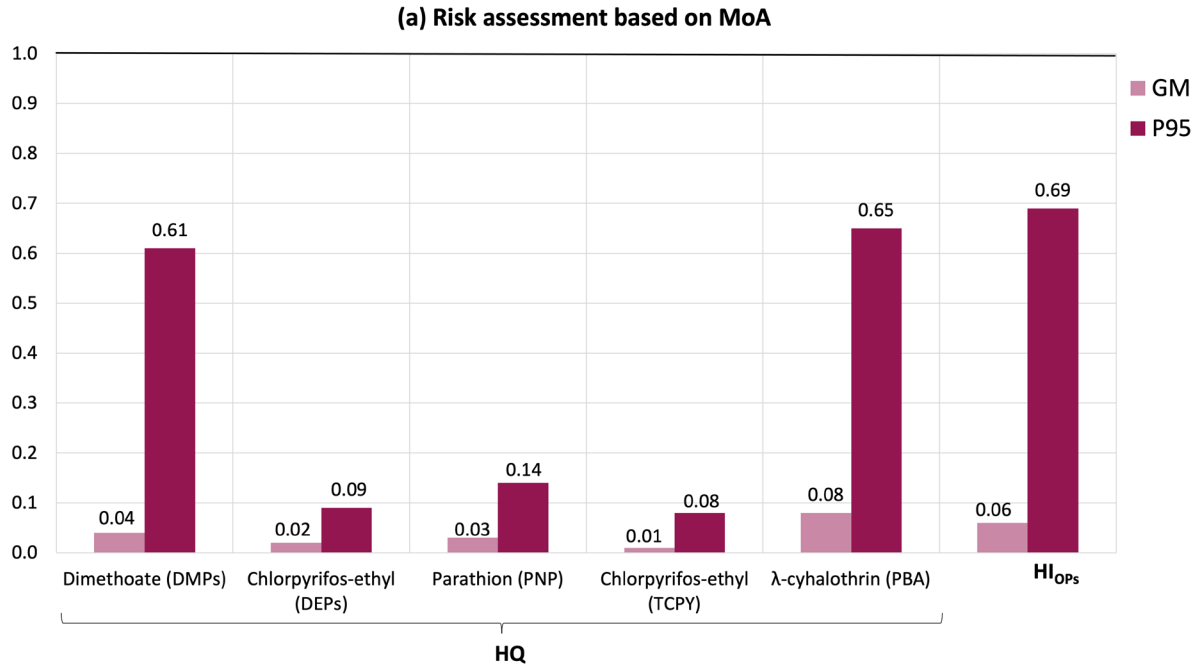

Figure 24a. Results of the risk assessment based on MoA at GM and P95 distribution levels for the exposure to non-persistent pesticides in the children of the BIOVAL study. HI was only calculated for OPs (sum of HQ of DMPs and DEPs). *MoA: mode of action; GM: geometric mean; P95: 95th percentile; HQ: hazard quotient; HI: hazard index.*

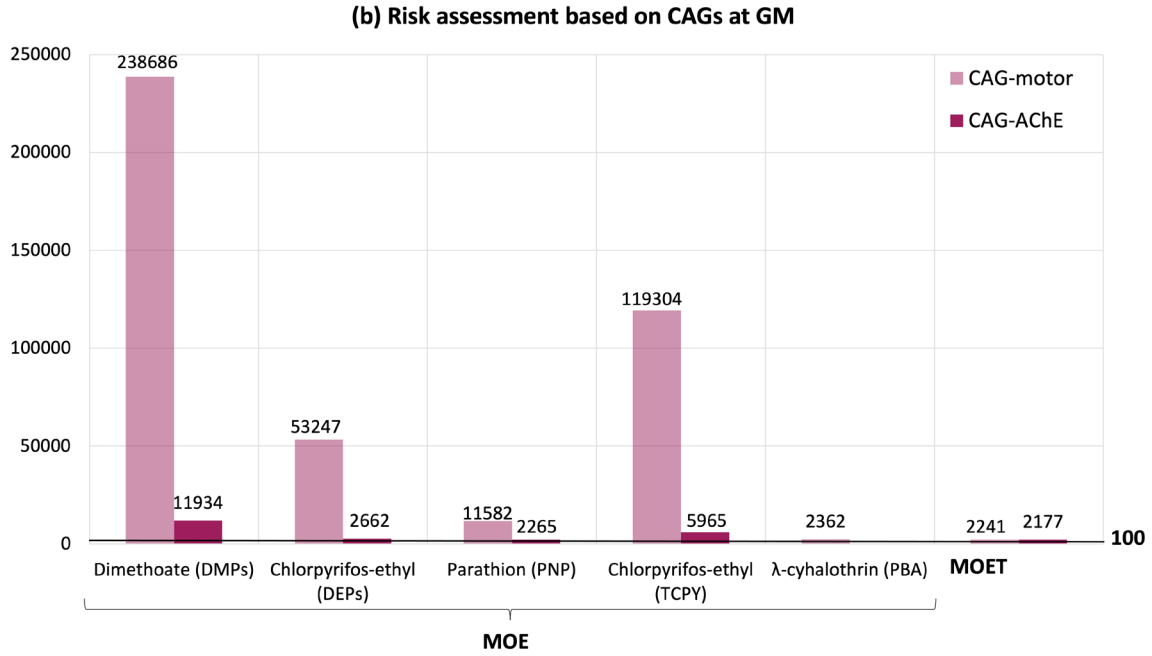

Figure 24b. Results of the risk assessment based on CAGs at GM for the exposure to non-persistent pesticides in the children of the BIOVAL study. MOET for CAG-AChE was only calculated for OPs (sum of MOE for CAG-AChE of DMPs and DEPs). *CAG: cumulative assessment group; GM: geometric mean; MOE: margin of exposure; MOET: total margin of exposure; CAG-motor: CAG of pesticides causing alterations of the motor system; CAG-AChE: CAG of pesticides affecting the acetylcholinesterase activity*.

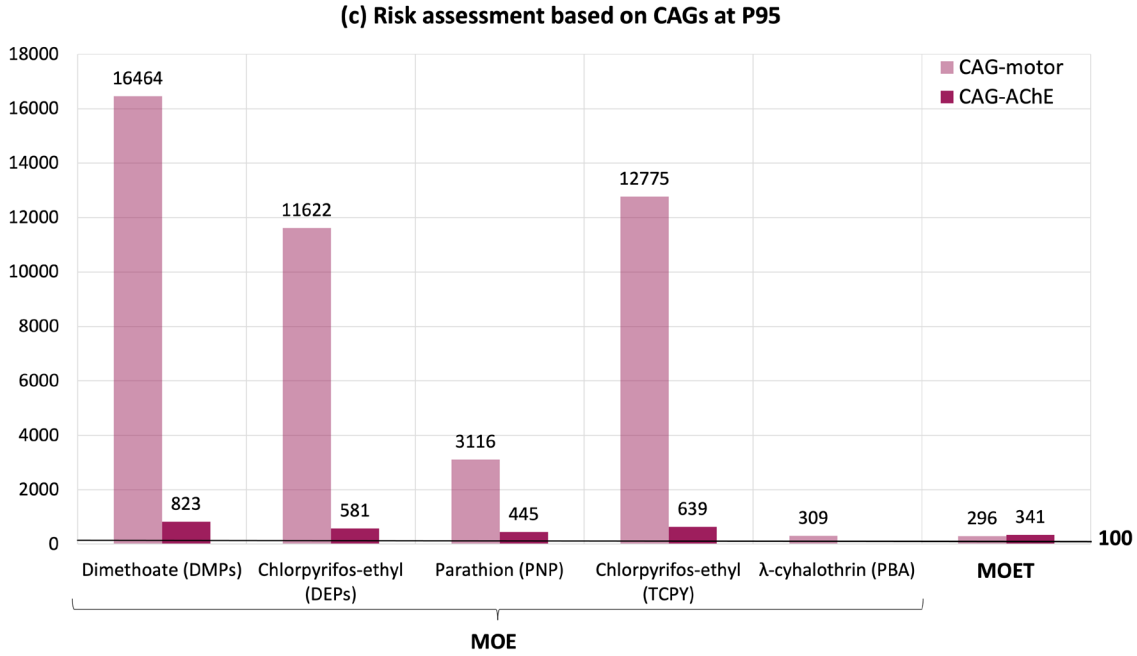

Figure 24c. Results of the risk assessment based on CAGs at P95 for the exposure to non-persistent pesticides in the children of the BIOVAL study. MOET for CAG-AChE was only calculated for OPs (sum of MOE for CAG-AChE of DMPs and DEPs). *CAG: cumulative assessment group; P95: 95th percentile; MOE: margin of exposure; MOET: total margin of exposure; CAG-motor: CAG of pesticides causing alterations of the motor system; CAG-AChE: CAG of pesticides affecting the acetylcholinesterase activity*.

In general, calculated MOETs were all above 100, which does not evidence a risk for the target population in any scenario. These results were compared with MOETs for children from the Netherlands, France, and Bulgaria calculated by EFSA using an external exposure approach[313], showing that MOETs observed in the present study are higher than EFSA's estimations for both CAGs, most likely because of the more conservative approach used by EFSA.

To sum up, similar conclusions are reached with both risk assessment approaches, given that no significant risk for the target population is observed using either strategy: all HQs and HIs were < 1, and MOETs were > 100. However, these findings must be interpreted with caution, and more detailed analyses would be recommended in coming HBM studies. It should be noted that the presence of some pesticide metabolites in urine could be due to the exposure to more than one parent pesticide, to pesticide degradation products, or to the metabolites themselves[314]. However, it is still unidentified to what extent these possible parent compounds play a role in systemic exposure, measured as urinary excretion[274]. Therefore, this study did not differentiate among them and, to facilitate the risk assessment, it was assumed that the presence of the pesticide metabolites in the analyzed urine samples was only due to the exposure to one parent pesticide. For this reason, the calculated EDI and risk assessment parameters are surely overestimated due to the likely contribution of the same metabolites coming from other pesticides or their breakdown products in the environment.

4.3.2 CONCLUSIONS

In this research, six non-specific metabolites of OPs and twenty specific metabolites of OPs, Pyr, and Herb were analyzed in the urine samples of 568 children (aged between 5 and12 years old), participating in the BIOVAL program (Spain). Five or more pesticide biomarkers were detected in 85 % of the children. The multiple robust models revealed that recent vegetable, legume, and cereal intakes were significant predictors of exposure to Pyr and OPs. Nevertheless, despite the results of the pesticide risk assessment not showing health risk evidence for children living in Spain, the biomonitoring of pesticides is highly advisable in order to monitor their exposure over time, due to the extensive use of pesticides in this country. However, it is necessary to increase the number of available health-based guidance values (e.g., BE, HBM-I and HBM-II) for the different pesticides in use to properly interpret the associated exposure in a health risk assessment context.

In addition, further efforts should be made to fully understand pesticide exposure in this region, starting with the design of more detailed and extensive questionnaires, as well as with the harmonization of methodologies used for estimating the cumulative risk. Furthermore, multi-residue methods, including as many pesticide metabolites as possible, are highly recommended to carry out more accurate cumulative risk estimations.

4.3.3 ARTICLE I.3: *EXPOSURE AND CUMULATIVE RISK ASSESSMENT TO NON-PERSISTENT PESTICIDES IN SPANISH CHILDREN USING BIOMONITORING*

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Exposure and cumulative risk assessment to non-persistent pesticides in Spanish children using biomonitoring

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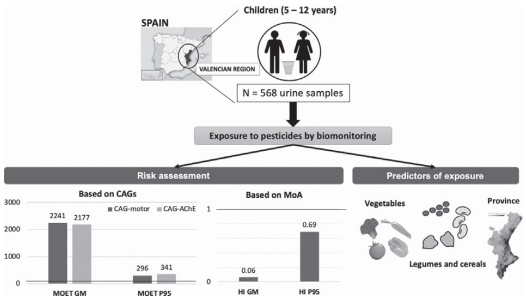
HIGHLIGHTS

- 26 pesticide metabolites were analyzed in urine samples from 568 Spanish children.
- Five pesticide biomarkers presented detection frequencies higher than 40%.
- Recent vegetable consumption was a major predictor of exposure to pyrethroids.
- Risk assessment estimations showed HQ and HI < 1, and MOETs > 100.

GRAPHICAL ABSTRACT



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ABSTRACT

The main objective of the present research is to evaluate the exposure to pesticides in children (n = 568) from the Valencian Region (Spain). Six non-specific and 20 specific metabolites of organophosphate pesticides (OPs), herbicides (Herb), and pyrethroids (Pyr) were analyzed in urine samples. The biomarkers with the highest detection frequencies (>70%) were diethyl phosphate, p-nitrophenol, 3-phenoxybenzoic acid, and 3,5,6-trichloro-2-pyridinol, whose geometric mean concentrations (ng·ml-n1) were 1.53, 1.03, 1.51 and 1.19, respectively. Robust regression models showed that the province of residence and the recent consumption of vegetables, legumes and cereals were some of the most important predictors of pesticide exposure. Pesticide risk assessment is estimated using two different strategies: one based on the pesticides' mode of action (MoA) and the other based on cumulative assessment groups (CAGs), proposed by EFSA. The estimated daily intakes (EDIs) ranged from 0.08 (chlorpyrifos) to 1.62 μg·kg bw-1 (λ-cyhalothrin). The MoA approach resulted in hazard quotients ranging from 0.01 (chlorpyrifos) to 0.65 (λ-cyhalothrin), and a hazard index for OPs lower than 1. Similarly, the risk assessment based on CAGs led to total margins of exposure (MOETs) far from 100. In conclusion both risk assessment strategies does not reveal any evidence of a potential health risk due to pesticide exposure in Spanish children.

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1. Introduction

Pesticides are chemical substances used to control harmful organisms (insects, rodents, fungi, and unwanted weeds) or vector-borne diseases (e.g., carried through mosquitoes), as well as to protect plants during production, storage, and transport, with the objective of achieving higher yields (European Commission, 2019a; World Health Organization, 2018a). On the one hand, pesticide applications in agriculture allow us to produce enough food to support the continuing growth of the global population, which is expected to have grown 30% by 2050 (World Health Organization, 2018b). However, industrial agriculture releases these substances not only into the food chain but also into the soil, water, and air, threatening human health and our environment (Rotterdam Convention, 2019). Apart from agricultural activities, pesticides can also occasionally be used for domestic purposes and in local public places, such as parks and gardens.

Pyrethroids (Pyr), organophosphates (OPs), and some herbicides (Herb) are the major types of pesticides used in the EU and worldwide (EUROSTAT, 2018). Some of these substances have neurotoxic and endocrine disrupting properties and, due to their presence in the environment, wildlife and humans can be negatively affected by the exposure to them (Sidhu et al., 2019). In fact, chlorpyrifos, one of the most used insecticides in Europe in the last few years, has recently been banned and classified as toxic for reproduction (category 1B) by the European Food Safety Authority (EFSA, 2019a).

As a result of their extensive use, the general population is exposed to pesticide residues through several routes and pathways. The principal route of exposure to pesticides is the ingestion of food, mainly by fruit and vegetable consumption, and water (Kim et al., 2017). Nevertheless, non-dietary routes, such as inhalation and dermal contact via direct contact or dust, may also play an important role (Kim et al., 2017; López et al., 2017). The exposure to pesticides has been related to several adverse effects on humans, depending on the toxicity and the level of exposure (Kim et al., 2017). In general, they have been linked to the following health disorders: cancer (e.g. bladder and colon cancer), respiratory problems (e.g. asthma), Parkinson, Alzheimer, reproductive disorders (e.g. male infertility), among others (Singh et al., 2018). In addition, several neurodevelopmental effects of pesticides on humans have been reported, concluding that exposure to these substances during pregnancy and childhood may have negative effects on children's neurodevelopment (Liu and Schelar, 2012). For this reason, children and pregnant women are considered the most vulnerable population groups.

The risk assessment associated with pesticide exposure is traditionally performed by an external approach (monitoring), which considers the pesticide residue levels in food and the consumption data of the target population to be able to obtain an estimated dietary intake (Dujardin and Bocca, 2019). But this methodology is not accurate enough, since other possible routes of exposure that could be relevant, like for instance, inhalation or dermal contact, are not considered in this approach. In this context, human biomonitoring (HBM) is gaining increasing importance as a tool for exposure assessment of environmental pollutants. HBM of pesticides involves the measurement of the target pesticide and/or its metabolites (biomarkers of exposure) in biological matrices, such as urine, blood, or hair, in order to estimate the internal exposure to these contaminants (Barr, 2008). Therefore, a more suitable risk assessment of these substances can be achieved by combining biomonitoring data with toxicological data and the toxicokinetic properties of the pesticides since exposure to pesticides through all possible routes and pathways can thereby be taken into account (Katsikantami et al., 2019). OPs, Pyr, and Herb are non-persistent pesticides, which means that these compounds do not bioaccumulate in the body, and their metabolites are mainly excreted through urine in <72 h after exposure (ATSDR, 2003; Garfitt et al., 2002; Kaloyanova and El Batawi, 1991; Nolan et al., 1984). On account of this, HBM of these compounds is usually carried out by analyzing their metabolites (biomarkers of exposure) in human urine (Fernández et al., 2020; Jing and Kannan, 2018; Roca et al., 2014b, 2014a; Yusa et al., 2015).

The general population is exposed every day to combinations of different pesticides (Bopp et al., 2018). Thus, exposure to these substances should not be addressed individually, since the potential toxicity of their mixture could be underestimated (Nicolopoulou-Stamati et al., 2016). Also, interactions between multiple pesticide residues or different chemical families should be considered in a cumulative risk assessment (EFSA, 2019b). The conventional way of assessing the individual risk of pesticide exposure is by calculating the hazard quotient (HQ), which is the ratio of the potential exposure to a substance and the level at which no adverse effects are expected (EFSA, 2013). The HQs of pesticides that have the same mechanism of action (MoA) are added to obtain a hazard index (HI), with which the cumulative risk can be estimated (EFSA, 2013). In 2019, EFSA proposed a new risk assessment methodology which considers the synergistic effects of pesticides in cumulative assessment groups (CAGs), whereby those active substances that affect the same target organ/system are grouped together despite having a different MoA (EFSA, 2019b).

According to Eurostat, Spain was the European country with the highest sales of pesticides in 2016 while also one of the main agricultural producers in the European Union (EUROSTAT, 2018). In 2010, a previous pilot HBM study was conducted to obtain preliminary data on contemporary exposure of Spanish school-aged children to pesticides (CIPAV project), providing evidence of their exposure to these substances (Roca et al., 2014b). The results and lessons learned from this pilot study were used to carry out a larger cross-sectional HBM study in 2015 with children from the same region, in order to assess their exposure and risk of being exposed to pesticides and other contaminants which are present in their diet (BIOVAL program) (Pérez et al., 2017).

The goals of the present study are: i) to determine the levels of non-persistent pesticide metabolites in urine samples of children (from 5 to 12 years of age) participating in the BIOVAL program; ii) to identify the main predictors of exposure to these pesticides; and, iii) to characterize the cumulative risk of the exposure to pesticides in the target population using two different approaches.

2. Materials and methods

2.1. Study design and target population

The BIOVAL study design was described in depth by Pérez et al. (2017). In brief, the parents and guardians of primary school children from the Valencian Community (Castellón, Alicante, and Valencia provinces) were asked to participate in the BIOVAL program in 2016. The inclusion criteria of the participants were: i) children aged between 5 and 12 years old, ii) no metabolic disease or urinary excretion problems, and iii) residence in the Valencian Community (Castellón, Alicante, or Valencia). A total of 666 children were recruited from 25 primary schools in municipalities of the Valencian Region.

2.2. Sample and data collection

First-morning void (FMV) urine samples were selected to assess short-term exposure to pesticides in the BIOVAL program because it was the most practical collection method accepted by the scientific community (Hioki et al., 2019; Kissel et al., 2005) and generally used by large biomonitoring programs, such as the Canadian Health Measurement Survey (Health Canada, 2019) and the NHANES (National Health and Nutrition Examination Survey) (CDC, 2019). FMV samples were collected by the participants themselves. For this purpose, they were provided with a kit containing disposable gloves, a sterile 100-ml polypropylene bottle, and urine collection instructions. All samples were collected after informed consent had been signed by the parents of the participants. The urine samples were kept at 4 °C in a

portable refrigerator, and within 2 h, they were transported to the Biobank for Biomedical Research and Public Health of the Valencian Community (IBSP-CV Biobank) (PT13/0010/0064) integrated in the Spanish National Biobank Network and the Valencian Biobanking Network, where they were divided into 10-ml aliquots and stored at −80 °C until analysis. A questionnaire with detailed information on the socio-demographic characteristics and food habits was completed by the volunteers' parents or guardians. These variables are summarized in Table 1. All personal data of the participants were protected and stored in a secure place, which could only be accessed by authorized people. Moreover, the BIOVAL study and the sampling protocol had been approved by the Scientific Ethics Committee of the Valencian Research Centre for Public Health (FISABIO) of the Valencian Government (Directorate-General of Public Health).

2.3. Chemical analysis

For this study, five metabolites of Herb, four metabolites of Pyr, and seventeen metabolites of OPs (11 specific and 6 non-specific metabolites) were selected to be analyzed in the urine (Table S1). Dialkyl phosphates (DAPs), non-specific biomarkers of OPs, were determined by liquid-liquid extraction, and the rest of the biomarkers by QuEChERS, followed by a liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) detection, as described in two previous papers (Fernández et al., 2019; Roca et al., 2014a) (see SM1 and SM2). Creatinine was measured using Jaffé's reaction at the University Hospital Doctor Peset in Valencia (Spain). Urine samples with urinary concentrations of creatinine out of the range 30–300 mg·dl^{-1} were excluded from our study (Barr et al., 2005).

Both quality assurance and quality control procedures followed in these analyses are described in detail in Fernández et al. (2020, 2019). Briefly, reagent and sample blanks, and in-house prepared quality control samples were used to check the extraction efficiency and ensure a proper quantification of the real samples in each analytical sequence. A matrix-matched calibration curve was used, and analogous isotopic labeled internal standards were also added to all samples so as to minimize the effect of the urine matrix and to correct losses during the sample treatment. In addition, the laboratory participates in the German External Quality Assessment Scheme (G-EQUAS) intercomparison programs in order to check the validity of the results. Results of G-EQUAS materials for DAPs and for some specific metabolites of OPs and Pyr (PBA, DBCA, FPBA, TCPY and PNP) can be found in Fernández et al. (2019) and Table S2, respectively.

2.4. Data and statistical analysis

The statistical analysis was performed using R4 software (version 3.5.2). Pesticide metabolite levels in urine below the procedural quantification limit (LoQ) were estimated and replaced by values between 0 and the LoQ (Vrijheid et al., 2017), following the maximum likelihood estimation method suggested by EFSA (2010). This method assumes that data are distributed according to a parametric distribution and estimates the parameters of this distribution maximizing the probability of obtaining the observed sample. A log-normal distribution was assumed for the pesticides' biomarker levels in the urine.

First, a descriptive analysis of all studied variables was performed. Qualitative variables were described by absolute frequencies and percentages, and quantitative variables by their median and range (Table 1). Additionally, urinary levels were summarized by calculating the detection frequency (DF, % samples > LoQ), 25th, 50th, 75th, and 95th percentiles (P25, P50, P75, and P95), the geometric mean (GM), and maximum, as can be seen in Table 2. The relationship of the biomarkers with the qualitative and quantitative characteristics of the target population was described by the median and range of the metabolite urinary levels in each category and Spearman's correlation, respectively. In addition, Spearman's test was used to study correlations among metabolites.

Robust multiple linear regression models were built to assess the relationship of the urinary levels of the most frequently detected metabolites (DF > 40%) with the lifestyle, socio-demographic characteristics and food consumption habits of the participants. The reference categories for the qualitative variables were as follows: “Alicante” for the variable “Province”, “Urban area” for “Home location”, “Male” for “Sex”, “Spain” for “Country of birth”, “3 or more days per week” for “Physical activity”, “Without studies of primary studies” for “Maximum level of education of the parents”, and “Unemployed” for “Employment situation”. To ensure that creatinine levels did not affect the statistical significance of other variables in the model, urinary concentrations of metabolites without adjustment for creatinine were included in the regression analysis and creatinine was added as a separate quantitative variable (Barr et al., 2005). DAPs were considered as a sum of dimethyl phosphate (DMP), dimethyl thiophosphate (DMTP), dimethyl

Table 1
Lifestyle, sociodemographic characteristics and food consumption habits of the children participating in the BIOVAL program (N = 568).

Variable	n (%)	Median (minimum – maximum)
Children's characteristics		
Body Mass Index (BMI, kg·m^{-2})		17 (12–45)
Missing data	17 (3%)	
Age (years)		8 (5–12)
Province		
Alicante	204 (36%)	
Castellón	126 (22%)	
Valencia	238 (42%)	
Home location		
Urban area	444 (78%)	
Countryside	22 (4%)	
Other	101 (17.8%)	
Missing data	1 (0.2%)	
Sex		
Male	287 (51%)	
Female	281 (49%)	
Country of birth		
Spain	557 (98%)	
Foreign	8 (2%)	
Missing data	3 (0%)	
Physical activity		
3 or more days per week	299 (53%)	
1 or 2 days per week	209 (37%)	
Occasionally or never	60 (10%)	
Parents' characteristics		
Country of birth		
Spain	492 (87%)	
Foreign	60 (10%)	
Missing data	16 (3%)	
Maximum level of education		
Without studies or primary studies	58 (10%)	
Secondary studies	169 (30%)	
University studies	341 (60%)	
Employment situation		
Unemployed	38 (7%)	
Employed	530 (93%)	
Food consumption in the last 72 h by groups (grams)		Median (minimum – maximum)
Eggs		60 (0–360)
Meat products		470 (0–2295)
Fishery products		220 (0–1730)
Dairy products		1008 (0–2820)
Vegetables		337.5 (0–1798.5)
Fruits		560 (0–2625)
Nuts		0 (0—210)
Legumes and cereals		524 (0–2248)
Canned vegetables, fruits and legumes		0 (0–1465)
Oils and fats		10 (0—120)
Miscellany (snacks, pre-cooked meals, pastries and sauces)		85 (0–420)

Table 2
Urinary levels of the analyzed pesticide metabolites (ng·ml^{-1}) in the target population from the Valencian Region (n = 568).[a,b]

Biomarker	LoQ	DF (%)	P25	P50	GM	P75	P95	Maximum
Metabolites of herbicides								
2,4-D	0.25	35	<LoQ	<LoQ	<LoQ	0.40 (0.39)	1.30 (1.32)	12.36 (9.51)
ATZM	0.25	4	–	–	–	–	–	0.80 (1.49)
ALAM	0.125	4	–	–	–	–	–	1.12 (0.99)
METM	0.125	3	–	–	–	–	–	1.42 (2.01)
2,4,5-T	0.125	0.2	–	–	–	–	–	0.30 (0.56)
Metabolites of pyrethroids								
PBA (non-specific metabolite)	0.50	79	0.63 (0.73)	1.63 (1.65)	1.51 (1.56)	3.60 (3.70)	11.57 (11.66)	130.31 (127.68)
DCCA	5.00	20	<LoQ	<LoQ	<LoQ	<LoQ	46.65 (46.33)	256.00 (338.62)
DBCA	1.25	14	<LoQ	<LoQ	<LoQ	<LoQ	5.56 (6.85)	219.10 (241.56)
FPBA	0.125	4	–	–	–	–	–	2.52 (2.82)
Specific metabolites of OPs								
PNP	0.25	85	0.52 (0.57)	1.30 (1.34)	1.03 (1.06)	2.25 (2.19)	5.23 (4.99)	11.80 (15.90)
TCPY	0.50	74	<LoQ	1.13 (1.23)	1.19 (1.22)	3.20 (3.49)	11.08 (13.02)	102.20 (91.66)
DEAMPY	0.25	37	<LoQ	<LoQ	<LoQ	0.67 (0.69)	4.00 (3.81)	49.77 (58.69)
METHA	0.125	27	<LoQ	<LoQ	<LoQ	0.14 (0.16)	0.79 (0.84)	3.60 (2.86)
IMPY	0.25	9	–	–	–	–	0.75 (0.81)	7.00 (11.67)
MDA	1.25	7	–	–	–	–	1.39 (1.64)	12.39 (10.55)
MNP	0.25	7	–	–	–	–	0.55 (0.57)	11.63 (28.72)
DIMET	0.25	3	–	–	–	–	–	2.78 (3.84)
OMET	0.25	2	–	–	–	–	–	2.60 (3.39)
CHMC	0.50	1	–	–	–	–	–	1.54 (1.20)
ACEP	0.50	1	–	–	–	–	–	1.60 (1.73)
Non-specific metabolites of OPs (dialkyl phosphates)								
DEP	0.50	92	0.91 (0.89)	1.52 (1.51)	1.53 (1.58)	2.45 (2.80)	5.90 (7.78)	21.38 (55.10)
DMP	0.50	65	<LoQ	0.88 (0.97)	0.95 (0.98)	3.02 (3.19)	12.94 (14.55)	81.76 (74.10)
DMTP	0.50	33	<LoQ	<LoQ	<LoQ	0.88 (0.92)	14.68 (16.07)	218.82 (179.40)
DMDTP	0.50	27	<LoQ	<LoQ	<LoQ	0.60 (0.58)	2.51 (3.25)	180.63 (257.46)
DETP	0.50	21	<LoQ	<LoQ	<LoQ	<LoQ	3.60 (3.29)	113.83 (122.13)
DEDTP	0.50	3	–	–	–	–	–	1.48 (1.40)
∑DMPs[c,d]		–	6.31 (6.22)	14.14 (14.76)	16.45 (16.99)	35.87 (38.26)	238.44 (251.58)	2774.13 (2346.87)
∑DEPs[c,e]		–	8.25 (7.60)	12.45 (13.09)	13.39 (13.83)	19.86 (23.72)	61.36 (67.32)	759.09 (814.48)
∑DAPs[c,f]		–	18.20 (17.91)	30.83 (32.48)	37.16 (38.38)	61.03 (70.33)	284.77 (302.52)	2784.45 (2394.68)

LoQ = Quantification Limit; DF = Detection Frequency (% samples > LoQ); P25 = 25th percentile; AM = Arithmetic mean; GM = Geometric mean; P75 = 75th percentile; P95 = 95th percentile; SD = Standard deviation.
[a] Some parameters were not calculated due to the low DF of some metabolites.
[b] Values within parenthesis are the urinary levels adjusted by creatinine (μg g Cre^{-1}).
[c] Units: nmol·L^{-1} (nmol·g Cre^{-1}).
[d] ∑DMPs = DMP + DMTP + DMDTP.
[e] ∑DEPs = DEP + DETP + DEDTP.
[f] ∑DAPs = ∑DEPs + ∑DMPs.

dithiophosphate (DMDTP), diethyl phosphate (DEP), diethyl thiophosphate (DETP), and diethyl dithiophosphate (DEDTP) in nmol·l^{-1}. The logarithmic transformation of urinary concentrations was taken into account to obtain the normality of the response variable. Robust regression was considered as an alternative to the ordinary least squares (OLS) estimation methods, given the presence of some outlier values in the data. The models were built following a backward step-by-step variable selection procedure based on the Robust Final Prediction Error criterion (Maronna et al., 2018). The numerical explanatory variables were centered and divided by twice their standard deviation so that the magnitude of regression coefficients could be directly comparable (Gelman, 2008). Confidence intervals at 95% significance were calculated for the model coefficients, and a statistically significant relationship between variables at 5% significance was considered.

2.5. Risk assessment

In this research, children's exposure to pesticides was evaluated by an internal dose approach. For this purpose, the intake of parent pesticides based on GM and P95 urinary concentrations of their biomarkers was estimated as follows (Katsikantami et al., 2019):

$$EDI = \frac{C_U \times V_{24h} \times MW_P}{F_{UE} \times BW} \quad (1)$$

where EDI is the estimated daily intake of the parent pesticide (μg·kg bw^{-1}·day^{-1}), C_U the molar concentration of the pesticide metabolite in urine (μmol·l^{-1}), V_{24h} the total urinary volume excreted within 24 h (0.66 l for children) (Aylward et al., 2011), MW_P the molecular weight of the selected parent pesticide (g·mol^{-1}), F_{UE} the urinary excretion factor of the parent pesticide (ranging between 0.30 and 0.90) (Bouchard et al., 2005; Gosselin et al., 2004; Katsikantami et al., 2019; Khemiri et al., 2017), and BW the mean body weight of the target population (33 kg). Parameters selected for the calculation of EDI are shown in Table S3. It was assumed that the presence of pesticide metabolites in the children's urine was mainly due to their exposure to the parent pesticide, and not to the metabolites themselves. Furthermore, only parent pesticides whose metabolites have a high detection frequency (DF > 40%) were considered in this calculation. The selection of the parent compound for those metabolites which have various parent pesticides was based on the following criteria:

a) An active substance approved by the Regulation (EC) No 1107/2009.
b) The pesticide with the lowest toxicological reference value was chosen.

Once the EDIs of the GM and P95 levels were determined, two different strategies were performed to carry out the risk assessment: one based on the pesticides' MoA (Katsikantami et al., 2019); and the

other based on CAGs, as has been recently proposed by EFSA (Dujardin and Bocca, 2019; EFSA, 2019b).

2.5.1. Risk assessment based on MoA

In this approach, each pesticide's EDI is compared to its acceptable daily intake (ADI), as a toxicological reference value, in order to obtain a HQ as follows:

$$HQ_i = \frac{EDI_i}{ADI_i} \quad (2)$$

If the HQ of a single pesticide is lower than 1, there is no risk of exposure to that pesticide. The HQs of pesticides with the same MoA can be added up to obtain a HI, which is used to evaluate the cumulative risk of exposure to a particular group of pesticides (formula (3)). A HI below 1 in a certain family of pesticides means that there is a low risk for human health:

$$HI_{MoA} = \sum HQ_i \quad (3)$$

Hence, in the study's context, exposure to OPs, Pyr and Herb have to be assessed separately, since they have different MoAs (EFSA, 2019b). Therefore, this method does not take into account the synergies between the different pesticide families (EFSA, 2019b), leading to an underestimation of the risk.

2.5.2. Risk assessment based on CAGs

After adapting EFSA's procedure for assessing the cumulative exposure to pesticides that affect the nervous system, it was applied in this HBM study (Dujardin and Bocca, 2019; EFSA, 2019b). Consequently, the estimated exposure for two CAGs was calculated: pesticides that cause functional alterations of the motor division of the nervous system (CAG-motor) and pesticides that lead to brain and/or erythrocyte acetylcholinesterase (AChE) inhibition (CAG-AChE), assuming that substances from the same CAG combine their effects by dose addition. Firstly, the EDI for each pesticide was compared with its no observed adverse effect level (NOAEL) of acute exposure to each CAG, using this level as a health-based reference point. For this purpose, the individual margin of exposure (MOE_i) for each active substance and CAG was calculated as follows:

$$MOE_{i,CAG} = \frac{NOAEL_{i,CAG}}{EDI_i} \quad (4)$$

The cumulative risk was assessed using the total margin of exposure (MOET), obtained by the addition of the reciprocal of MOEs, as shown in formula (5). Generally, a MOET above 100 is not considered a risk for humans.

$$\frac{1}{MOET_{CAG}} = \sum \frac{1}{MOE_{i,CAG}} \quad (5)$$

3. Results

Out of the 666 recruited children, only 572 urine samples were analyzed because 94 participants did not collect a sufficient amount of urine. In addition, four urine samples were removed from the current study because they had a creatinine level below 30 $mg \cdot dl^{-1}$. As a result, 568 urine samples were considered in the descriptive and statistical analyses. Creatinine concentration in these samples ranged from 32.6 to 265.9 $mg \cdot dl^{-1}$, with a median value of 98.3 $mg \cdot dl^{-1}$. The socio-demographic characteristics and information about the food consumption of the participants in the 72 h before collecting the sample are presented in Table 1. Most of the children (93%) had at least one parent employed, 78% of them were living in urban areas, and 60% had at least one parent with university studies.

3.1. Levels of urinary pesticide biomarkers

The descriptive analysis for each metabolite is summarized in Table 2. In general, detection frequencies ranged from <1% (2,4,5-Trichlorophenoxyacetic acid; 2,4,5-T) to 92% (DEP). The highest GM was obtained for DEP (1.53 $ng \cdot ml^{-1}$), one of the non-specific metabolites of OPs. As can be seen in Table 2, the specific metabolites of parathion and chlorpyrifos (p-nitrophenol, PNP; and 3,5,6-trichloro-2-pyridinol, TCPY; respectively) and the non-specific metabolite of Pyr (3-phenoxybenzoic acid; PBA) were found in >74% of the samples with GMs higher than 1 $ng \cdot ml^{-1}$. Another frequently detected non-specific biomarker of OPs was DMP, with a DF of 65%. On the other hand, the highest maximum detected urinary level (256 $ng \cdot ml^{-1}$) was for 2,2-dichlorovinyl-2,2,-dimethylcicloprопane-1-carboxylic acid (DCCA), a metabolite of cyfluthrin, cypermethrin, and permethrin. The rest of metabolites were detected in <37% of the urine samples.

It is worth noting that 85% of the children had five or more pesticide metabolites in their urine samples. Indeed, at least one metabolite of OPs and Pyr was detected in 97% and 83% of the children, respectively. In contrast, biomarkers of Herb were only detected in 41% of the children.

3.2. Correlations and predictors of pesticide exposure

Correlations among the most frequently detected biomarkers (PBA, TCPY, PNP, and ∑DAPs) and all parameters related to the characteristics of the target population from Table 1 were statistically studied as is fully described in Section 2.3. The Spearman correlation matrix obtained to explore the relationships among TCPY, PNP, PBA, the sum of dimethyl phosphates (∑DMPs) and the sum of diethyl phosphates (∑DEPs) are shown in Table S4. As can be observed, TCPY, PBA, ∑DMPs, and ∑DEPs had a significant correlation with PNP. In the case of TCPY, it was also positively correlated with PBA, as was this last one with the sum of dimethyl phosphates (∑DMPs). Moreover, a significant relationship was found between ∑DMPs and the sum of diethyl phosphates (∑DEPs).

Table 3 shows the median urinary levels of biomarkers distributed among the categories of qualitative variables and their Spearman correlation coefficients with quantitative parameters. The resulting regression models with their standardized coefficients and their corresponding 95% CI are represented in Fig. 1. Further statistical information about these models is described in Table 4.

By means of the regression models shown in Fig. 1, a significant relationship was found between urinary PBA and fresh vegetable consumption, showing higher concentrations in children who had eaten more vegetables during the last 72 h. The same effect was observed between the consumption of fresh or dried legumes and cereals and ∑DAPs. Concerning canned vegetables, legumes, and fruits, two different tendencies were noticed: PNP levels in urine were higher as their consumption increased, but the opposite happened in the case of DAPs.

Regarding the association with socio-demographic characteristics, TCPY and PNP concentrations were lower in those children and parents born in countries other than Spain, respectively, probably because these families have a different diet or food sources than the Spanish population, but since <10% of the participants were foreign, this finding was not considered relevant. In the case of ∑DAPs, a negative correlation was found with the age of children.

However, given that the robust regression models for TCPY, PNP, PBA and ∑DAPs presented in Table 4 accounted for only between 5 and 12% of the variance (R^2), the results could not be conclusive.

3.3. Risk assessment

EDI for chlorpyrifos-ethyl, dimethoate, parathion, and λ-cyhalothrin were calculated based on their urinary metabolite concentrations at GM and P95 levels. In the case of chlorpyrifos-ethyl, EDI was calculated

Table 3
Descriptive analysis of metabolite levels in urine according to the lifestyle, sociodemographic characteristics and food consumption habits of the participants in ng·ml^{-1}.

Variable	TCPY Median (minimum - maximum)	PNP Median (minimum - maximum)	PBA Median (minimum - maximum)	∑DAPs Median (minimum - maximum)[a]
Province				
Alicante	0.82 (0.02–35.60)	1.50 (0.02–11.80)	1.84 (0.03–72.80)	29.61 (5.03–2784.45)
Castellón	1.71 (0.04–102.20)	1.02 (0.04–6.47)	1.71 (0.07–130.31)	20.37 (5.52–1781.11)
Valencia	1.10 (0.02–14.50)	1.30 (0.08–9.50)	1.49 (0.05–101.37)	44.88 (5.23–1563.73)
Home location				
Urban area	1.11 (0.02–102.20)	1.32 (0.02–11.80)	1.54 (0.06–113.00)	32.27 (5.03–1781.11)
Countryside	0.87 (0.09–24.20)	1.01 (0.04–5.51)	2.86 (0.03–21.41)	36.58 (11.46–2278.74)
Other	1.40 (0.02–27.40)	1.20 (0.04–9.21)	1.83 (0.11–130.31)	25.31 (5.18–2784.45)
Children's characteristics				
Sex				
Male	0.90 (0.02–52.00)	1.37 (0.04–11.80)	1.51 (0.03–113.00)	32.20 (5.03–2784.45)
Female	1.38 (0.02–102.20)	1.23 (0.02–9.30)	1.70 (0.06–130.31)	29.87 (5.18–1563.73)
BMI[b]	0.081	−0.054	0.006	−0.039
Age[b]	0.073	−0.009	0.019	−0.048
Country of birth				
Spain	1.19 (0.02–102.20)	1.29 (0.02–11.80)	1.55 (0.03–130.31)	30.40 (5.03–2784.45)
Foreign	0.57 (0.08–1.04)	1.47 (0.24–4.67)	2.41 (0.28–9.86)	46.11 (10.84–287.48)
Physical activity				
3 or more days per week	1.36 (0.02–102.20)	1.40 (0.04–8.42)	1.71 (0.06–118.63)	30.13 (5.03–2278.74)
1 or 2 days per week	1.00 (0.04–35.60)	1.25 (0.02–11.80)	1.40 (0.03–130.31)	31.27 (5.18–1781.11)
Occasionally or never	1.00 (0.05–52.00)	1.00 (0.04–7.80)	1.82 (0.06–17.64)	36.82 (5.52–2784.45)
Creatinine[b]	0.068	0.250	0.164	−0.005
Food consumption in the last 72 h[b]				
Eggs	−0.017	0.023	−0.025	0.040
Meat products	−0.002	0.002	0.056	−0.030
Fishery products	−0.034	0.074	0.027	−0.065
Dairy products	−0.101	0.031	−0.105	−0.042
Vegetables	−0.055	0.070	0.094	0.084
Nuts	0.026	−0.053	0.020	0.003
Fruits	−0.060	0.031	−0.001	−0.042
Legumes and cereals	0.039	0.014	0.017	0.008
Miscellany	−0.101	0.007	0.004	0.001
Oils and fats	0.062	−0.128	−0.058	−0.109
Canned vegetables, fruits and legumes	0.013	0.068	0.004	−0.112
Parent's characteristics				
Country of birth				
Spain	1.19 (0.02–102.20)	1.30 (0.04–11.80)	1.52 (0.03–130.31)	31.95 (5.03–2784.45)
Foreign	0.86 (0.02–13.89)	0.99 (0.02–7.30)	2.00 (0.15–36.12)	26.30 (5.23–475.37)
Maximum level of education				
Without studies or primary studies	1.21 (0.06–35.60)	0.95 (0.02–6.07)	1.94 (0.07–20.23)	21.06 (5.03–784.74)
Secondary studies	1.04 (0.02–52.00)	1.24 (0.04–8.90)	1.60 (0.06–39.61)	34.83 (5.23–1563.73)
University studies	1.18 (0.02–102.20)	1.36 (0.04–11.80)	1.51 (0.03–130.31)	30.40 (5.18–2784.45)
Employment situation				
Unemployed	0.96 (0.08–35.60)	0.97 (0.02–3.73)	1.81 (0.06–101.37)	22.53 (5.93–399.93)
Employed	1.16 (0.02–102.20)	1.32 (0.04–11.80)	1.58 (0.03–130.31)	32.03 (5.03–2784.45)

[a] Units: nmol·l^{-1}.
[b] Spearman correlation coefficient.

twice: with the concentrations of its specific metabolite (TCPY) and its non-specific metabolite (ΣDEPs) because it was the only approved pesticide that had been recognized as a precursor of both biomarkers when the BIOVAL study took place. The resulting EDIs of the selected pesticides are shown in Table 5.

As can be seen in Table 5, the highest EDI_{GM} (0.21 μg·kg bw^{-1}·day^{-1}) was obtained for λ-cyhalothrin, since its non-specific metabolite PBA had the lowest F_{UE} (0.30) and one of the highest urinary concentration levels. On the other hand, the lowest EDI_{GM} (0.08 μg·kg bw^{-1}·day^{-1}) was obtained for dimethoate and chlorpyrifos-ethyl (based on TCPY levels).

3.3.1. Risk assessment based on MoA

The HQs and HI for OPs are shown in Table 5. Regarding HQs, a range from 0.01 (chlorpyrifos-ethyl) to 0.08 (λ-cyhalothrin) was obtained based on the GM level, and between 0.08 (chlorpyrifos-ethyl) and 0.65 (λ-cyhalothrin) for the 95th percentile. It should be highlighted that the dissimilarities between the HQs were due mainly to the big differences between the ADI of the evaluated pesticides, which ranged from 2 (dimethoate) to 10 μg·kg bw^{-1}·day^{-1} (chlorpyrifos-ethyl).

The cumulative risk based on MoA was only assessed for OPs because Pyr are not AChE inhibitors and they could not be added together with the OPs. The HI for OPs was calculated adding the HQs of chlorpyrifos-ethyl (∑DEPs) and dimethoate (∑DMPs) since their EDI was calculated from the DAP concentrations, i.e., the representative metabolites of almost the entire OPs family. HIs for the GM and P95 levels of OPs were 0.06 and 0.69, respectively.

3.3.2. Risk assessment based on CAGs

Table 5 presents the results of the cumulative exposure calculations for the two CAGs: CAG-motor and CAG-AChE. In the same way than in the risk assessment based on MoA, MOEs were calculated for both CAGs using EDIs at the GM and P95 levels, ranging from 309 ($MOE_{P95,motor}$ for λ-cyhalothrin) to 238,686 ($MOE_{GM,motor}$ for dimethoate).

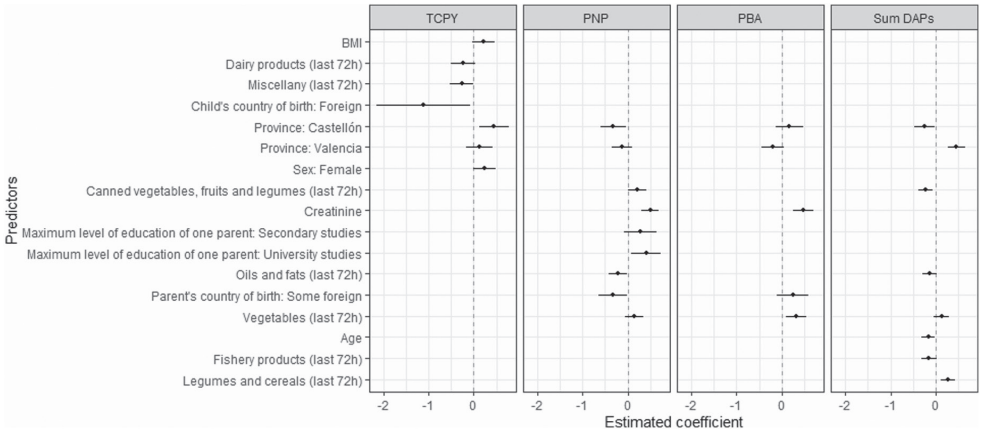

Fig. 1. Standardized coefficients of the Robust linear regression models fitted for the log-transformed urinary concentrations of TCPY, PNP, PBA and ΣDAPs, with their corresponding 95% confidence intervals.

MOETs for the CAG-motor group were calculated considering MOEs for chlorpyrifos-ethyl (∑DEPs), dimethoate (∑DMPs), and λ-cyhalothrin, which acted as representatives of the OPs and Pyr families, respectively; while the MOETs for the CAG-AChE group were estimated taking into account only MOEs for chlorpyrifos-ethyl (∑DEPs) and dimethoate (∑DMPs), as in the cumulative risk assessment based on MoA, since Pyr pesticides does not have the same MoA as OPs.

The calculated MOETs ranged from 296 (CAG-AChE at P95) to 2241 (CAG-AChE at GM). Regarding CAG-AChE, chlorpyrifos-ethyl was the most significant contributor (82%) at the GM level, since it has the lowest $NOAEL_{AChE}$ and urinary levels of ∑DEPs were one of the highest among the evaluated metabolites. In the case of CAG-motor, λ-cyhalothrin made the greatest contribution (>95%) to both levels of the distribution.

Table 4
Results of the robust linear regression models for log-transformed concentrations of pesticide metabolite in urine.[a]

Biomarker	Variable	Standardized coefficients (95% CI)	Standard error	P-value	Multiple R-squared
TCPY	Intercept	−0.0734 (−0.3219–0.1751)	0.1268	0.5631	0.0520
	Province: Castellón	0.4520 (0.1149–0.7891)	0.1720	0.0088*	
	Province: Valencia	0.1237 (−0.1651–0.4125)	0.1473	0.4015	
	Sex: Female	0.2412 (−0.0125–0.4949)	0.1294	0.0630	
	BMI	0.2175 (−0.0363–0.4713)	0.1295	0.0937	
	Child's country of birth: Foreign	−1.1215 (−2.1686 to −0.0744)	0.5342	0.0363*	
	Dairy products (last 72 h)	−0.2362 (−0.497–0.0246)	0.1331	0.0765	
	Miscellany (last 72 h)	−0.2637 (−0.5202 to −0.0072)	0.1308	0.0443*	
PNP	Intercept	−0.0787 (−0.404–0.2466)	0.1660	0.6355	0.1011
	Province: Castellón	−0.3196 (−0.6075 to −0.0317)	0.1469	0.0300*	
	Province: Valencia	−0.1304 (−0.3545–0.0937)	0.1143	0.2546	
	Creatinine	0.4968 (0.3023–0.6913)	0.0993	<0.0001*	
	Parent's country of birth: Some foreign	−0.3368 (−0.6548 to −0.0188)	0.1622	0.0383*	
	Maximum level of education of one parent: Secondary studies	0.2813 (−0.074–0.6366)	0.1813	0.1212	
	Maximum level of education of one parent: University studies	0.4131 (0.0812–0.745)	0.1693	0.0150*	
	Vegetables (last 72 h)	0.1420 (−0.0624–0.3464)	0.1043	0.1740	
	Oils and fats (last 72 h)	−0.2148 (−0.4218 to −0.0078)	0.1056	0.0425*	
	Canned vegetables, fruits and legumes (last 72 h)	0.2072 (0.0032–0.4112)	0.1041	0.0470*	
PBA	Intercept	0.4327 (0.2435–0.6219)	0.0965	<0.0001*	0.0469
	Province: Castellón	0.1698 (−0.1383–0.4779)	0.1572	0.2805	
	Province: Valencia	−0.198 (−0.448–0.052)	0.1275	0.1211	
	Creatinine	0.4872 (0.2646–0.7098)	0.1136	<0.0001*	
	Parent's country of birth: Some foreign	0.2519 (−0.0972–0.601)	0.1781	0.1578	
	Vegetables (last 72 h)	0.3159 (0.0881–0.5437)	0.1162	0.0068*	
∑DAPs	Intercept	3.3484 (3.2125–3.4843)	0.0693	<0.0001*	0.1173
	Province: Castellón	−0.2477 (−0.4742 to −0.0212)	0.1155	0.0324*	
	Province: Valencia	0.4655 (0.2808–0.6502)	0.0942	<0.0001*	
	Age	−0.1638 (−0.3213 to −0.0063)	0.0803	0.0419*	
	Fishery products (last 72 h)	−0.1472 (−0.3208–0.0264)	0.0886	0.0971	
	Vegetables (last 72 h)	0.131 (−0.0473–0.3093)	0.0909	0.1504	
	Legumes and cereals (last 72 h)	0.2757 (0.1073–0.4441)	0.0859	0.0014*	
	Oils and fats (last 72 h)	−0.1307 (−0.2944–0.033)	0.0835	0.1181	
	Canned vegetables, fruits and legumes (last 72 h)	−0.2209 (−0.3797 to −0.0621)	0.0810	0.0066*	

CI: confidence intervals.
[a] Only biomarkers with a detection frequency > 40% were considered in order to build a regression model.
* *P*-value ≤ 0.05.

Table 5
Estimated daily intakes of the selected pesticides and results of the risk assessment estimations based on MoA (HQ and HI) and based on CAGs (MOE and MOET).[a]

Metabolite	Selected pesticide	EDI[s] (μg·kg bw^{-1}·day^{-1})		Risk assessment based on MoA[b]				Risk assessment based on CAGs[c]							
								CAG-motor				CAG-AChE			
		EDI_{GM}	EDI_{P95}	HQ_{GM}	HQ_{P95}	HI_{GM}[d]	HI_{P95}[d]	MOE_{GM}	MOE_{P95}	$MOET_{GM}$[e]	$MOET_{P95}$[e]	MOE_{GM}	MOE_{P95}	$MOET_{GM}$[f]	$MOET_{P95}$[f]
∑DEPs	Chlorpyrifos-ethyl	0.19	0.86	0.02	0.09	0.06	0.69	53,247	11,622	2241	296	2662	581		
∑DMPs	Dimethoate	0.08	1.21	0.04	0.61			238,686	16,464			11,934	823		
TCPY	Chlorpyrifos-ethyl	0.08	0.78	0.01	0.08			119,304	12,775			5965	639	2177	341
PNP	Parathion	0.11	0.56	0.03	0.14			11,582	3116			2265	445		
PBA	λ-cyhalothrin	0.21	1.62	0.08	0.65			2362	309			[g]			

EDI: estimated daily intake; HQ: hazard quotient; HI: hazard index; MoA: mode of action; CAG: cumulative assessment group; CAG-motor: cumulative assessment group of pesticides that cause functional alterations of the motor division of the nervous system; CAG-AChE: cumulative assessment group of pesticides that lead to brain and/or erythrocyte acetylcholinesterase (AChE) inhibition; MOE: margin of exposure; MOET: total margin of exposure; GM: geometric mean of the metabolite urinary levels; P95: 95th percentile of the metabolite urinary levels.

[a] EDIs were calculated with a total urinary volume excreted within 24 h of 0.66 l (Aylward et al., 2011) and the mean body weight of the target population (33 kg).

[b] ADI was selected as a health-based reference value.

[c] NOAEL was selected as a health-based reference value.

[d] HI was calculated only for OPs, since pyrethroids do not have the same mechanism of action. HI was calculated based on DEPs and DMPs, as representatives of the OP group: $HQ_{chlorpyrifos\text{-}ethyl} + HQ_{dimethoate}$.

[e] 1/MOET for pesticides causing alterations on the motor division was calculated as follows: $1/MOE_{chlorpyrifos\text{-}ethyl} + 1/MOE_{dimethoate} + 1/MOE_{cyhalothrin}$.

[f] 1/MOET for pesticides causing AChE inhibition was calculated based on DEPs and DMPs, as representatives of the OP group: $1/MOE_{chlorpyrifos\text{-}ethyl} + 1/MOE_{dimethoate}$.

[g] This pesticide does not affect the AChE activity, so it does not have a MOE associated to this effect, and does not contribute to the $MOET_{AChE}$.

4. Discussion

The most frequently detected pesticide biomarkers in the urine of children were four metabolites of OPs (DEP, DMP, TCPY, and PNP) and one metabolite of Pyr (PBA). The comparison of the GM urinary levels of these metabolites with other HBM studies of children from other countries is presented in Fig. 2.

The present research found lower concentrations of urinary TCPY than Australia (Li et al., 2019) and Thailand (Panuwet et al., 2009), but higher TCPY urinary levels than other countries, such as Italy (Bravo et al., 2019), Slovenia (Bravo et al., 2020), and China (Wang et al., 2016). In fact, due to the extensive exposure of the European population to chlorpyrifos and the epidemiological evidence related to neurodevelopmental outcomes in children, EFSA considered that the approval criteria which are applicable to human health, as laid down in Article 4 of Regulation (EC) No 1107/2009, were not met and consequently, the use of chlorpyrifos has been recently banned in Europe (2019) (EFSA, 2019c).

In the case of PBA, the present study reports the highest concentration of this urinary metabolite in comparison with countries worldwide. This finding is consistent with the data provided by Eurostat, which affirms that Spain, a leading agricultural producer in the EU, was also one of the greatest consumers of pesticides in 2016 (EUROSTAT, 2018). In contrast, urinary DAP levels seem to be lower in children in Spain than in other parts of the world.

Finally, the urinary levels of PNP obtained in this research were around the mean level of the GM reported by other countries around the world, but much higher levels of PNP were observed in children from Thailand (Panuwet et al., 2009), probably because the survey was conducted before the prohibition of parathion (2008). However, bearing in mind that both parathion and methyl-parathion are banned in the EU, and that they are not the only source of urinary PNP levels, as also exposure to nitrobenzene could be a cause, no more conclusions could be drawn.

Comparing our results with two previous HBM studies conducted with children from Spain in 2009 (González-Alzaga et al., 2015) and 2010 (Roca et al., 2014b), exposure to chlorpyrifos appears to have decreased from 2010 to 2016 (our study), since the levels of urinary TCPY are found to be lower in the present study. On the other hand, the opposite trend occurs for Pyr exposure: levels of urinary PBA seem to have increased over the years in Spain. These findings are consistent with the decrease of 6.5% in the number of food samples with chlorpyrifos and chlorpyrifos-methyl residues detected in the Valencian Community from 2010 to 2015 (Generalitat Valenciana, Conselleria de Sanitat Universal i Salut Pública, 2019, 2015), and with the increase of 46% in sales of Pyr (in tons) between 2011 and 2016 in Spain (Ministerio de Agricultura y Medio Ambiente, 2011, 2017). In the case of ∑DAPs, the reason for this increase was not clear because there was no available data about the total amount of OPs used in these years in Spain.

According to the obtained Spearman matrix, PBA was positively correlated with TCPY and ∑DMPs, probably because Pyr and chlorpyrifos, together with other OPs, could be used together to control different insect pests in the same type of crops. The relationship between ∑DMPs and ∑DEPs may be explained not only by the same cause mentioned above, but also by the drift of application of different OPs in contiguous cultivation areas (Generalitat Valenciana. Conselleria de Sanitat Universal i Salut Pública, 2019, 2015). In the case of PNP, we did not find any explanation for its correlation with all metabolites, as the use of its parent pesticides are banned in Europe.

Regarding predictors of exposure to PBA, a significant relationship was observed with recent vegetable intake (Fig. 1). This finding is consistent with the results of the Food Safety monitoring program carried out in the Valencian Community in 2015, in which residues of deltamethrin, cypermethrin and λ-cyhalothrin (parent compounds of PBA) were detected in 15%, 12%, and 6% of the food samples analyzed, respectively (Generalitat Valenciana, Conselleria de Sanitat Universal i Salut Pública, 2019). In the case of ∑DAPs, the ingestion of legumes and cereals seemed to have a significant impact on their urinary concentrations, as Fernández et al. (2020) have described recently. On the other hand, while ∑DAPs presented a negative correlation with the consumption of canned vegetables, fruits, legumes, and cereals, PNP presented a positive one. However, no explanation was found for these findings. In contrast, when observing the results obtained with the regression models, no association is found between the TCPY urinary levels and fruit or vegetable consumption, which is not consistent with the results reported by the Valencian Government (Generalitat Valenciana, Conselleria de Sanitat Universal i Salut Pública, 2019), given that chlorpyrifos-ethyl was detected in >40% of food samples from the Valencian Community in 2015. This probably is related to the fast elimination of TCPY in urine (half-live about 27 h) (Nolan et al., 1984), whereas questionnaires on food consumption covered 72 h before sampling. Therefore, these different time windows could be a limitation to find better correlations.

In terms of exposure, similar tendencies were found in a previous biomonitoring study carried out with lactating mothers from the Valencian Region in 2015 (Fernández et al., 2020), which concluded

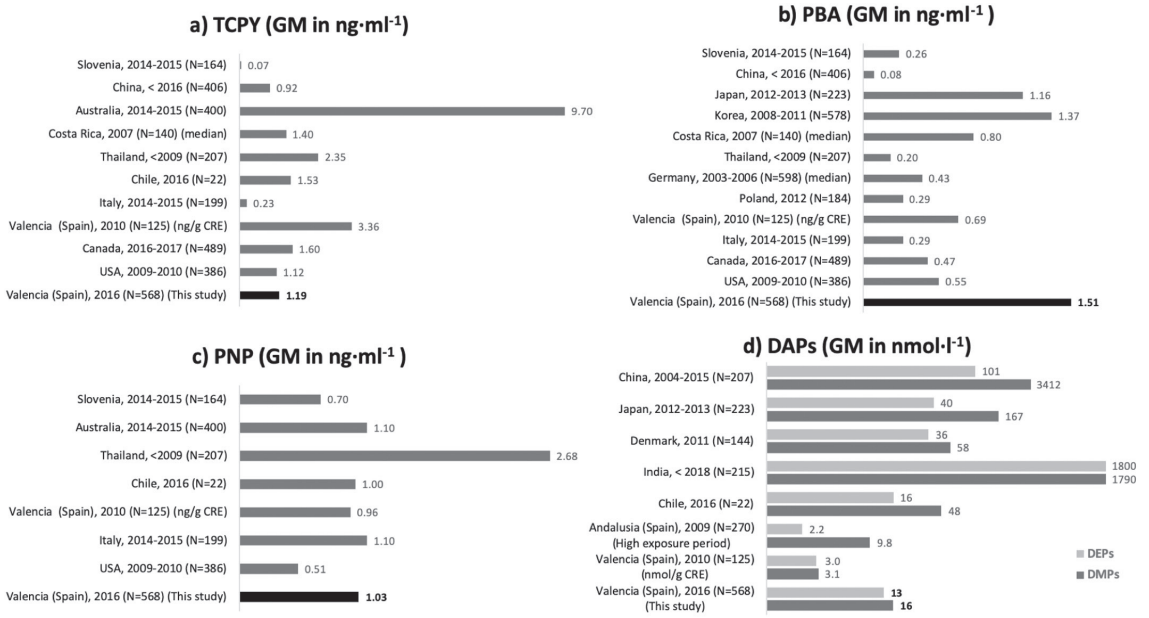

Fig. 2. Comparison of the urinary levels (geometric mean) of a) TCPY, b) PBA, c) PNP and d) ΣDAPs among different countries around the world: Slovenia (Bravo et al., 2020), China (Wang et al., 2016; Yu et al., 2016), Australia (Li et al., 2019), Costa Rica (van Wendel de Joode et al., 2016), Thailand (Panuwet et al., 2009), Chile (Muñoz-quezada et al., 2019), Italy (Bravo et al., 2019), Spain (González-Alzaga et al., 2015; Roca et al., 2014b), Canada (Health Canada, 2019, 2013), USA (CDC, 2019), Japan (Osaka et al., 2016), Korea (Lee et al., 2019), Germany (Schulz et al., 2009), Poland (Wielgomas and Piskunowicz, 2013), Denmark (The Danish Environmental Protection Agency, 2016) and India (Sinha and Banda, 2018).

that the Spanish population seemed to be more exposed to Pyr than to OPs. In terms of risk based on MoA, all the HQs and HIs were <1. However, rather high HQs and HIs were obtained in the children participating in this research compared to women from the same region of Spain (Fernández et al., 2020), most likely because of the differences in body burden. The highest HQ among OPs was that of dimethoate, as has also been observed in other populations around the world (Katsikantami et al., 2019), although lower HQs and a lower HI for OPs were found in the present study. In fact, in 2019, EFSA announced the non-renewal of the approval of dimethoate as an active substance in plant protection products in Europe since the endocrine disrupting potential of this substance could not be excluded (European Commission, 2019b).

According to the risk assessment based on CAGs, the calculated MOETs were all above 100, which does not evidence a risk for the target population in any scenario. These results were compared with MOETs for children from the Netherlands, France, and Bulgaria calculated by EFSA using an external exposure approach (Dujardin and Bocca, 2019). MOETs observed in the present study were higher than EFSA's estimations for both CAGs, most likely because of the more conservative assumptions made by EFSA in its estimations.

To sum up, similar conclusions were reached with both risk assessment approaches, given that no significant risk for the target population was found using either strategy: all HQs and HIs were <1, and MOETs were >100. However, these findings have to be interpreted with caution, and more detailed analyses would be recommended in coming HBM studies. It should be noted that the presence of some pesticide metabolites in urine could be due to the exposure to more than one parent pesticide, to pesticide degradation products, or to the metabolites themselves (Needham, 2005). However, it is still unidentified to what extent these sources of exposure play a role in systemic exposure, measured as urinary excretion (Wielgomas and Piskunowicz, 2013). Therefore, this study did not differentiate among them and, in order to facilitate the risk assessment, it was assumed that the presence of the pesticide metabolites in the analyzed urine samples was only due to the exposure to one parent pesticide. For this reason, the calculated EDI and risk assessment parameters were surely overestimated due to the likely contribution of the same metabolites coming from other pesticides or their breakdown products in the environment.

In addition, further efforts have to be made to fully understand pesticide exposure in this region, starting with the design of more detailed and extensive questionnaires and harmonizing methodologies used for estimating the cumulative risk. Furthermore, multi-residue methods, including as many pesticide metabolites as possible, are highly recommended in order to carry out a more accurate cumulative risk estimation.

5. Conclusions

In this research, six non-specific metabolites of OPs and twenty specific metabolites of OPs, Pyr, and Herb were analyzed in the urine samples of children (aged between 5 and12 years old), participating in the BIOVAL program (Spain). Five or more pesticide biomarkers were detected in 85% of the children. The multiple robust models revealed that recent vegetable, legume, and cereal intakes were significant predictors of exposure to Pyr and OPs. Nevertheless, despite the results from the pesticide risk assessment not showing a health risk evidence for children living in Spain, the biomonitoring of pesticides is highly advisable in order to be able to observe the population's exposure in Spain over time, due to the extensive use of pesticides in this country.

However, it is necessary to increase the number of available health-related guidance values (e.g. biomonitoring equivalents, HBM-I and HBM-II) for the different pesticides in use, in order to properly interpret exposure in a health risk assessment context.

6. Study limitations

The employment situation of the participants' parents was skewed toward employed people and the majority of participants lived in urban areas. Another shortcoming of the study is the lack of some specific information, such as the type of food the participants consumed (such as whether they ate organic food), if they peeled, washed or treated the fruits and vegetables before eating them, or the use of pesticides in the participants' household. Likewise, it should be noted that the presence of pesticide metabolites in urine may be due to the exposure to the main metabolites in the environment (e.g. from pesticides breakdown products), other parent pesticides, and/or other chemical compounds. This could result in an overestimation of the risk evaluation. On the other hand, a mean BW and a theoretical value of V_{24h} were used in the calculation of EDIs, but metabolism and excretion of pesticides can be highly different between children.

CRediT authorship contribution statement

Sandra F. Fernández: Methodology, Validation, Investigation, Writing - original draft, Visualization. **Olga Pardo:** Writing - review & editing, Supervision, Funding acquisition. **Francisca Corpas-Burgos:** Software, Formal analysis, Data curation. **Vicent Yusà:** Conceptualization, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

All authors report no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140983.

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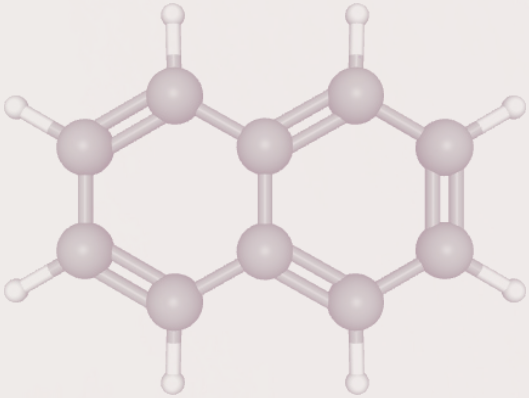
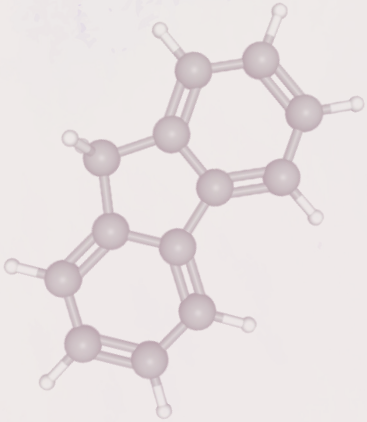
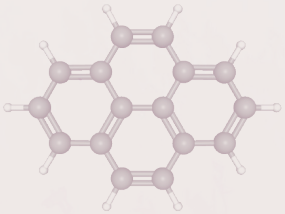
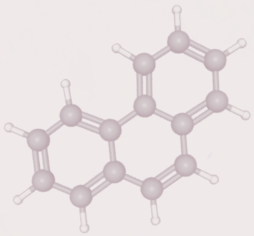

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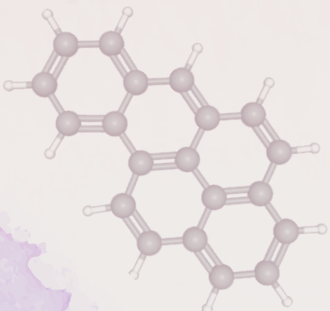
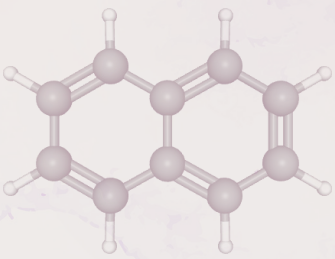
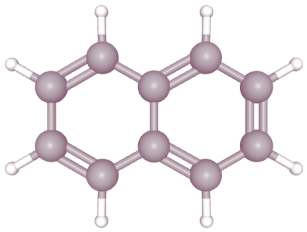
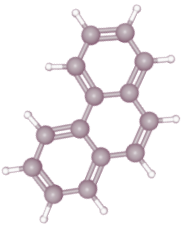
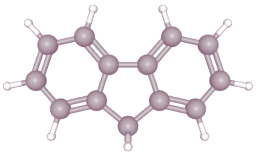
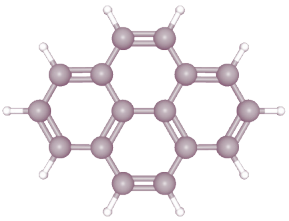
CHAPTER II

BIOMONITORING OF POLYCYCLIC AROMATIC HYDROCARBONS IN CHILDREN AND MOTHERS: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT

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CHAPTER II

5.1 BIOMONITORING OF POLYCYCLIC AROMATIC HYDROCARBONS IN MOTHERS: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT

5.1.1 RESULTS AND DISCUSSION

The urines of the BETTERMILK project were analyzed using a methodology based on LLE and LC-MS/MS for the determination of 11 OHPAHs. The figures of merit of the developed method are presented in Table 15. In addition, the laboratory passed the European interlaboratory comparison investigations and external quality assurance schemes (HBM4EU ICI/EQUAS) for the analysis of 1OHNAP, 2OHNAP, 2OHFLU, 1OHPHEN, 2OHPHEN, 3OHPHEN, 4OHPHEN & 1OHPYR, and participated in several rounds of the G-EQUAS intercomparison programs for 1OHNAP, 2OHNAP & 1OHPYR analysis in urine with successful performance. Additionally, two CRMs, named *SRM 3672 – Organic Contaminants in Smokers' Urine* and *SRM 3673 – Organic Contaminants in Non-smokers' Urine* from the NIST, were used as internal QCs for all OHPAHs (except from 3OHBAP), obtaining $|Z| \leq 2$ in all analytical sequences.

Table 15. Results of the parameters evaluated in the validation of the method (n = 5) for the analysis of 11 PAH metabolites in urine: linearity, accuracy, precision, and specificity.

Analyte	LoQ ($ng \cdot ml^{-1}$)	Linearity		Accuracy (%R)			Precision (%CV)			%S
		R^2	Residuals	LoQ level	Medium level	High level	LoQ level	Medium level	High level	
1OHNAP	0.01	0.985 – 0.999	0.2 – 18.0	102	101	97	14.8	6.6	3.1	12.7
2OHNAP	0.01	0.972 – 0.998	0.9 – 17.6	108	92	106	17.9	7.5	6.4	11.0
2OHFLU	0.01	0.983 – 0.999	0.0 – 16.9	108	99	103	13.0	2.6	2.0	8.6
3OHFLU	0.05	0.984 – 0.996	0.1 – 16.7	82	95	103	16.6	6.6	2.9	10.7
1OHPHEN	0.01	0.981 – 0.997	0.8 – 13.9	100	103	102	7.1	3.6	2.1	11.3
2OHPHEN	0.01	0.987 – 0.998	0.1 – 16.6	100	97	107	9.9	5.8	4.7	11.4
3OHPHEN	0.01	0.989 – 0.998	0.1 – 19.4	100	98	104	7.1	4.1	4.3	7.4
4OHPHEN	0.01	0.990 – 0.999	0.0 – 17.8	100	105	95	8.2	3.0	9.6	6.9
9OHPHEN	0.01	0.987 – 0.997	0.3 – 15.9	102	96	104	14.8	5.0	8.7	17.3
1OHPYR	0.01	0.99 – 1.00	0.1 – 14.8	110	103	109	8.4	2.0	5.6	11.8
3OHBAP	0.05	0.979 – 0.993	0.0 – 19.4	100	101	97	9.9	8.0	9.0	13.0

LoQ = limit of quantification; %R = relative recovery (%); %CV: coefficient of variation (%); Medium level = 0.5 $ng \cdot ml^{-1}$ in urine; High level = 50 $ng \cdot ml^{-1}$ in urine; %S: selectivity (%)

In total, 120 women participated in the study, and 119 provided a urine sample. However, only 113 urine samples were examined for the presence of 11 OHPAHs because the volume of six of them was insufficient to carry out the analysis. Moreover, three urine samples had a concentration of creatinine out of the range of 30 – 300 $mg \cdot dl^{-1}$, and consequently, these samples were excluded from the present study. Finally, 110 urine samples with creatinine concentrations between 30 and 240 $mg \cdot dl^{-1}$ (mean of 100 $mg \cdot dl^{-1}$), were considered in the present study. Information about lifestyle, socio-demographic characteristics, and food consumption habits of the target population is summarized in Table 16. Most of the women (91 %) lived in an urban area, 83 % of them were employed, and all of them had good or medium-good adherence to the Mediterranean diet. In addition, only 8 % of the participants were smokers.

Table 16. Socio-demographic characteristics, lifestyle and food consumption habits of the lactating women participating in the BETTERMILK project and included in the analysis of PAH metabolites in urine (n =110).

Socio-demographic characteristics	n (%)	Mean (minimum – maximum)
Primipara	-	-
Yes	64 (58 %)	-
No	46 (42 %)	-
Age (years)	-	**34 (20 – 45)**
BMI before pregnancy ($kg \cdot m^{-2}$)	-	**23 (17 – 35)**
Missing data	2 (2 %)	-
Country of birth	-	-
Spain	95 (88 %)	-
Foreign	13 (12 %)	-
Missing data	2 (2 %)	-
Place of residence	-	-
Urban	100 (91 %)	-
Rural	10 (9 %)	-
Educational level	-	-
Without studies or primary studies	13 (12 %)	-
Secondary studies	23 (21 %)	-
University studies	74 (67 %)	-
Occupational status	-	-
Employed	91 (83 %)	-
Unemployed	19 (17 %)	-

Socio-demographic characteristics	n (%)	Mean (minimum – maximum)
Physical activity	-	-
Frequently (3 or more days/week)	36 (33 %)	-
Occasionally (1 or 2 days/week)	46 (42 %)	-
Never	28 (25 %)	-
Smoking habit	-	-
Currently smokes	9 (8 %)	-
Former smoker	45 (41 %)	-
Never have smoked	56 (51 %)	
Time with the windows open during the day (hours)	-	**10.4 (1.0 – 24.0)**

Food consumption frequency ($g \cdot month^{-1}$)	n (%)	Mean (minimum – maximum)
Dairy products	-	15010 (1400 – 43400)
Eggs	-	682 (30 – 1800)
Meat products	-	5570 (737 – 13700)
Fishery products	-	4770 (1040 – 15600)
Non-smoked fish[a]	-	4025 (980 – 15160)
Smoked fish[a]	-	453 (40 – 960)
Vegetables	-	14600 (1180 – 38900)
Fruits	-	18500 (3720 – 84500)
Legumes	-	1295 (165 – 9155)
Cereals	-	1797 (470 – 4530)
Oils and fats	-	846 (152 – 1540)
Bakery products	-	1610 (171 – 7300)
Miscellany (snacks and sauces)	-	2100 (343 – 29700)
Drinks ($ml \cdot month^{-1}$)	-	42900 (1730 – 76600)
Water[b]	-	34139 (200 – 54000)
Alcoholic beverages[b]	-	1532 (480 – 10710)
Soft drinks[b]	-	3348 (522 – 52065)
Juices and vegetable-based drinks[b]	-	2537 (400 – 34500)
Coffee[b]	-	968 (50 – 6775)
Tea[b]	-	331 (75 – 4500)
Missing data	6 (5 %)	-

Food consumption frequency (g·month^{-1})	n (%)	Mean (minimum – maximum)
Adherence to the Mediterranean diet (Med-DQI)	-	-
Good	46 (44 %)	-
Medium-good	58 (56 %)	-
Missing data	6 (5 %)	-
Saturated fatty acids energy (%SFA)	-	**3.0 (0.2 – 10.6)**
Missing data	6 (5 %)	-
Daily cholesterol intake (mg·day^{-1})	-	**371 (93 – 838)**
Missing data	6 (5 %)	-

[a]Subdivision of the group Fishery products

[a]Subdivision of the group Drinks

5.1.1.1 URINARY LEVELS OF PAH METABOLITES

The descriptive statistics of the OHPAH urinary levels of the target population (in ng·ml^{-1} and µg·g creatinine^{-1}) are presented in Table 17. From the target PAH metabolites, 2OHNAP and 1OHPYR, urinary metabolites of naphthalene and pyrene, respectively, were the most abundant compounds found in 100 % of the urine samples. Another naphthalene metabolite, 1OHNAP, was detected in 96 % of the samples, while OHFLUs were detected in more than 70 % of the samples, and DFs for OHPHENs ranged from 90 % (9OHPHEN) to 99 % (3OHPHEN). On the other hand, the urinary metabolite of benzo(a)pyrene, 3OHBAP, was only found in one sample, as it has a low urinary excretion proportion (4 – 12 %) because urine is not its primary route of elimination[145,315].

Regarding concentrations, the highest urinary levels were found for 1OHNAP and 2OHNAP, with GMs of 0.8 and 7.1 ng·ml^{-1}, and 95th percentiles of 5.7 and 31.2 ng·ml^{-1}, respectively. Similar GM levels were observed for 2OHFLU (0.17 ng·ml^{-1}), 3OHFLU (0.13 ng·ml^{-1}), and 1OHPYR (0.12 ng·ml^{-1}). In general, concentrations of OHPHENs, with GMs ranging from 0.04 (4OHPHEN) to 0.14 ng·ml^{-1} (1OHPHEN), were the lowest ones. It is worth noting that 100 % of the women had seven or more PAH metabolites at quantifiable levels in their urine samples.

Table 17. Descriptive statistics of the urinary OHPAH concentrations in ng·ml^{-1} (µg·g Cre^{-1}) in breastfeeding mothers of the BETTERMILK study (n =110).

Metabolite	LoQ (ng·ml^{-1})	DF (%)	P25	P50
1OHNAP	0.01	96	0.3 (0.4)	0.7 (0.8)
2OHNAP	0.01	100	3.6 (4.5)	7.4 (7.0)
2OHFLU	0.01	98	0.10 (0.10)	0.15 (0.16)
3OHFLU	0.05	70	0.05 (0.04)	0.14 (0.17)
1OHPHEN	0.01	98	0.08 (0.09)	0.15 (0.14)
2OHPHEN	0.01	95	0.04 (0.04)	0.07 (0.07)
3OHPHEN	0.01	99	0.06 (0.06)	0.10 (0.09)
4OHPHEN	0.01	92	0.02 (0.02)	0.04 (0.04)
9OHPHEN	0.01	90	0.05 (0.06)	0.11 (0.09)
1OHPYR	0.01	100	0.08 (0.09)	0.12 (0.12)
[a]3OHBAP	0.05	<1	-	-
[b,c]∑OHNAPs	-	-	31 (38)	61 (57)
[b,d]∑OHFLUs	-	-	1.0 (1.1)	2.0 (1.9)
[b,e]∑OHPHENs	-	-	1.7 (1.7)	2.4 (2.3)
[b,f]∑OHPAHs	-	-	36 (42)	66 (62)

LoQ = Quantification Limit; DF = Detection Frequency (% samples > LoQ); P = percentile; AM = Arithmetic mean; GM= Geometric mean; SD = Standard deviation; Min. = minimum; Max. = maximum

[a]Some parameters were not calculated due to the low DF of this metabolite.

[b]Units: nmol·l^{-1} (nmol·g Cre^{-1})

[c]∑OHNAPs = 1OHNAP + 2OHNAP

[d]∑OHFLUs = 2OHFLU + 3 OHFLU

[e]∑OHPHENs = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN

[f]∑OHPAHs = ∑OHNAPs + ∑OHFLUs + ∑OHPHENs + 1OHPYR + 3OHBAP

AM	GM	P75	P95	Range (Min. – Max.)	SD
2.0 (1.8)	0.8 (0.8)	1.8 (1.7)	5.7 (7.6)	<0.01 – 25.3 (<0.01 – 19.8)	4.0 (3.3)
10.0 (9.3)	7.1 (7.2)	11.9 (11.2)	31.2 (24.6)	1.5 – 46.7 (1.59 – 41.3)	9.4 (3.2)
0.30 (0.30)	0.17 (0.18)	0.31 (0.27)	1.15 (0.96)	<0.01 – 2.23 (<0.01 – 2.88)	0.40 (0.44)
0.27 (0.26)	0.13 (0.13)	0.35 (0.33)	1.01 (0.84)	<0.05 – 2.18 (<0.05 – 2.78)	0.37 (0.36)
0.22 (0.22)	0.14 (0.15)	0.24 (0.25)	0.70 (0.62)	<0.01 – 1.26 (<0.01 – 1.63)	0.22 (0.26)
0.08 (0.09)	0.06 (0.06)	0.10 (0.10)	0.20 (0.25)	<0.01 – 0.45 (<0.01 – 0.46)	0.06 (0.08)
0.12 (0.12)	0.09 (0.09)	0.16 (0.14)	0.35 (0.30)	<0.01 – 0.49 (<0.01 – 0.72)	0.10 (0.11)
0.05 (0.06)	0.04 (0.04)	0.07 (0.07)	0.16 (0.17)	<0.01 – 0.29 (<0.01 – 0.42)	0.05 (0.07)
0.14 (0.14)	0.08 (0.09)	0.16 (0.18)	0.44 (0.47)	<0.01 – 0.87 (<0.01 – 0.67)	0.15 (0.14)
0.16 (0.16)	0.12 (0.13)	0.18 (0.17)	0.41 (0.41)	0.03 – 1.06 (0.03 – 0.69)	0.15 (0.13)
-	-	-	-	-	-
83 (77)	58 (59)	90 (92)	250 (223)	11 – 456 (13 – 344)	84 (64)
3.1 (3.1)	2.0 (2.0)	3.6 (3.4)	10.9 (10.2)	0.4 – 22.2 (0.4 – 30.2)	3.9 (3.9)
3.2 (3.2)	2.6 (2.6)	4.3 (3.7)	8.4 (8.1)	0.7 – 11.8 (0.8 – 16.8)	2.4 (2.7)
90 (84)	65 (66)	97 (101)	259 (239)	16 – 483 (16 – 363)	89 (68)

To put the urinary levels of OHPAHs obtained in the present study in an international context, a comparison with levels reported in other HBM studies on general population, females, mothers and pregnant women from different countries was performed (Figures 25a-d). In general, the same pattern in detection frequencies as well as in the urinary levels of OHPAHs was observed, since 1OHNAP and 2OHNAP were the most frequently detected metabolites with the highest urinary levels in most of the countries.

In the present study, levels of urinary 1OHNAP were generally found to be lower than in other countries (Figure 25a), such as Italy[316], Australia[304] and the USA[301,317], although they were higher than in women from Denmark[318] and China[154,319]. In general, higher urinary concentrations of 2OHNAP were observed in the Spanish women under study when compared to other parts of the world. Nevertheless, some studies carried out in the USA[301,317] reported higher urinary levels of this naphthalene metabolite in smoker and fertile American women than those obtained in this research. By contrast, concentrations of urinary 2OHFLU obtained in the target population were lower than those obtained in other countries, such as in Czech mothers[320], and those observed in some studies from the USA[301,317,321] and Canada[322,323], but higher than those found in Chinese women[154,319] (Figure 25b). On the other hand, levels of urinary 3OHFLU in the women participating in the BETTERMILK study were comparable to those reported in other studies around the world, except for smoker American females[301], who presented the highest concentrations (see Figure 25b). On the whole, urinary levels of OHPHENs and 1OHPYR of the target population were in the range of the rest of the countries, the highest concentrations having been observed in women from the Czech Republic[320] and Poland[324], followed by USA[301,321] and Israel[325]; and the lowest levels having been found in Danish women[318], the general population in Australia[304], and non-smoker American females[301] (Figures 25c and 25d). It is important to note that similar 1OHPYR concentrations were found among Spanish studies[178,179], although lower levels were observed in non-smoker Spanish females[179] (see Figure 25d).

The difference in urinary OHPAH levels among countries could mostly be explained by the differences in dietary patterns, smoking habits, and air pollution levels[326], as well as, different study designs, populations, laboratory analysis, races, among other factors. However, other possible parent compounds may influence urinary OHPAH levels if the biomarkers of exposure are non-specific to PAH exposure. This is the case of 1OHNAP, which is also a biomarker of exposure to the pesticide carbaryl[304].

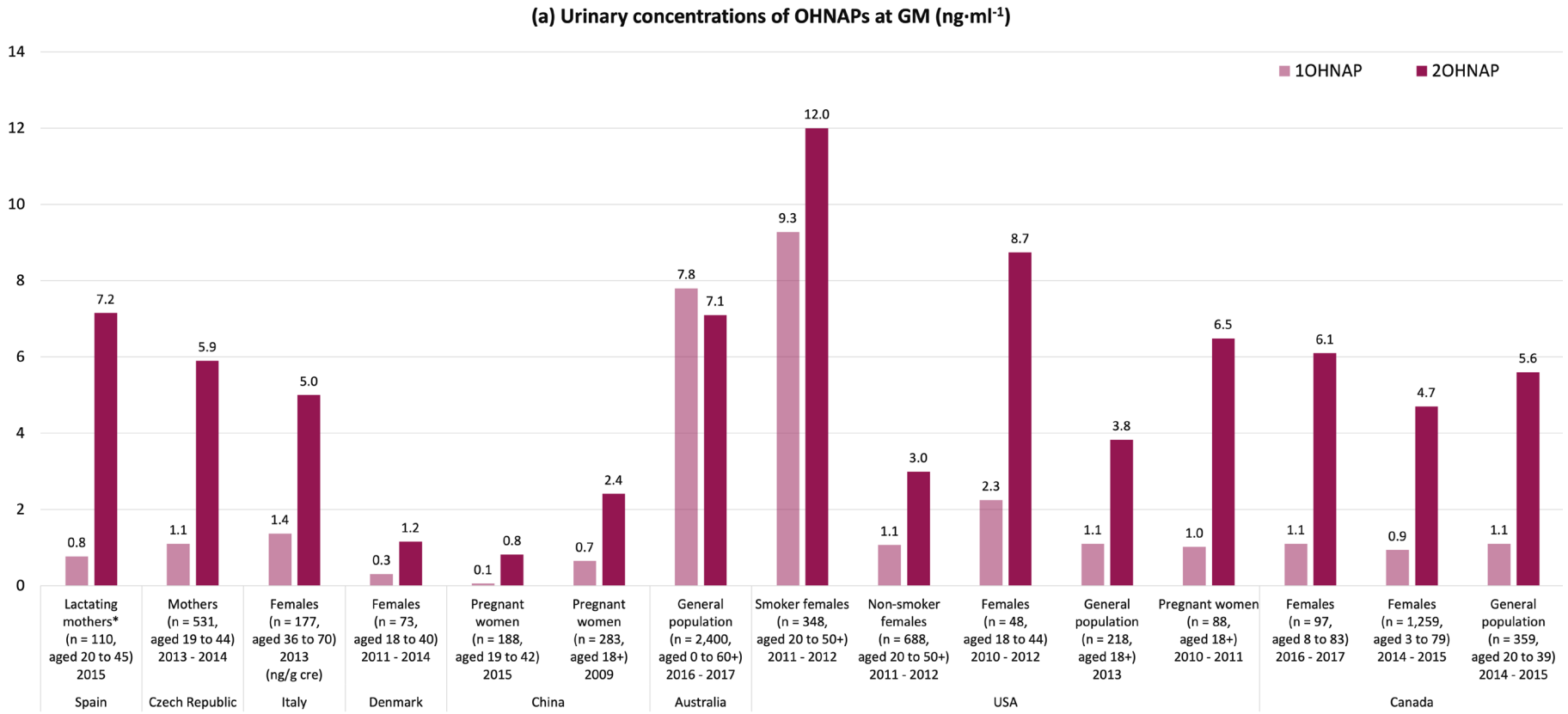

Figure 25a. Comparison of existing HBM data of urinary 1OHNAP and 2OHNAP (ng·ml^{-1}) in mothers, females (smokers and non-smokers), pregnant women, and general population at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain (Present study*), Czech Republic[320], Italy[316], Denmark[318], China[154,319], Australia[304], USA[301,317,321] and Canada[322,323]. *GM: geometric mean; '+' means 'older than'; cre: creatinine.*

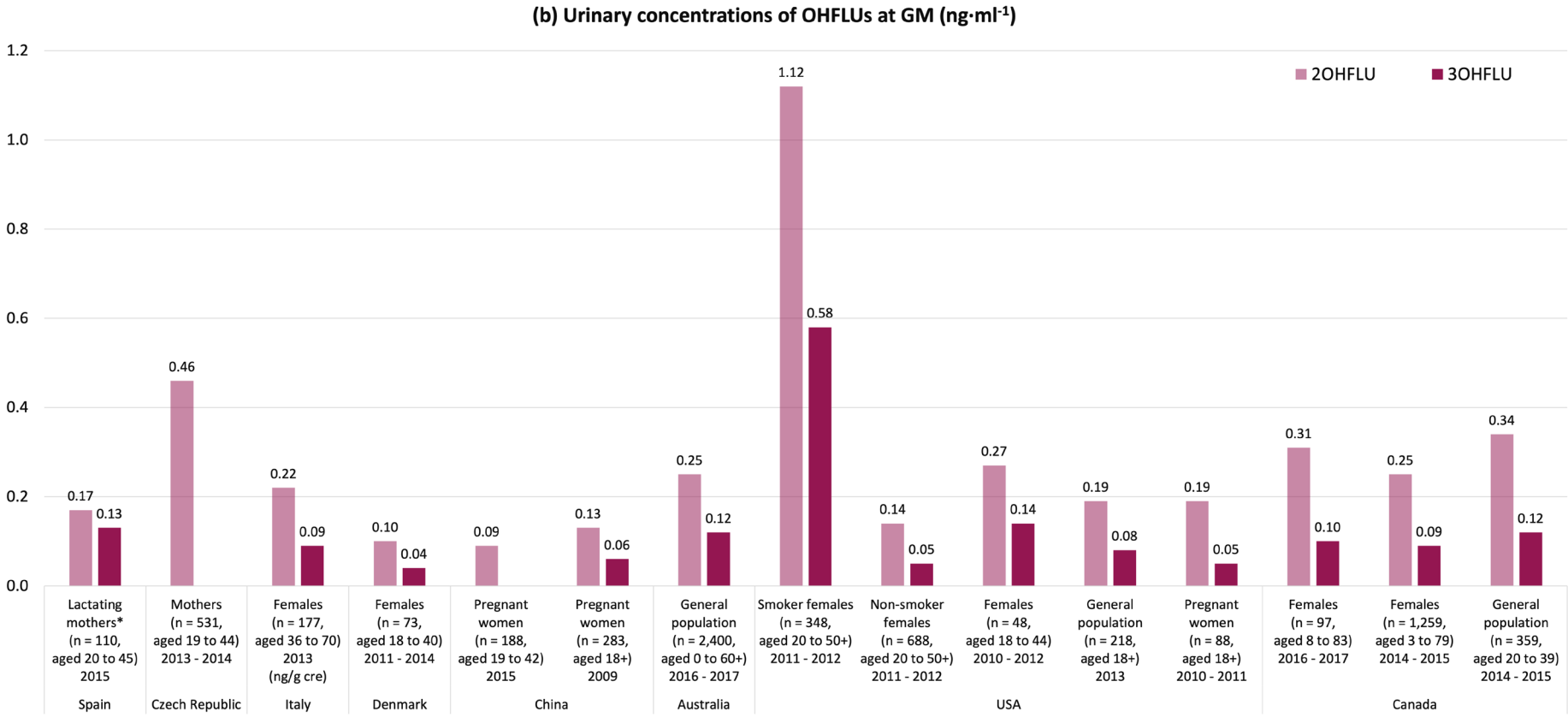

Figure 25b. Comparison of existing HBM data of urinary 2OHFLU and 3OHFLU (ng·ml^{-1}) in mothers, females (smokers and non-smokers), pregnant women, and general population at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain (Present study*), Czech Republic[320], Italy[316], Denmark[318], China[154,319], Australia[304], USA[301,317,321] and Canada[322,323]. *GM: geometric mean; '+' means 'older than'; cre: creatinine.*

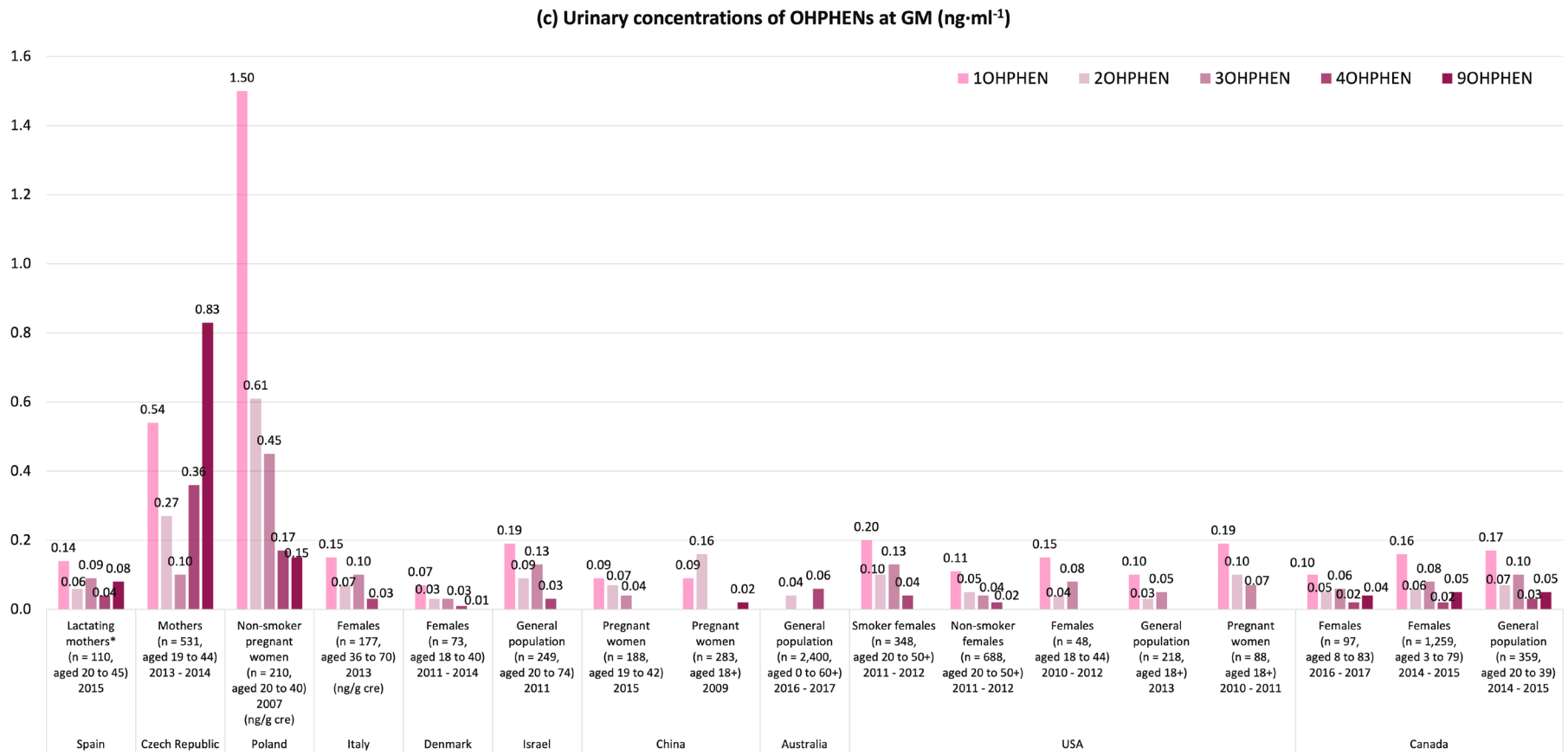

Figure 25c. Comparison of existing HBM data of urinary 1OHPHEN, 2OHPHEN, 3OHPHEN, 4OHPHEN and 9OHPHEN (ng·ml^{-1}) in mothers, pregnant women, females (smokers and non-smokers), and general population at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain (Present study*), Czech Republic[320], Poland[324], Italy[316], Denmark[318], Israel[325], China[154,319], Australia[304], USA[301,317,321] and Canada[322,323]. *GM: geometric mean; '+' means 'older than'; cre: creatinine.*

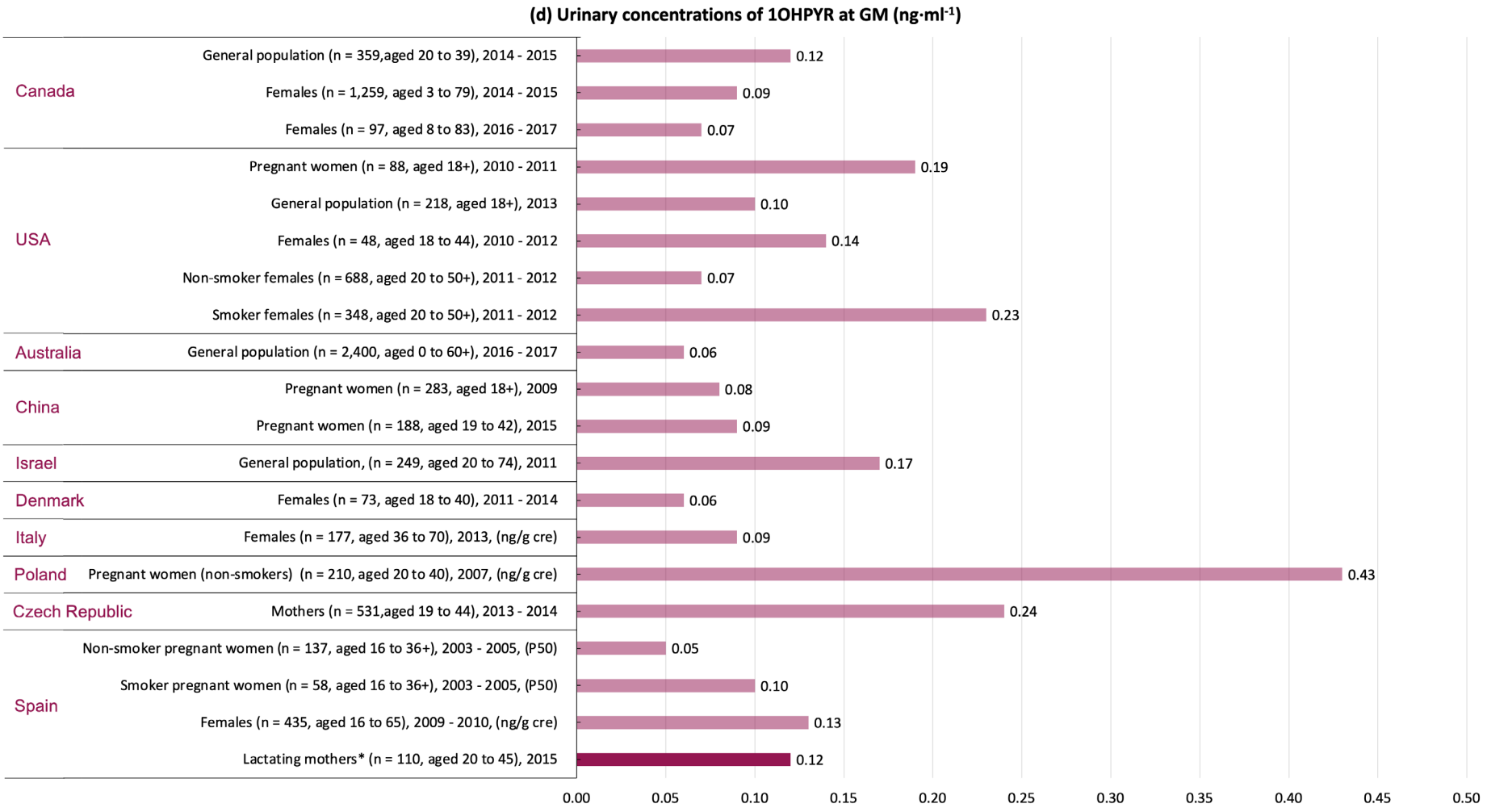

Figure 25d. Comparison of existing HBM data of urinary 1OHPYR (ng·ml^{-1}) in mothers, females (smokers and non-smokers), pregnant women, and general population at different sample collection years (number of samples and age range within parenthesis) across the following countries: Spain[178,179], Czech Republic[320], Poland[324], Italy[316], Denmark[318], Israel[325], China[154,319], Australia[304], USA[301,317,321] and Canada[322,323]. *Present study; *GM: geometric mean; P50: 50th percentile; '+' means 'older than'; cre: creatinine.*

5.1.1.2 STATISTICAL ANALYSIS

First, a Spearman matrix was obtained to explore correlations among ∑OHNAPs, ∑OHFLUs, ∑OHPHENs and 1OHPYR, showing that all OHPAHs were positively related to one another ($p < 0.01$), indicating common exposure sources.

The resulting multiple linear regression models that represent the relationships of each PAH family with socio-demographic characteristics and general dietary habits of the target population are presented in Figure 26a. Additional regression models were constructed including also the subgroups of *Fishery products* (Non-smoked and Smoked fish) and *Drinks* (Water, Alcoholic beverages, Soft drinks, Juices and Vegetable-based drinks, Coffee and Tea), which are represented in Figure 26b. Overall, the fitted regression models were weak but accounted for 26.5 – 35.5 % of the variance (R^2).

According to the fitted models presented in Figure 26a and 26b, the smoking habit markedly influenced OHPAHs levels (p-values ≤ 0.01), despite the low percentage of smokers (8 %), Therefore, the smoking was the determinant of major significance for all OHPAHs, due to the high regression coefficients observed for this variable (β ranging from 0.632 to 1.421) comparing with the others in the fitted models (β ranging from 0.203 to 0.807). This is consistent with the risk assessment estimations, which revealed higher HQs and HIs of PAHs in smoker women compared to non-smokers (see section 5.1.1.3). These findings corroborated that tobacco smoke is one of the primary factors contributing to PAH exposure in smokers, as previously reported by several authors[145,174,178,323,324,327].

Apart from that, food consumption habits appeared to be also relevant factors in PAH exposure in the target population. Indeed, diet is found to account for almost 90 % of PAH daily intake[114]. In the present study, the multiple regression models showed that a high ingestion of vegetables lead to lower ∑OHNAPs urinary levels, most likely because this food group is a minor contributor to PAH exposure[328], thus an increase in the consumption of vegetables might lead to a decrease in the PAH exposure[329]. Similar results were found in two previous HBM studies on adolescents[330] and occupationally exposed population[331], in which vegetable intake was associated with lower levels of OHPAHs in urine. This may also be related to a healthy lifestyle, such as following a Mediterranean diet[332], which was found to have a negative relationship with urinary concentrations of ∑OHNAPs, although it is not statistically significant to reach a conclusion (p-value > 0.05).

On the other hand, oils and fats ingestion seemed to increase levels of urinary ∑OHNAPs. This fact can be explained by the lipophilic characteristics of PAHs, that tend to accumulate in the fat content of biosystems and food chain[333]. In addition, according to several diet studies on the Spanish population[165], oils and fats are one of the major contributors to the exposure to PAHs by dietary intake. What is more, oils and fats have been reported to be important precursors in the formation of PAHs in foods[334], as these substances are generated from SFAs under high heating conditions[319,335]. This could also be the reason why a significant and positive association was observed between %SFA (% energy) and urinary levels of ∑OHFLUs and OHPHENs.

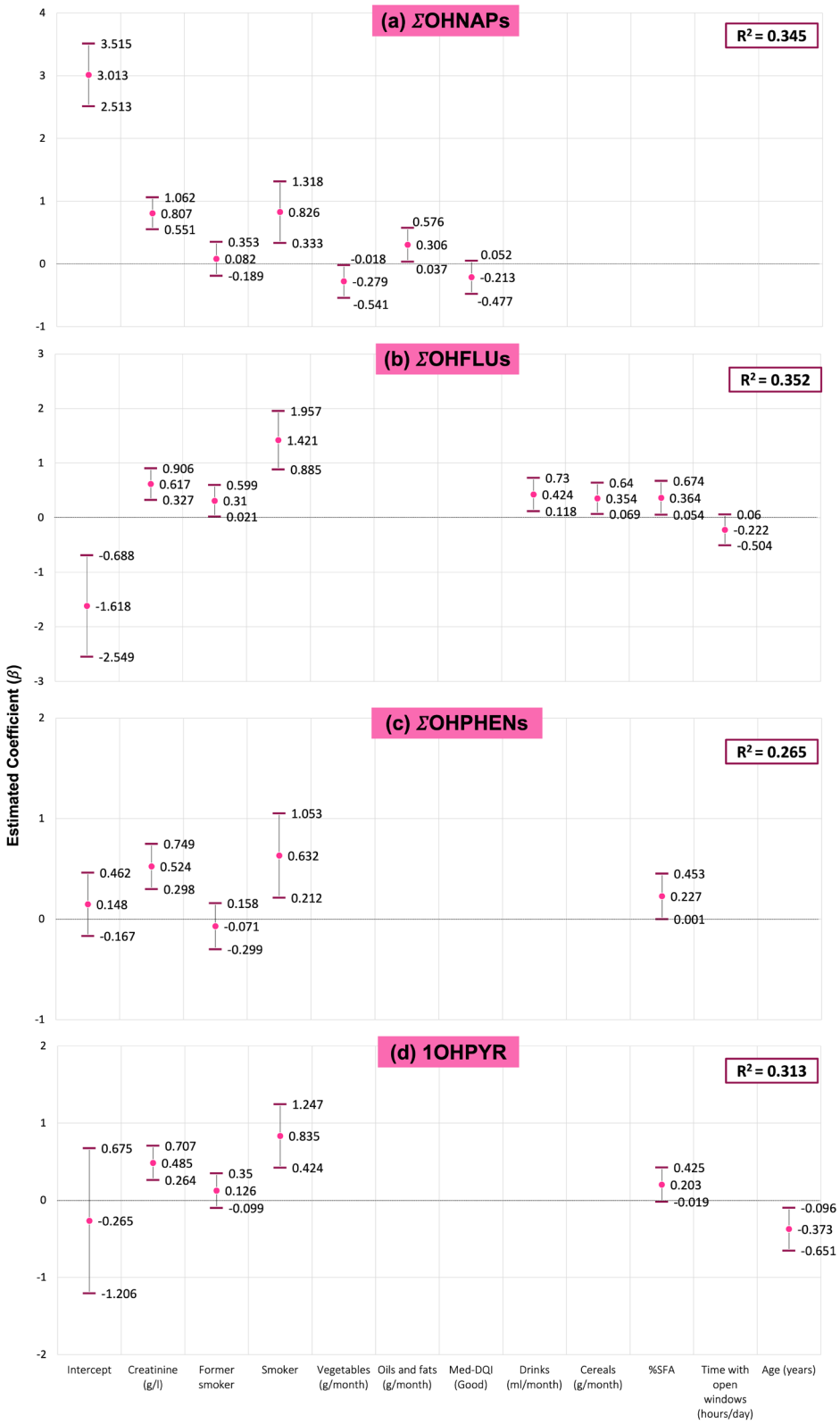

Figure 26a. Linear regression models fitted for the log-transformed (a) ∑OHNAPs, (b) ∑OHFLUs, (c) ∑OHPHENs, and (d) 1OHPYR concentrations considering aggregated food groups (n = 104, only participants with a completed questionnaire were considered), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2) for the women participating in the BETTERMILK project. *Med-DQI: adherence to the Mediterranean diet; %SFA: saturated fatty acid energy.*

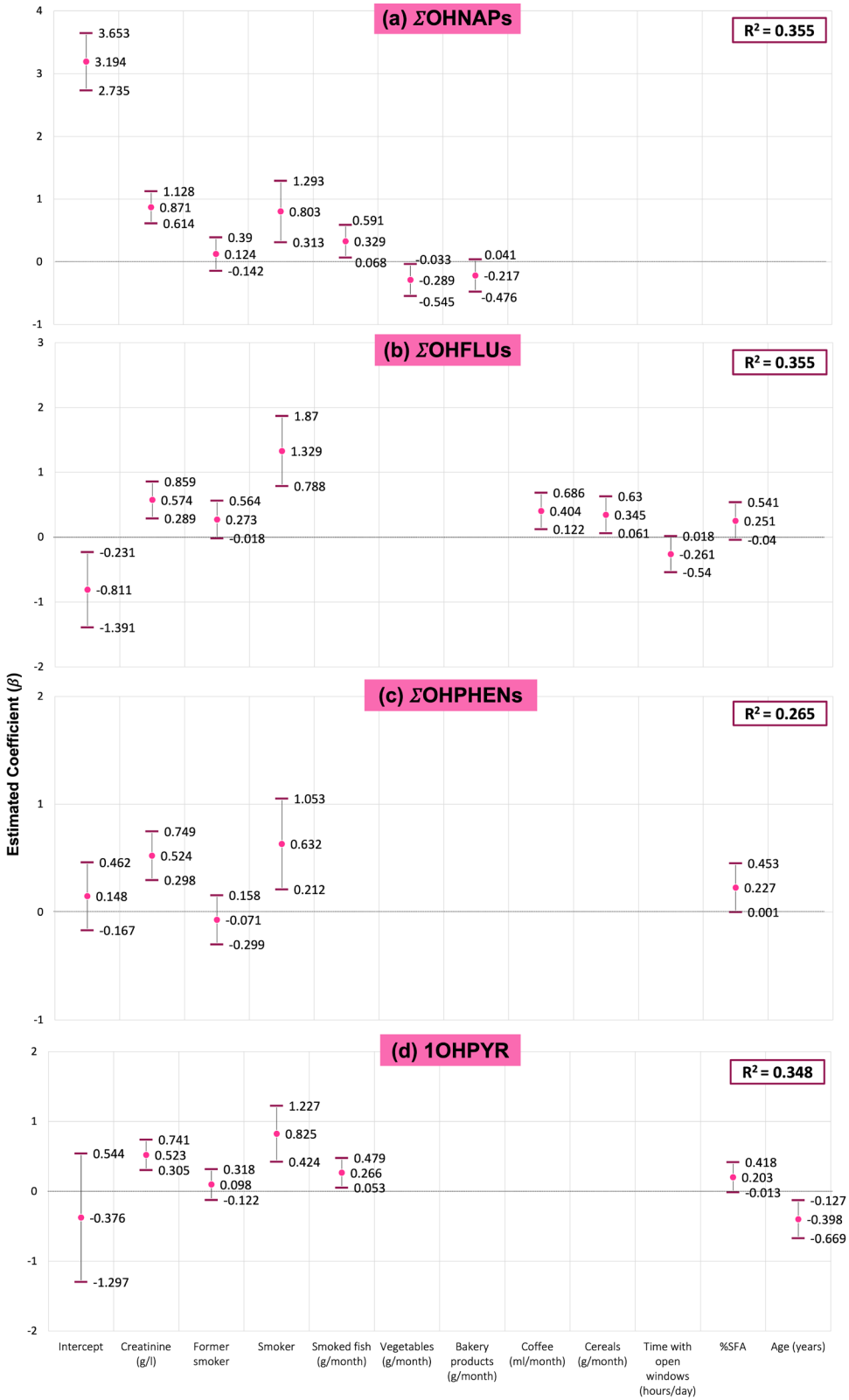

Figure 26b. Linear regression models fitted for the log-transformed (a) ∑OHNAPs, (b) ∑OHFLUs, (c) ∑OHPHENs, and (d) 1OHPYR concentrations considering disaggregated food groups (n = 104, only participants with a completed questionnaire were considered), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2) for the women participating in the BETTERMILK project. *%SFA: saturated fatty acid energy.*

The regression models for ∑OHFLUs revealed that a high consumption of cereals and coffee had a positive effect on their urinary levels. Indeed, cereals are considered one of the highest contributors to the total dietary intake of PAHs by EFSA and other authors[145,165,328,336]. In the case of coffee, two recent HBM studies have also reported a positive association between the coffee ingestion and increased concentrations of fluorene metabolites in urine[316,330]. The reason might be that low molecular weight PAHs are prevalent in coffee brews when high temperatures are used during the roasting process[145,337].

According to EFSA, seafood and seafood products are significant contributors to dietary exposure to high molecular weight PAHs. In addition, a survey carried out in Catalonia in 2006 suggested that this food group is also an important contributor of exposure to low molecular weight PAHs, such as phenanthrene and naphthalene[165]. However, in the present study only significant associations were found with ∑OHNAPs and 1OHPYR when the group 'Fishery products' was divided in two subgroups (non-smoked and smoked fish) (Figure 26b). In this way, ingestion of smoked fish was associated with increased levels of these metabolites in urine. This finding is consistent with Llop et al. (2008), who reported that the intake of smoked food was an important predictor of exposure to 1OHPYR in Spanish pregnant women[179]. Likewise, a HBM study carried out in Japan found that a high fish consumption increased urinary levels of 1OHPYR[331].

Other foodstuffs that are contributors of great importance in PAH exposure are meat and meat products[165,331,336]. Notwithstanding the latter, no association at all was found with consumption of meat products in the women participating in the present study. This may be due to the lack of detailed information in the food frequency questionnaire, such as the recent intake of specific food or the cooking practices used by the participants.

With regard to other relevant findings, a negative association was found between urinary 1OHPYR and the age of individuals, as already reported by other authors[169,330,338]. However, to date, there is no scientific evidence to support this.

5.1.1.3 RISK ASSESSMENT

Given that 3OHBAP was detected in less than 1 % of the samples, only EDIs for naphthalene, fluorene, phenanthrene, and pyrene were calculated. The estimated daily intakes based on unadjusted urinary levels (EDI_{Vol}) and creatinine-adjusted concentrations (EDI_{Cre}) were calculated stratifying by the smoking habit of the participants.

Among assessed PAHs, the highest mean EDI_{Vol} were obtained for naphthalene, ranging from 173 (non-smokers) to 446 $ng \cdot kg^{-1} \cdot day^{-1}$ (smokers), since its metabolites were found at much higher concentration levels than the rest of the OHPAHs. Conversely, fluorene was

the PAH compound that presented the lowest mean EDI_{Vol} values, which ranged from 9 (non-smokers) to 38 $ng \cdot kg^{-1} \cdot day^{-1}$ (smokers). In general, mean EDI_{Cre} values, ranging from 6 (fluorene in non-smokers) to 604 $ng \cdot kg^{-1} \cdot day^{-1}$ (naphthalene in smokers), were lower than those obtained using unadjusted concentrations. Similar tendencies were observed when considering the 95th percentile. As expected, the highest EDIs were found for participants who were smokers, except for EDI_{Cre} of phenanthrene at P95, in which non-smokers showed higher values (433 $ng \cdot kg^{-1} \cdot day^{-1}$) than smokers (370 $ng \cdot kg^{-1} \cdot day^{-1}$). However, no significant differences were observed between EDIs of non-smokers and former smokers.

Comparing with other countries, EDIs for pyrene obtained here were lower than those observed in Mexican women[339], several Asian countries[172,340], such as China, India, Kuwait and Vietnam, and other European countries[341], such as the Netherlands, Greece and Denmark; although they were higher than those in Belgium (adolescents) and Sweden[341]. On the other hand, similar EDIs were found among adults living in France, Italy, Germany, Czech Republic[341], and the assessed Spanish population. For naphthalene, fluorene and phenanthrene, higher EDIs were observed in the present study compared to primiparas and pregnant women in China[154,172].

The results of the risk assessment (HQ and HI) are presented in Figure 27. As can be seen, the calculation for the non-cancer risk assessment with unadjusted EDIs resulted in HQ_{GM} between 0.0002 (fluorene) and 0.02 (naphthalene). In the case of HQ_{P95}, the values ranged from 0.0010 (fluorene) to 0.08 (naphthalene). The individual risk estimated with the creatinine-adjusted levels showed HQ_{Cre} of the same order of magnitude but slightly lower than unadjusted HQs.

Regarding the cumulative risk calculations, HI of smokers was approximately twice the HI of non-smokers, although in all cases HI was lower than 1. As a result, exposure to PAHs should not be considered a potential toxic hazard for the Spanish population under study. However, considering that humans are exposed to PAH mixtures containing more than 100 chemical substances, and in the present study the cumulative risk was only assessed for 4 PAHs (naphthalene, fluorene, phenanthrene and pyrene), there may be an underestimation of the risk.

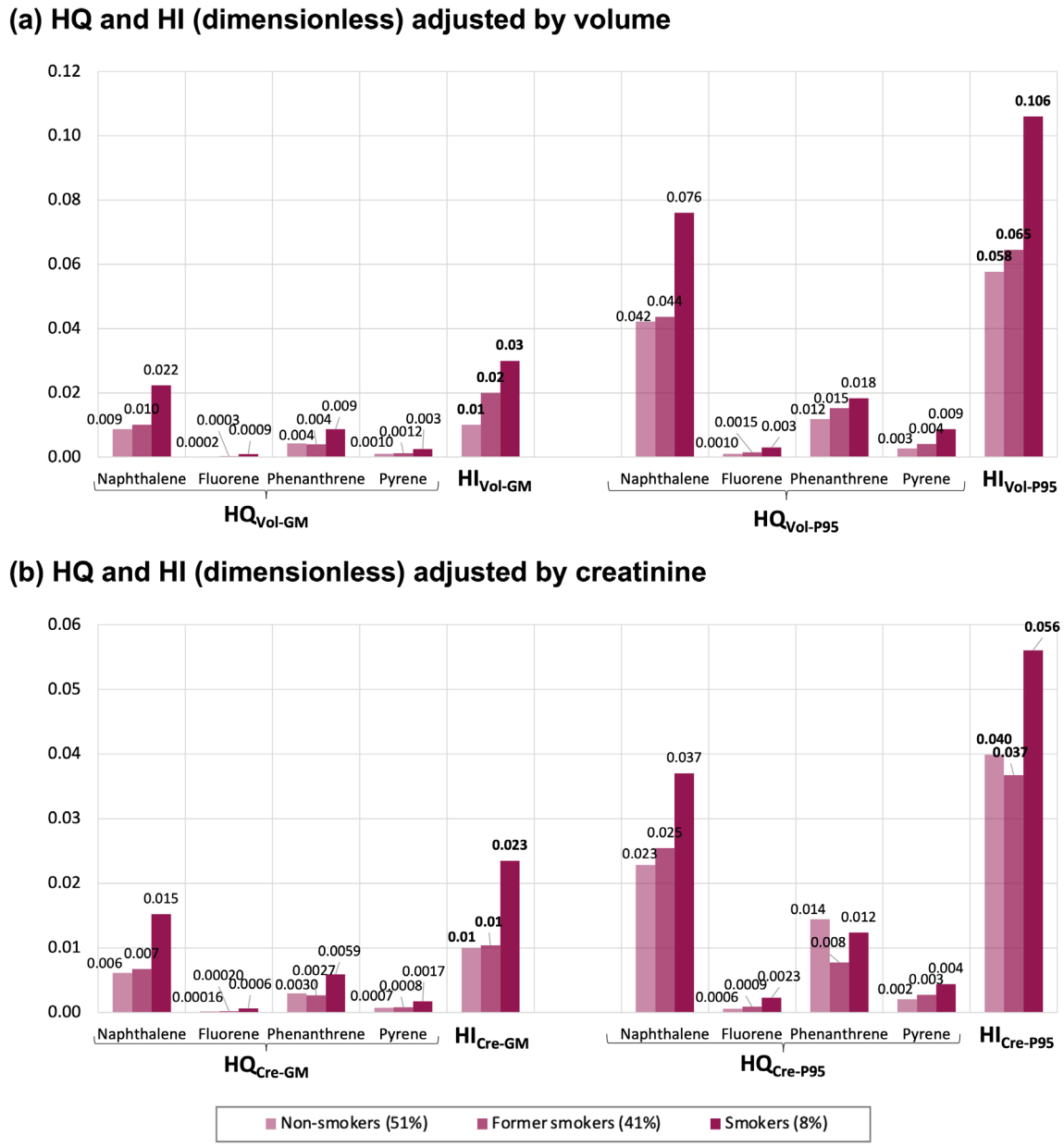

Figure 27. Results of the non-cancer risk assessment stratified by the smoking habit of the mothers of the BETTERMILK study: HQ of individual PAHs and HI adjusted (a) by volume and (b) by creatinine. *HQ: hazard quotient; HI: hazard index; GM: geometric mean; P95: 95th percentile; Vol: adjusted by volume; Cre: adjusted by creatinine.*

Despite there being no evidence of a non-cancer health risk, HBM and epidemiological studies are highly recommended to obtain more extensive data about nutritional and environmental influencing factors in PAH exposure, as well as to understand the real health impact on vulnerable population, such as pregnant women and children.

5.1.2 CONCLUSIONS

In the present study, high detection frequencies (> 70 %) were observed for ten of the analyzed urinary PAH metabolites, with GMs ranging from 0.04 (4OHPHEN) to 7.1 ng·ml^{-1} (2OHNAP), in the Spanish lactating mothers under study (n = 110). The highest urinary concentrations were found for two naphthalene metabolites, i.e. 1OHNAP and 2OHNAP. In general, the obtained OHPAH urinary levels were in the same concentration range as those reported in similar studies from other countries. According to the fitted regression models, smoking and dietary habits were the most influential determinants of exposure to PAHs. In an exposure context, naphthalene was observed the most relevant contributor to PAH exposure. The risk assessment estimations showed that there was no excess health risk expected due to cumulative exposure to PAHs in the target population, since HIs were far below 1.

5.1.3 ARTICLE II.1: *BIOMONITORING OF POLYCYCLIC AROMATIC HYDROCARBONS IN THE URINE OF LACTATING MOTHERS: URINARY LEVELS, ASSOCIATION WITH LIFESTYLE FACTORS, AND RISK ASSESSMENT*

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Biomonitoring of polycyclic aromatic hydrocarbons in the urine of lactating mothers: Urinary levels, association with lifestyle factors, and risk assessment☆

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are pollutants produced during incomplete combustion of organic matter and several industrial processes. Humans can be exposed to PAHs through ingestion of food, inhalation of tobacco smoke or polluted air, and dermal contact, causing immunologic, developmental, and reproductive problems.

In the present research, eleven metabolites of PAHs were analyzed in the urine of 110 lactating women living in Spain (2015). PAH metabolites were extracted from the urine samples by liquid-liquid extraction and their determination was performed by liquid chromatography coupled to tandem mass spectrometry. In addition, information on lifestyle and dietary habits of the participants was collected using a questionnaire.

All the PAH metabolites were detected in more than 70% of the samples, except for 3-hydroxybenzo(a)pyrene which was detected in less than 1% of the samples. The highest urinary levels were found for naphthalene metabolites, with geometric means of 0.8 (1-hydroxynaphthalene) and 7.1 ng ml^{-1} (2-hydroxynaphthalene). The statistical analysis showed that smoking status, as well as the ingestion of certain food groups (vegetables, cereals, oils and fats, smoked fish and coffee), were the main influencing factors of exposure to PAHs.

The estimated daily intake (EDI) was calculated for naphthalene, fluorene, phenanthrene, and pyrene, ranging from 6 to 1522 ng $kg^{-1} \cdot day^{-1}$. The non-cancer risk associated to PAH exposure was estimated, showing hazard quotients (HQs) and hazard indexes (HIs) below 1. Therefore, it did not reveal a significant health risk for Spanish women due to PAH exposure.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a large group of highly lipophilic contaminants that are made up of two or more fused aromatic rings (EFSA, 2008; Pruneda-Álvarez et al., 2016). They are mainly produced and released during incomplete combustion of organic matter and several industrial processes (EFSA, 2008; Lee et al., 2019). The formation of PAHs is generally associated with natural sources (e.g., volcanoes and forest fires), anthropogenic activities (e.g., waste incineration, processing of coal, crude oil, petroleum, and natural gas, vehicle exhaust emissions, tobacco smoke, etc.), and some culinary practices (e.g., drying, grilling, roasting, frying, and smoking) (Health Canada, 2017; Urbancova et al., 2017).

Numerous epidemiological studies have identified some PAHs as genotoxic substances, carcinogens, mutagens, and teratogens (Knafla et al., 2011; Prado et al., 2012; WHO, 2010) since they can cause damage to DNA, proteins, and lipids via oxidative stress (Eom et al., 2013). Moreover, they also have adverse immunologic, developmental, and reproductive effects on humans (Kim et al., 2011; Li et al., 2015; Lou et al., 2019). In fact, the International

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Agency for Research on Cancer (IARC) classified benzo(a)pyrene as carcinogenic to humans (Group 1) in 2012 (IARC, 2020). Other PAHs have been identified as possibly carcinogenic to humans (Group 2 B), such as chrysene and naphthalene, or not classifiable as to their carcinogenicity to humans (Group 3) (IARC, 2020). In addition, the United States Environmental Protection Agency (US EPA) has developed a list of 16 PAHs, including benzo(a)pyrene, naphthalene, fluorene, pyrene and phenanthrene among others, which should be prioritized for monitoring because of their potential human health and/or ecological toxicity (US EPA, 2014). In recent years, PAHs have become a public health concern, leading the Agency for Toxic Substances and Disease Registry (ATSDR) and the Human Biomonitoring for Europe project (HBM4EU) to also include PAHs in their respective lists of priority substances (ATSDR, 2020; HBM4EU, 2019), as they are considered potentially one of the most significant threatening substances for human health due to their frequency, toxicity, and potential for human exposure, and which will be candidates for further research.

Humans can be exposed to PAHs through three pathways: ingestion of contaminated food, which is the main source of exposure to PAHs in non-smokers; inhalation of tobacco smoke and polluted air; and contact with these substances through the skin in occupationally exposed individuals, such as firefighters or asphalt workers (EFSA, 2008; European Commission, 2016). Once absorbed by the body, PAHs are oxidized to hydroxy-PAHs (OHPAHs) by the P450 cytochrome and then rapidly excreted through urine (low molecular weight PAHs) or feces (high molecular weight PAHs) as free metabolites or glucuronic acid and sulfate compounds (Hoseini et al., 2018). In this way, urine is the most analyzed matrix since it reflects recent exposure due to the relative short elimination half-lives of most PAHs (Li et al., 2012).

The wide exposure of the population to PAHs and their toxicity has led to a growing interest in evaluating the body burdens of PAHs in the most sensitive sub-population groups, such as children and pregnant women (Kim et al., 2011; Li et al., 2015; Lou et al., 2019). Another group of special concern is lactating mothers, not only because exposure to PAHs can influence their health but also the health of their children through breastfeeding (Berlin et al., 2002), since PAHs have also been detected in human milk (Acharya et al., 2019).

As the primary source of exposure to PAHs is food, the conventional way of assessing PAH exposure is external, that is through diet studies (EFSA, 2008; Martorell et al., 2012). However, given that exposure to PAHs may also occur through other multiple sources and pathways, further understanding of the overall exposure is necessary. In this context, human biomonitoring (HBM) is extremely useful for assessing the total human exposure to PAHs by measuring the internal dose in biological samples (CDC, 2019; Health Canada, 2017). A more suitable risk assessment of PAHs can thereby be achieved by combining HBM data with toxicological data and toxicokinetic properties of PAHs since exposure through all possible sources and pathways are taken into account (Katsikantami et al., 2019). An additional advantage is that HBM data can help evaluate the effectiveness of the measures adopted in Europe in order to reduce the presence of PAHs in food, water, and ambient air by monitoring the general population's exposure to PAHs (HBM4EU, 2018).

In Europe, the HBM4EU initiative aims to generate knowledge on exposure trends and associated health effects of PAHs by using OHPAHs as internal indicators of exposure (biomarkers) in urine samples (HBM4EU, 2018). In the case of Spain, the BIOAMBIENT. ES project was the first national-level HBM survey to estimate urinary levels of 1-hydroxypyrene and several hydroxyphenanthrenes in a representative sample of Spanish adults in 2010 (Bartolomé et al., 2015).

In this context, the goals of the present study are the following: i) to determine the urinary concentration profiles of 11 metabolites of naphthalene, fluorene, phenanthrene, pyrene, and benzo(a)pyrene in a group of lactating women living in Valencia (Spain); ii) to investigate the relationships between urinary OHPAH levels, diet, and socio-demographic characteristics; and iii) to estimate the exposure to PAHs and the cumulative risk of this exposure in the target population.

2. Materials and methods

2.1. Study design, sample and data collection

2.1.1. Study population

The present study is part of the BETTERMILK project described elsewhere (Yusà et al., 2017), whose main objective was to know the exposure of Valencian breastfeeding mothers to environmental and food contaminants. In brief, the recruitment of participants took place between June and November 2015 at the University and Polytechnic Hospital "La Fe" (Valencia, Spain), period in which these women came to the hospital for giving birth. The selection criteria for participating in the study were the following:

i) Good health status of mother and newborn.
ii) Gestation (pregnancy and delivery) without complications, such as gestational diabetes, preeclampsia or hypertensive disorders, chronic pathology requiring medication incompatible with lactation, infections (chorioamnionitis, endometritis, hepatitis C), and uncontrolled pregnancy.
iii) Living in Valencia for more than 10 years.
iv) Decision to maintain breastfeeding for at least 2 months.

In total, 120 lactating women who matched the selection criteria volunteered to participate in the BETTERMILK project.

2.1.2. Urine samples collection

Each donor collected their own first-morning urine void sample between 2 and 8 weeks after childbirth. A 125-ml polypropylene container and the instructions for a proper urine collection was supplied to the participants. The urine samples remained at 4 °C in their households until they were transported to the Biobank for Biomedical Research and Public Health of the Valencian region (IBSP-CV Biobank, Spain), where they were aliquoted in 10-ml tubes and stored at −80 °C until analysis. In total, 119 urine samples were analyzed in the Public Health Laboratory of Valencia.

2.1.3. Data collection

The same day, after the urine collection, detailed information on physical and socio-demographic characteristics, lifestyle, household features, and dietary habits (Food Consumption Frequency) of the volunteers was obtained by means of a questionnaire administered by a trained fieldworker. A set of the information it provided is summarized in Table 1. Food Consumption Frequency was calculated by transforming individual food consumption items into monthly food intakes ($g \cdot month^{-1}$, or $ml \cdot month^{-1}$ in drinks), using estimations from Table S1 together with formula (1):

$$Food\ intake\left(g\ or\ ml \cdot month^{-1}\right) = Portion\ size\ (g\ or\ ml) \times Intake\ frequency\left(portions \cdot month^{-1}\right) \quad (1)$$

The monthly intake of individual foods was then summed up in each of the following groups: dairy products, eggs, meat products, fishery products, vegetables, fruits, legumes, cereals, oils and fats,

Table 1
Lifestyle, sociodemographic characteristics and dietary habits of the studied population (n = 110).

Sociodemographic characteristics	n (%)	Mean (minimum – maximum)
Primipara	–	–
Yes	64 (58%)	–
No	46 (42%)	–
Age (years)	–	34 (20–45)
BMI before pregnancy (kg·m^{-2})	–	23 (17–35)
Missing data	2 (2%)	–
Country of birth	–	–
Spain	95 (88%)	–
Foreign	13 (12%)	–
Missing data	2 (2%)	–
Place of residence	–	–
Urban	100 (91%)	–
Rural	10 (9%)	–
Educational level	–	–
Only primary school	13 (12%)	–
Secondary school	23 (21%)	–
University degree	74 (67%)	–
Occupational Status	–	–
Employed	91 (83%)	–
Unemployed	19 (17%)	–
Weekly amount of physical activity	–	–
Frequently (3 or more days/week)	36 (33%)	–
Occasionally (1 or 2 days/week)	46 (42%)	–
Never	28 (25%)	–
Smoking habit	–	–
Currently smokes	9 (8%)	–
Former smoker	45 (41%)	–
Never have smoked	56 (51%)	
Time with the windows open during the day (hours)	–	10.4 (1.0–24.0)
Dietary habits	**n (%)**	**Mean (minimum – maximum)**
Food consumption frequency (g·month^{-1})		
Dairy products	–	15010 (1400–43400)
Eggs	–	682 (30–1800)
Meat products	–	5570 (737–13700)
Fishery products	–	4770 (1040–15600)
Non-smoked fish[a]	–	4025 (980–15160)
Smoked fish[a]	–	453 (40–960)
Vegetables	–	14600 (1180–38900)
Fruits	–	18500 (3720–84500)
Legumes	–	1295 (165–9155)
Cereals	–	1797 (470–4530)
Oils and fats	–	846 (152–1540)
Bakery products	–	1610 (171–7300)
Miscellany (snacks and sauces)	–	2100 (343–29700)
Drinks (ml·month^{-1})	–	42900 (1730–76600)
Water[b]	–	34139 (200–54000)
Alcoholic beverages[b]	–	1532 (480–10710)
Soft drinks[b]	–	3348 (522–52065)
Juices and vegetable-based drinks[b]	–	2537 (400–34500)
Coffee[b]	–	968 (50–6775)
Tea[b]	–	331 (75–4500)
Missing data	6 (5%)	–
Adherence to the Mediterranean diet (Med-DQI)	–	–
Good	46 (44%)	–
Medium-good	58 (56%)	–
Missing data	6 (5%)	–
Saturated fatty acids energy (% SFA)	–	3.0 (0.2–10.6)
Missing data	6 (5%)	–
Daily cholesterol intake (mg·day^{-1})	–	371 (93–838)
Missing data	6 (5%)	–

[a] Subdivision of the group Fishery products.
[b] Subdivision of the group Drinks.

bakery products, drinks, and miscellany (snacks and sauces).

Adherence to the Mediterranean diet was estimated by means of the Mediterranean Diet Quality Index (Med-DQI), following the methodology proposed by Gerber (2006). In brief, the following items were calculated for each participant: % saturated fatty acids (SFA) energy, mg cholesterol·day^{-1}, g meat·day^{-1}, ml olive oil·day^{-1}, g fish·day^{-1}, g cereals and legumes·day^{-1}, and g vegetables and fruits·day^{-1}. The diet of the participants was then classified as "good" (1–4 arbitrary units "a. u."), "medium-good" (5–7 a. u.), "medium-poor" (8–10 a. u.) and "poor" (11–14 a. u.), following the criteria presented in Table S2.

2.2. Ethical approval

The study received ethical clearance by the Clinical Research Ethics Committee of the Public Health Directorate, the Center for

Public Health Research of the Valencian Government, and the Biomedical Scientific Ethics Committee of the University and Polytechnic Hospital "La Fe". All samples were collected after informed consent of each subject was obtained. Samples from donors included in the present study were stored in the Biobank for Biomedical and Public Health Research (IBSP-CV BioBank; PT13/0010/0064), which is a member of the Spanish National Biobanks Network and the Valencian Biobanking Network. All personal data were processed (collected, handled, transferred, and analyzed) in a secure setting and were not kept for longer than necessary for processing. Data was processed compliant with all national and EU level ethical and legal considerations, in particular the General Data Protection Regulation.

2.3. Reagents and chemicals

Hexane (for pesticide residue analysis), methanol (LC-MS grade), water (HPLC grade), and ethyl acetate (for pesticide residue analysis) were supplied by VWR Prolabo (Barcelona, Spain). Acetic acid and β-glucuronidase/arylsulfatase (from Helix pomatia; β-glucuronidase activity 30 $U \cdot ml^{-1}$, and sulphatase activity 60 $U \cdot ml^{-1}$) were obtained from Merck (Barcelona, Spain). Silanization solution I (~5% dimethyldichlorosilane in heptane), Supel™ QuE Z-Sep (300 mg Z-Sep + 900 mg $MgSO_4$, 12 ml), and sodium acetate (>99%) were purchased from Sigma-Aldrich (Madrid, Spain).

2.4. Standards and solutions

Certified standards of 1-hydroxynaphthalene (1OHNAP, 100 μg ml^{-1} in acetonitrile) and 2-hydroxynaphthalene (2OHNAP) were obtained from Neochema (Bodenheim, Germany) and Dr. Ehrenstorfer (Augsburg, Germany), respectively. 2-hydroxyfluorene (2OHFLU), 1-hydroxyphenanthrene (1OHPHEN), 2-hydroxyphenanthrene (2OHPHEN), 3-hydroxyphenanthrene (3OHPHEN), 4-hydroxyphenanthrene (4OHPHEN), and 3-hydroxybenzo(a)pyrene (3OHBAP, 50 μg ml^{-1} in toluene) were supplied by Chiron SA (Trondheim, Norway). 3-Hydroxyfluorene (3OHFLU), 9-hydroxyphenanthrene (9OHPHEN), and 1-hydroxypyrene were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada), Sigma-Aldrich, and AccuStandard® (Connecticut, USA), respectively. Isotopically labeled analogues, specifically $^{13}C_6$-1OHNAP (50 μg ml^{-1} in toluene), $^{13}C_6$-2OHFLU (50 μg ml^{-1} in toluene), $^{13}C_6$-2OHPHEN (50 μg ml^{-1} in toluene), and $^{13}C_6$-1OHPYR (50 μg ml^{-1} in toluene) were supplied by Cambridge Isotope Laboratories, Inc. (Massachusetts, USA), $^{2}H_7$-2OHNAP by Sigma-Aldrich, and $^{2}H_8$-9OHPHEN and $^{2}H_{11}$-3OHBAP by Toronto Research Chemicals, Inc. The purity of all certified standards and their internal standards (IS's) was at least 98%. The preparation and concentration of stock and working solutions is described in the supplementary material (SM1). The CAS number and other chemical information of the analyzed OHPAHs and their corresponding IS's are presented in Table S3.

2.5. Chemical analysis

2.5.1. Sample preparation

The analysis of the 11 PAH urinary metabolites was performed according to the procedure developed by Lankova et al. (2016) but with a few modifications. Firstly, all glass materials were precleaned with hexane and then silanized with the silanization solution I in an ultrasonic bath for 15 min in order to avoid OHPAH losses during the hydrolysis and extraction steps as a result of their absorption by the glass wall (Campo et al., 2008). An amount of 2.5 ml of the homogenized sample was then transferred into a 10-ml amber glass tube and it was spiked with 25 μl of the IS working solution. After 10 μl of β-glucuronidase/arylsulfatase and 1 ml of acetate buffer (pH 5) were added, the mixture was incubated overnight (37 °C, 15 h) so that OHPAHs might be released from the conjugated forms. After the mixture had cooled down, 4 ml of ethyl acetate was added to the enzymatic hydrolysate sample and it was vigorously shaken for 1 min. The tube was centrifuged at 3000 rpm and 15 °C for 10 min to facilitate phase separation. The liquid-liquid extraction with ethyl acetate was carried out twice, and then the total organic layer was taken and transferred to the Supel™ QuE Z-Sep tube. The mixture was shaken again for 1 min and centrifuged at 3500 rpm and 20 °C for 10 min. After that, the purified extract was transferred to an empty 10-ml amber glass tube and evaporated under a N_2 stream (25 °C, 7 bar) until dry. Then, the residue was rapidly dissolved in 100 μl of water/methanol (40:60) containing 0.1% of acetic acid. The reconstituted extract was transferred into an injection vial for its analysis with the high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS).

The PAH metabolites in the extracts were determined by using a Vanquish HPLC system (Thermo Fisher Scientific, Germany) equipped with a KINETEX Core-shell technology F5 (Phenomenex, USA) column (150 mm × 2.1 mm, 2.6 μm). Detection of the 11 OHPAHs was performed on a Finnigan TSQ Quantum Ultra detector (San José, CA, USA) equipped with an electrospray ionization source (ESI) in the negative ion mode. The OHPAHs were determined by using selected reaction monitoring (SRM) and data acquisition was carried out using the Thermo Scientific Trace Finder TM 3.2 software.

The limit of quantification (LoQ) was 0.05 ng ml^{-1} for 3OHFLU and 3OHBAP, and 0.01 ng ml^{-1} for the rest of the OHPAHs. Conditions of the HPLC-MS/MS method for analysis of OHPAHs are fully described in SM2. Figure S1 and S2 show the HPLC-MS/MS chromatograms of the highest level of the matrix-matched calibration curve (50 ng ml^{-1}) and of a real urine sample, respectively.

2.5.2. Quality assurance/Quality control (QA/QC) and validation

The laboratory works under a highly consolidated quality system established by UNE-EN ISO/IEC 17025:2017. In order to ensure a proper quantification, a procedural matrix-matched calibration curve was included in each set of samples by spiking synthetic urine at different concentration levels from the LoQ to 50 ng ml^{-1}. In addition, each batch included quality control (QC) samples (spiked synthetic urine at the LoQ, 0.5, and 50 ng ml^{-1} levels), as well as a reagent and a urine blank to assess for any background contribution during the whole experimental procedure. Before enzymatic deconjugation, analogous isotopically labeled IS's for PAH metabolites (Table S3) were added to compensate for the influence of the matrix or experimental losses of the targeted OHPAHs. The acceptance criteria for QA/QC and the validation of the method were based on the Bioanalytical Method Validation Guidance for Industry (US Department of Health and Human Services Food and Drug Administration, 2018), as is described in SM3. Results for the evaluation of the method validation parameters are shown in Table S4.

Additionally, to ensure the method's quality, external quality controls were performed by participating in two German External Quality Assessment Scheme (G-EQUAS) intercomparing programs per year, including 1OHNAP, 2OHNAP, and 1OHPYR analysis. The rest of the OHPAHs, including all the mentioned above, were internally evaluated using the Standard Reference Materials® 3672 and 3673 (organic contaminants in the urine of smokers and non-smokers, respectively) supplied by the US National Institute of Standards and Technology (NIST, USA). In the case of 3OHBAP, for which there was no certified reference material, artificially spiked

urine samples at three levels of concentration (0.05, 0.5, and 50 ng ml^{-1}) were analyzed.

2.6. Urinary creatinine measurement

Creatinine concentration was determined using Jaffé's reaction at the University and Polytechnic Hospital "La Fe" (Valencia, Spain). According to the World Health Organization (WHO), urine samples with a creatinine concentration outside the range of 30–300 mg dl^{-1} are not considered valid for the evaluation of chemical exposures and, therefore, samples not meeting this criterion were excluded from the present study (Barr et al., 2005). Creatinine measurements were also used to correct variability in the dilution of urine samples by transforming urinary levels of OHPAHs into $\mu g \cdot g^{-1}$ creatinine.

2.7. Statistical analysis

The statistical analysis was carried out using the version 26.0 of the IBM SPSS® Statistics software (2019). The maximum likelihood estimation (MLE) method proposed by the European Food Safety Authority (EFSA, 2010) was used to statistically impute missing OHPAH concentrations: those not detected or detected below the LoQ. Firstly, the detection frequency (DF), arithmetic mean (AM), geometric mean (GM), 25th, 50th, 75th, and 95th percentiles (P25, P50, P75, and P95), minimum and maximum (range), and standard deviation (SD), were calculated in order to summarize the obtained urinary OHPAH levels in $ng \cdot ml^{-1}$ and $\mu g \cdot g^{-1}$ creatinine. In addition, OHPAHs which came from the same PAH parent were converted to $nmol \cdot l^{-1}$ and $nmol \cdot g^{-1}$ creatinine, and added together as follows: the sum of hydroxynaphthalenes (∑OHNAPs) = 1OHNAP + 2OHNAP; the sum of hydroxyfluorenes (∑OHFLUs) = 2OHFLU + 3OHFLU; and the sum of hydroxyphenanthrenes (∑OHPHENs) = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN.

The studied variables obtained from the participant's questionnaires were described as can be seen in Table 1: qualitative variables with absolute frequencies and percentages, and quantitative variables by their mean, minimum and maximum values. The Spearman's test was used to study correlations among PAH metabolites.

Additionally, one linear regression model for each PAH chemical family (∑OHNAPs, ∑OHFLUs, ∑OHPHENs and 1OHPYR) was built to be able to explore their relationship with the socio-demographic, lifestyle, and dietary habits of the mothers. A forward stepwise selection procedure based on the corrected Akaike information criterion (AICc) (Konishi and Kitagawa, 2008) was used to construct the prediction models considering all variables from Table 1. To ensure that creatinine levels did not affect the statistical significance of other variables in the model, urinary concentrations of OHPAHs without adjustment for creatinine (in $nmol \cdot l^{-1}$) were used in the regression analysis and creatinine was added as a separate quantitative variable (Barr et al., 2005). The logarithmic transformation of urinary concentrations in $nmol \cdot l^{-1}$ was taken into account to obtain a normal distribution of the response variables. The numerical explanatory variables were divided by twice their SD in order to obtain directly-comparable regression coefficients (Gelman et al., 2008). Confidence intervals at 95% significance were calculated for the model coefficients, and a statistically significant relationship between variables at 5% significance was considered. Finally, the regression assumptions were verified using Shapiro-Wilk test (normality of residuals), Durbin-Watson statistic (no autocorrelation), scatter plots (homoscedasticity), and tolerance and condition indexes (no multicollinearity).

2.7.1. Sensitivity analysis

A comprehensive sensitivity analysis was carried out to see whether the methodology used for handling concentration data below the LoQ influenced the results obtained for OHPAHs with DF between 40 and 100%. For this purpose, different methods were used in the handling of the data:

a) "Discard": all data < LoQ were discarded.
b) "Upper-bound": all data < LoQ were replaced by the corresponding LoQ.
c) "Lower-bound": all data < LoQ were replaced by "0".
d) "LoQ/2": all data < LoQ were replaced by LoQ/2.
e) "LoQ/$\sqrt{2}$": all data < LoQ were replaced by LoQ/$\sqrt{2}$.
f) "MLE": data < LoQ were imputed from the distribution that best fitted the observed values above the LoQ, compatibly with the percentage of data < LoQ. This method was applied 5 times to explore differences between different imputations.

Descriptive statistics of OHPAHs levels were calculated and regression models for ∑OHNAPs, ∑OHFLUs and ∑OHPHENs were fitted from each data set.

2.8. Exposure to PAHs and risk assessment

In the present study, exposure to PAHs was evaluated by an internal dose approach. Given that the non-occupational population and non-smokers are exposed to PAHs mainly through dietary ingestion (EFSA, 2008; European Commission, 2016), exposure to PAHs was only assessed for dietary intake. For this purpose, the urinary levels of OHPAHs were transformed into an oral estimated daily intake (EDI) of the parent PAH compound. EDI was calculated at GM and P95 concentration levels stratified by the smoking habit of the participants (non-smokers, former smokers and currently smokers), and unadjusted and adjusted by creatinine, using the following equations (Koch et al., 2003; Peng et al., 2020):

$$EDI\left(\frac{ng}{kg\ bw \cdot day}\right) = \frac{C_U \times V_{24h} \times MW}{F_{UE} \times BW} \quad (2)$$

$$EDI_{Cre}\left(\frac{ng}{kg\ bw \cdot day}\right) = \frac{C_U \times Cre_{24h} \times MW}{F_{UE}} \quad (3)$$

where C_U = molar concentration (GM or P95) of the OHPAH in urine ($nmol \cdot l^{-1}$ for EDI, or $nmol \cdot g\ Cre^{-1}$ for EDI_{Cre}); V_{24h} = total urinary volume excreted within 24 h for adults ($1.6\ l \cdot day^{-1}$) (Aylward et al., 2011); Cre_{24h} = total urinary creatinine excreted within 24 h standardized by body weight for women ($18\ mg\ kg\ bw^{-1} \cdot day^{-1}$) (Koch et al., 2003); MW = molecular weight of the parent PAH compound ($g \cdot mol^{-1}$); F_{UE} = fraction of the ingested dose of PAH which is eliminated as urinary OHPAH (values ranged from 0.08 to 1) (Li et al., 2012; Motorykin et al., 2015); BW = mean body weight of the participants before pregnancy (60 kg).

The worst-case scenario was chosen, since the BW before pregnancy was used to calculate the EDI and not the postpartum BW. In addition, due to the existing scientific gap on the metabolism of PAHs in humans, the used F_{UE}s were the mean of the F_{UE}s reported by two studies on dietary exposure to PAHs (Li et al., 2012; Motorykin et al., 2015), involving only 9 adults (non-smokers and no known occupational PAH exposure), which may lead to possible uncertainties affecting the results of the risk assessment.

The EDI was estimated only for the most frequently detected biomarkers (DF > 40%), taking into account that concentrations of OHPAHs which came from the same PAH parent were aggregated

and then assessed jointly. Therefore, EDIs were calculated for naphthalene, fluorene, phenanthrene, and pyrene, assuming that their urinary metabolites (∑OHNAPs, ∑OHFLUs, ∑OHPHENs, and OHPYR, respectively) came solely from the oral intake of these parental compounds. The parameters for the calculation of EDIs for each PAH are shown in Table S5.

The calculated EDIs were used to assess the non-carcinogenic risks associated with PAH exposure for the population under review. For this purpose, a hazard quotient (HQ) was used to assess the individual risk of exposure to PAHs by ingestion. It was calculated as the ratio of the EDI for each PAH to its reference dose for oral exposure (RfD), ranging from 20 (naphthalene) to 40 $\mu g\,kg^{-1}\cdot day^{-1}$ (fluorene) (TCEQ, 2017; US EPA, 1998, 1990a, 1990b), as a toxicological reference value (see Equation (3)) (Miri et al., 2018; Peng et al., 2020). If the HQ was less than one, it was unlikely that there was any adverse health effect from a single exposure. The hazard index (HI) was calculated to assess the cumulative risk of dietary exposure to PAH mixtures. To this end, the individual HQs of the PAHs were added up according to Equation (4) (Peng et al., 2020), with a HI below 1 meaning a low risk for the target population due to its cumulative exposure to the analyzed PAHs.

$$HQ_i = \frac{EDI_i}{RfD_i} \quad (4)$$

$$HI_{PAHs} = \sum HQ_i \quad (5)$$

The carcinogenic risk of a PAH mixture is often expressed by its benzo(a)pyrene equivalent concentration using toxicity equivalency factors (TEFs) (Duan et al., 2016). However, according to EFSA Panel on Contaminants in the Food Chain (CONTAM Panel), the TEF approach to risk assessment is not scientifically valid due to its poor prediction of the carcinogenic potency of PAH mixtures, the lack of oral carcinogenicity studies for the different PAHs, and their different modes of action (EFSA, 2008). Thus, the assessment of cancer risk of PAHs was not conducted in the present study.

3. Results

In total, 120 women participated in the study, and 119 provided a urine sample. However, only 116 urine samples were examined for the presence of 11 OHPAHs because six donors did not collect a sufficient volume of urine to carry out the analysis. Moreover, three urine samples had a concentration of creatinine out of the range of 30–300 $mg\,dl^{-1}$, and consequently, these samples were excluded from the present study. Finally, 110 urine samples with creatinine concentrations between 0.3 and 2.4 $g\,l^{-1}$ (mean value of 1.0 $g\,l^{-1}$), were considered in the descriptive and statistical analyses. Information about lifestyle, socio-demographic characteristics, and food consumption habits of the target population is summarized in Table 1. Most of the women (91%) lived in an urban area, 83% of them were employed, and all of them had good or medium-good adherence to the Mediterranean diet. In addition, only 8% of the participants were smokers.

3.1. Urinary concentrations of PAH metabolites

The descriptive statistics of the OHPAH urinary levels of the target population (in $ng\cdot ml^{-1}$ and $\mu g\cdot g$ $creatinine^{-1}$) are presented in Table 2. From the target PAH metabolites, 2OHNAP and 1OHPYR, urinary metabolites of naphthalene and pyrene, respectively, were the most abundant compounds found in 100% of the urine samples. Another naphthalene metabolite, 1OHNAP, was detected in 96% of the samples, while OHFLUs were detected in more than 70% of the samples, and detection frequencies for OHPHENs ranged from 90% (9OHPHEN) to 99% (3OHPHEN). On the other hand, the urinary metabolite of benzo(a)pyrene, 3OHBAP, was only found in one sample, the reason being that it had a low urinary excretion proportion (4–12%) given that this is not its main excretion route (Chien and Yeh, 2012; EFSA, 2008).

Regarding concentrations, the highest urinary levels were found for 1OHNAP and 2OHNAP, with GMs of 0.8 and 7.1 $ng\,ml^{-1}$, and a 95th percentile of 5.7 and 31.2 $ng\,ml^{-1}$, respectively. Similar GM levels were observed for 2OHFLU (0.17 $ng\,ml^{-1}$), 3OHFLU (0.13 $ng\,ml^{-1}$), and 1OHPYR (0.12 $ng\,ml^{-1}$). In general, concentrations of OHPHENs, whose GM ranged from 0.04 (4OHPHEN) to 0.14 $ng\,ml^{-1}$ (1OHPHEN), were the lowest ones. It is worth noting that 100% of the women had seven or more PAH metabolites in their urine samples.

3.2. Associations with personal factors

The Spearman matrix that was obtained to explore correlations among ∑OHNAPs, ∑OHFLUs, ∑OHPHENs and 1OHPYR is shown in Table S6. As can be seen, all OHPAHs were positively related to one another ($p < 0.01$).

The resulting multiple linear regression models that represent the relationships of each PAH family with sociodemographic characteristics and dietary habits of the target population are presented in Table 3. Additional regression models were constructed including also the subgroups of Fishery products (non-smoked and smoked fish) and Drinks (water, alcoholic beverages, soft drinks, juices and vegetable-based drinks, coffee and tea), which are shown in Table S7.

According to the fitted models presented in Table 3, creatinine concentration had a positive relationship with all PAH metabolites. In addition, despite the low percentage of smokers (8%), the smoking habit markedly influence OHPAHs levels (p-value ≤ 0.01), showing that smoker women had significantly higher OHPAHs urinary levels than non-smokers. Due to its significantly higher regression coefficients (β ranging from 0.632 to 1.421) compared to the rest of variables (β ranging from 0.203 to 0.807), the smoking habit appeared to be the factor most affecting the urinary concentrations of the PAH metabolites under study. In the case of 1OHPYR, a negative association was also found with the age of the mother.

Regarding the food consumption habits of the participants, women who consumed a higher quantity of vegetables per month had lower ∑OHNAPs levels in urine. On the contrary, women who consumed more oils and fats were found to have higher levels of urinary ∑OHNAPs. A further finding from the statistical analysis presented in Table S7 is that ∑OHNAPs and 1OHPYR levels were also positively related with the consumption of smoked fish. Likewise, cereals and coffee intakes seemed to increase urinary levels of ∑OHFLUs. In addition, a significant and positive association was found with the percentage of SFA energy in the case of ∑OHFLUs and ∑OHPHENs.

Other relevant but not statistically significant findings (p-values > 0.05) were that a good adherence to the Mediterranean diet and having the windows opened more hours during the day appeared to be related to lower urinary levels of ∑OHNAPs and ∑OHFLUs, respectively.

Overall, the fitted regression models were weak but accounted for 26–35% of the variance (R^2).

3.2.1. Sensitivity analysis

Results of the descriptive statistics for each method used in the sensitivity analysis is presented in Table S8. In addition, the resulting regression models can be shown in Table S9.

Table 2
Descriptive analysis of the OHPAHs levels (ng·ml^{-1}) in urine from Spanish breastfeeding mothers (n = 110)[a].

Metabolites	LoQ	DF (%)	P25	P50	AM	GM	P75	P95	Range[b]	SD
1OHNAP	0.01	96	0.3 (0.4)	0.7 (0.8)	2.0 (1.8)	0.8 (0.8)	1.8 (1.7)	5.7 (7.6)	<0.01–25.3 (<0.01–19.8)	4.0 (3.3)
2OHNAP	0.01	100	3.6 (4.5)	7.4 (7.0)	10.0 (9.3)	7.1 (7.2)	11.9 (11.2)	31.2 (24.6)	1.5–46.7 (1.59–41.3)	9.4 (3.2)
2OHFLU	0.01	98	0.10 (0.10)	0.15 (0.16)	0.30 (0.30)	0.17 (0.18)	0.31 (0.27)	1.15 (0.96)	<0.01–2.23 (<0.01–2.88)	0.40 (0.44)
3OHFLU	0.05	70	0.05 (0.04)	0.14 (0.17)	0.27 (0.26)	0.13 (0.13)	0.35 (0.33)	1.01 (0.84)	<0.05–2.18 (<0.05–2.78)	0.37 (0.36)
1OHPHEN	0.01	98	0.08 (0.09)	0.15 (0.14)	0.22 (0.22)	0.14 (0.15)	0.24 (0.25)	0.70 (0.62)	<0.01–1.26 (<0.01–1.63)	0.22 (0.26)
2OHPHEN	0.01	95	0.04 (0.04)	0.07 (0.07)	0.08 (0.09)	0.06 (0.06)	0.10 (0.10)	0.20 (0.25)	<0.01–0.45 (<0.01–0.46)	0.06 (0.08)
3OHPHEN	0.01	99	0.06 (0.06)	0.10 (0.09)	0.12 (0.12)	0.09 (0.09)	0.16 (0.14)	0.35 (0.30)	<0.01–0.49 (<0.01–0.72)	0.10 (0.11)
4OHPHEN	0.01	92	0.02 (0.02)	0.04 (0.04)	0.05 (0.06)	0.04 (0.04)	0.07 (0.07)	0.16 (0.17)	<0.01–0.29 (<0.01–0.42)	0.05 (0.07)
9OHPHEN	0.01	90	0.05 (0.06)	0.11 (0.09)	0.14 (0.14)	0.08 (0.09)	0.16 (0.18)	0.44 (0.47)	<0.01–0.87 (<0.01–0.67)	0.15 (0.14)
1OHPYR	0.01	100	0.08 (0.09)	0.12 (0.12)	0.16 (0.16)	0.12 (0.13)	0.18 (0.17)	0.41 (0.41)	0.03–1.06 (0.03–0.69)	0.15 (0.13)
3OHBAP[c]	0.05	<1	–	–	–	–	–	–	–	–
[d,e]∑OHNAPs	–	–	31 (38)	61 (57)	83 (77)	58 (59)	90 (92)	250 (223)	11 - 456 (13–344)	84 (64)
[d,f]∑OHFLUs	–	–	1.0 (1.1)	2.0 (1.9)	3.1 (3.1)	2.0 (2.0)	3.6 (3.4)	10.9 (10.2)	0.4–22.2 (0.4–30.2)	3.9 (3.9)
[d,g]∑OHPHENs	–	–	1.7 (1.7)	2.4 (2.3)	3.2 (3.2)	2.6 (2.6)	4.3 (3.7)	8.4 (8.1)	0.7–11.8 (0.8–16.8)	2.4 (2.7)
[d,h]∑OHPAHs	–	–	36 (42)	66 (62)	90 (84)	65 (66)	97 (101)	259 (239)	16 - 483 (16–363)	89 (68)

LoQ = Quantification Limit; DF = Detection Frequency (% samples > LoQ); P = percentile; AM = Arithmetic mean; GM = Geometric mean; SD = Standard deviation.
[a] Values within parenthesis are the urinary levels adjusted by creatinine (μg·g Cre^{-1}).
[b] Minimum – maximum.
[c] Some parameters were not calculated due to the low DF of this metabolite.
[d] Units: nmol·l^{-1} (nmol·g Cre^{-1}).
[e] ∑OHNAPs = 1OHNAP + 2OHNAP.
[f] ∑OHFLUs = 2OHFLU + 3 OHFLU.
[g] ∑OHPHENs = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN.
[h] ∑OHPAHs = ∑OHNAPs + ∑OHFLUs + ∑OHPHENs + 1OHPYR + 3OHBAP.

Table 3
Results of linear regression model with influencing factors on the log-transformed PAH metabolite concentrations (n = 104).

Metabolite	Variable	Standardized regression coefficients (95% CI)	Standard error	p-value	R^2	adjR^2
ΣOHNAPs	Intercept	3.013 (2.513–3.515)	0.252	**<0.001**	0.383	0.345
	Creatinine (g·l^{-1})	0.807 (0.551–1.062)	0.129	**<0.001**		
	Smoking habit (Former smoker)	0.082 (−0.189 – 0.353)	0.136	0.549		
	Smoking habit (Currently smokes)	0.826 (0.333–1.318)	0.248	**0.001**		
	Vegetables (g·month^{-1})	−0.279 (−0.541–−0.018)	0.132	**0.037**		
	Oils and fats (g·month^{-1})	0.306 (0.037–0.576)	0.136	**0.026**		
	Adherence to the Mediterranean diet (Good)	−0.213 (−0.477 – 0.052)	0.133	0.115		
ΣOHFLUs	Intercept	−1.618 (−2.549–−0.688)	0.426	**0.001**	0.396	0.352
	Smoking habit (Former smoker)	0.310 (0.021–0.599)	0.146	**0.036**		
	Smoking habit (Currently smokes)	1.421 (0.885–1.957)	0.270	**<0.001**		
	Creatinine (g·l^{-1})	0.617 (0.327–0.906)	0.146	**<0.001**		
	Drinks (ml·month^{-1})	0.424 (0.118–0.730)	0.154	**0.007**		
	Cereals (g·month^{-1})	0.354 (0.069–0.640)	0.144	**0.015**		
	Saturated fatty acids energy (% SFA)	0.364 (0.054–0.674)	0.156	**0.022**		
	Time with the windows open during the day (hours)	−0.222 (−0.504 – 0.060)	0.142	0.121		
ΣOHPHENs	Intercept	0.148 (−0.167 – 0.462)	0.158	0.353	0.293	0.265
	Creatinine (g·l^{-1})	0.524 (0.298–0.749)	0.114	**<0.001**		
	Smoking habit (Former smoker)	−0.071 (−0.299 – 0.158)	0.115	0.541		
	Smoking habit (Currently smokes)	0.632 (0.212–1.053)	0.212	**0.004**		
	Saturated fatty acids energy (% SFA)	0.227 (0.001–0.453)	0.030	**0.049**		
1OHPYR	Intercept	−0.265 (−1.206 – 0.675)	0.474	0.577	0.347	0.313
	Creatinine (g·l^{-1})	0.485 (0.264–0.707)	0.112	**<0.001**		
	Smoking habit (Former smoker)	0.126 (−0.099 – 0.350)	0.113	0.269		
	Smoking habit (Currently smokes)	0.835 (0.424–1.247)	0.207	**<0.001**		
	Age (years)	−0.373 (−0.651–−0.096)	0.140	**0.009**		
	Saturated fatty acids energy (% SFA)	0.203 (−0.019 – 0.425)	0.112	0.072		

According to Table S8, similar results were obtained in the descriptive statistics of the different data sets considered in the sensitivity analysis. As expected, slightly higher and lower values were obtained in the description of the data with discarded values below the LoQ ("discard" method) and imputed using the "lower-bound" method compared to the rest of methods, respectively.

Regarding the regression models presented in Table S9, similar results were also obtained in the fit of each data set. Only the "discard" and "lower-bound" methods gave different results in the models obtained for ∑OHFLUs. On the other hand, similar results were obtained for all the five different imputations of MLE.

Substitution methods are biased and do not consider the overall distribution of detected samples. Different evaluation studies on statistical treatments of left-censored data show that the MLE method is one of the best approaches from a methodological perspective (EFSA, 2010) and this is why it was selected for handling data below the LoQ in the present study.

3.3. Risk assessment

Given that 3OHBAP was detected in less than 1% of the samples, only EDIs for naphthalene, fluorene, phenanthrene, and pyrene were calculated. The estimated daily intakes based on unadjusted urinary levels (EDI) and creatinine-adjusted concentrations

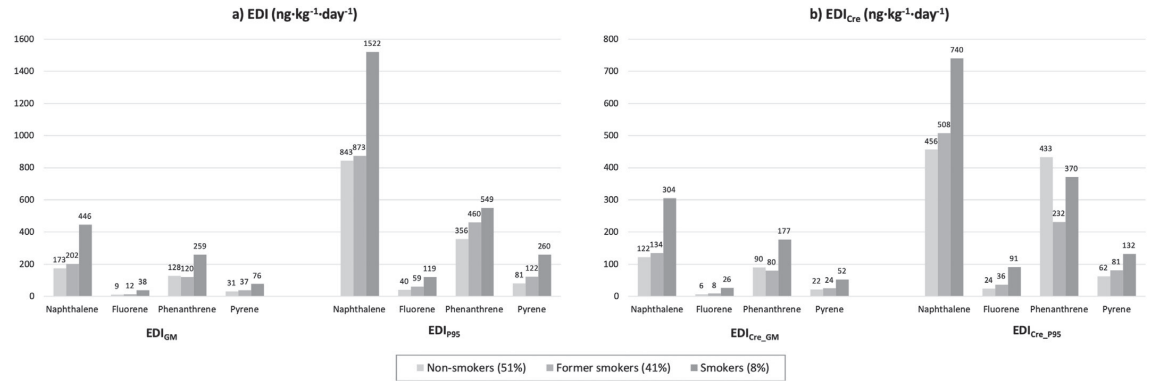

Fig. 1. Estimated daily intakes (EDI, in ng·kg^{-1}·day^{-1}) of individual PAHs unadjusted (a) and adjusted by creatinine (b), stratified by the smoking habit of the participants.

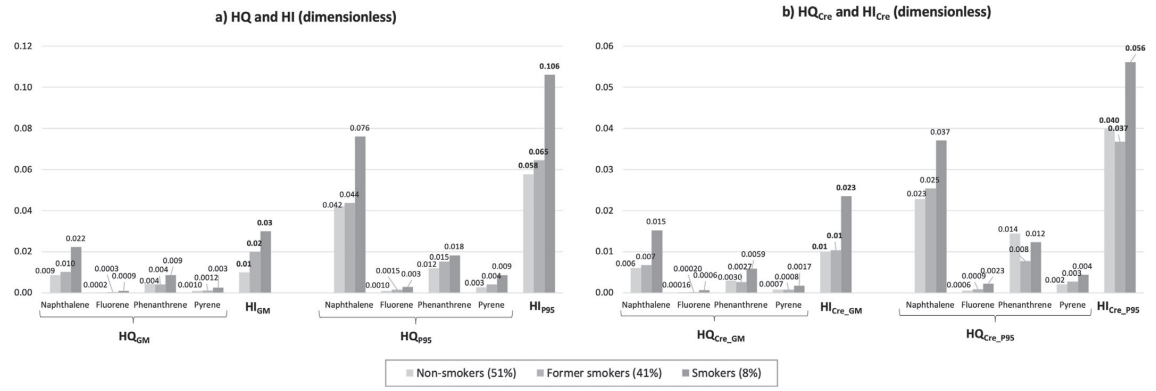

Fig. 2. Results of the non-cancer risk assessment stratified by the smoking habit of the participants: Hazard Quotients of individual PAHs and Hazard Indexes unadjusted (a) and adjusted by creatinine (b).

(EDI$_{Cre}$), as well as stratified by the smoking habit of the participants, are shown in Fig. 1. The results of the risk assessment (HQ and HI) are presented in Fig. 2.

As can be observed in Fig. 1, the highest EDI was obtained for naphthalene, ranging from 173 (GM) to 1522 ng kg^{-1}·day^{-1} (P95), since its metabolites were found at much higher concentration levels than the rest of the OHPAHs. Conversely, fluorene was the PAH compound that presented the lowest EDI values, which ranged from 9 (GM) to 119 ng kg^{-1}·day^{-1} (P95). In general, EDI$_{Cre}$ values, ranging from 6 (GM for fluorene) to 740 ng kg^{-1}·day^{-1} (P95 for naphthalene), were lower than those obtained using unadjusted concentrations. As expected, the highest EDIs were found for participants who were smokers, except for EDI$_{Cre}$ of phenanthrene at P95. However, no significant differences were observed between EDIs of non-smokers and former smokers.

As can be seen in Fig. 2, the calculation for the non-cancer risk assessment with unadjusted EDIs resulted in HQ$_{GM}$ between 0.0002 (fluorene) and 0.02 (naphthalene). In the case of HQ$_{P95}$, the values ranged from 0.0010 (fluorene) to 0.08 (naphthalene). The individual risk estimated with the creatinine-adjusted levels showed HQ$_{Cre}$ of the same order of magnitude but slightly lower than unadjusted HQs. Regarding the cumulative risk calculations, HI of smokers was approximately twice the HI of non-smokers, although in all cases it was lower than 1.

4. Discussion

To put the urinary levels of OHPAHs obtained in the present study in an international context, a comparison with levels reported in other HBM studies with adults, women, mothers, pregnant women, and the general population from different countries was made (Fig. 3). The same pattern in detection frequencies as well as in the urinary levels of OHPAHs was observed in most of the countries: 1OHNAP and 2OHNAP were the most frequently detected metabolites with the highest urinary levels.

In the present study, levels of urinary 1OHNAP were generally found to be lower than in other countries, such as Australia (Thai et al., 2020) and the USA (CDC, 2019; Luderer et al., 2017), although they were higher than in women from Denmark (Rosofsky et al., 2017) and China (Lou et al., 2019; Nie et al., 2019). In the case of 2OHNAP, in general, higher urinary concentrations were observed in our study when compared to other parts of the world. Nevertheless, some studies carried out in the USA (CDC, 2019; Luderer et al., 2017) reported higher urinary levels of this naphthalene metabolite than those obtained in this research. In contrast, concentrations of urinary 2OHFLU obtained in the target

Study number	Country	Period time	n	Population	Reference
1	Spain	2015	110	Mothers	Present study
2	Spain	2009 – 2010	435	Women (ng·g Cre^{-1})	Bartolome et al., 2015
3	Spain	2003 – 2005	58	Pregnant women (median of smokers)	Llop et al., 2008
4	Spain	2003 – 2005	137	Pregnant women (median of non-smokers)	
5	Czech Republic	2013 – 2014	531	Mothers	Urbancova et al., 2017
6	Poland	2007	210	Pregnant women (ng·g Cre^{-1})	Polanska et al., 2014
7	Italy	2013	177	Women (ng·g Cre^{-1})	Iamiceli et al., 2020
8	Denmark	2011 – 2014	73	Fertile women	Rosofsky et al., 2017
9	Israel	2011	249	Adults	Berman et al., 2013
10	China	2015	188	Pregnant women	Lou et al., 2019
11	China	2009	283	Pregnant women	Nie et al., 2019
12	Australia	2016 – 2017	2400	General population	Thai et al., 2020
13	USA	2011 – 2012	348	Women (smokers)	CDC, 2019
14	USA	2011 – 2012	688	Women (non-smokers)	
15	California (USA)	2010 – 2012	48	Fertile women	Luderer et al., 2017
16	California (USA)	2013	218	Adults	Biomonitoring California, 2018
17	California (USA)	2010 – 2011	88	Pregnant women	
18	Canada	2016 – 2017	97	Women	Ratelle et al., 2020
19	Canada	2014 – 2015	1259	Women	Health Canada, 2017
20	Canada	2014 – 2015	359	Adults	

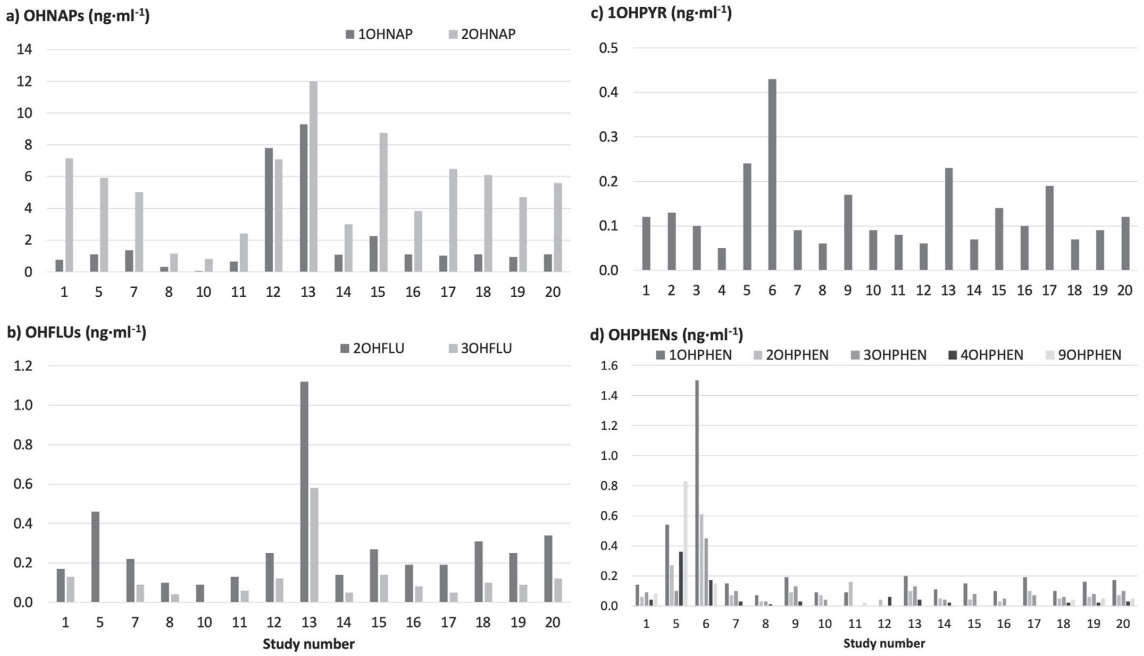

Fig. 3. Comparison of the urinary levels (geometric mean in ng·ml^{-1}) of a) OHNAPs, b) OHFLUs, c) 1OHPYR and d) OHPHENs among different countries around the world (each number represents a different study).

population were lower than those obtained in other countries, such as the Czech Republic (Urbancova et al., 2017), and also those observed in some studies from the USA (Biomonitoring California, 2018; CDC, 2019; Luderer et al., 2017) and Canada (Health Canada, 2017; Ratelle et al., 2020). Similarly to our study, lower levels of 2OHFLU have also been found in Chinese women (Lou et al., 2019; Nie et al., 2019). On the other hand, levels of urinary 3OHFLU in the women participating in the present study were comparable to those reported in other studies around the world, with the exception of American female smokers (CDC, 2019). On the whole, urinary levels of OHPHENs and 1OHPYR of the target population were in the range of the rest of the countries, the highest concentrations having been observed in women from the Czech Republic (Urbancova et al., 2017) and Poland (Polanska et al., 2014), followed by USA (Biomonitoring California, 2018; CDC, 2019) and Israel (Berman et al., 2013); and the lowest levels having been found in Danish women (Rosofsky et al., 2017), the general population in Australia (Thai et al., 2020), and American female non-smokers (CDC, 2019). It is important to note that similar 1OHPYR concentrations were found in Spanish studies (Bartolomé et al., 2015; Llop et al., 2008), and lower levels were observed in Spanish female non-smokers (Llop et al., 2008).

The difference in urinary OHPAH levels among countries could mostly be explained by the differences in dietary patterns, smoking habits, and air pollution levels (HBM4EU, 2018), as well as, different study designs, populations, laboratory analysis, races, among other factors. However, other possible exposure sources may influence urinary OHPAH levels if the biomarkers of exposure are non-specific to PAH exposure. This is the case of 1OHNAP, which is also a biomarker of exposure to the pesticide carbaryl.

Regarding the statistical analysis, the smoking habit of the women was the determinant of major significance for all OHPAHs, due to the high regression coefficients observed for this variable comparing with the others in the fitted models. This is consistent with the risk assessment estimations, which revealed higher HQs and HIs of PAHs in smoker women compared to non-smokers. These findings corroborated that tobacco smoke is one of the primary factors contributing to PAH exposure in smokers, as previously reported by several authors (Bartolomé et al., 2015; Cao et al., 2020; Dobraca et al., 2018; EFSA, 2008; Polanska et al., 2014; Ratelle et al., 2020).

Apart from that, dietary sources appeared to be also relevant factors in PAH exposure of the target population. Indeed, diet is found to account for almost 90% of PAH daily intake (HBM4EU, 2019). In the present study, the multiple regression models showed that the ingestion of vegetables had a negative effect on ∑OHNAPs urinary levels, most likely because this food group is a minor source of exposure to PAHs (Falcó et al., 2003), thus an increase in the consumption of vegetables might lead to a decrease in the PAH exposure (Martorell et al., 2012). Similar results were found in two previous HBM studies on adolescents (Jain, 2020) and occupationally exposed population (Kawamoto et al., 2007), in which vegetable intake was associated with lower levels of OHPAHs in urine. This may also be related to a healthy lifestyle, such as following a Mediterranean diet (Gerber, 2006), which was found to have a negative relationship with urinary concentrations of ∑OHNAPs, although it was not statistically significant to reach a final conclusion (p-value > 0.05).

On the other hand, oils and fats ingestion seemed to increase levels of urinary∑OHNAPs. This fact can be explained by the lipophilic characteristics of PAHs, that tend to accumulate in the fat content of biosystems and food chain (Bansal and Kim, 2015). In addition, according to several diet studies on the Spanish population (Domingo and Nadal, 2015), oils and fats are one of the major contributors to the exposure to PAHs by dietary intake. What is more, oils and fats have been reported to be important precursors in the formation of PAHs in foods (EFSA, 2007), as these substances are generated from SFAs under high heating conditions (Nie et al., 2019; Pitts, 1983). This could also be the reason why a significant and positive association was observed between SFA (% energy) and urinary levels of ∑OHFLUs and OHPHENs.

The regression models for ∑OHFLUs revealed that a high consumption of cereals and coffee had a positive effect on their urinary levels. Indeed, cereals have been considered one of the highest contributors to the total dietary intake of PAHs by EFSA and other authors (Domingo and Nadal, 2015; EFSA, 2008; Falcó et al., 2003; Ibáñez et al., 2005). In the case of coffee, two recent HBM studies also reported a positive association between the coffee ingestion and concentrations of fluorene metabolites in urine (Iamiceli et al., 2020; Jain, 2020). The reason is that low molecular weight PAHs are prevalent in coffee brews when high temperatures are used during the roasting process (EFSA, 2008; Orecchio et al., 2019).

According to EFSA, seafood and seafood products are also significant contributors to dietary exposure to high molecular weight PAHs. In addition, a survey carried out in Catalonia in 2006 suggested that this food group is also an important source of exposure to low molecular weight PAHs, such as phenanthrene and naphthalene (Domingo and Nadal, 2015). However, in the present study only significant associations were found with ∑OHNAPs and 1OHPYR when the group "Fishery products" was divided in two subgroups (non-smoked and smoked fish). In this way, ingestion of smoked fish was associated with increased levels of these metabolites in the urine. This finding was consistent with Llop et al. (2008), who reported that the intake of smoked food was an important predictor of exposure to 1OHPYR in Spanish pregnant women. Likewise, a HBM study carried out in Japan showed that a high fish consumption increased urinary levels of 1OHPYR (Kawamoto et al., 2007).

Other foodstuffs that are considered to be contributors of great importance in PAH exposure are meat and meat products (Domingo and Nadal, 2015; Ibáñez et al., 2005; Kawamoto et al., 2007). Notwithstanding the latter, no association at all was found with consumption of meat products in the women participating in the present study. This may be due to the lack of detailed information in the food frequency questionnaire, such as the recent intake of specific food or the cooking practices used by the participants.

With regard to other relevant findings, a negative association was found between urinary 1OHPYR and the age of individuals, as already reported by other authors (Hoseini et al., 2018; Jain, 2020; Yu et al., 2016). However, to date, there is no scientific evidence to support this.

In an exposure context, the EDIs for pyrene obtained here were lower than those observed in Mexican women (Pruneda-Álvarez et al., 2016), several Asian countries, such as China, India, Kuwait and Vietnam (Guo et al., 2013; Peng et al., 2020), and other European countries, such as the Netherlands, Greece and Denmark (HBM4EU, 2019); although they were higher than those in Belgium (adolescents) and Sweden (HBM4EU, 2019). On the other hand, similar EDIs were found among adults living in France, Italy, Germany, Czech Republic (HBM4EU, 2019), and the assessed Spanish population. For naphthalene, fluorene and phenanthrene, higher EDIs were observed in the present study compared to primiparas and pregnant women in China (Lou et al., 2019; Peng et al., 2020).

Fortunately, given that the obtained HQs and HIs were far below 1, exposure to PAHs should not be considered a potential toxic hazard for the Spanish population. However, taking into account that humans are exposed to PAH mixtures containing more than 100 chemical substances, and in the present study the cumulative risk was assessed for only 4 PAHs (naphthalene, fluorene, phenanthrene and pyrene), there may be an underestimation of the risk.

Despite there being no evidence of a non-cancer health risk, HBM and epidemiological studies are highly recommended to obtain more extensive data about nutritional and environmental influencing factors in PAH exposure, as well as to understand the real health impact on the vulnerable population, such as pregnant women and children.

5. Study limitations

This study presents the following limitations:

i) The recruitment of the participants was conducted in just one hospital located in the Valencian Community, covering only a limited area of this region.
ii) The sample size (n = 110) corresponds to a pilot study, thus the conclusions of the present study should not be extrapolated to the entire population of the Valencian Region.
iii) The maximum level of education of the mothers was biased toward university studies.

iv) The questionnaire did not inquire into what culinary practices the participants used, and about the exposure of second-hand smoke or the intensity of smoking habit.
v) PAH metabolites were analyzed in first-morning urine samples, which may not capture the long-term exposure status of the target population.

6. Conclusions

In the present study, high detection frequencies (>70%) were obtained for ten of the analyzed urinary PAH metabolites, with geometric means ranging from 0.04 (4OHPHEN) to 7.1 ng ml^{-1} (2OHNAP). The highest urinary concentrations were found for the two naphthalene metabolites (1OHNAP and 2OHNAP). In general, the obtained OHPAH urinary levels were in the same concentration range as those reported in similar studies from other countries.

According to the fitted regression models, smoking and dietary habits were the most influential determinants of exposure to PAHs.

In an exposure context, there was no excess health risk expected due to cumulative exposure to PAHs in the target population.

Author statement

Sandra F. Fernández: Methodology, Validation, Investigation, Writing - original draft, Visualization, Software, Formal analysis, Data curation; Agustín Pastor: Writing - review & editing, Supervision; Olga Pardo: Writing - review & editing, Supervision, Funding acquisition; Vicent Yusà; : Conceptualization, Writing - review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.115646.

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5.2 BIOMONITORING OF POLYCYCLIC AROMATIC HYDROCARBONS IN CHILDREN: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT

5.2.1 RESULTS AND DISCUSSION

Urine samples of the BIOVAL study were analyzed using the same validated method as in the BETTERMILK project. In the end, a total of 566 urine samples with a creatinine concentration ranging from 33 to 266 mg·dl^{-1} (mean of 104 mg·dl^{-1}) were considered in the descriptive and statistical analysis of PAH metabolites. Table 18 summarizes the variables obtained from the questionnaires regarding the socio-demographic characteristics, lifestyle habits, and the dietary patterns of the donors that were used in this research. Out of the 566 children, 50 % were boys and 50 % were girls; 51 % were aged between 5 and 8 years old and 49 % between 9 and 12 years old. Most of the children (93 %) had at least one parent employed, and 78 % of them lived in urban areas. Anomalous BMIs were found in 16 children (3 %), who had a BMI > 25 kg·m^{-2}, four of which presented a BMI > 30 kg·m^{-2}. A BMI lower than 18 kg·m^{-2} was observed in 62 % of the children.

Table 18. Socio-demographic characteristics, lifestyle and food consumption habits of the children of the BIOVAL program included in the analysis of PAH metabolites in urine (n = 566).

Parents' characteristics	n (%)
Country of birth	
Both parents were Spanish	490 (87 %)
At least one of the parents was foreign	58 (10 %)
Missing data	18 (3 %)
Maximum level of education	
Without studies or primary studies	58 (10 %)
Secondary studies	167 (30 %)
University studies	341 (60 %)
Employment situation	
Both unemployed	38 (7 %)
Only one parent employed	164 (29 %)
Both employed	364 (64 %)

Children's characteristics	n (%)	Mean (minimum – maximum)
BMI ($kg \cdot m^{-2}$)	-	**18 (12 – 45)**
Missing data	16 (3 %)	-
Age (years)	-	**8 (5 – 12)**
Province	-	-
Alicante	205 (36 %)	-
Castellón	126 (22 %)	-
Valencia	235 (42 %)	-
Place of residence	-	-
Urban area	441 (78 %)	-
Rural area	22 (4 %)	-
Other	102 (18 %)	-
Missing data	1 (0.2 %)	-
Sex	-	-
Male	285 (50 %)	-
Female	281 (50 %)	-
Country of birth	-	-
Spain	555 (98 %)	-
Foreign	8 (1 %)	-
Missing data	3 (1 %)	-
Physical activity	-	-
Frequently (3 or more days/ week)	299 (53 %)	-
Occasionally (1 or 2 days/week)	207 (36.5 %)	-
Never	60 (10.5 %)	-

Food consumption in the last 72 h (grams)	Mean (minimum – maximum)
Eggs	65 (0 – 360)
Meat products	484 (0 – 1545)
Fishery products	262 (0 – 1300)
Dairy products	996 (0 – 2820)
Vegetables	399 (0 – 1798)
Potato fried products (French fries, chips and other snacks)	26 (0 – 210)
Fruits and nuts	840 (0 – 2835)
Legumes	101 (0 – 1000)
Cereals	498 (0 – 1655)
Bakery products	75 (0 – 350)
Oils and fats	17 (0 – 120)

Frequency of consumption of packaged food (number of products·month^{-1})	Mean (minimum – maximum)
Canned food	0.4 (0 – 4)
Plastic-packed food	6 (0 – 21)
Carton-packed food	0.9 (0 – 4)
Food in glass containers	0.2 (0 – 6)

5.2.1.1 URINARY LEVELS OF PAH METABOLITES

Table 19 shows the descriptive statistics of the urinary concentrations of OHPAH (in ng·ml^{-1} and µg·g creatinine^{-1}) in the target population. As can be observed, all PAH metabolites were detected in more than 78 % of the urine samples, except for 3OHBAP (5 %) and 3OHFLU (37 %), partially due to the fact that these two metabolites had the highest LoQ (0.05 ng·ml^{-1}). In the case of 3OHBAP, the metabolite of the only known carcinogenic PAH (benzo(a)pyrene), the low detection rate observed here was in line with other HBM studies performed in the Czech Republic[177], Spain (BETTERMILK project), Portugal[342], Canada[322,323], and China[167], despite having different LoQs. The main reason is that high-molecular weight PAHs, those with more than four fused rings, are largely eliminated through feces[168,343].

Table 19. Descriptive statistics of urinary OHPAHs concentrations in ng·ml^{-1} (µg·g Cre^{-1}) in children of the BIOVAL program (n = 566).

Metabolite	LoQ (ng·ml^{-1})	DF (%)	P25	P50
1OHNAP	0.01	87	0.2 (0.3)	0.6 (0.6)
2OHNAP	0.01	100	5 (6)	11 (11)
2OHFLU	0.01	99	0.11 (0.12)	0.17 (0.17)
[a]3OHFLU	0.05	37	-	-
1OHPHEN	0.01	97	0.06 (0.07)	0.11 (0.11)
2OHPHEN	0.01	96	0.02 (0.03)	0.04 (0.04)
3OHPHEN	0.01	99	0.05 (0.06)	0.08 (0.09)
4OHPHEN	0.01	78	0.01 (0.01)	0.02 (0.02)
9OHPHEN	0.01	86	0.02 (0.02)	0.04 (0.04)
1OHPYR	0.01	98	0.07 (0.07)	0.11 (0.10)
[a]3OHBAP	0.05	5	-	-
[b,c]∑OHNAPs	-	-	42 (48)	81 (80)
[b,d]∑OHFLUs	-	-	0.8 (0.8)	1.2 (1.2)
[b,e]∑OHPHENs	-	-	1.1 (1.2)	1.6 (1.6)
[b,f]∑OHPAHs	-	-	46 (51)	85 (84)

LoQ = Quantification Limit; DF = Detection Frequency (% samples > LoQ); P = percentile; AM = Arithmetic mean; GM= Geometric mean; SD = Standard deviation; Min. = minimum; Max. = maximum

[a]Some parameters were not calculated due to the low DF of this metabolite

[b]Units: nmol·l^{-1} (nmol·g Cre^{-1})

[c]∑OHNAPs = 1OHNAP + 2OHNAP

[d]∑OHFLUs = 2OHFLU + 3 OHFLU

[e]∑OHPHENs = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN

[f]∑OHPAHs = ∑OHNAPs + ∑OHFLUs + ∑OHPHENs + 1OHPYR + 3OHBAP

AM	GM	P75	P95	Range (Min. – Max.)	SD
1.0 (1.0)	0.3 (0.4)	1.2 (1.1)	3.1 (3.2)	<0.01 – 17.2 (<0.01 – 12.4)	1.4 (1.4)
18 (18)	10 (11)	20 (20)	53 (52)	0.03 – 382 (0.02 – 265)	28 (24)
0.25 (0.25)	0.17 (0.18)	0.27 (0.25)	0.55 (0.55)	<0.01 – 16.99 (<0.01 – 17.61)	0.73 (0.75)
0.10 (0.10)	-	0.07 (0.07)	0.29 (0.30)	<0.05 – 2.42 (<0.05 – 2.55)	0.26 (0.29)
0.16 (0.16)	0.10 (0.10)	0.18 (0.17)	0.39 (0.41)	<0.01 – 4.35 (<0.01 – 4.93)	0.26 (0.28)
0.06 (0.06)	0.04 (0.04)	0.07 (0.07)	0.15 (0.14)	<0.01 – 1.26 (<0.01 – 1.31)	0.08 (0.08)
0.12 (0.11)	0.08 (0.09)	0.14 (0.13)	0.30 (0.28)	<0.01 – 2.47 (<0.01 – 2.56)	0.15 (0.15)
0.04 (0.04)	0.02 (0.02)	0.04 (0.04)	0.11 (0.10)	<0.01 – 0.60 (<0.01 – 0.62)	0.05 (0.05)
0.06 (0.06)	0.03 (0.03)	0.07 (0.07)	0.18 (0.19)	<0.01 – 1.65 (<0.01 – 1.87)	0.10 (0.10)
0.13 (0.13)	0.10 (0.10)	0.16 (0.15)	0.29 (0.29)	<0.01 – 0.84 (<0.01 – 1.20)	0.10 (0.10)
-	-	-	-	-	-
132 (129)	79 (82)	151 (150)	389 (368)	2647 (1836)	196 (165)
1.9 (1.9)	1.2 (1.3)	1.9 (1.7)	5.2 (5.0)	106.5 (110.4)	4.7 (4.9)
2.2 (2.2)	1.7 (1.7)	2.6 (2.4)	5.0 (4.9)	39.0 (40.4)	2.7 (2.8)
137 (133)	85 (88)	157 (158)	398 (374)	2653 (1840)	198 (166)

Regarding OHPAH urinary concentrations, the highest levels were found for the metabolites of naphthalene, 1OHNAP and 2OHNAP, with GMs of 0.3 and 10 ng·ml^{-1}, respectively. By contrast, the phenanthrene metabolites 4OHPHEN, 9OHPHEN, and 2OHPHEN had the lowest GMs of all the metabolites: 0.02, 0.03, and 0.04 ng·ml^{-1}, respectively. The urinary concentrations of the remaining OHPAHs ranged from 0.08 (3OHPHEN) to 0.17 ng·ml^{-1} (2OHFLU), while P95 (considered as the RV_{95} of the target population) were in the range of 0.11 (4OHPHEN) to 53 ng·ml^{-1} (2OHPHEN). Overall, more than seven metabolites of PAHs were detected in approximately 90 % of the children. The predominant metabolite in the PAHs' total internal dose in the present study was 2OHNAP, accounting for 87 % of the ∑OHPAHs concentrations, followed by 1OHNAP (7 %), ∑OHPHENs (3 %), ∑OHFLUs (2 %), and 1OHPYR (1 %). This composition profile was very similar to the results obtained in children in Portugal[342], Poland[158], Germany[176], China[344], Canada[322,323], USA[301], and Australia[304].

In a global context, several similarities and differences were observed between urinary PAH concentrations obtained here and those reported in children from other countries. Figures 28a-d show the mean OHPAH concentrations observed in children at school age in countries around the world, and Figure 28e represents the RV_{95} used to compare the exposure of children from the BIOVAL study with the following population-representative surveys: the German Environmental Survey, 2014-2017 (GerES V)[176]; the National Health and Nutrition Examination Survey (NHANES)[301]; and the Canadian Health Measures Survey (CHMS)[322]. It should be considered that these studies are not methodologically homogeneous, as they included different types of urine samples, age ranges of children and sampling years. Moreover, the analytical methods, including their corresponding LoQs, employed in each study differed from each other.

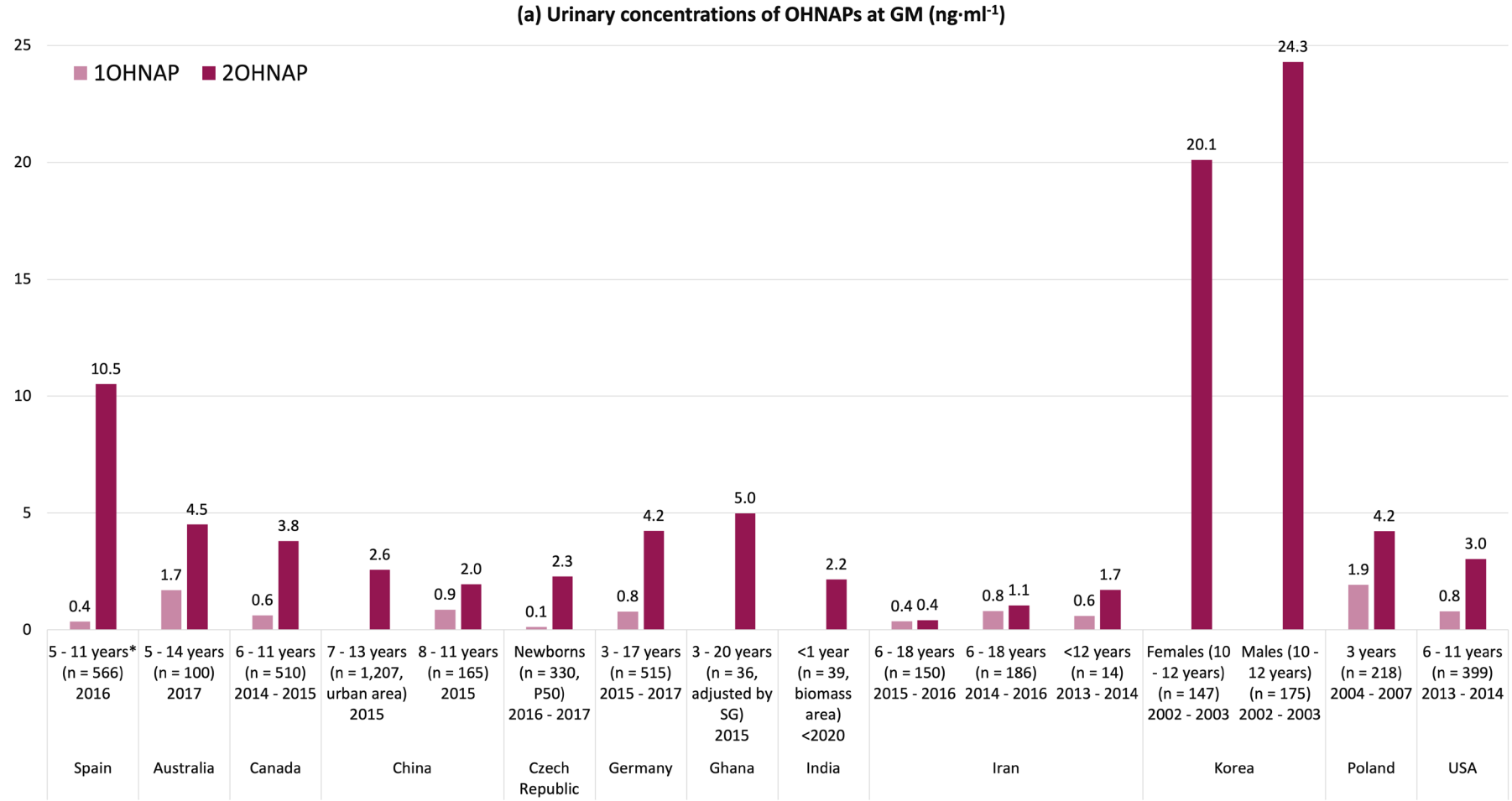


Figure 28a. Comparison of existing HBM data of urinary 1OHNAP and 2OHNAP (ng·ml^{-1}) in children of different ages at different sample collection years (number of samples within parenthesis) across the following countries: Spain (*Present study), Australia[304], Canada[322], China, Czech Republic[177], Germany[176], Ghana[345], India[346], Iran[169,347,348], Korea[349], Poland[158], and USA[301] . *GM: geometric mean; P50: 50th percentile; SG: specific gravity.*

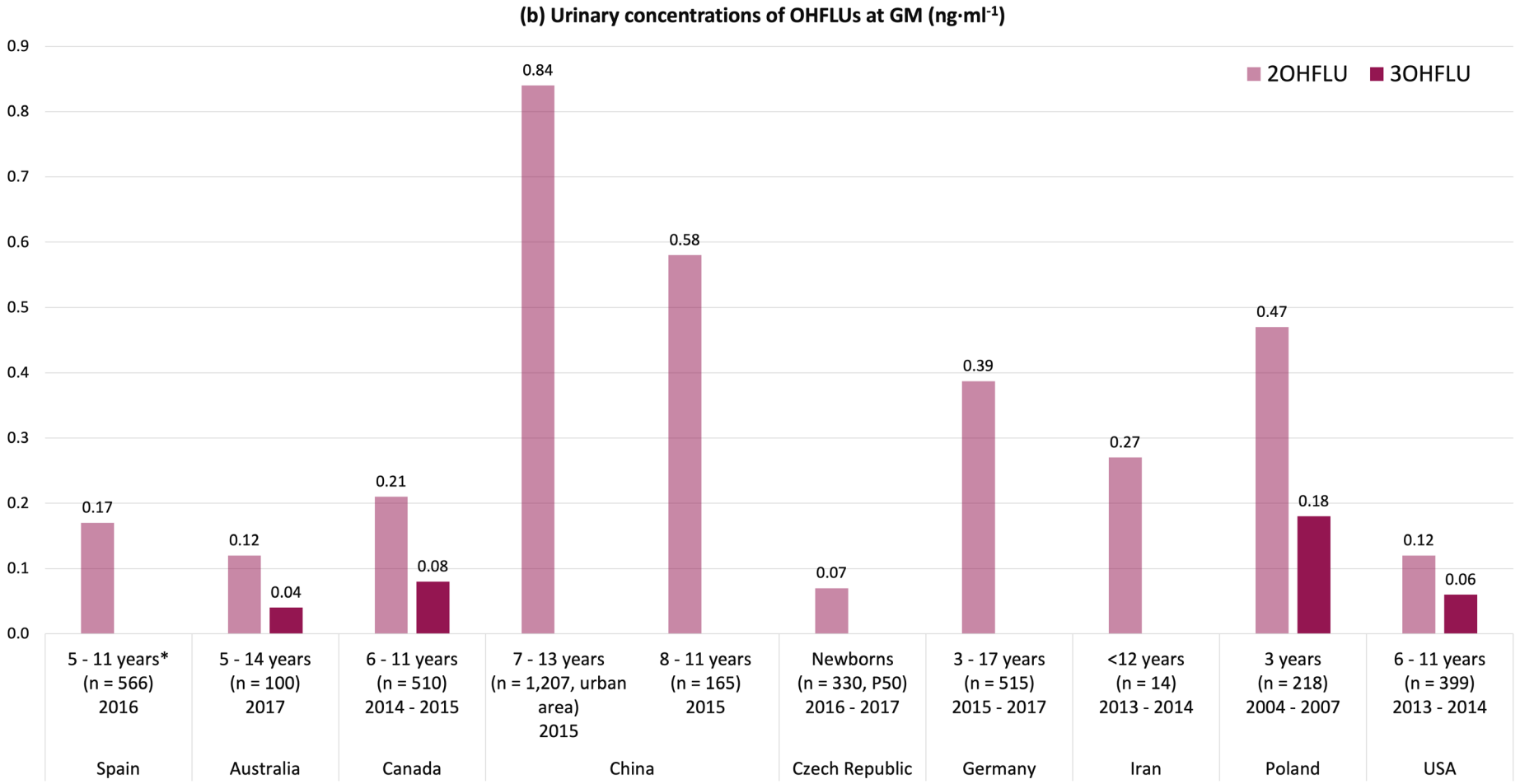


Figure 28b. Comparison of existing HBM data of urinary 2OHFLU and 3OHFLU (ng·ml^{-1}) in children of different ages at different sample collection years (number of samples within parenthesis) across the following countries: Spain (*Present study), Australia[304], Canada[322], China[344,350], Czech Republic[177], Germany[176], Iran[169], Poland[158], and USA[301]. *GM: geometric mean; P50: 50th percentile.*

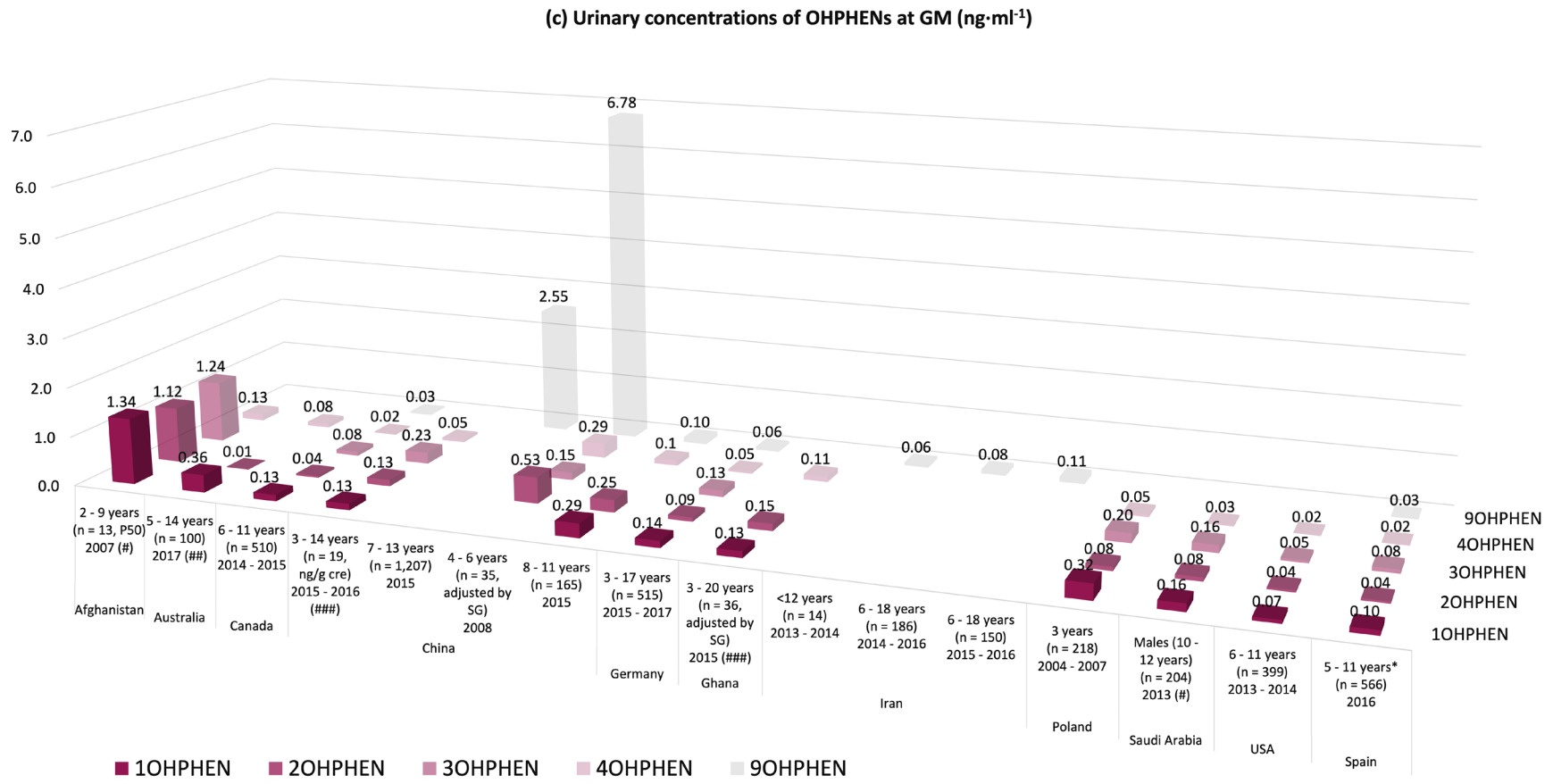


Figure 28c. Comparison of existing HBM data of urinary 1OHPHEN, 2OHPHEN, 3OHPHEN, 4OHPHEN and 9OHPHEN (ng·ml^{-1}) in children of different ages at different sample collection years (number of samples within parenthesis) across the following countries: Afghanistan[351], Australia[304], Canada[322], China[167,344,350,352], Germany[176], Ghana[345], Iran[169,347,348], Poland[158], Saudi Arabia[353], USA[301], and Spain (*Present study). *GM: geometric mean; P50: 50th percentile; cre: creatinine; SG: specific gravity; (#:) 2OHPHEN is the sum of 2OHPHEN and 9OHPHEN; (##): 1OHPHEN is the sum of 1OHPHEN and 3OHPHEN; (###): 1OHPHEN is the sum of 1OHPHEN and 9OHPHEN.*

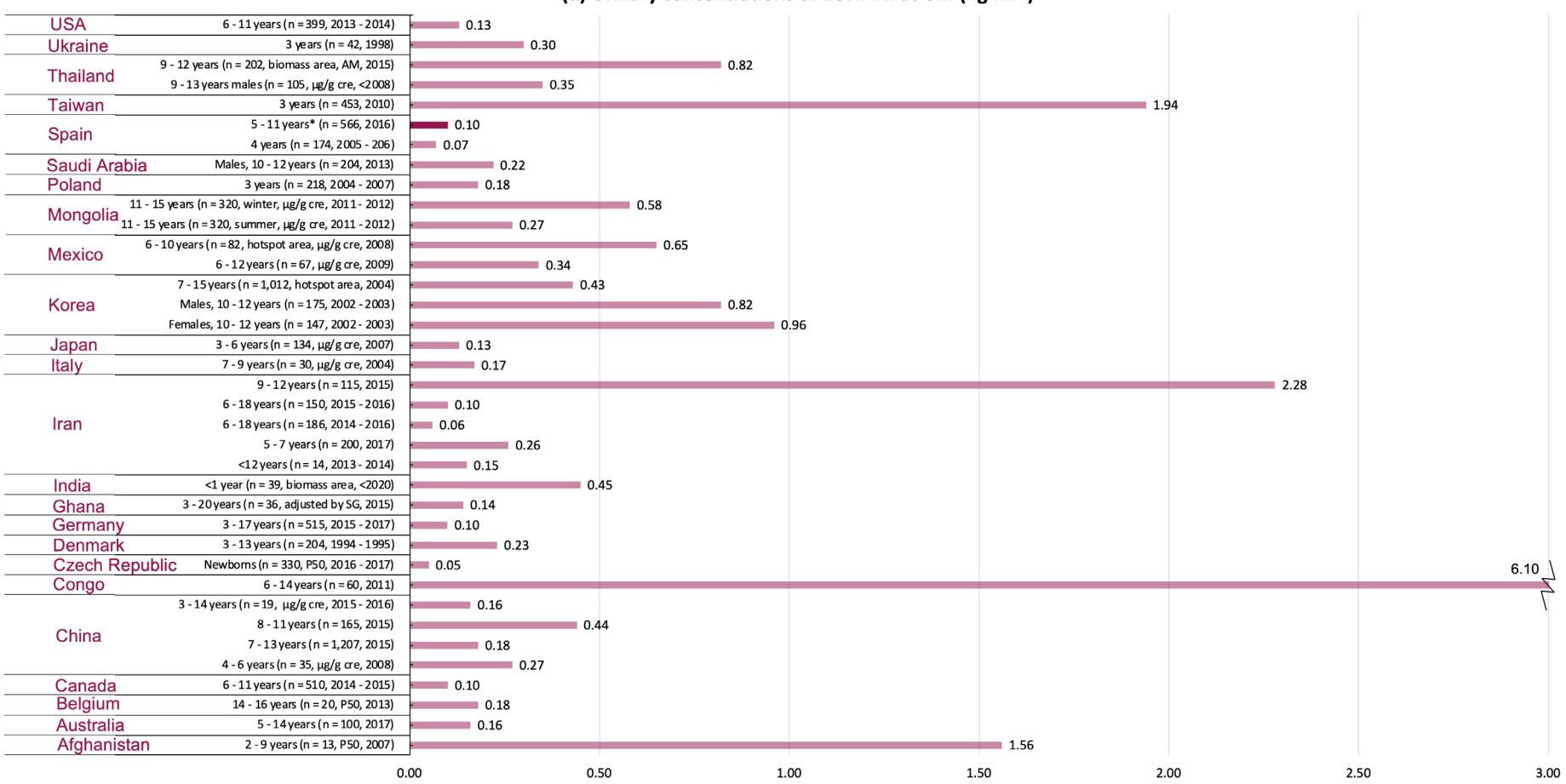


Figure 28d. Comparison of existing HBM data of urinary 1OHPYR (ng·ml^{-1}) in children of different ages (number of samples and sample collection years within parenthesis) across the following countries: USA[301], Ukraine[354], Thailand[355,356], Taiwan[357], Spain[178,180], Saudi Arabia[353], Poland[158], Mongolia[358], Mexico[359,360], Korea[349,361], Japan[362], Italy[363], Iran[147,169,347,348,364], India[346], Ghana[345], Germany[176], Denmark[365], Czech Republic[177], Congo[366], China[167,344,350,352], Canada[322], Belgium[367], Australia[304] and Afghanistan[351]. *Present study; *GM: geometric mean; AM: arithmetic mean; cre: creatinine; SG: specific gravity; P50: 50th percentile.*

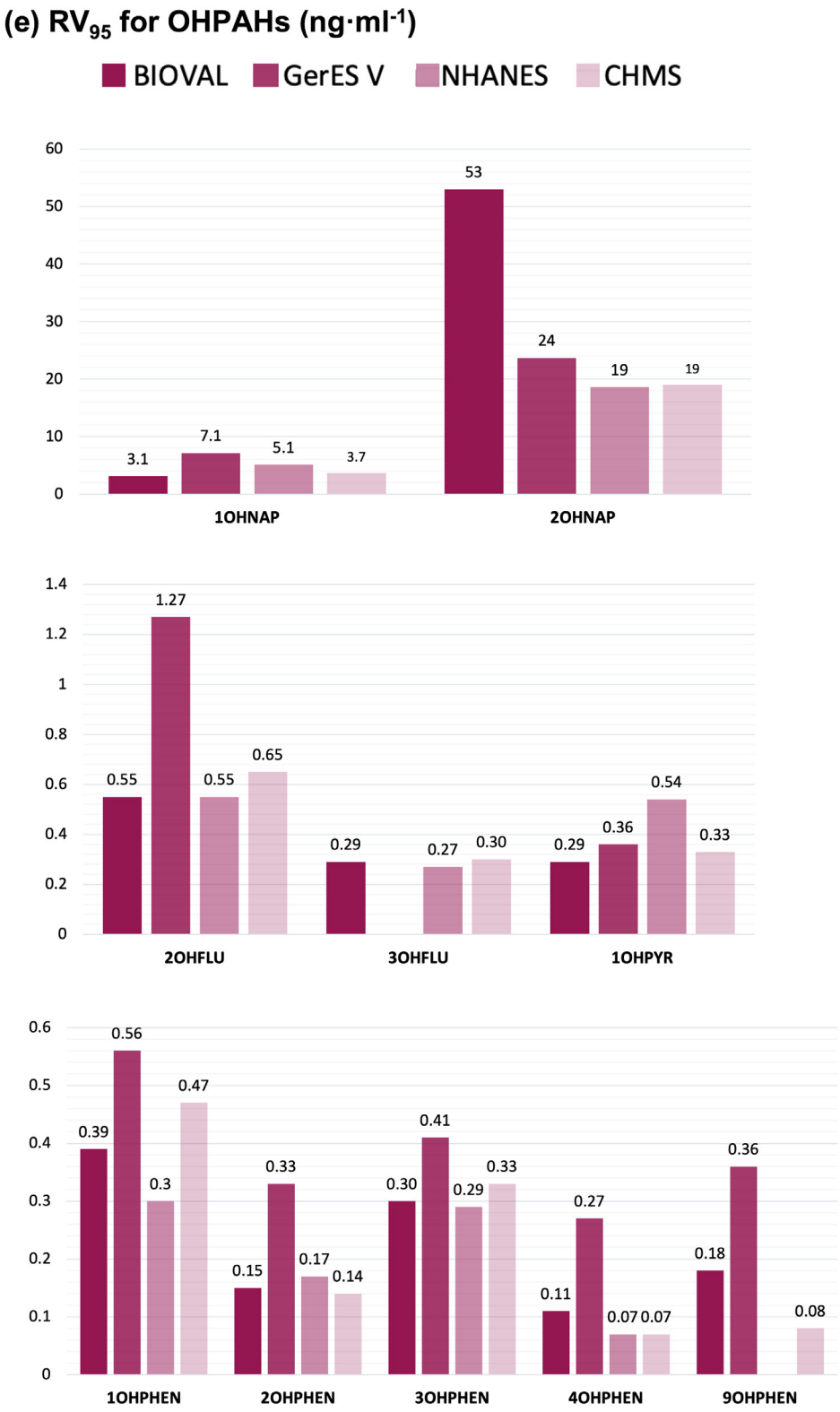


Figure 28e. Comparison of RV_{95} of urinary OHPAHs obtained in the present study with those from existing representative HBM programs in USA (NHANES)[301], Germany (GerES V, 2OHFLU is the sum of 2OHFLU and 9OHFLU)[176] and Canada (CHMS)[322]. *RV_{95}: reference values.*

Firstly, the present study reported mean levels of 2OHNAP between 2 and 25 times higher than levels observed in children from other countries of America, Europe, Africa, and Asia (see Figure 28a), these levels only being lower than those of children living in polluted areas located in China[167,352] and Korea[349]. In fact, the RV_{95} derived for 2OHNAP in the present study was approximately twice the RV_{95} observed for children in Germany, USA, and Canada (Figure 28e), showing that exposure of Spanish children to naphthalene is apparently high and should be reduced[368] due to its potential carcinogenic properties[122]. The higher exposure to PAHs in Spanish children compared to other countries could be attributed to many reasons, such as a higher exposure to environmental tobacco smoke (ETS), differences in food intake and in cooking methods, or a worse indoor/outdoor air quality[155,169,173,176,369]. However, further analyses are required to draw firm conclusions. By contrast, the opposite trend was found to be the case for 1OHNAP, with children from the BIOVAL study having the lowest levels compared to the rest of studies, except for newborns living in the Czech Republic[177]. In addition, the lowest RV_{95} for 2OHNAP was observed for the children under study compared to those values reported in the existing representative HBM programs.

Secondly, North American[301,322] and Australian children[304] showed similar RV_{95} and mean levels of 2OHFLU and 3OHFLU, respectively, to those obtained in the present research. On the other hand, there were some Asian[167,169,344,352] and European countries (Germany and Poland)[158,176] which reported higher mean concentrations of these fluorene metabolites in children (see Figure 28b). Furthermore, the GerES V survey reported the highest RV_{95} for fluorene metabolites in children of a similar age (Figure 28e), probably because it is referred to the sum of 2- and 9-hydroxyfluorene[176].

In general, lower levels of OHPHENs were observed in the children under study, except from those living in the USA[301] and Australia[304], in which lower levels of 1OHPHEN and 3OHPHEN, and 2OHPHEN were reported, respectively (see Figure 28c). Likewise, RV_{95} derived for OHPHENs in Spanish children appeared to be lower than or in the same range as those reported in German, American, and Canadian children.

For 1OHPYR, mean levels and RV_{95} in the studied children were generally lower than or comparable to other nationalities. The highest mean concentrations of 1OHPYR in children were found in Mexico[359,360], and some countries belonging to Asia[349,351,356,357,364] and Africa[366] (see Figure 28d), probably because people from less developed countries are more likely to be more exposed to PAH pollution due to deficient air pollution control in these areas and the use of biomass fuels in households[351,370,371]. When comparing the findings obtained here to another study carried out on Spanish children, urinary concentrations of 1OHPYR were slightly higher in the Valencian children of the present study (2016) than in children living in Granada (2005-2006)[180]. In addition, similar 1OHPYR concentrations were observed between the studied children and the Spanish adult population (see Figure 25d), including lactating mothers of the BETTERMILK study.

5.2.1.2 STATISTICAL ANALYSIS

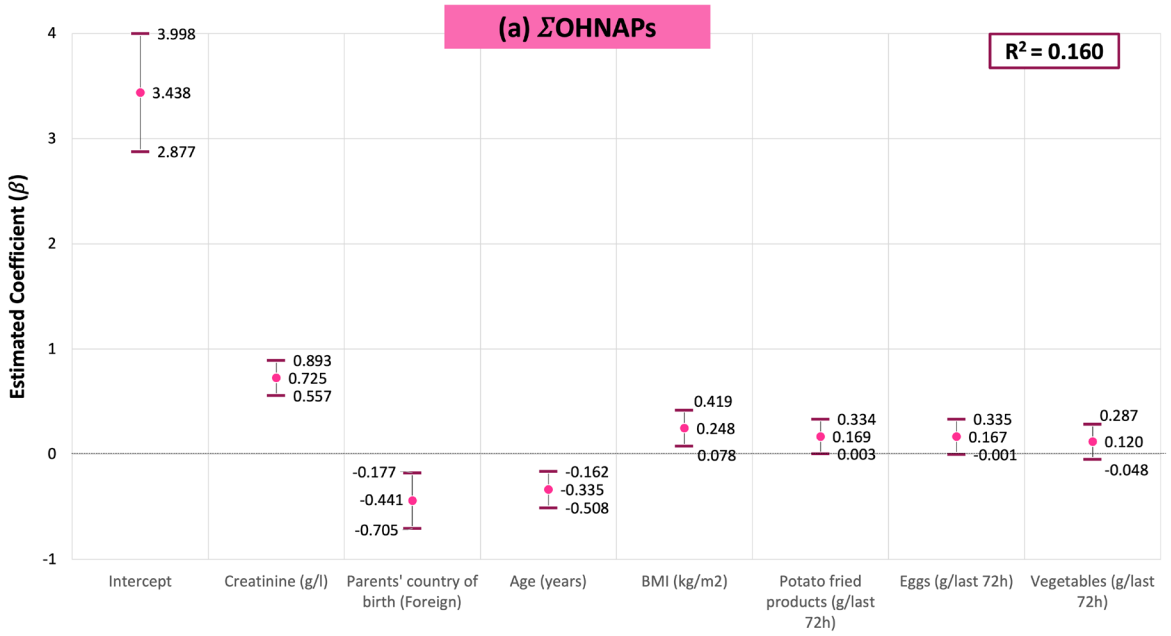
According to the Spearman's correlation matrix, all PAH metabolites were highly and directly related to each other (p-values > 0.01; correlation coefficients between 0.393 and 0.737), indicating, in all probability, similar exposure media, as it was previously observed in the BETTERMILK project.

Figure 29 represents the results of the multiple regression model obtained for each PAH along with its standardized coefficients (β) and their corresponding 95 % CIs. The obtained regression coefficients (R^2) were between 0.160 – 0.227. This prediction model showed that consumption of potato fried products, legumes, and pre-packaged food, as well as several socio-demographic characteristics, such as sex, BMI, age, educational level, and province of residence, were the most relevant factors affecting OHPAH urinary levels in children. Possible reasons for these findings are discussed in the following paragraphs.

Concerning food consumption habits, a positive association was observed between ∑OHNAPs and the intake of potato fried products. This is in accordance with Purcaro et al. (2006), who affirmed that fried food is an influential factor of exposure to low molecular PAHs[372]. This association was also observed in a HBM project conducted on Spanish pregnant women[179]. Surprisingly, the opposite trend was found for 1OHPYR. This fact may be explained by the high variability of PAH concentrations produced after the frying process, which can be very different depending on the cooking conditions[372].

Legumes were a food group contributing to ∑OHFLUs levels in the present study, since the more intake of legumes, the higher the urinary levels of fluorene metabolites in children. In fact, Bartolomé et al. (2015) found similar associations between OHPAHs and consumption of legumes[178]. However, several Spanish total diet studies reported showed that legumes are minor contributors of PAHs[165,336,373]. Another relevant finding with a clear tendency to significance (p-values close to 0.05) were that eggs consumption appeared to be related to higher urinary levels of ∑OHNAPs, ∑OHPHENs and 1OHPYR.

In addition, the consumption frequency of plastic-wrapped food was highly and positively related with all OHPAH concentrations, except for ∑OHNAPs, which might indicate that it could be a possible determinant of exposure to PAHs in the children under study. Similarly, children participating in the GerES V showed higher urinary OHPAH levels if they tended to chew plastic objects[176]. This is in line with recent studies by Li et al. (2017) and Schweighuber et al. (2019), who studied the presence of PAHs in polystyrene-based products in contact with foodstuffs, concluding that low molecular weight PAHs can be generated in the production and processing of these materials, as well as being present in the dye used for their coloring[374,375]. In addition, they also reported that PAH migration from these plastic materials into foods is possible, which requires further attention[374,375].



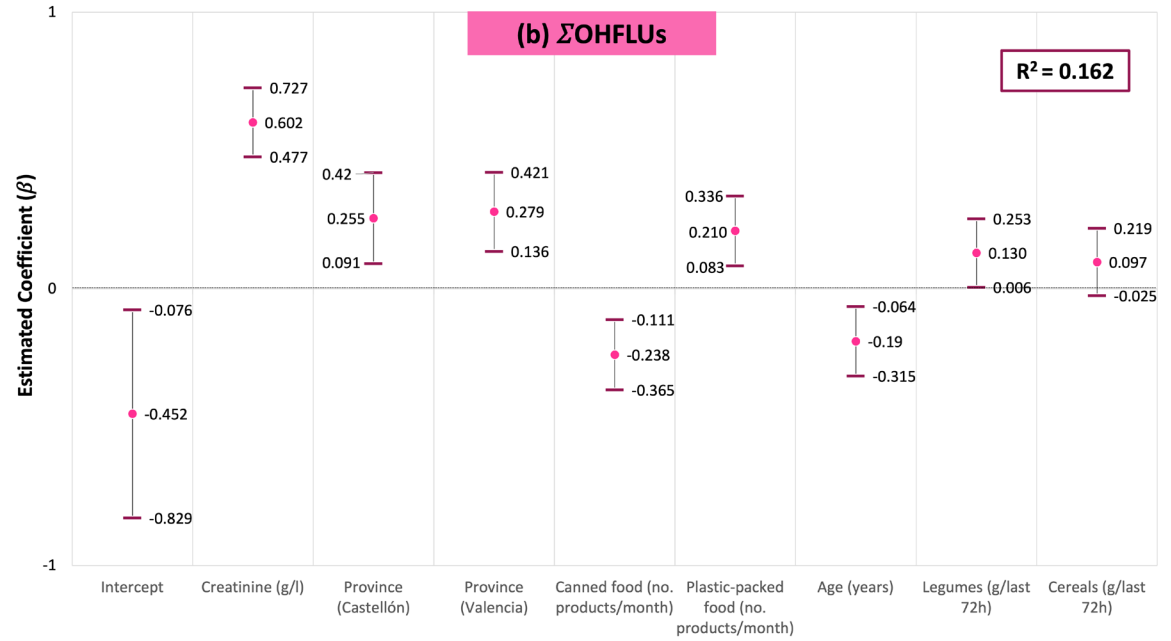
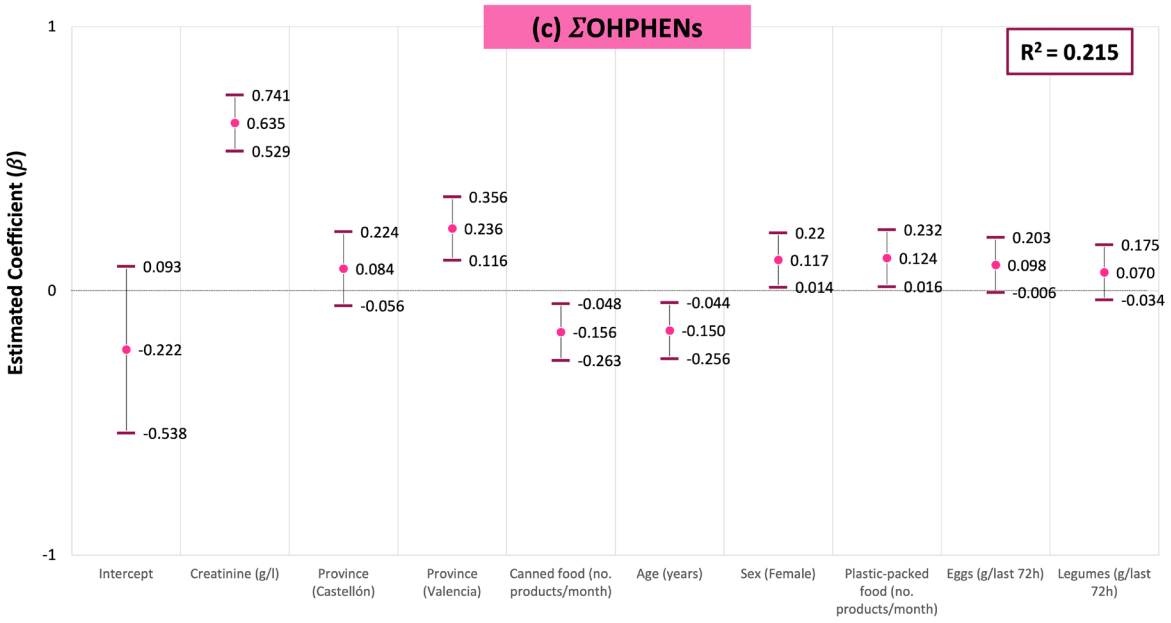
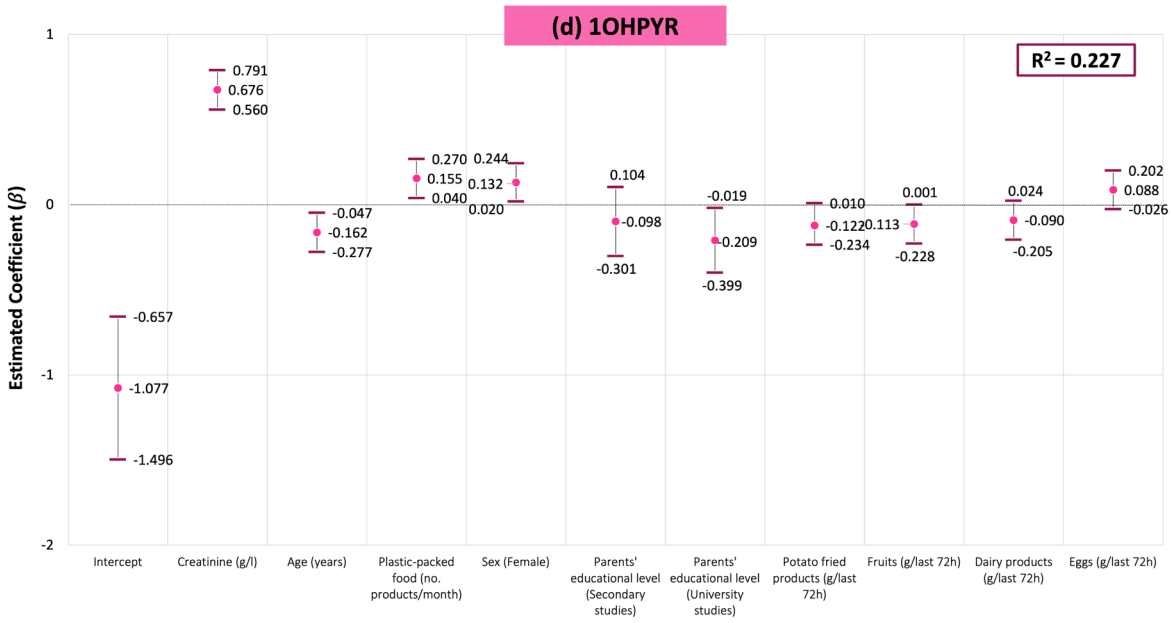


Figure 29. Linear regression models fitted for the log-transformed levels of (a) ∑OHNAPs, (b) ∑OHFLUs, (c) ∑OHPHENs and (d) 1OHPYR of the children participating in the BIOVAL study (n = 547), only donors with a completed questionnaire were considered), with its corresponding regression coefficients, 95 % confidence intervals and coefficients of determination (R^2). *no.: number.*


(c) ΣOHPHENs
R² = 0.215
Estimated Coefficient (β)
1
0
-1
0.093
-0.222
-0.538
0.741
0.635
0.529
0.224
0.084
-0.056
0.356
0.236
0.116
-0.048
-0.156
-0.263
-0.044
-0.150
-0.256
0.22
0.117
0.014
0.232
0.124
0.016
0.203
0.098
-0.006
0.175
0.070
-0.034
Intercept
Creatinine (g/l)
Province (Castellón)
Province (Valencia)
Canned food (no. products/month)
Age (years)
Sex (Female)
Plastic-packed food (no. products/month)
Eggs (g/last 72h)
Legumes (g/last 72h)


(d) 1OHPYR
R² = 0.227
Estimated Coefficient (β)
1
0
-1
-2
-0.657
-1.077
-1.496
0.791
0.676
0.560
-0.047
-0.162
-0.277
0.270
0.155
0.040
0.244
0.132
0.020
0.104
-0.098
-0.301
-0.019
-0.209
-0.399
0.010
-0.122
-0.234
0.001
0.113
-0.228
0.024
-0.090
-0.205
0.202
0.088
-0.026
Intercept
Creatinine (g/l)
Age (years)
Plastic-packed food (no. products/month)
Sex (Female)
Parents' educational level (Secondary studies)
Parents' educational level (University studies)
Potato fried products (g/last 72h)
Fruits (g/last 72h)
Dairy products (g/last 72h)
Eggs (g/last 72h)

As shown by Figure 29, the age of the children was negatively related to all OHPAHs. This has been also observed in other HBM studies on children from other countries[330,338,342,358,361,362,376,377]. The reason for this could be that small children have not fully developed metabolic and physiologic functions, leading to a reduced capability to degrade PAHs and more susceptibility to these substances[169,173,338,342,376]. Also, very young children frequently play on the floor, increasing the inhalation or dermal absorption of PAHs from carpets and soil[155,169,173,347]. However, other authors report no associations between urinary OHPAHs and age[178,179,345,358], while one study found that this association was positive[350].

In the case of ∑OHNAPs, children who had higher BMIs tended to have higher urinary concentrations of these metabolites than those with lower BMIs. This correlation has been previously described by other authors[176,178,330,347,353], revealing the fat-soluble properties of PAHs[350]. If sex differences are considered, females presented higher ∑OHPHENs and 1OHPYR levels in their urines than males in the present study. Despite several authors not reporting any relationship with this aspect[178,354,358,362,363,377,378], differences between both sexes have been supported by many other studies[169,173,338,345,350,379]. This finding is possibly explained by sex-related differences in absorption and metabolism of PAHs[342,379], as well as by other behaviors (e.g. participation in different indoor and outdoor activities)[338].

With regard to inhalation of polluted indoor and outdoor air, several studies support that it may play an important role in the internal exposure to PAHs[180,342,344,367]. However, although many authors observed higher OHPAHs concentrations in children living in urban areas (high pollution) compared to those living in rural areas (low pollution)[155,344,355,363,365], no differences between children living in these locations were observed in the present research. By contrast, it was observed that the province where the children lived had a significant effect on their ∑OHFLUs and ∑OHPHENs concentrations, as reported in other previous studies[174,380], with participants living in Valencia, and to a lesser extent in Castellón, appearing to be more exposed to fluorene and phenanthrene than children living in *Alicante*. Therefore, the association of OHPAH levels with location of residence may suggest that airborne exposure is an important contributor, but no conclusions can be drawn with this information alone.

In recent studies, low household income has been associated with higher levels of 1OHPYR and other OHPAHs in urine[330,350,379], but this characteristic was not considered in the present research. Nevertheless, lower concentrations of 1OHPYR were found in children whose parents had a high level of education, which is also related to the socio-economic status of the family[174,367,381]. Chuang et al. (1999) suggested that the increased PAH exposure of low-income people is likely to be related to poor air quality, which may be considerably influenced by ETS and household location (proximity to industrial or dense traffic areas)[382].

5.2.1.3 RISK ASSESSMENT

For risk assessment estimations, EDIs, HQs, and HIs were calculated for naphthalene, fluorene, phenanthrene, and pyrene. Exposure to benzo(a)pyrene was not assessed due to the low DF (5 %) of its urinary metabolite (3OHBAP) in the analyzed samples.

According to the EDI calculations, naphthalene was the PAH with the highest EDI_{GM} and EDI_{P95} (204 and 997 ng·kg-bw^{-1}·day^{-1}, respectively), followed by phenanthrene (66 and 199 ng·kg-bw^{-1}·day^{-1}), pyrene (23 and 67 ng·kg-bw^{-1}·day^{-1}), and finally, fluorene (5 and 22 ng·kg-bw^{-1}·day^{-1}). Broadly speaking, EDI_{GM} and EDI_{P95} of children aged 5 to 8 years old, which ranged from 7 to 269 ng·kg-bw^{-1}·day^{-1} and from 38 to 1363 ng·kg-bw^{-1}·day^{-1}, respectively, were approximately twice that of children aged 9 to 12 years old, ranging from 4 to 162 ng·kg-bw^{-1}·day^{-1} and from 17 to 807 ng·kg-bw^{-1}·day^{-1}, respectively, evidencing the results of the statistical analysis regarding the influence of age on PAH exposure. In comparison with other studies, lower EDIs were found in the present study when comparing the median daily intakes of pyrene with those reported in most European regions (0.05 µg·kg-bw^{-1}·day^{-1})[341] and several Asian countries[340,344].

Concerning HQs and HIs represented in Figure 30, HQs were in the range of 0.00013 (fluorene) and 0.010 (naphthalene) at GM level, and of 0.00056 (fluorene) and 0.050 (naphthalene) at P95 level, resulting in a cumulative risk to PAHs, calculated as HI, far below 1. In this context, naphthalene was the major contributor to the risk of exposure to the assessed PAH mixture at both distribution levels. As expected, higher values of HQ and HI were observed in children from 5 to 8 years old compared to children aged 9 to 12 and non-smoker women of the BETTERMILK study (see Chapter 5.1), evidencing again that young children might be at greater risk of exposure to PAHs.

Given that all HQs and HIs were lower than 1, no apparent non-cancer health risk associated with the individual or cumulative exposure to PAHs in the target children could be determined. In any case, due to the high urinary levels of 2OHNAP obtained in the present research, further studies are recommended to monitor PAH internal exposure over time, especially in young children, as well as to identify other significant factors contributing to PAH exposure in children living in Spain.

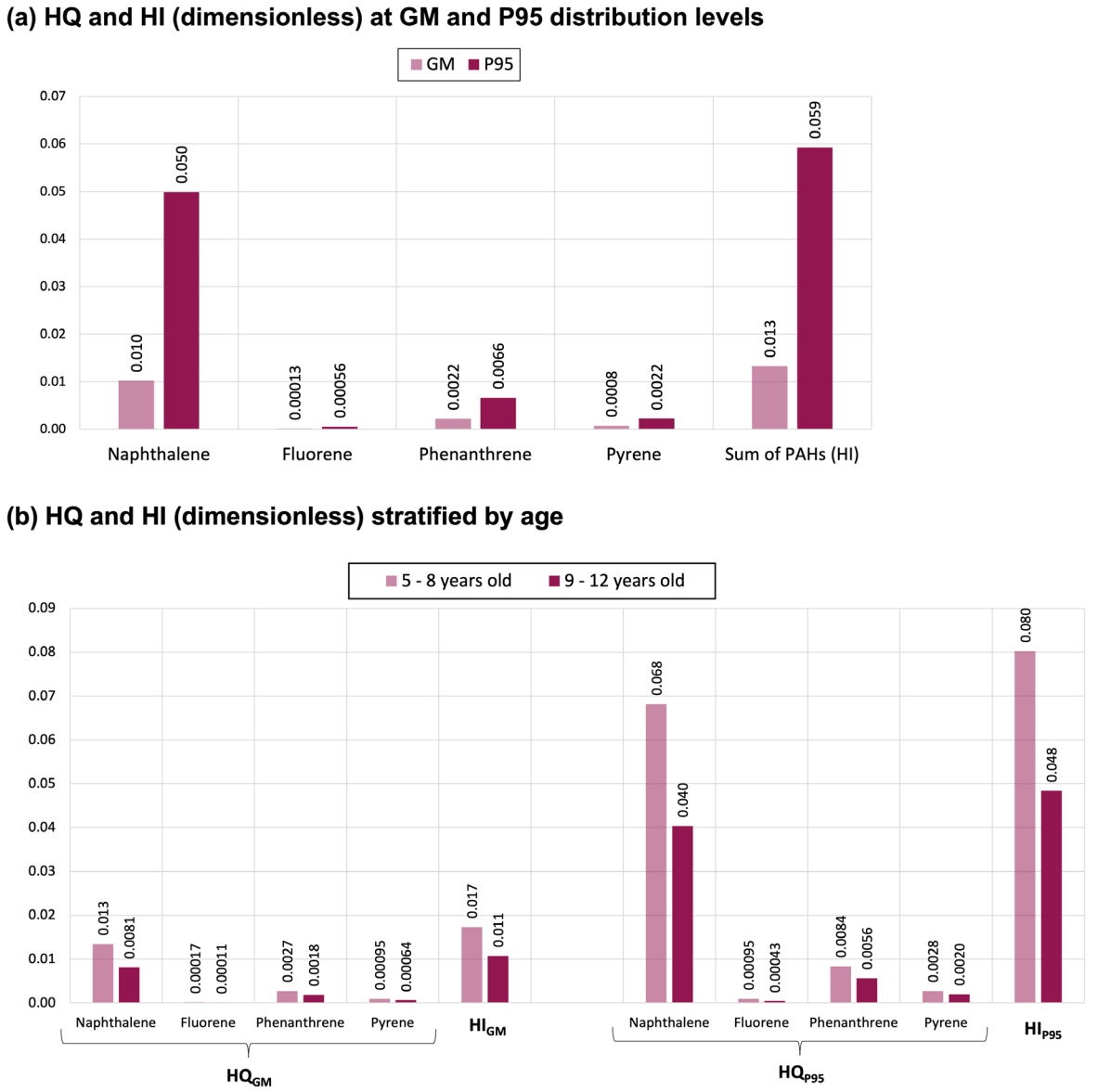


Figure 30. HQ for naphthalene, fluorene, phenanthrene and pyrene, and HI for the sum of PAHs at GM and P95 distribution levels in the children of the BIOVAL study considering (a) the entire study population and (b) stratified age groups (5 – 8 and 9 – 12 years old). *HQ: hazard quotient; HI: hazard index; GM: geometric mean; P95: 95th percentile.*

5.2.2 CONCLUSIONS

In the present research, nine urinary biomarkers of exposure to PAHs were detected in more than 78 % of children living in the Valencian Region (Spain). RV_{95} for urinary metabolites of naphthalene, fluorene, phenanthrene and pyrene of Spanish children aged 5-12 years (n = 566) were derived from the present study. According to the urinary composition profiles, the highest levels were found for metabolites of naphthalene, which were responsible for 94 % of the total internal exposure to PAHs. Overall, the obtained OHPAH concentrations were lower or in the same range as children of other nationalities. Nonetheless, 2OHNAP levels were much higher than the average levels described worldwide, showing that more attention should be paid to Spanish children's exposure to naphthalene. Age, BMI, dietary habits, province of residence, and plastic-wrapped food intake were identified as relevant predictors of exposure to PAHs. In terms of exposure, no potential health risk due to PAH exposure was found for the children under study.

5.2.3 ARTICLE II.2. *CHILDREN'S EXPOSURE TO POLYCYCLIC AROMATIC HYDROCARBONS IN THE VALENCIAN REGION (SPAIN): URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT*

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Children's exposure to polycyclic aromatic hydrocarbons in the Valencian Region (Spain): Urinary levels, predictors of exposure and risk assessment

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are pollutants that are released into the environment during incomplete combustion of organic matter and which can have a negative effect on human health. PAHs enter the human body mostly through ingestion of food or inhalation of tobacco smoke. The purpose of the present study is to evaluate the internal levels of PAHs that children living in the Valencian Region (Spain) are exposed to.

In total, we measured eleven biomarkers of exposure to naphthalene, fluorene, phenanthrene, pyrene, and benzo(a)pyrene in the urine of 566 children aged 5–12. The analytical method was based on a liquid-liquid extraction of the PAH metabolites from the urine samples, followed by their determination by liquid chromatography coupled to tandem mass spectrometry. In addition, we used a questionnaire to collect the socio-demographic characteristics and 72 h dietary recall information of the participants in our study.

Overall, we detected PAH metabolites in more than 78% of the children, with the exception of 3-hydroxyfluorene and 3-hydroxybenzo(a)pyrene, which were found in less than 37% of the analyzed samples. The most abundant biomarker found was 2-hydroxynaphthalene, with a geometric mean of 10 $ng{\cdot}ml^{-1}$. Reference values (RV_{95}) ranging from 0.11 (4-hydroxyphenanthrene) to 53 $ng{\cdot}ml^{-1}$ (2-hydroxynaphthalene) in urine of Spanish children were derived from the present study. According to the statistical analysis, the factors that were significantly associated with the internal exposure to PAHs were province of residence, body mass index (BMI), children's age, consumption of plastic-wrapped food, and dietary habits. The estimated daily intakes in geometric mean terms ranged from 5 (fluorene) to 204 $ng{\cdot}kg\text{-}bw^{-1}{\cdot}day^{-1}$ (naphthalene). Risk assessment calculations showed higher hazard quotients and hazard indexes for children aged 5–8 than those aged 9–12, but all were below 1.

In conclusion, no potential non-cancer health risk due to PAH exposure was observed in children living in Spain.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a complex mixture of over 100 different organic compounds composed of two or more aromatic rings (EFSA, 2008). These substances are released into the environment during incomplete combustion of organic matter originating from natural sources (e.g., volcanic eruptions, forest fires), and anthropogenic activities, such as tobacco smoke, vehicle exhausts, processing of fuels, forest fires, and some culinary practices (drying, grilling, roasting, frying, and smoking food) (EFSA, 2008; Liu et al., 2016; Martínez-Salinas et al., 2010; Miri et al., 2018). Additionally, they are used in the manufacture of plasticizers, pigments, drying agents, and pesticides (Martínez-Salinas et al., 2010).

In general, the main pathway of human exposure to PAHs is the ingestion of contaminated foodstuffs (EFSA, 2008). According to the European Food Safety Authority (EFSA), the two highest contributors to the PAH dietary exposure are cereals and seafood (EFSA, 2008), although they have been also detected in meat, eggs and dairy products,

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among others (Domingo and Nadal, 2015; Luzardo et al., 2013). However, inhalation of tobacco smoke, passive smoking and dermal contact are also significant contributors to PAH exposure in smokers and the occupationally exposed population (e.g., firefighters, asphalt workers, etc.), respectively (EFSA, 2008; Fan et al., 2012). With regard to inhalation of polluted air, this is a marginal contributor to total PAH exposure (<10%), except for people living in areas close to refineries, certain industries or waste incinerators (HBM4EU, 2019).

PAHs have been associated with a wide range of toxicological effects, such as reproductive and developmental toxicity, immunotoxicity, and carcinogenic and genotoxic potencies (Huang et al., 2019a). More specifically, human exposure to PAHs has been linked to many diseases including cancer, diabetes, cardiovascular diseases, and hypertension (HBM4EU, 2018; Oliveira et al., 2019). Indeed, the United States Environmental Protection Agency (USEPA) has classified 16 PAH compounds as priority pollutants (US EPA, 2014), and the International Agency for Research on Cancer (IARC) has classified benzo(a)pyrene as a human carcinogen (Group 1). Other PAHs, such as naphthalene and chrysene, have been deemed as "possibly carcinogenic to humans" (Group 2B), while other PAH compounds (e.g., fluorene, phenanthrene, pyrene, etc.) have been considered as unclassifiable with regard to their carcinogenicity risk to humans (Group 3), because of limited or inadequate experimental evidence (IARC, 2021). In this context, children are considered one of the most vulnerable population groups, since they have a less efficient detoxification system, lower body weight and higher inhalation rates compared to adults, resulting in higher exposure to PAHs (Oliveira et al., 2019), which may disrupt their growth and development process (Sochacka-Tatara et al., 2018).

To control and mitigate PAH exposure in the general population, the European Union (EU) has implemented several regulatory measures for PAHs in food and environment. In 2006, maximum levels (MLs) for certain PAHs in foods were established by Commission Regulation No 1881/2006 (European Commission, 2006). Furthermore, several PAHs are now deemed as priority hazardous substances under the Directives 2000/60/EC and 2016/2284, which aim to improve the quality of water and ambient air, respectively (The European Parliament and the Council of the European Union, 2016, 2000). The Commission Regulation No 1272/2013 also limited the PAH content in rubbers and plastic components of consumer articles (European Commission, 2013).

Human biomonitoring (HBM), defined as the measurement of concentrations of chemicals or their metabolites in biological samples such as blood or urine, has been considered as a relevant tool to evaluate the PAH exposure of different population groups, since the internal dose of these pollutants accounts for all sources and pathways of exposure (Ganzleben et al., 2017). In the EU, the Human Biomonitoring for Europe (HBM4EU) initiative included PAHs in its first round of priority substances and is currently working on determining the internal exposure of European citizens to these pollutants and their adverse effects with a view to supporting policy making (Ganzleben et al., 2017; HBM4EU, 2018).

For the HBM of PAHs, it should be taken into account that these compounds are absorbed and rapidly metabolized in the human body (Li et al., 2012). After exposure, they are biotransformed in the liver into reactive epoxides by the cytochrome P450, and reduced or hydrolyzed to hydroxylated PAHs (OHPAHs) (Hoseini et al., 2018). After that, these OHPAHs are conjugated to glucuronic acid or sulphate compounds to increase their water solubility and, finally, excreted through the bile, urine, or feces (HBM4EU, 2018). Specifically, because low-molecular-weight PAHs have relatively short elimination half-lives and are primarily excreted in urine (Li et al., 2012; Sochacka-Tatara et al., 2018), the presence of OHPAHs in urine reflects recent exposure to PAHs (Sochacka-Tatara et al., 2018).

Among all OHPAHs, the metabolite of pyrene, 1-hydroxypyrene (1OHPYR), is the most common biomarker used as indicator of exposure to PAHs (Huang et al., 2019a; Oliveira et al., 2019). Nevertheless, given that humans are exposed to a mixture of PAHs, the HBM of other OHPAHs along with 1OHPYR would provide a better overall assessment of PAH exposure (Dobraca et al., 2018; Fan et al., 2012). In fact, urinary metabolites of naphthalene, fluorene, phenanthrene, chrysene, and benzo(a)pyrene are also commonly used as biomarkers of exposure to PAHs in many HBM studies (CDC, 2019; Health Canada, 2017; Hoseini et al., 2018; Murawski et al., 2020; Urbancova et al., 2020). In Spain, the BIOAMBIENT.ES project was the first national-level HBM survey to show urinary levels of OHPAHs in a representative sample of Spanish adults in 2009 (Bartolomé et al., 2015). But in the case of children, only one HBM study on PAHs has been conducted so far in Spain (Freire et al., 2009), and thus there is a lack of updated data on their exposure to PAHs.

The main objective of our study was to evaluate the exposure of children living in the Valencian Region (Spain) to PAHs by measuring 11 urinary OHPAHs levels in their urine samples. We also investigated the influence of dietary habits and socio-demographic characteristics on PAH exposure of children. Likewise, hazard quotients and hazard indexes were estimated to assess the risk associated to PAH exposure.

2. Materials and methods

2.1. Study design

The present research is part of the BIOVAL study, a cross-sectional HBM program carried out by the Public Health Directorate of the Regional Government of València (Spain) in 2016, whose objective was to assess the risk and the exposure to PAHs and other food pollutants for children between 5 and 12 years of age living in the Valencian Region. The complete study design was previously described by Pérez et al., (2017). In short, parents of children who studied in Valencian primary schools were asked to participate in the BIOVAL study. The participants had to meet the following inclusion criteria:

(i) Children aged between 5 and 12 years old.
(ii) No metabolic disease or urinary problems.
(iii) Residence in the Valencian Region: Castelló de la Plana, València or Alacant provinces.

In total, 666 children were randomly recruited from 25 primary schools located in 16 municipalities of the entire Valencian territory. However, only 566 children provided a sufficient volume of urine or had a creatinine concentration within the range 30–300 mg·dl^{-1} (Barr et al., 2005) to carry out the analysis of the 11 PAH metabolites. The representativeness of the sample was guaranteed because: i) as recommended by the International Federation of Clinical Chemist (IFCC), more than 120 randomly-selected children were included in the BIOVAL study (Pérez et al., 2017); ii) the percentage of participants recruited by province was in accordance with the proportionality of the geographical distribution of this population (Pérez et al., 2017) (Fig. 1); ii) 50% of the children aged between 6 and 8 years old, and 50% between 9 and 11 years old; and, iii) sex equality was achieved: 50% of children were males and 50% were females.

2.2. Sample and data collection

Prior to the sample collection, an informed consent form was signed by the parents who showed interest in their children taking part in the study. The parents certified in this document that they were accurately and adequately informed about the BIOVAL study and voluntarily consented to their children's participation in it.

Each participant was provided with a kit containing the urine collection instructions, disposable gloves and a sterile 100-ml polypropylene bottle. They collected their first-morning void urine samples at home, which were then kept at 4 °C in a portable refrigerator, before being transported to the Biobank for Biomedical Research and Public Health of the Valencian Region (IBSP-CV Biobank, Spain; PT13/0010/

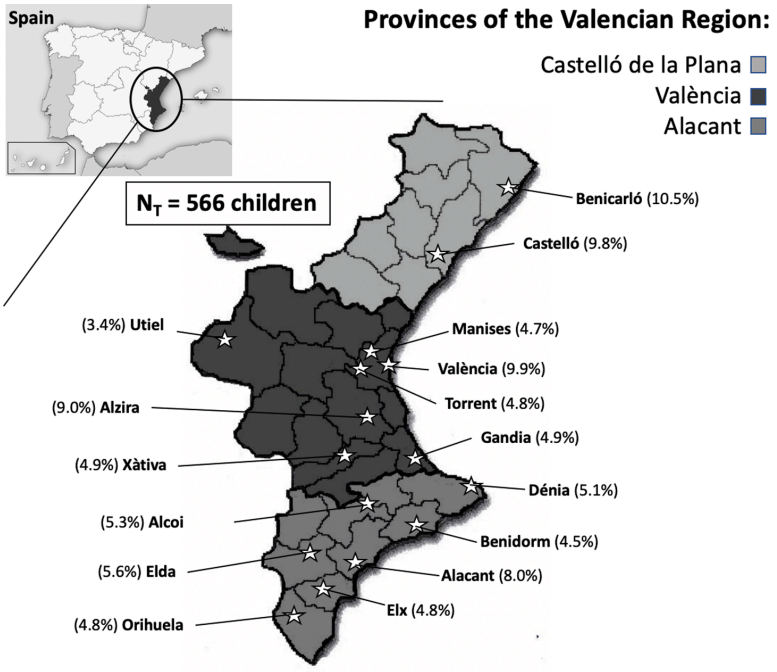


Fig. 1. Geographical distribution of the children participating in the BIOVAL program along the three provinces of the Valencian Region.

0064). The samples were integrated into the Spanish National Biobank Network and the Valencian Biobanking Network, where they were stored in 10-ml aliquots at −80 °C until analysis.

The parents of the donors were asked to complete a questionnaire on dietary data, lifestyle, and general socio-demographic characteristics of their children. The purpose of this questionnaire was to gather information on the participants in order to statistically relate it to the measurements conducted on their urine samples. Table 1 summarizes the socio-demographic characteristics and eating habits that were extracted from the participants' questionnaires for the PAH study.

2.3. Ethical approval xxx

The study received ethical clearance from the Ethics Committee for Clinical Research of the Directorate General of Public Health and Centre for Advanced Research in Public Health of the Valencian Government. After informed consent was obtained from each participant, all the samples were collected and then stored in the IBSP-CV BioBank. All personal data were dealt with (collected, handled, transferred, and analyzed) in a secure setting and were not kept for a period longer than necessary for the information to be processed. The actual processing of the data was compliant with all ethics and legal considerations at national and EU level, in particular the General Data Protection Regulation.

2.4. Chemical analysis

We analyzed the urine samples from the BIOVAL program in the Public Health Laboratory of València (Spain) using the method, reagents and the standard solutions fully described in Fernández et al., (2021). Table S1 lists the 11 OHPAHs determined in the present study, together with their corresponding internal standards (IS).

In brief, 2.5 ml of urine was spiked with 10 μL of β-glucuronidase/arylsulfatase, 1 ml of acetate buffer (pH = 5) and the IS working solution (150–500 $ng{\cdot}ml^{-1}$). The mixture was incubated 15 h at 37 °C. After the enzymatic hydrolysis, the mixture was subjected to a liquid–liquid extraction with ethyl acetate (2 × 4 ml). Then, the total organic layer was transferred to a Supel™ QuE Z-Sep tube and shaken for 1 min. After centrifugation, the purified extract was evaporated under a N_2 stream until dryness. Finally, the dry residue was dissolved in 100 μL of water: methanol (40:60) containing 0.1% of acetic acid. The final extract was transferred into an injection vial for analysis into a Vanquish high-performance liquid chromatography system (Thermo Fisher Scientific, Germany) equipped with a KINETEX Core-shell technology F5 (Phenomenex, USA) column (150 mm × 2.1 mm, 2.6 μm), and coupled to a Finnigan TSQ Quantum Ultra detector (San José, CA, USA) tandem mass spectrometry system equipped with an electrospray ionization source (ESI) in the negative ion mode. The limits of quantification (LoQ) of the target metabolites are presented in Table 2. The procedure and criteria for quality assurance/quality control (QA/QC) and method validation are fully described in Fernández et al. (2021).

We measured urinary creatinine using the Jaffé's reaction at the University Hospital Doctor Peset in València (Spain) in order to correct differences in the OHPAH levels due to urine dilution. In accordance with international guidelines, samples with urinary concentrations of creatinine out of the range 30–300 $mg{\cdot}dl^{-1}$ were excluded from the study (Barr et al., 2005).

2.5. Statistical analysis

To perform the statistical analysis, we used IBM SPSS® Statistics (version 26.0) and R software (version 3.5.2). First, the unquantified OHPAH concentrations (<LoQ) were estimated by applying the maximum likelihood estimation method, recommended by the European Food Safety Authority (EFSA, 2010). The obtained OHPAH levels in $ng{\cdot}ml^{-1}$ and $\mu g{\cdot}g$ $creatinine^{-1}$ were summarized by the following descriptors: detection frequency (DF), arithmetic mean (AM), geometric mean (GM), 25th, 50th, 75th, and 95th percentiles (P25, P50, P75, and

Table 1
Lifestyle, sociodemographic characteristics and food consumption habits of the children participating in the BIOVAL programme ($n_T = 566$).

Variable	n (%)	Mean (minimum–maximum)
Children's characteristics		
Body Mass Index (BMI, kg·m^{-2})		18 (12–45)
Missing data	16 (3%)	
Age (years)		8 (5–12)
Province		
Alacant	205 (36%)	
Castelló de la Plana	126 (22%)	
València	235 (42%)	
Home location		
Urban	441 (78%)	
Rural	22 (4%)	
Other	102 (18%)	
Missing data	1 (0.2%)	
Sex		
Male	285 (50%)	
Female	281 (50%)	
Country of birth		
Spain	555 (98%)	
Foreign	8 (1%)	
Missing data	3 (1%)	
Physical activity		
3 or more days per week	299 (53%)	
1 or 2 days per week	207 (36.5%)	
Occasionally or never	60 (10.5%)	
Parents' characteristics		
Country of birth		
Both parents were Spanish	490 (87%)	
At least one of the parents was foreign	58 (10%)	
Missing data	18 (3%)	
Maximum level of education		
Without studies or primary studies	58 (10%)	
Secondary studies	167 (30%)	
University studies	341 (60%)	
Employment situation		
Both unemployed	38 (7%)	
Only one parent employed	164 (29%)	
Both employed	364 (64%)	
Food consumption in the last 72 h (grams)		**Mean (minimum–maximum)**
Eggs		65 (0–360)
Meat products		484 (0–1545)
Fishery products		262 (0–1300)
Dairy products		996 (0–2820)
Vegetables		399 (0–1798)
Chips		26 (0–210)
Fruits and nuts		840 (0–2835)
Legumes		101 (0–1000)
Cereals		498 (0–1655)
Bakery products		75 (0–350)
Oils and fats		17 (0–120)
Frequency of consumption of packaged food (number of products·month^{-1})		**Mean (minimum–maximum)**
Canned food		0.4 (0–4)
Plastic-packed food		6 (0–21)
Carton-packed food		0.9 (0–4)
Food in glass containers		0.2 (0–6)

P95), and maximum and standard deviation (SD). In addition, OHPAHs with the same PAH parent were converted to nmol·l^{-1} and added together as follows: sum of hydroxynaphthalenes (∑OHNAPs) = 1OHNAP + 2OHNAP; sum of hydroxyfluorenes (∑OHFLUs) = 2OHFLU + 3OHFLU; and sum of hydroxyphenanthrenes (∑OHPHENs) = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN. To be able to study correlations among the PAH metabolites, we used Spearman's test. While qualitative variables were described with absolute frequencies and percentages, quantitative variables were summarized by their median, minimum and maximum values (Table 1).

As the data was not normally distributed, we applied the logarithm (log) of each OHPAH chemical family to build an exposure prediction model including urinary creatinine levels and variables from Table 1 as independent variables, which were treated as continuous if they were quantitative parameters (age, BMI and food consumption habits), or as dichotomous (sex of the children) or polychotomous variables (province, home location, country of birth, physical activity, and parents' characteristics), depending on their number of categories. For this purpose, we performed linear regression models for log-transformed ∑OHNAPs, ∑OHFLUs, ∑OHPHENs, and 1OHPYR concentrations, using a forward stepwise selection procedure based on the corrected Akaike information criterion (AICc) (Konishi and Kitagawa, 2008). Additionally, we divided the numerical explanatory variables by two times their SD to obtain directly-comparable regression coefficient magnitudes (Gelman et al., 2008). A p-value less than or equal to 0.05 was considered as statistically significant. The coefficients estimated for each variable included in the final model reflected the change in the log of PAH metabolite levels in the urine of children.

Finally, the following assumptions were verified: normality of the residuals with the Shapiro-Wilk test, non-autocorrelation using the Durbin-Watson statistic, homoscedasticity by scatter plots, and non-multicollinearity of explanatory variables with tolerance and condition indexes.

2.6. Risk assessment

Due to the fact that the main source of PAH exposure in non-smokers is diet (EFSA, 2008), we assessed the risk of exposure to PAHs in the target children by considering only the dietary ingestion route. To this end, we calculated the estimated daily intake (EDI) of naphthalene, fluorene, phenanthrene, and pyrene by combining urinary levels of their metabolites (∑OHNAPs, ∑OHFLUs, ∑OHPHENs and 1OHPYR, respectively) with their toxicokinetic properties in the human body. GM and P95 distribution levels were used to calculate EDIs, which were stratified according to the age of the participants (5–8 years old, and 9–12 years old), using equation (1) below (Peng et al., 2020) and the parameter values presented in Table S2.

$$EDI = \frac{C_U \times V_{24h} \times MW}{F_{UE} \times BW} \quad (1)$$

EDI = estimated daily intake of the PAH parent compound (ng·kg-bw^{-1}·day^{-1})
C_U = molar concentration of the sum of OHPAHs with the same PAH parent in urine (GM or P95 in nmol·l^{-1})
V_{24h} = total urinary volume excreted within 24 h for children (0.66 l·day^{-1}) (Aylward et al., 2011)
MW = molecular weight of the parent PAH compound (g·mol^{-1})
F_{UE} = fraction of the oral intake dose of PAH excreted as urinary OHPAH (ranging from 0.08 to 1) (Li et al., 2012; Motorykin et al., 2015)
BW = mean body weight of the target population (27 kg for 5–8 years old children, and 39 kg for 9–12 years old children)

The non-cancer risk from a single exposure was assessed by means of a hazard quotient (HQ), calculated as the ratio of EDI for each PAH to its reference dose for oral exposure (RfD) (Table S2), as follows (Miri et al., 2018; Peng et al., 2020):

$$HQ_i = \frac{EDI_i}{RfD_i} \quad (2)$$

Additionally, we evaluated the cumulative risk of exposure to PAH mixtures by adding up individual HQs of PAHs according to equation (3) (Peng et al., 2020). This parameter is known as the hazard index (HI):

$$HI_{PAHs} = \sum HQ_i \quad (3)$$

A HQ < 1 is interpreted as an acceptable risk from an exposure to a

Table 2
Descriptive analysis of the OHPAHs levels (ng·ml^{-1}) in urine from Valencian children (n = 566).[a]

Parent compound	Metabolites	LoQ	DF (%)	P25	P50	AM	GM	P75	P95	Maximum	SD
Naphthalene	1OHNAP	0.01	87	0.2 (0.3)	0.6 (0.6)	1.0 (1.0)	0.3 (0.4)	1.2 (1.1)	3.1 (3.2)	17.2 (12.4)	1.4 (1.4)
	2OHNAP	0.01	100	5 (6)	11 (11)	18 (18)	10 (11)	20 (20)	53 (52)	382 (265)	28 (24)
	[c,d]∑OHNAPs	–	–	42 (48)	81 (80)	132 (129)	79 (82)	151 (150)	389 (368)	2647 (1836)	196 (165)
Fluorene	2OHFLU	0.01	99	0.11 (0.12)	0.17 (0.17)	0.25 (0.25)	0.17 (0.18)	0.27 (0.25)	0.55 (0.55)	16.99 (17.61)	0.73 (0.75)
	3OHFLU	0.05	37	-[b]	-[b]	0.10 (0.10)	-[b]	0.07 (0.07)	0.29 (0.30)	2.42 (2.55)	0.26 (0.29)
	[c,e]∑OHFLUs	–	–	0.8 (0.8)	1.2 (1.2)	1.9 (1.9)	1.2 (1.3)	1.9 (1.7)	5.2 (5.0)	106.5 (110.4)	4.7 (4.9)
Phenanthrene	1OHPHEN	0.01	97	0.06 (0.07)	0.11 (0.11)	0.16 (0.16)	0.10 (0.10)	0.18 (0.17)	0.39 (0.41)	4.35 (4.93)	0.26 (0.28)
	2OHPHEN	0.01	96	0.02 (0.03)	0.04 (0.04)	0.06 (0.06)	0.04 (0.04)	0.07 (0.07)	0.15 (0.14)	1.26 (1.31)	0.08 (0.08)
	3OHPHEN	0.01	99	0.05 (0.06)	0.08 (0.09)	0.12 (0.11)	0.08 (0.09)	0.14 (0.13)	0.30 (0.28)	2.47 (2.56)	0.15 (0.15)
	4OHPHEN	0.01	78	0.01 (0.01)	0.02 (0.02)	0.04 (0.04)	0.02 (0.02)	0.04 (0.04)	0.11 (0.10)	0.60 (0.62)	0.05 (0.05)
	9OHPHEN	0.01	86	0.02 (0.02)	0.04 (0.04)	0.06 (0.06)	0.03 (0.03)	0.07 (0.07)	0.18 (0.19)	1.65 (1.87)	0.10 (0.10)
	[c,f]∑OHPHENs	–	–	1.1 (1.2)	1.6 (1.6)	2.2 (2.2)	1.7 (1.7)	2.6 (2.4)	5.0 (4.9)	39.0 (40.4)	2.7 (2.8)
Pyrene	1OHPYR	0.01	98	0.07 (0.07)	0.11 (0.10)	0.13 (0.13)	0.10 (0.10)	0.16 (0.15)	0.29 (0.29)	0.84 (1.20)	0.10 (0.10)
Benzo(a)pyrene	3OHBAP	0.05	5	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]	-[b]
[c,g]∑OHPAHs		–	–	46 (51)	85 (84)	137 (133)	85 (88)	157 (158)	398 (374)	2653 (1840)	198 (166)

LoQ = Quantification Limit; DF = Detection Frequency (% samples > LoQ); P = percentile; AM = Arithmetic mean; GM = Geometric mean; SD = Standard deviation.
[a] Values within parenthesis are the urinary levels adjusted by creatinine (μg·g Cre^{-1}).
[b] This parameter was not calculated due to the low DF of this metabolite.
[c] Units: nmol·l^{-1} (nmol·g Cre^{-1}).
[d] ∑OHNAPs = 1OHNAP + 2OHNAP.
[e] ∑OHFLUs = 2OHFLU + 3 OHFLU.
[f] ∑OHPHENs = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN.
[g] ∑OHPAHs = ∑OHNAPs + ∑OHFLUs + ∑OHPHENs + 1OHPYR + 3OHBAP.

specific PAH and a HI < 1 means that the cumulative exposure to a certain PAH mixture is little concern to public health.

According to the Panel on Contaminants in the Food Chain (CONTAM Panel), only PAHs for which oral carcinogenicity data are available can help to assess the carcinogenic potency of a PAH mixture (EFSA, 2008). For this reason, the only possible indicators of cancer risk are the following eight PAHs (PAH8): benzo(a)pyrene, benz(a)anthracene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(ghi)perylene, chrysene, dibenz(a,h)anthracene, and indeno(1,2,3-cd)pyrene (EFSA, 2008). And therefore, since naphthalene, fluorene, phenanthrene, and pyrene are not included in this group, the risk of cancer from exposure to PAHs could not be assessed in the present study.

3. Results

In the end, a total of 566 urine samples with a creatinine concentration ranging from 33 to 266 mg·dl^{-1} (mean = 104 mg creatinine ·dl^{-1} urine) were considered in the descriptive and statistical analysis. Table 1 summarizes the parameters obtained from the questionnaires regarding the socio-demographic characteristics, lifestyle habits, and the dietary patterns of the donors used in the research. Out of the 566 children, 50% were boys and 50% were girls; 51% were aged between 5 and 8 years old and 49% between 9 and 12 years old. Most of the children (93%) had at least one parent employed, and 78% of them lived in urban areas. Anomalous BMI were found in 16 children (3%), who had a BMI greater than 25 kg·m^{-2}, four of which presented a BMI greater than 30 kg·m^{-2}. In contrast, 62% of the children had a BMI lower than 18 kg·m^{-2}.

3.1. Urinary levels of OHPAHs

Table 2 shows the descriptive statistics of the urinary concentrations of OHPAH (in ng·ml^{-1} and μg·g creatinine^{-1}) in the target population. As can be observed, all PAH metabolites were detected in more than 78% of the urine samples, with the exception of 3OHBAP and 3OHFLU, with DFs of 5% and 37%, respectively, since these two metabolites had the highest LoQ (0.05 ng·ml^{-1}). It is worth noting that 2OHNAP was found in 100% of the analyzed samples.

Regarding OHPAH urinary concentrations, the highest levels were found for the metabolites of naphthalene, 1OHNAP and 2OHNAP, with GMs of 0.3 and 10 ng·ml^{-1}, respectively. By contrast, the phenanthrene metabolites 4OHPHEN, 9OHPHEN, and 2OHPHEN had the lowest GMs of all the metabolites: 0.02, 0.03, and 0.04 ng·ml^{-1}, respectively. The urinary concentrations of the remaining OHPAHs ranged from 0.08 (3OHPHEN) to 0.17 ng·ml^{-1} (2OHFLU), while reference values (RV_{95}) were in the range of 0.11 (4-hydroxyphenanthrene) to 53 ng·ml^{-1} (2-hydroxynaphthalene). Overall, more than seven metabolites of PAHs were detected in approximately 90% of the children.

3.2. Correlations and predictors of exposure to PAHs

Table S3 includes the Spearman's correlation matrix calculated for the urinary levels of the OHPAHs. According to this matrix, all PAH metabolites were highly and directly related to each other, indicating, in all probability, similar sources.

Fig. 2 represents the results of the multiple regression model obtained for each PAH family along with its standardized coefficients (β) and their corresponding 95% confidence intervals (CIs). The final regression coefficients (R^2) obtained were between 0.160 and 0.227. The obtained regression formulas for predicting OHPAHs concentrations in urine and more detailed information on these prediction models are shown in Table S4.

As shown by Fig. 2, the age of the children was negatively related to all OHPAHs. In contrast, a positive correlation was found with urinary creatinine. In the case of OHNAPs, children who had foreign parents tended to have lower urinary concentrations of these metabolites than those with Spanish parents, but given that only 10% of the participants

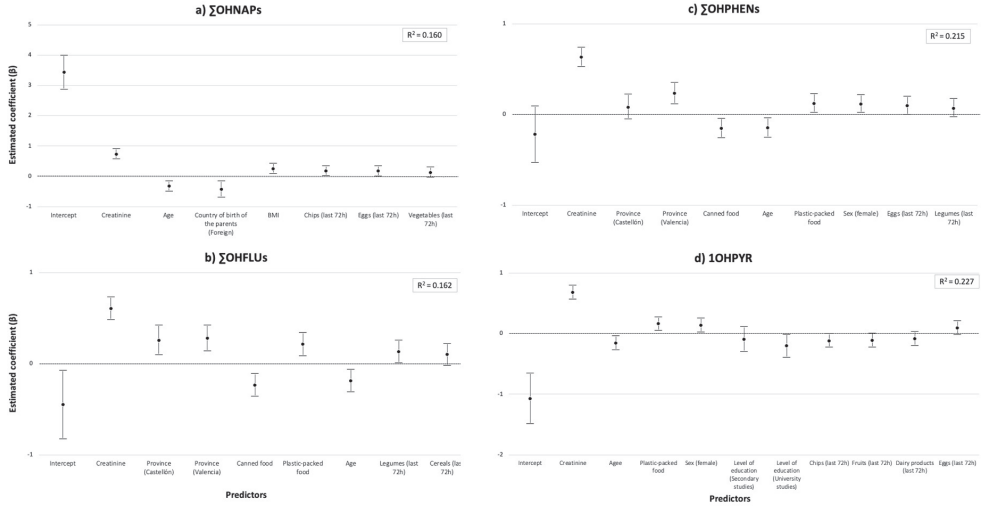


Fig. 2. Standardized coefficients of the linear regression models fitted for the log-transformed urinary concentrations of a) ΣOHNAPs, b) ΣOHFLUs, c) ΣOHPHENs, and d) 1OHPYR, with their corresponding 95% confidence intervals and coefficients of determination (R^2).

were foreign, this finding was not considered highly relevant. Moreover, higher OHNAPs levels were found in children with higher BMIs. In addition, urinary levels of ∑OHFLUs and ∑OHPHENs were significantly different depending on the province where the children lived. In general, participants living in the València province, and to a lesser extent in Castelló de la Plana, had higher levels of these metabolites in their urine samples. If sex differences are taken into account, it can be observed that girls presented higher ∑OHPHENs and 1OHPYR concentrations than boys. Furthermore, the higher the parents' education level was, the lower the 1OHPYR urinary levels were.

Regarding dietary habits of the participants, children who consumed a higher number of chips and legumes in the last 72 h presented higher ∑OHNAPs and ∑OHFLUs concentrations in their urine, respectively. By contrast, a negative association was found between the intake of chips and urinary levels of 1OHPYR. Moreover, the consumption frequency of food packaged in plastic was highly and positively related with all OHPAH concentrations, except for ∑OHNAPs.

Another relevant finding with a clear tendency to significance (p-values close to 0.05) were that eggs consumption appeared to be related to higher urinary levels of ∑OHNAPs, ∑OHPHENs and 1OHPYR.

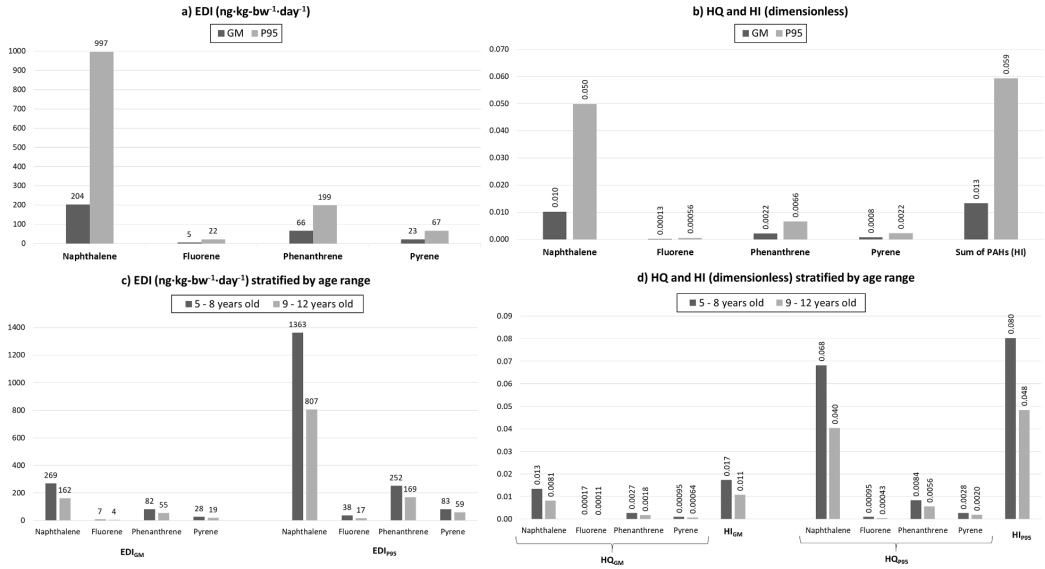


Fig. 3. a) Estimated daily intakes (EDI, in ng·kg-bw^{-1}·day^{-1}) at GM and P95 for naphthalene, fluorene, phenanthrene and pyrene; and, b) Hazard quotients and hazard indexes at GM and P95 for PAHs, c) Estimated daily intakes (EDI, in ng·kg-bw^{-1}·day^{-1}) stratified by age range; and, d) Hazard quotients and hazard indexes stratified by age range.

3.3. Risk assessment

EDIs, HQs, and HIs calculated for naphthalene, fluorene, phenanthrene, and pyrene are represented in Fig. 3. We did not assess exposure to benzo(a)pyrene due to the low DF (5%) of its urinary metabolite (3OHBAP) in the analyzed samples.

As illustrated by Fig. 3, naphthalene was the PAH with the highest EDI (204 and 997 ng·kg-bw^{-1}·day^{-1} for GM and P95, respectively), followed by phenanthrene (66 and 199 ng·kg-bw^{-1}·day^{-1}), pyrene (23 and 67 ng·kg-bw^{-1}·day^{-1}), and finally, fluorene (5 and 22 ng·kg-bw^{-1}·day^{-1}). Broadly speaking, EDIs of children aged 5–8 years old were approximately twice that of children aged 9–12 years old.

Concerning the risk estimation represented in Fig. 3, HQs were in the range of 0.00013 (fluorene) and 0.010 (naphthalene) at GM level, and of 0.00056 (fluorene) and 0.050 (naphthalene) at P95 level. Similarly, the cumulative risk to PAHs resulted in HIs far below 1, with naphthalene being the major contributor to the risk of exposure to the assessed PAH mixture at both distribution levels. As expected, children from 5 to 8 years old obtained higher values of HQ and HI than children aged 9–12. Fortunately, given that all HQs and HIs were lower than 1, no apparent non-cancer health risk associated with the exposure to PAHs in the target children could be determined.

4. Discussion

In the present study, 2OHNAP was the predominant metabolite in the PAHs' total internal dose, accounting for 87% of the ∑OHPAHs, followed by 1OHNAP (7%), ∑OHPHENs (3%), ∑OHFLUs (2%), and 1OHPYR (1%). This composition profile is very similar to what was obtained in children in Portugal (Oliveira et al., 2017), Poland (Sochacka-Tatara et al., 2018), Germany (Murawski et al., 2020), China (Yu et al., 2021), Canada (Health Canada, 2017; Ratelle et al., 2020), USA (CDC, 2019), and Australia (Thai et al., 2020). In the case of 3OHBAP, the metabolite of the only known carcinogenic PAH (benzo(a)pyrene), we observed very low detection rates (5%), which is in line with other HBM studies in the Czech Republic (Urbancova et al., 2020), Spain (Fernández et al., 2021), Portugal (Oliveira et al., 2017), Canada (Health Canada, 2017; Ratelle et al., 2020), and China (Fan et al., 2012), despite having different LoQs. The main reason is that high-molecular weight PAHs, those with more than four fused rings, are largely eliminated through feces (Li et al., 2012; Likhachev et al., 1992).

If we consider the obtained concentrations in a global context, we can find several similarities and differences among children around the world. Table 3 shows the mean OHPAH concentrations observed in children at school age in countries around the world.

In addition, Fig. 4 represents the RV_{95} used to compare the exposure of children from the BIOVAL study with the following population-representative surveys: the German Environmental Survey, 2014–2017 (GerES V) (Murawski et al., 2020); the National Health and Nutrition Examination Survey (NHANES) (CDC, 2019); and the Canadian Health Measures Survey (CHMS) (Health Canada, 2017). It should be taken into account that these studies are not methodologically homogeneous, in that they include different types of urine samples, age ranges of children, and sampling years, as well as the fact that they employ other analytical methods and/or report different LoQs.

Firstly, our study reports mean levels of 2OHNAP between 2 and 25 times higher than levels observed in children from other countries of America, Europe, Africa, and Asia, these levels only being lower than those of children living in polluted areas located in China (Fan et al., 2012; Lin et al., 2020) and Korea (Yoon et al., 2012). In fact, the RV_{95} derived for 2OHNAP in the present study (53 ng·ml^{-1}) is approximately twice the RV_{95} observed for children in Germany (24 ng·ml^{-1}), USA (19 ng·ml^{-1}), and Canada (19 ng·ml^{-1}), showing that exposure of Spanish children to naphthalene is apparently high and should be reduced (Schulz et al., 2011), given that naphthalene is considered by the International Agency for Research on Cancer as a possible carcinogenic substance (IARC, 2021). This fact could be attributed to a higher exposure to PAH sources in Spanish children compared to other countries, such as environmental tobacco smoke (ETS), food and cooking methods, or indoor/outdoor air quality (Hoseini et al., 2018; Huang et al., 2019b; Morgan et al., 2015; Murawski et al., 2020; Oliveira et al., 2019). However, further analyses are required to draw firm conclusions.

By contrast, the opposite trend was found to be the case for 1OHNAP, with children from the BIOVAL study having the lowest levels compared to the rest of the world, except for newborns living in the Czech Republic (Urbancova et al., 2020). In addition, the lowest RV_{95} for 2OHNAP was observed for children living in Spain.

Secondly, North-American (CDC, 2019; Health Canada, 2017) and Australian children (Thai et al., 2020) showed similar RV_{95} and mean levels of 2OHFLU and 3OHFLU, respectively, to those obtained in the present research. On the other hand, there were some Asian (Fan et al., 2012; Hoseini et al., 2018; Lin et al., 2020; Yu et al., 2021) and European countries (Murawski et al., 2020; Sochacka-Tatara et al., 2018) which reported higher mean concentrations of these fluorene metabolites. Furthermore, the GerES V survey reported the highest RV_{95} for fluorene metabolites in children of a similar age (Fig. 4), probably because it is referred to the sum of 2OHFLU and 9-hydroxyfluorene (9OHFLU) (Murawski et al., 2020).

In general, lower levels of OHPHENs have been observed in children under study, except from those living in the USA (CDC, 2019) and Australia (Thai et al., 2020), in which lower levels of 1OHPHEN, 3OHPHEN, and 2OHPHEN were reported, respectively. Likewise, RV_{95} derived for OHPHENs in Spanish children appeared to be lower than or in the same range as those reported for German, American, and Canadian children.

Finally, the children in our study obtained mean levels and RV_{95} of 1OHPYR that were generally lower than or comparable to other nationalities. The highest mean concentrations of 1OHPYR were found in Mexico (Martínez-salinas et al., 2012; Sánchez-Guerra et al., 2012), and some countries belonging to Asia (Hemat et al., 2012; Naksen et al., 2017; Shahsavani et al., 2017; Wang et al., 2017; Yoon et al., 2012) and Africa (Tuakuila et al., 2015), probably because people from less developed countries are more exposed to PAH pollution due to deficient air pollution control in these areas and the use of biomass fuels in households (Adeniji et al., 2018; EUROSTAT, 2020; Hemat et al., 2012).

When comparing our findings to another study carried out on Spanish children, urinary concentrations of 1OHPYR were slightly higher in the Valencian children of the present study (2016) than in children living in Granada (2005–2006) (Freire et al., 2009).

The statistical analysis showed that consumption of chips, legumes, and pre-packaged food, as well as several socio-demographic characteristics, such as sex, BMI, age, educational level, and home location, were the most relevant factors affecting OHPAH urinary levels in children. Possible reasons for these findings are discussed in the following paragraphs.

Concerning food consumption habits, a positive association was observed between ∑OHNAPs and the intake of potato chips. This is in accordance with Purcaro et al., 2006, who affirmed that fried food was an important source of exposure to low-molecular PAHs. This association was also observed in an HBM project conducted on Spanish pregnant women (Llop et al., 2008). Surprisingly, the opposite trend was found for 1OHPYR. This fact may be explained by the high variability of PAH concentrations produced after the frying process, which can be very different depending on the cooking conditions (Purcaro et al., 2006).

Legumes were a food group contributing to ∑OHFLUs levels in the present study, since the more intake of legumes, the higher the urinary levels of fluorene metabolites in children. In fact, Bartolomé et al., (2015) found associations between OHPAHs and legumes similar to those of the present study. However, as reported by several Spanish total diet studies, legumes are minor contributors of PAHs (Domingo and Nadal, 2015; Ibáñez et al., 2005; Martí-Cid et al., 2008).

Another important finding was that plastic-wrapped food was

Table 3
OHPAHs concentrations (geometric mean) reported in children at school age and other population groups in countries around the world.*

Continent	Reference	Country, City/ Region	Target population	Sampling year	N	Units	1OHNAP	2OHNAP	2OHFLU	3OHFLU	1OHPHEN	2OHPHEN	3OHPHEN	4OHPHEN	9OHPHEN	1OHPYR
Europe	**This study**	Spain, Valencian Region	Children (5–11 years old)	2016	566	ng/ml (μg/g Cre)	0.3 (0.4)	10 (11)	0.17 (0.18)	<0.05	0.10 (0.10)	0.04 (0.04)	0.08 (0.09)	0.02 (0.02)	0.03 (0.03)	0.10 (0.10)
	Freire et al., (2009)	Spain, Granada	Children (4 years old)	2005–2006	174	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.07
	Sochacka-Tatara et al., (2018)	Poland, Krakow	Children (3 years old)	2004–2007	202/218	ng/ml	1.92	4.22	0.47	0.18	0.32	0.08	0.20	0.05	NA	0.18
	Murawski et al., (2020)	Germany	Children (3–17 years old)	2015–2017	516	ng/ml	0.79	4.23	0.39	NA	0.14	0.09	0.13	0.05	0.06	0.10
	Urbancova et al., (2020)[c]	Czech Republic	Newborns	2016–2017	330	ng/ml	0.12	2.28	0.07	NA	NA	NA	NA	NA	NA	0.05
	Cirillo et al. (2006)[a]	Italy, Campania	Children (7–9 years old)	2004	30	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.17
	Hansen et al., (2006)[b]	Denmark	Children (3–13 years old)	1994–1995	204	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.23
	De Craemer et al., (2016)[c]	Belgium	Adolescents (14–16 years old)	2013	20	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.18[j]
	Mucha et al., (2006)	Ukraine, Kiev (city)	Children (3 years old)	1998	42	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.30
	Mucha et al., (2006)	Ukraine, Mariupol (steel mill and coking facility)	Children (3 years old)	1998	48	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.52
America	CDC (2019)	USA	Children (6–11 years old)	2013–2014	399	ng/ml	0.80	3.03	0.12	0.06	0.07	0.04[d]	0.05[d]	0.02[d]	NA	0.13
	Health Canada (2017)	Canada	Children (6–11 years old)	2014–2015	510	ng/ml	0.62	3.80	0.21	0.08	0.13	0.04	0.08	0.02	0.03	0.10
	Martínez-salinas et al. (2012)[a,e]	Mexico (rural areas exposed to biomass combustion)	Children (6–12 years old)	2009	90	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	1.54
	Martínez-salinas et al. (2012)[a],f	Mexico (urban areas exposed to traffic and brick furnace emissions)	Children (6–12 years old)	2009	67	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.34
	Sánchez-Guerra et al., (2012)[a]	Mexico (petrochemical areas)	Children (6–10 years old)	2008	82	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.65
Oceania	Thai et al., (2020)	Australia, South East Queensland region	Children (5–14 years old)	2017	100	ng/ml	1.70	4.50	0.12	0.04	0.36[k]	0.01	0.36[k]	0.08	NA	0.16
Asia				2015	165		0.86	1.95	0.58	NA	0.29	0.25	NA	0.10	0.10	0.44

(*continued on next page*)

Table 3 (*continued*)

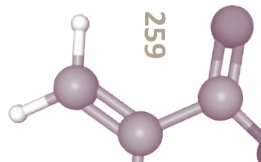
Continent	Reference	Country, City/ Region	Target population	Sampling year	N	Units	1OHNAP	2OHNAP	2OHFLU	3OHFLU	1OHPHEN	2OHPHEN	3OHPHEN	4OHPHEN	9OHPHEN	1OHPYR
	Yu et al., (2021)	China, Shenzhen, Guangdong Province	Children (8–11 years old)			ng/ml										
	Lin et al., (2020)	South China (e-waste recicling area)	Children (3–14 years old)	2015–2016	19	μg/g Cre	9.55	10.60	0.90	0.05	0.13[l]	0.13	0.23	0.05	0.13[l]	0.16
	Liu et al., (2017)	China, Chongqing (urban area)	Children (7–13 years old)	2015	1207	ng/ml	NA	2.57	0.84	NA	NA	NA	NA	NA	2.55	0.18
	Fan et al., (2012)[a]	China (residential area)	Children (4–6 years old)	2008	35	μg/g Cre	NA	5.91	3.58	NA	NA	0.53	0.15	0.29	6.78	0.27
	Fan et al., (2012)[a]	China (polluted area)	Children (4–6 years old)	2008	39	μg/g Cre	NA	11.60	6.00	NA	NA	0.55	0.64	0.40	4.13	1.23
	Hoseini et al., (2018)	Iran, Tehran	Children (<12 years old)	2013–2014	14	ng/ml	0.60	1.71	0.27	NA	NA	NA	NA	NA	0.06	0.15
	Shahsavani et al., (2017)[g]	Iran, Shiraz	Children (9–12 years old)	2015	115	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	2.28
	Miri et al., (2018)	Iran	Children (5–7 years old)	2017	200	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.26
	Poursafa et al. (2018)[e]	Iran, Isfahan	Children (6–18 years old)	2014–2016	186	ng/ml	0.81	1.05	NA	NA	NA	NA	NA	NA	0.08	0.06
	Kelishadi et al., (2018)	Iran, Isfahan	Children (6–18 years old)	2015–2016	150	ng/ml	0.36	0.42	NA	NA	NA	NA	NA	NA	0.11	0.10
	Naksen et al., (2017)[h]	Thailand (biomass burning-exposed area)	Children (9–12 years old)	2015	202	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.82
	Ruchirawat et al., (2007)[a]	Thailand, Chonburri (rural area)	Male children (9–13 years old)	NR	61	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.19
	Ruchirawat et al., (2007)[a]	Thailand, Bangkok (urban area)	Male children (9–13 years old)	NR	105	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.35
	Hemat et al., (2012)[c]	Afghanistan	Children (2–9 years old)	2007	13	ng/ml	NA	NA	NA	NA	1.34	1.12[m]	1.24	0.13	1.12[m]	1.56
	Lee et al., (2007)	Korea (areas near a steel mill)	Children (7–15 years old)	2004	1012	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.43
	Yoon et al., (2012)[f]	Korea, Seoul/ Incheon (metropolitan area)	Children (10–12 years old)	2002–2003	197	ng/ml	NA	18.55	NA	NA	NA	NA	NA	NA	NA	0.80[n]
	Yoon et al., (2012)[f]			2002–2003	125	ng/ml	NA	27.8	NA	NA	NA	NA	NA	NA	NA	1.14[n]

(*continued on next page*)

Table 3 (*continued*)

Continent	Reference	Country, City/ Region	Target population	Sampling year	N	Units	1OHNAP	2OHNAP	2OHFLU	3OHFLU	1OHPHEN	2OHPHEN	3OHPHEN	4OHPHEN	9OHPHEN	1OHPYR
		Korea, Pohang (industrial area)	Children (10–12 years old)													
	Puttaswamy et al., (2020)	India (rural areas with solid biomass fuel cooking)	Children (<1 years old)	NR	39	ng/ml	NA	2.15	NA	NA	NA	NA	NA	NA	NA	0.45
	Wang et al., (2017)	Taiwan	Children (3 years old)	2010	453	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	1.94
	Alghamdi et al., (2015)[i]	Saudi Arabia (area near to an oil refinery)	Male children (10–12 years old)	2013	167	ng/ml	NA	NA	NA	NA	0.19	0.08[m]	0.18	0.04	0.08[m]	0.30
	Alghamdi et al., (2015)[i]	Saudi Arabia (area near to a highway)	Male children (10–12 years old)	2013	187	ng/ml	NA	NA	NA	NA	0.13	0.07[m]	0.15	0.03	0.07[m]	0.19
	Alghamdi et al., (2015)[i]	Saudi Arabia (area near to the Red Sea)	Male children (10–12 years old)	2013	132	ng/ml	NA	NA	NA	NA	0.15	0.08[m]	0.14	0.03	0.08[m]	0.17
	Mori et al., (2011)	Japan, Tokio (suburban area)	Children (3–6 years old)	2007	134	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.13
	Chen et al., (2015)[a]	Mongolia, Ulaanbaatar (warm/ moderate season)	Children (11–15 years old)	2011–2012	320	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.27
	Chen et al., (2015)[a]	Mongolia, Ulaanbaatar (cold season)	Children (11–15 years old)	2011–2012	320	μg/g Cre	NA	NA	NA	NA	NA	NA	NA	NA	NA	0.58
Africa	Bortey-Sam et al., (2017)[j]	Ghana, Kumasi	Young people (3–20 years old)	2015	36	ng/ml	NA	4.99	NA	NA	0.13[l]	0.15	NA	0.11	0.13[l]	0.14
	Tuakuila et al., (2015)	Congo, Kinshasa	Children (6–14 years old)	2011	60	ng/ml	NA	NA	NA	NA	NA	NA	NA	NA	NA	6.10

NA: not analyzed.
NR: not reported.
[*] 3OHBAP was not included in this table because we did not have statistically comparable data to compare our results with other studies. In addition, very few articles reported levels for this metabolite.
[a] Value converted from μmol/mol creatinine to μg/g Creatinine using the molecular weight of creatinine and OHPAHs.
[b] Value converted from μmol/mol creatinine to ng/ml using the mean of creatinine levels (0.3 mmol/l) and molecular weight of 1OHPYR.
[c] Median values.
[d] These values correspond to a survey conducted between 2011 and 2012.
[e] Calculated mean of 4 different groups.
[f] Calculated mean of 2 different groups.
[g] Value converted from ng/g creatinine to ng/ml using the mean of creatinine levels (156 mg/dl).
[h] Arithmetic mean.
[i] Calculated mean of 3 different groups.
[j] Adjusted by specific gravity.
[k] Sum of 1OHPHEN and 3OHPHEN.
[l] Sum of 1OHPHEN and 9OHPHEN.
[m] Sum of 2OHPHEN and 9OHPHEN.
[n] Conjugated 1OHPYR.



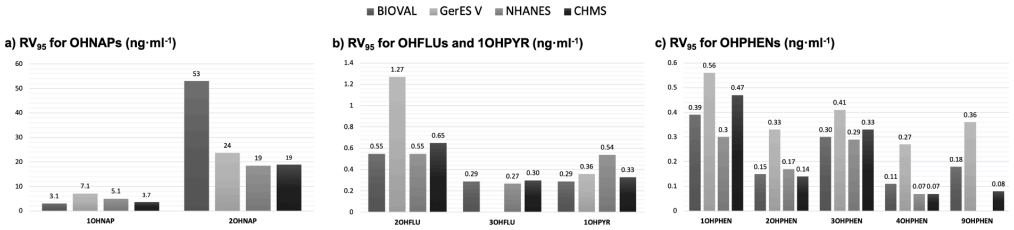


Fig. 4. Comparison among reference values (RV_{95}) in children reported by the following HBM surveys: the GerES V study (Germany, 2015–2017)* (Murawski et al., 2020); the NHANES (USA, 2013–2014) (CDC, 2019); the CHMS (Canada, 2014–2015) (Health Canada, 2017); and, the BIOVAL study (Spain, 2016) (Present study), for urinary OHPAHs: a) OHNAPs, b) OHFLUs and 1OHPYR, and c) OHPHENs.

identified as a possible source of exposure to PAHs in the children under study. Similarly, children participating in the GerES V showed higher urinary OHPAH levels if they tended to chew plastic objects (Murawski et al., 2020). This is in line with recent studies by Li et al. (2017) and Schweighuber et al. (2019), who studied the presence of PAHs in polystyrene-based products in contact with foodstuffs, concluding that low-molecular weight PAHs can be generated in the production and processing of these materials, as well as being present in the dye used for their coloring. In addition, they also reported that PAH migration from these plastic materials into foods is possible, which requires further attention (Li et al., 2017; Schweighuber et al., 2019).

With regard to inhalation of polluted indoor and outdoor air, several studies support that it may play an important role in the internal exposure to PAHs (De Craemer et al., 2016; Freire et al., 2009; Oliveira et al., 2017; Yu et al., 2021). However, although many authors observed higher OHPAHs concentrations in children living in urban areas (high pollution) compared to those living in rural areas (low pollution) (Cirillo et al., 2006; Hansen et al., 2006; Oliveira et al., 2019; Ruchirawat et al., 2007; Yu et al., 2021), our results showed no differences between children living in these locations. By contrast, it was observed that the province where the children lived had a significant effect on their OHPAH concentrations, as reported in other previous studies (Dobraca et al., 2018; Lee et al., 2009), with participants living in València appearing to be more exposed to PAHs than children living in Castelló de la Plana or Alacant. Therefore, the association of OHPAH levels with location of residence may suggest that airborne exposure is an important contributor, but no conclusions could be drawn with this information alone.

In recent studies, low household income has been associated with higher levels of 1OHPYR and other OHPAHs in urine (Jain, 2020; Keir et al., 2020; Liu et al., 2017), but this characteristic was not considered in the present research. Nevertheless, lower concentrations of 1OHPYR were found in children whose parents had a high level of education, which is also related to the socio-economic status of the family (De Craemer et al., 2016; Dobraca et al., 2018; Lampert et al., 2018). Chuang et al. (1999) suggested that the increased PAH exposure of low-income people is likely to be related to poor air quality, which may be considerably influenced by ETS and household location (proximity to industrial or dense traffic areas).

Another relevant finding was that the urinary levels of PAH metabolites were lower in older children. This has been also observed in other HBM studies on children from other countries (Chen et al., 2015; Jain, 2020; Lee et al., 2007; Mori et al., 2011; Oliveira et al., 2017; Palazzi et al., 2019; Thai et al., 2016; Yu et al., 2016). The reason for this could be that small children have not fully developed metabolic and physiologic functions, leading to a reduced capability to degrade PAHs and more susceptibility to these substances (Hoseini et al., 2018; Huang et al., 2019a; Oliveira et al., 2017; Palazzi et al., 2019; Yu et al., 2016). Also, very young children frequently play on the floor, increasing the inhalation or dermal absorption of PAHs from carpets and soil (Hoseini et al., 2018; Huang et al., 2019a; Oliveira et al., 2019; Poursafa et al., 2017). However, other authors report no associations between urinary OHPAHs and age (Bartolomé et al., 2015; Bortey-Sam et al., 2017; Chen et al., 2015; Llop et al., 2008), while one study finds that this association is positive (Liu et al., 2017).

Regarding sex, females presented higher ∑OHPHENs and 1OHPYR levels in their urines. Despite several authors not reporting any relationship with this aspect (Bartolomé et al., 2015; Chen et al., 2015; Cirillo et al., 2006; Huang et al., 2006; Mori et al., 2011; Mucha et al., 2006; Thai et al., 2016), differences between the sexes have been supported by many recent studies (Bortey-Sam et al., 2017; Hoseini et al., 2018; Huang et al., 2019a; Keir et al., 2020; Liu et al., 2017; Yu et al., 2016). This finding is possibly explained by sex-related differences in absorption and metabolism of PAHs (Keir et al., 2020; Oliveira et al., 2017), as well as by other behaviors (e.g. participation in different indoor and outdoor activities) (Yu et al., 2016).

In addition, BMI tended to increase ∑OHNAPs concentrations in the children under study. This correlation between some OHPAHs and BMI has been previously described by other authors (Alghamdi et al., 2015; Bartolomé et al., 2015; Jain, 2020; Murawski et al., 2020; Poursafa et al., 2017), revealing the fat-soluble properties of PAHs (Liu et al., 2017).

In a risk assessment context, lower EDIs were found in the present study when comparing the median daily intake of pyrene of most European countries (0.05 μg/kg-day) (HBM4EU, 2019) and several Asian countries (Guo et al., 2013; Yu et al., 2021). In addition, the children aged 5–8 years old in our research presented higher EDIs and HQs for naphthalene, and higher HIs at both distribution levels (GM and P95) than the children aged 9–12 years old and female non-smokers living in València (2015) (Fernández et al., 2021), evidencing that younger children might be at greater risk of exposure to PAHs.

However, the cumulative risk of exposure to PAHs expressed by HI values was far below 1 in all cases, suggesting that this exposure is unlikely to have a significant impact on children's health. In any case, given the high urinary levels of 2OHNAP obtained in the present research, further studies are recommended to monitor PAH internal exposure over time, especially in younger children, as well as to identify other significant factors contributing to PAH exposure in children living in Spain.

5. Conclusions

In the present research, nine urinary biomarkers of exposure to PAHs were detected in more than 78% of children living in the Valencian Region (Spain). RV_{95} for urinary metabolites of naphthalene, fluorene, phenanthrene and pyrene of Spanish children aged 5–12 years were derived from the present study. According to the urinary composition profiles, the highest levels were found for metabolites of naphthalene, which were responsible for 94% of the total internal exposure to PAHs. Overall, the obtained OHPAH concentrations were lower or in the same range as children of other nationalities. Nonetheless, 2OHNAP levels are much higher than the average levels described worldwide, showing that more attention should be paid to Spanish children's exposure. Age, BMI,

dietary habits, province of residence, and plastic-wrapped food intake were identified as relevant predictors of exposure to PAHs. In terms of exposure, no potential health risk due to PAH exposure was found for Spanish children.

6. Strengths and limitations

The questionnaire used for the data collection was a general questionnaire designed for the assessment of exposure to several food and environmental contaminants. Therefore, some specific questions related to PAHs exposure, such as ETS, were not collected and consequently, they were not included in the statistical analysis.

On the other hand, a major strength of the present study was the collection of urine samples from a large children population with wide geographical distribution, being a representative sample of the children living in this region. Thus, the sample size of the study together with the high detection frequencies observed for the majority of OHPAHs led to a high statistical power of the study results. Finally, this is the first study to report urinary levels of 11 OHPAHs in Spanish children, highly contributing to the knowledge of PAH exposure in this country.

CRediT authorship contribution statement

Sandra F. Fernández: Methodology, Validation, Investigation, Writing - original draft, Visualization, Software, Formal analysis, Data curation. **Olga Pardo:** Writing - review & editing, Supervision, Funding acquisition. **Cristina S. Hernández:** Writing - original draft. **Borja Garlito:** Writing - review & editing. **Vicent Yusà:** Conceptualization, Writing - review & editing, Supervision, Project, administration. **:** .

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106535.

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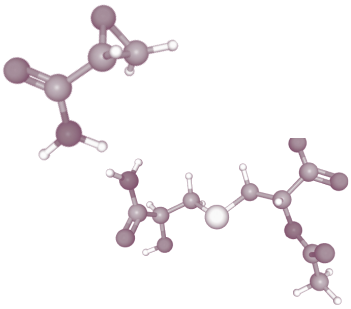
CHAPTER III

BIOMONITORING OF ACRYLAMIDE IN CHILDREN AND MOTHERS: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT



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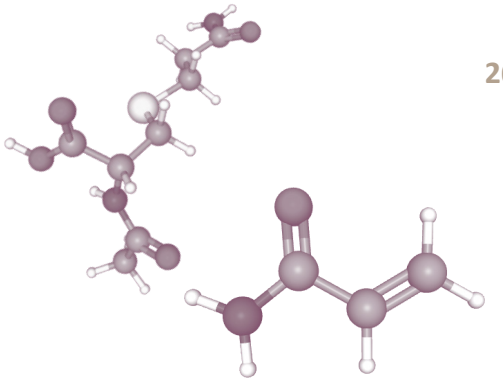

CHAPTER III

6.1 BIOMONITORING OF ACRYLAMIDE IN MOTHERS: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT

6.1.1 RESULTS AND DISCUSSION

Urinary mercapturic acids of AA were analyzed in the present study using an analytical method based on 'dilute-and-shoot' and LC-MS/MS, which met all the acceptance criteria of the validation parameters described in section 3. Results of the method validation conducted in 5 different batches are presented in Table 20. As external QA/QC, the laboratory participated in the intercomparison program G-EQUAS 66/2020 during the analysis of the field urine samples, obtaining |Z| between 0.15 and 1.35 for AAMA, and between 0.32 and 0.59 for GAMA3, which were classified as satisfactory results ($|Z| \leq 2$). Additionally, CRMs from the NIST (SRM 3672 and SRM 3673) were used as internal QCs for AAMA and GAMA3, obtaining $|Z| \leq 2$ in all analytical sequences.

Table 20. Results of the validation (n = 5) in terms of linearity, accuracy, precision, and specificity of the analytical method used for the analysis of the three urinary acrylamide metabolites in the present research.

Analyte	LoQ ($ng \cdot ml^{-1}$)	Linearity		Accuracy (%R)			Precision (%CV)			%S
		R^2	Residuals (%)	LoQ level	Medium level	High level	LoQ level	Medium level	High level	
AAMA	0.25	0.9877 – 0.9904	0.55 – 17.65	105	114	108	3.7	1.3	1.7	28
GAMA3	0.25	0.9935 – 0.9967	0.05 – 14.66	98	102	96	9.7	1.1	1.2	28
AAMA-Sul	0.25	0.9970 – 0.9983	0.01 – 8.17	97	102	107	6.8	1.0	1.6	28

LoQ = limit of quantification; %R = relative recovery (%); %CV: coefficient of variation (%); Medium level = 50 $ng \cdot ml^{-1}$ in urine; High level = 1000 $ng \cdot ml^{-1}$ in urine; %S = specificity (%)

In total, 117 out of the 119 urine samples collected in the BETTERMILK project were analyzed in this study, since two donors did not provide enough volume of urine to carry out the analysis. In addition, three urine samples were excluded due to an abnormal creatinine concentration (lower than 30 or higher than 300 $mg \cdot dl^{-1}$). In total, 114 urine samples with creatinine levels ranging from 30 to 240 $mg \cdot dl^{-1}$ (mean = 110 $mg \cdot dl^{-1}$) were included in the present research. Table 21 summarizes the socio-demographic characteristics and dietary habits related to AA exposure of the population under study. The mean age of the subjects was 34 years old, and more than 80 % of them lived in an urban area and were employed. Of the 114 women, only 9 were active smokers at the time of the sample collection. Potatoes and coffee products intake in the last 72 h were not recorded in the questionnaire of the BETTERMILK project, so the monthly frequency of consumption of these food groups were considered instead.

Table 21. Socio-demographic characteristics, lifestyle and food consumption habits of the lactating mothers participating in the BETTERMILK project included in the analysis of acrylamide metabolites in urine (n = 114).

Socio-demographic characteristics	n (%)	Mean (minimum – maximum)
Primipara	-	-
Yes	67 (59 %)	-
No	47 (41 %)	-
Age (years)	-	**34 (20 – 45)**
Missing data	1 (1 %)	-
BMI before pregnancy ($kg \cdot m^{-2}$)	-	**23 (17 – 35)**
Missing data	2 (2 %)	-
Country of birth	-	-
Spain	99 (87 %)	-
Foreign	13 (11 %)	-
Missing data	2 (2 %)	-
Place of residence	-	-
Urban	99 (87 %)	-
Rural	11 (10 %)	-
Missing data	4 (3 %)	-
Educational level	-	-
Without studies or primary studies	9 (8 %)	-
Secondary studies	28 (25 %)	-
University studies	77 (67 %)	-
Occupational status	-	-
Employed	94 (82 %)	-
Unemployed	18 (16 %)	-

Socio-demographic characteristics	n (%)	Mean (minimum – maximum)
Missing data	2 (2 %)	-
Physical activity	-	-
Frequently (3 or more days/week)	19 (17 %)	-
Occasionally (1 or 2 days/week)	18 (16 %)	-
Never	74 (64 %)	-
Missing data	3 (3 %)	-
Smoking habit	-	-
Currently smokes	9 (8 %)	-
Former smoker	47 (41 %)	-
Never have smoked	58 (51 %)	-
Use of sunscreen	-	-
Yes	43 (38 %)	-
No	71 (62 %)	-
Frequency of sunscreen's use	-	-
Never/Occasionally	74 (65 %)	-
Weekly	10 (9 %)	-
Daily	26 (23 %)	-
Missing data	4 (3 %)	-
Use of body cream	-	
Yes	89 (78 %)	-
No	25 (22 %)	-
Frequency of body cream's use	-	-
Never/Occasionally	25 (22 %)	-
Weekly	10 (17 %)	-
Daily	67 (59 %)	-
Missing data	2 (2 %)	-
Dietary habits related to AA exposure	**n (%)**	**Mean (minimum – maximum)**
Cereal products (portions in the last 72 h)	-	6 (2 – 20)
Bakery products (portions in the last 72 h)	-	2 (0 – 18)
Precooked food (portions in the last 72 h)	-	0.2 (0 – 2)
Packaged products (portions in the last 72 h)	-	14 (1 – 39)
Monthly coffee intake (ml·month^{-1})	-	970 (50 – 6770)
Monthly potatoes intake (g·month^{-1})		1584 (140 – 5820)
Missing data	6 (5 %)	-

6.1.1.1 URINARY LEVELS OF ACRYLAMIDE METABOLITES

Descriptive statistics for AAMA, GAMA3 and AAMA-Sul levels found in the urine samples unadjusted and adjusted by creatinine are presented in Table 22. All metabolites were above the LoQ in 100 % of the analyzed urines. The highest concentrations were found for AAMA, the major urinary biomarker of AA, with 50th and 95th percentiles of 73 and 266 ng·ml^{-1}, respectively. Lower urinary levels were observed for AAMA-Sul and GAMA3, with medians of 36 and 14 ng·ml^{-1}, respectively. Very similar concentrations were obtained when adjusted by creatinine.

Table 22. Descriptive analysis of the concentrations of urinary acrylamide metabolites in the breastfeeding mothers participating in the BETTEERMILK study (n = 114).

Metabolite	LoQ	DF (%)	P25	P50	AM
AAMA	0.25	100	41	73	99
AAMA-Sul	0.25	100	20	36	44
GAMA3	0.25	100	9	14	19
[a]∑UAAM	-	100	297	534	672
Ratio GAMA3/AAMA	-	-	0.16	0.21	0.22
$AAMA_{Cre}$	-	-	45	69	89
$AAMA\text{-}Sul_{Cre}$	-	-	24	32	40
$GAMA3_{Cre}$	-	-	10	14	17
[b]$\sum UAAM_{Cre}$	-	-	330	495	608

LoQ = Limit of quantification; DF = Detection Frequency (% samples > LoQ); Min. = minimum; P = percentile; AM = Arithmetic mean; GM = Geometric mean; Min. = minimum; Max. = maximum; SD = Standard deviation

[a]∑UAAM = AAMA + AAMA-Sul + GAMA3

[b]$\sum UAAM_{Cre}$ = $AAMA_{Cre}$ + $AAMA\text{-}Sul_{Cre}$ + $GAMA3_{Cre}$

GM	P75	P95	Range (Min – Max.)	SD	Units
70	117	266	10 – 627	94	$ng \cdot ml^{-1}$
33	55	109	3 – 276	39	
15	22	47	4 – 112	15	
500	833	1803	73 – 4231	603	$nmol \cdot l^{-1}$
0.21	0.27	0.39	0.06 – 0.48	0.08	Dimensionless
71	112	204	9 – 586	76	$\mu g \cdot g\ Cre^{-1}$
33	50	88	3 – 181	28	
15	21	32	4 – 73	10	
503	759	1332	67 – 3287	460	$nmol \cdot g\ Cre^{-1}$

Urinary levels of AA metabolites have been reported in other international HBM studies. Figure 31 summarizes the main data of recent studies on HBM of AA in adults. In general, AAMA is the most analyzed AA metabolite in urine, being detected in more than 98 % of the samples worldwide. As it can be observed in Figure 31a, some differences were observed among countries when comparing the median urinary levels of this metabolite. In general, most of the international studies reported AAMA levels up to three times lower than in the present study, showing a higher exposure in the Spanish population. On the contrary, a study focusing on a group of adults (21 – 45 years old) living in the USA showed the highest AAMA urinary levels[213], but it should be noted that more than 75 % of those adults were smokers. Taking into account median levels stratified by the smoking habit, it can be observed that non-smokers from Taiwan (men) [383–385] and USA[213,214,386] seemed to be more exposed to AA than non-smoker mothers from the BETTERMILK project. In addition, smokers from Poland (women)[387], Norway[388], Germany[389,390] and USA[213,386] presented higher urinary levels of AAMA than the studied smoker Spanish women, although the number of samples of smokers in the present study was too low to be statistically comparable. Regarding GAMA3 (Figure 31b), higher urinary levels were found in the North-American population[213,386], non-smoker females from Germany[224], smoker women from Poland[387], and Taiwanese men[391], compared to the population under study. The rest of the studies had similar or lower levels of urinary GAMA3 comparing with the present survey. Concerning AAMA-Sul, no other studies have been found reporting urinary levels of this metabolite in adults.

Due to the fact that GA is the genotoxic agent in AA exposure[392], the ratio GAMA3/AAMA, which reflects the rate of oxidative metabolism, was also determined with values ranging from 0.06 to 0.48, with a median of 0.21 in the women under study (Table 22). As some HBM studies did not report this value, it was calculated using the median concentrations reported for GAMA3 and AAMA in each study for comparison purposes. Similar ratios were found in the present study compared to those observed in the German population (0.15 – 0.23)[393] and North-American adults (0.18 – 0.23)[212,213]. However, there are other studies showing ratios higher than 0.30[224,386,387,394], and others showing lower ratio values, ranging from 0.04 to 0.16[211,388,389,395–397]. These considerable variations among populations were probably due to differences in the metabolism of individuals, in the exposure dose, or in the type of urine sample used in each study[398,399]. For smokers, GAMA3/AAMA ratios were lower than those calculated for non-smokers in the present study (0.16 vs 0.22), which was in line with the results reported by Mojska et al. (2016)[387] and Urban et al. (2006)[393], and ratios estimated for the rest of studies. This might be explained by a possible inhibition of the cytochrome P450 2E1 activity by nicotine from cigarette smoke[398].

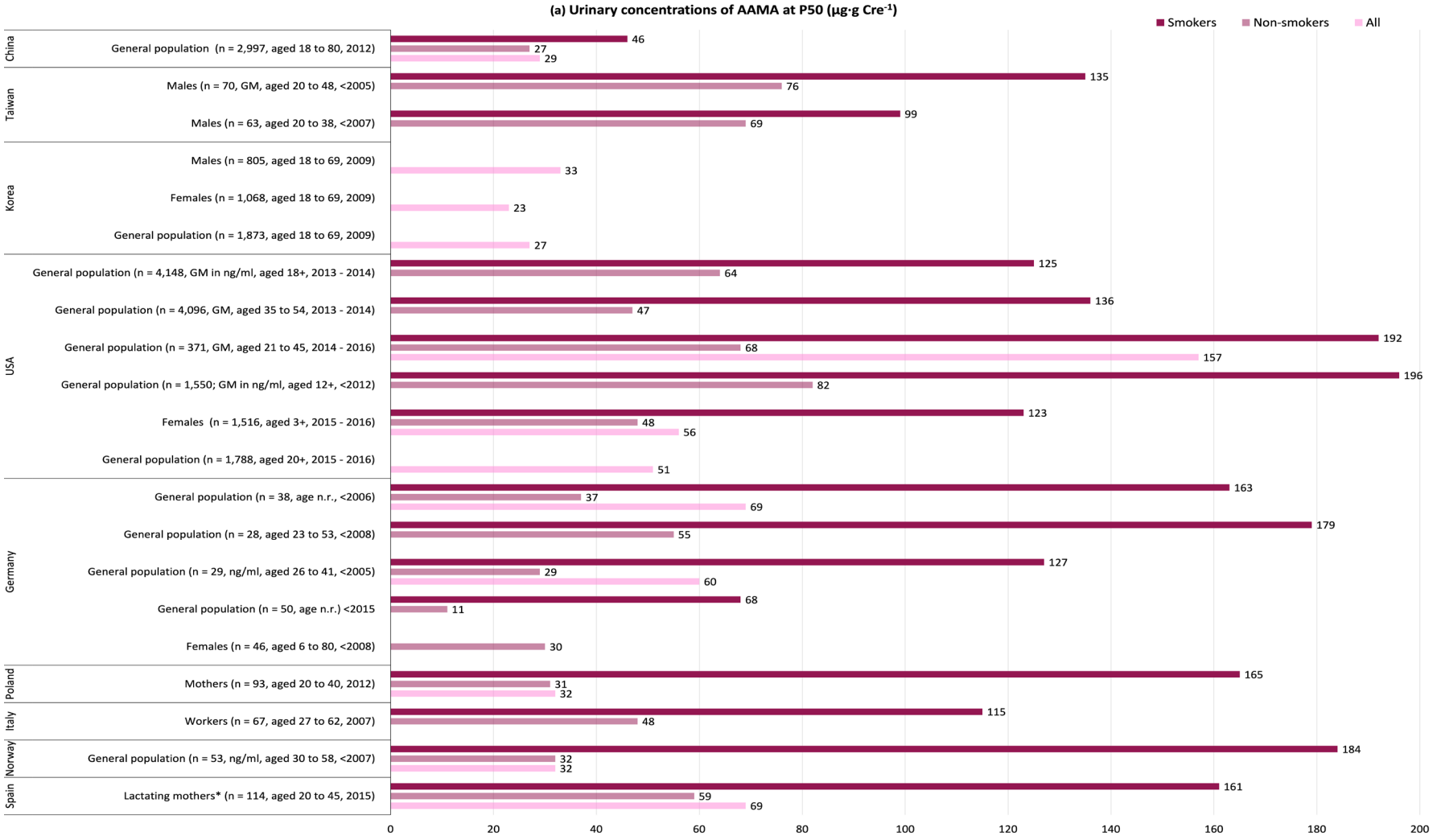

Figure 31a. Comparison of existing HBM data of urinary AAMA (µg·g Cre^{-1}) in mothers, general population, females, males, and workers (number of samples, age range and sample collection years within parenthesis) stratified by smoking habit across the following countries: Spain (*Present study), Norway[388], Italy[211], Poland[387], Germany[224,389,390,394,395], USA[212–214,386,400,401], Korea[402,403], Taiwan[383–385], and China[396,397]. *P50: 50th percentile; GM: geometric mean; cre: creatinine; '+' means 'older than'; n.r.: not reported.*

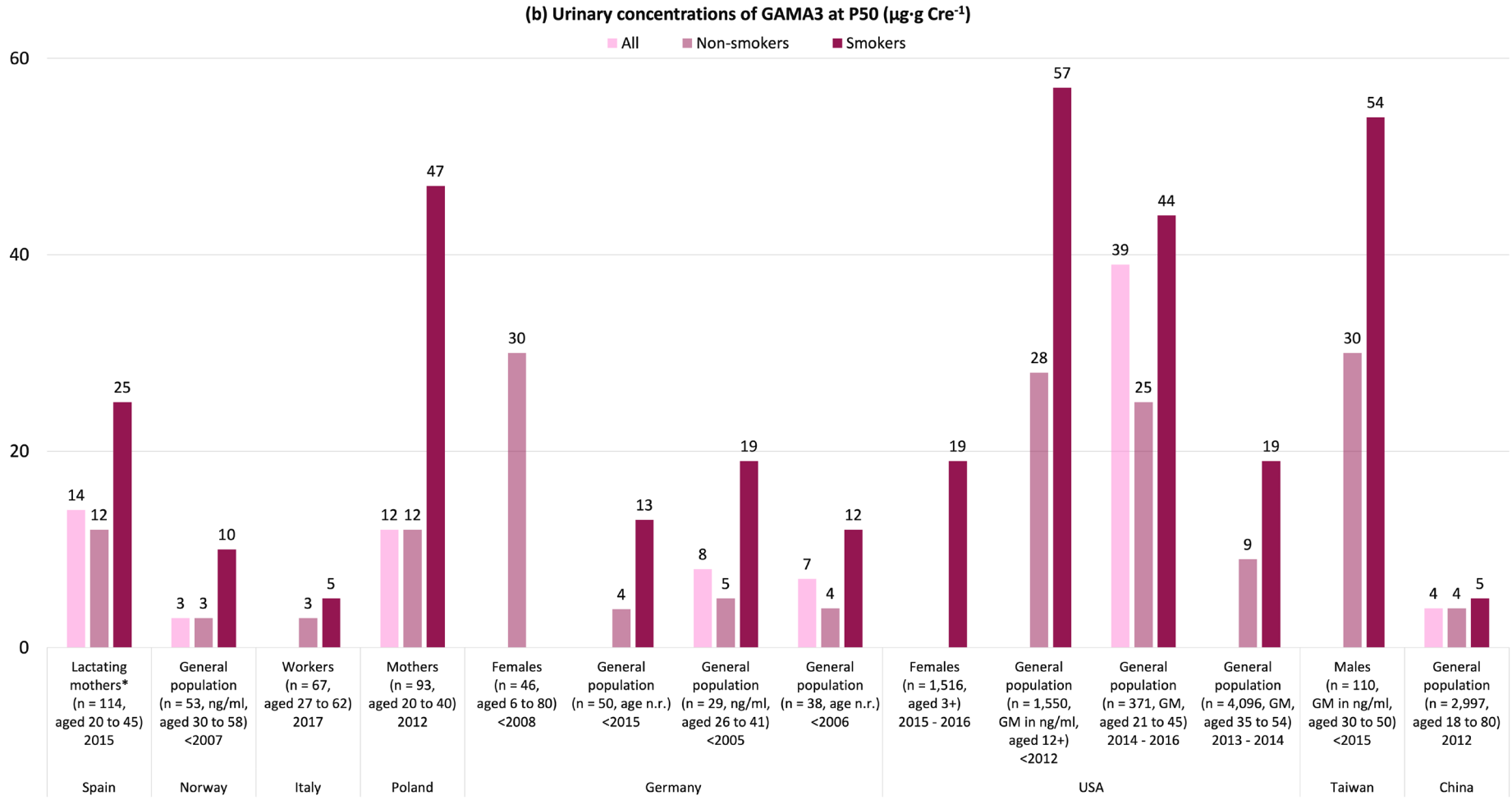

Figure 31b. Comparison of existing HBM data of urinary GAMA3 (µg·g Cre^{-1}) in mothers, general population, workers, females, and males at different sample collection years (number of samples and age range within parenthesis) stratified by smoking habit across the following countries: Spain (*Present study), Norway[388], Italy[211], Poland[387], Germany[224,389,394,395], USA[212,213,386,400], Taiwan[391] and China[396,397]. *P50: 50th percentile; GM: geometric mean; cre: creatinine; '+' means 'older than'; n.r.: not reported.*

6.1.1.2 STATISTICAL ANALYSIS

The Spearman correlation matrix, including AAMA, GAMA3 and AAMA-Sul, showed highly statistically significant correlations among all biomarkers (p-values < 0.01), with Spearman coefficients closer to 1. This fact indicated that AA was the parent compound for the three mercapturic acids analyzed in urine.

Figure 32 presents the results of the log-linear regression analysis obtained for the sum of the three AA metabolites (∑UAAM) (n = 91). In total, the variables included in the regression model were able to explain 61 % of the variability in ∑UAAM. Among the food groups studied by regression analysis, high consumption of coffee was found to be highly related to increased levels of ∑UAAM. This was not an unexpected finding, since previous HBM studies have identified the intake of coffee as an important predictor of AA levels in urine[220,388,404], being responsible for approximately 11 % of the AA intake in coffee drinkers[404]. Furthermore, EFSA showed that the highest AA levels among many food commodities are found in coffee, since it can be formed during the roasting process[181]. Therefore, as it was also shown in the present research, coffee intake is a major contributor to AA dietary exposure in adults[181]. By contrast, according to Molina Périz et al. (2016), coffee consumption is responsible for only 3 % of the daily intake of AA in the Spanish population[405].

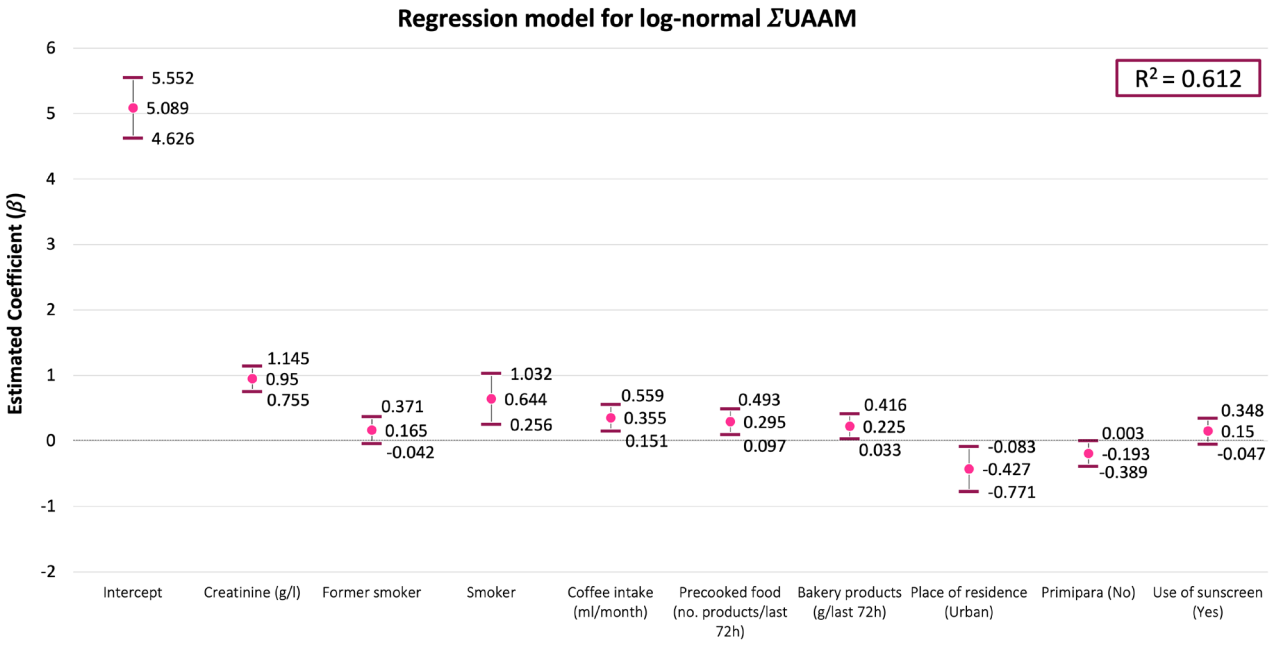

Figure 32. Linear regression model fitted for the log-transformed ∑UAAM (n = 91, only participants with a completed questionnaire were considered), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2) for the BETTERMILK study. *no.: number*.

Another relevant contributor to AA exposure observed in the present study was the intake of bakery products, as also previously reported by some HBM[226,404,406] and dietary studies[181,405]. The recent consumption of pre-prepared food was also significantly associated with higher ∑UAAM concentrations. This is in line with two studies on pre-cooked products[407,408], which found high AA concentrations in these foodstuffs even before heat treatment at home, due to their favorable environment for AA formation during processing and storage[407,408]. However, more research is needed on the occurrence of AA in this kind of food products.

Potatoes and cereal consumption were reported to be the main contributors to total dietary exposure to AA by many authors[181,223,226], but no relationship with ∑UAAM levels was observed in the present study. This could be due to differences in home-cooking habits of the participants[181] and the lack of data about the recent consumption of potato-based products in the present study, since AA metabolites are rapidly excreted in urine a few hours after dietary exposure[203].

Apart from diet, tobacco smoke seemed to be a strong contributor of AA internal exposure in smoker women, as it was observed in many recent studies [220,223,383,387,402]. However, in the present study, this subgroup sample size was too small (8 % of the women) to draw conclusive findings. Another significant socio-demographic characteristic contributing to AA exposure was the place of residence, showing higher ∑UAAM concentrations in those women who lived in rural areas compared to those living in urban areas. Nevertheless, although similar results were obtained in a study on Korean children[220], no explanation was found in the literature to interpret this observation. A positive association was also found between the use of sunscreen and ∑UAAM levels, but not at a significant level (p-value > 0.05), probably because it is a minor contributor to AA exposure as a residual monomer in cosmetic preparations[409].

6.1.1.3 RISK ASSESSMENT

In general, the risk characterization of AA exposure is carried out through dietary studies, taking into account only external exposure by food consumption[181]. A different perspective was used in the present study since the internal exposure to AA at GM and P95 distribution levels were considered in the risk evaluation using both reverse and forward dosimetry. For smokers, only the GM was considered, since the sample size was too low (n = 9) to calculate the P95.

For the reverse dosimetry, daily intakes of AA were estimated based on molar concentrations of $\sum UAAM_{Vol}$ and $\sum UAAM_{Cre}$ stratified by the smoking habit of the subjects. The mean EDIs adjusted by creatinine (EDI_{Cre}) and by volume (EDI_{Vol}) considering all women (smokers, non-smokers and former smokers) were 1.2 and 1.9 μg AA·kg^{-1}·day^{-1}, respectively, which were in the range of those EDIs derived for the European population (0.4 – 1.9 μg AA·kg bw^{-1}·day^{-1})[181] and higher than the EDIs derived from diet studies carried out in Spain (0.83 μg AA·kg bw^{-1}·day^{-1}) and, specifically, in adult women of the Valencian Region (0.54 μg AA·kg bw^{-1}·day^{-1})[405]. In the 95th percentile, EDI_{Cre} (3.3 μg

AA·kg bw^{-1}·day^{-1}) and EDI_{Vol} (6.8 µg AA·kg bw^{-1}·day^{-1}) in the present research were higher than those reported by EFSA (0.6 – 3.4 µg AA·kg bw^{-1}·day^{-1})[181]. These differences could be probably because in the present study not only dietary exposure was considered, but also other exposure determinants that contributed to the total AA exposure, such as tobacco smoke. In fact, EDI_{Cre} and EDI_{Vol} among smoker participants (2.6 and 3.9 µg AA·kg^{-1}·day^{-1}, respectively) in the BETTERMILK study were between two and three times higher than those who never smoked (1.0 and 1.5 µg AA·kg^{-1}·day^{-1}, respectively) or were former smokers (1.4 and 2.1 µg AA·kg^{-1}·day^{-1}, respectively). Comparing with other HBM studies, which reported EDIs ranging from 0.15 to 1.04 µg AA·kg bw^{-1}·day^{-1} [221,224,226,387,406], the EDIs obtained here were the highest reported so far, showing a higher exposure in the Spanish women under study. However, these discrepancies could also be due to the different assumptions made in each study to estimate the daily intake of AA based on HBM data.

Figure 33a shows the interpretation of the obtained EDIs in terms of risk using the MOE concept adjusted by volume (MOE_{Vol}) and by creatinine (MOE_{Cre}). In this approach, EDIs were compared to the $BMDL_{10}$ obtaining mean MOEs from 423 (MOE_{Cre} in non-smokers) to 109 (MOE_{Vol} in smokers), which were between 3 and 7 times lower than MOE values reported in European adults (1075 – 717)[181]. In general, MOEs at the P95 distribution level were lower than or close to 125, showing a possible health concern for neurotoxicity in the population under study.

In the forward dosimetry approach, a direct comparison of the obtained urinary concentrations of AAMA to the BEs was made. For this purpose, prioritization schemes representing BE and BE_{POD} values (see Figure 33b), and the calculation of HQs using BEs, were used as complementary alternatives to estimate the risk associated to AA internal exposure. Concerning the prioritization diagrams, mean AAMA levels found in the women participating in the BETTERMILK project were substantially higher than the BE, showing overall HQs from 4.4 to 5.5, but lower than the BE_{POD} for non-cancer risks. Consequently, the exposure to AA in the assessed Spanish population may be considered a medium priority for risk assessment follow-up. Similar results were found in HBM studies in Germany[223], USA[287] and Canada[229,410]. Taking the 95th percentile into account, the observed AAMA concentrations were about twice the BE_{POD} values (Figure 33b), with HQs between 10.5 and 22.1, exceeding the high priority line. Regarding the smoking status of the participants, non-smokers showed HQs between 3.5 and 4.5, while smokers had AAMA urinary concentrations similar to the BE_{POD}, resulting in HQs ranging from 10 to 12.

With respect to the different risk assessment strategies used in HBM studies, it should be noted that there is a need to harmonize methodologies for the risk evaluation criteria of HBM data in a global context. As it can be observed, forward dosimetry showed that AA exposure is a health concern for non-cancer risks at both distribution levels (GM and P95), since urinary levels of AAMA were above the BE with HQs >> 1. On the contrary, reverse dosimetry only indicated a concern with respect to non-neoplastic effects in the case of smokers and in the 95th percentile, where some MOEs were below 125.

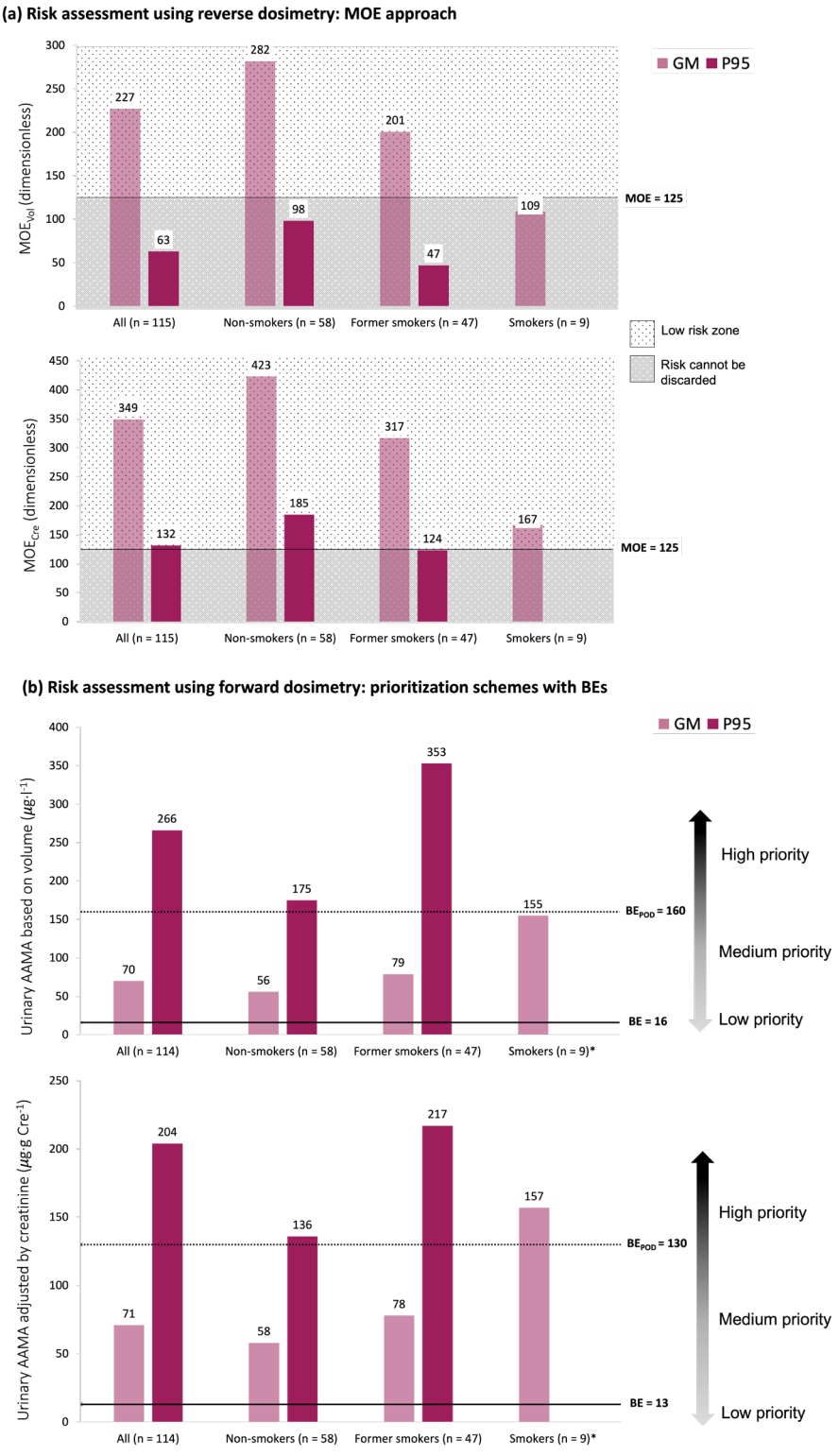

Figure 33. Interpretation of biomonitoring data of exposure to acrylamide at GM and P95 adjusted by volume and creatinine and stratified by the smoking habit of the mothers participating in the BETTERMILK study in a risk context using different screening tools: (a) Reverse dosimetry: representation of MOEs; and (b) Forward dosimetry: prioritization diagrams using BEs. *Number of smoker women was too low to calculate the risk at P95. *MOE: margin of exposure; Vol: adjusted by volume; Cre: adjusted by creatinine; GM: geometric mean; P95: 95th percentile; BE: biomonitoring equivalent; BE$_{POD}$: biomonitoring equivalent directly derived from a point of departure (POD) without considering intraspecies uncertainty factors.*

In summary, both risk assessment strategies showed that AA exposure might indicate a health concern with respect to its non-carcinogenic toxicity in the Spanish population. Therefore, it is concluded that there is a need to reduce this exposure and to follow-up the risk assessment in this country.

6.1.2 CONCLUSIONS

This research work is the first study reporting data on AA internal exposure in the Spanish population. The obtained results showed that 100 % of the Spanish women (n = 117) were exposed to AA, with mean EDIs of 1.2 – 1.9 μg AA·kg bw^{-1}·day^{-1}. The high intake of coffee, bakery and precooked products were strongly associated with increased urinary levels of AA metabolites. The risk assessment showed mean HQs ranging from 4.5 (all participants) to 12 (smokers), and mean MOEs between 423 (non-smokers) and 109 (smokers). These results confirm that AA exposure may present a concern with respect to its non-neoplastic effects in the target population, showing that further regulation and risk management activities, such as a risk assessment follow-up, are necessary to minimize AA exposure in Spain.

6.1.3 ARTICLE III.1. *EXPOSURE ASSESSMENT OF SPANISH LACTATING MOTHERS TO ACRYLAMIDE VIA HUMAN BIOMONITORING*

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Exposure assessment of Spanish lactating mothers to acrylamide via human biomonitoring

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ABSTRACT

Acrylamide (AA) is an organic compound classified as "Probably carcinogenic to humans" (Group 2 A) that can be found principally in processed carbohydrate-rich foods and tobacco smoke. In humans, after exposure, AA is rapidly metabolized and excreted in urine, predominantly as N-acetyl-S-(2-carbamoylethyl)-l-cysteine (AAMA), N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-l-cysteine (GAMA3) and N-Acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine (AAMA-Sul), which can be used as short-term biomarkers of exposure to AA.

In this study, the presence of AA metabolites in urine samples of lactating mothers living in Spain (n = 114) was analyzed by "dilute and shoot" and liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). All urinary metabolites were detected in 100% of the analyzed samples, with geometric means of 70, 33 and 15 ng ml^{-1}, for AAMA, AAMA-Sul and GAMA3, respectively. The consumption of coffee, bread and precooked food products were found to be significant predictors of internal exposure to AA. An estimated daily intake (EDI) of AA based on its urinary metabolites was calculated, obtaining mean values between 1.2 and 1.9 μg AA·kg bw^{-1}·day^{-1} in the target population. The risk assessment was evaluated using both reverse and forward dosimetry, showing an average margin of exposure (MOE) of 349 and a hazard quotient (HQ) of 5.5. Therefore, AA exposure should be considered a medium priority for risk assessment follow-up in the Spanish population, since a health concern with respect to non-neoplastic toxicity could not be discarded.

1. Introduction

Acrylamide (AA) or 2-propenamide (CAS number 79-06-01) is a low molecular weight, highly water-soluble organic compound, which is produced worldwide for numerous applications in the chemical industry (EFSA, 2015; World Health Organization, 2011). Commercially, it is used as a monomer in the synthesis of polyacrylamide polymers, which are employed in the paper and textile industries, as flocculant in water treatment, for building and construction purposes, in cosmetics, and in food packaging (EFSA, 2015). Moreover, AA is also present in tobacco products (McAdam et al., 2015).

In 2002, AA was discovered to be formed in carbohydrate-rich foods, mainly potatoes and some grains, when cooking under low moisture conditions and temperatures above 120 °C (e.g. by frying, baking and roasting), via Maillard reaction. This chemical reaction takes place between the amino group of certain amino acids (e.g. asparagine) and the carbonyl group of reducing sugars (e.g. glucose), naturally present in these foods (Mottram et al., 2002; Stadler et al., 2002). Indeed, according to the European Food Safety Authority (EFSA), the most important contributors to AA exposure in non-smokers are the consumption of cereal products, French fries and other potato-based products, bread, biscuits and coffee (EFSA, 2015).

Experimental studies have shown that AA produces neurotoxic, carcinogenic, mutagenic, genotoxic, and immunotoxic effects on

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animals (Eisenbrand, 2020). In humans, there are no consistent evidences about the carcinogenic effects of AA, but it has been found to have negative effects on children's development (Duarte-Salles et al., 2013; Pederson et al., 2012), since it is able to be transferred to the fetus through the placenta and blood-brain barriers, and through breast milk consumption in breastfed infants (Schettgen et al., 2004; Sörgel et al., 2002). In fact, Sörgel et al. (2002) found quantifiable AA levels in human milk, which could pose a risk to infants during breastfeeding, concluding that nursing mothers should avoid foods containing this substance. This makes breastfeeding mothers a vulnerable population group due to the exposure to AA and a target of future research.

Considering the available toxicological evidence, AA was classified in group 2 A as "Probably carcinogenic to humans" by the International Agency for Research on Cancer (IARC) (IARC, 1994). Moreover, some uses of AA are restricted under Annex XVII of REACH (Registration, Evaluation, Authorisation and Restriction of Chemicals) and it is included in the candidate list of Substances of Very High Concern for Authorization (European Chemicals Agency, 2021). In addition, due to its widespread exposure in general and vulnerable population (e.g. children and lactating/pregnant women) and its potential threat to health, different regulatory measures have been taken in Europe in order to mitigate the human exposure to AA. Since 2007, AA levels in food have been monitored in the European Union (EU) under the Recommendation 2007/331/EC (further extended by Recommendation (EU) 2019/188) (European Commission, 2007). In 2015, the Scientific Panel on Contaminants in the Food Chain (CONTAM panel) of the EFSA published a scientific opinion on AA, concluding that AA levels in food products were not consistently decreased in previous years, and dietary exposure to AA in the European citizens indicated a concern with respect to its carcinogenicity (EFSA, 2015). As a result, in 2017, the European Commission established a regulation containing "mitigation measures and benchmark levels for the reduction of the presence of acrylamide in food" (Regulation (EU) 2017/2158) (European Commission, 2017a). In addition to this, the use of AA was banned in cosmetic products (Regulation (EC) 1223/2009) (The European Parliament and The Council of the European Union, 2009) and in food-contact materials (Regulation (EU) 10/2011) (European Commission, 2011a), and a maximum level of 0.10 μg l^{-1} was established for AA residues in drinking water by the Council Directive 98/83/EC (The Council of the European Union, 1998).

General population is exposed to AA mainly by ingestion of food and inhalation of cigarette smoke (HBM4EU, 2019a). For exposed workers, the dermal uptake is also a relevant route of AA exposure (European Commission, 2011b). Once the exposure occurs, AA is rapidly absorbed and distributed to all body tissues. In phase I of its metabolism, AA is partly metabolized by cytochrome P450 2E1 to glycidamide (GA) (Kocadağli and Gökmen, 2016). In addition, AA and GA can form hemoglobin adducts (AAVal and GAVal, respectively), normally used as biomarkers of long-term exposure to AA (Goempel et al., 2017; Hagmar et al., 2005). In phase II, AA and GA are conjugated with glutathione and converted to their corresponding mercapturic acids (MA), N-acetyl-S-(2-carbamoylethyl)-l-cysteine (AAMA) and N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-l-cysteine (GAMA3), respectively, which are excreted in urine (Kocadağli and Gökmen, 2016). In humans, AAMA can be also oxidized to its corresponding sulfoxide (AAMA-Sul) (Zhang et al., 2015). About 50% of the AA is eliminated in the urine during the first 24 h after the ingestion, so its urinary metabolites can be used as biomarkers of recent exposure (Kocadağli and Gökmen, 2016).

Risk assessment of AA exposure in humans is commonly carried out by estimating its daily intake per kg of body weight based on its occurrence in food commodities, and comparing it with the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$) derived for animals as a health reference value (EFSA, 2015). However, this approach only takes into account the exposure derived from the dietary ingestion. Instead, human biomonitoring (HBM) could be a reliable alternative to obtain the total exposure dose to AA from all possible sources by analyzing its metabolites in urine or blood (Louro et al., 2019). For this purpose, Biomonitoring Equivalents (BEs) have been proposed as screening tools for HBM data interpretation in a risk assessment context. A BE is defined as the concentration of a chemical or its metabolites in a biological sample (blood, urine, etc.) that is consistent with an existing health-based exposure guidance value (Hays and Aylward, 2009). Hays and Aylward (2008) derived BEs for the interpretation of the concentration of AA metabolites in urine (13 μg AAMA·l^{-1}) and blood (8 and 13 fmol mg $globin^{-1}$ for AAVal and GAVal, respectively). In general, urine is the preferred specimen for AA biomonitoring. Its urinary metabolites are good biomarkers of recent exposure to AA since they are stable compounds that can be quantified with high specificity and sensitivity (HBM4EU, 2019a). In addition, urine is a non-invasive matrix, easy to obtain in large volumes.

Nevertheless, there are very few HBM data on AA exposure in the general population and vulnerable groups (Choi et al., 2019; Ji et al., 2013; Mojska et al., 2020; Schwedler et al., 2021), since most of the recent studies are focused on smokers (Frigerio et al., 2020; Goniewicz et al., 2018; Keith et al., 2020; Smith et al., 2020) or occupationally exposed population (Hagmar et al., 2001; Huang et al., 2011; Pennisi et al., 2013; Sams et al., 2015; S. Y. Wang et al., 2020). Thus, more HBM research is needed to gain knowledge of the internal AA exposure across the general and vulnerable population (HBM4EU, 2019a).

For the first time, data on urinary levels of AA metabolites in Spanish lactating women are presented and put in a risk assessment context using BEs. In addition, a daily intake of AA is estimated and potential predictors of exposure are investigated by multiple regression analysis.

2. Material and methods

2.1. Study design

The details of the BETTERMILK project were previously described in Yusà et al. (2017). In brief, 120 pregnant women aged 20–45 years were recruited from the University and Polytechnic Hospital "La Fe" (Valencia, Spain), where they gave birth between June and November 2015.

2.1.1. Data collection

All participants completed a questionnaire containing a) sociodemographic information, including the participants' age, body mass index (BMI), smoking status, use of cosmetics, among others; b) dietary data using a 72-h recall and c) the food consumption frequency during the previous months. A set of the provided information is summarized in Table 1, where qualitative variables are described with absolute frequencies and percentages, and quantitative variables by their mean, minimum and maximum values.

2.1.2. Sample collection

In total, 119 out of 120 women who agreed to participate in the study provided a first-morning urine sample, which was collected in a polypropylene container between 2 and 8 weeks after giving birth. All samples were kept at 4 °C in their households and then transferred to the Biobank for Biomedical and Public Health Research of the Valencian region (IBSP-CV Biobank, Spain, PT13/0010/0064), which is a member of the Spanish National Biobanks Network and the Valencian Biobanking Network, where they were aliquoted and stored at −80 °C. The University and Polytechnic Hospital "La Fe" (Valencia, Spain) was in charge of measuring creatinine concentration in urine samples using the Jaffé reaction colorimetric method. Those samples with a creatinine concentration outside the range of 30–300 mg dl^{-1} were not included in the present study (Barr et al., 2005).

Ethical approval

This study was approved by the Clinical Research Ethics Committee of the Public Health Directorate, the Center for Public Health Research of the Valencian Government, and the Biomedical Scientific Ethics

Table 1
Lifestyle, socio-demographic characteristics and dietary habits of the studied population (n = 114)[a].

Socio-demographic characteristics	n (%)	Mean (minimum – maximum)
Number of children	–	–
1	67 (59%)	–
2 or more	47 (41%)	–
Age (years)	–	34 (20–45)
Missing data	1 (1%)	–
Body mass index (BMI) before pregnancy ($kg{\cdot}m^{-2}$)	–	23 (17–35)
Missing data	2 (2%)	–
Country of birth	–	–
Spain	99 (87%)	–
Foreign	13 (11%)	–
Missing data	2 (2%)	–
Home location	–	–
Urban	99 (87%)	–
Rural	11 (10%)	–
Missing data	4 (3%)	–
Educational level	–	–
Only primary school	9 (8%)	–
Secondary school	28 (25%)	–
University degree	77 (67%)	–
Lifestyle characteristics	**n (%)**	
Occupational Status	–	–
Employed	94 (82%)	–
Unemployed	18 (16%)	–
Missing data	2 (2%)	–
Weekly amount of physical activity	–	–
Frequently (3 or more days/week)	19 (17%)	–
Occasionally (1 or 2 days/week)	18 (16%)	–
Hardly ever	74 (64%)	–
Missing data	3 (3%)	–
Smoking habit	–	–
Smoker	9 (8%)	–
Former smoker	47 (41%)	–
Non-smoker	58 (51%)	–
Use of sunscreen	–	–
Yes	43 (38%)	–
No	71 (62%)	–
Frequency of sunscreen's use	–	–
Never/Occasionally	74 (65%)	–
Weekly	10 (9%)	–
Daily	26 (23%)	–
Missing data	4 (3%)	–
Use of body cream	–	
Yes	89 (78%)	–
No	25 (22%)	–
Frequency of body cream's use	–	–
Never/Occasionally	25 (22%)	–
Weekly	10 (17%)	–
Daily	67 (59%)	–
Missing data	2 (2%)	–
Dietary habits related to AA exposure	**n (%)**	**Mean (minimum – maximum)**
Cereal products (portions in the last 72 h)	-	6 (2–20)
Bakery products (portions in the last 72 h)	–	2 (0–18)
Precooked food (portions in the last 72 h)	–	0.2 (0–2)
Packaged products (portions in the last 72 h)	–	14 (1–39)
Monthly coffe intake ($ml{\cdot}month^{-1}$)	–	970 (50–6770)
Monthly potatoes intake ($g{\cdot}month^{-1}$)		1584 (140–5820)
Missing data	6 (5%)	–

[a] Two donors did not collect enough volume of urine to carry out the analysis and 3 of them were excluded due to abnormal creatinine concentration (out of the range 30–300 mg dl^{-1}).

Committee of the University and Polytechnic Hospital "La Fe". All participants provided a written informed consent prior to the data and sample collection. All personal data were processed (collected, handled, transferred, and analyzed) in a secure setting and were not kept for longer than necessary for processing. Data was processed in accordance with all national and EU legal and ethical considerations, in particular the General Data Protection Regulation.

2.2. Chemicals

Water (LC-MS and HPLC grade) and acetic acid were obtained from VWR Prolabo (Barcelona, Spain) and Merck (Barcelona, Spain), respectively. Certified standards of AAMA, GAMA3 dicyclohexylammonium Salt, AAMA-Sul sodium salt, and their isotopically labelled analogues, specifically AAMA-d_3, GAMA3-d_3 dicyclohexylammonium salt, and AAMA-Sul-d_3 sodium salt, were purchased from Toronto Research Chemicals, Inc. (Toronto, Canada). All certified standards and their internal standards (IS) were of analytical purity (>97%). Standard Reference Materials® (SRM) 3672 and 3673 were supplied by the US National Institute of Standards and Technology (NIST, USA). Chemical information of the analyzed AA metabolites and their corresponding IS are presented in Table S1. Stock and working solutions were prepared as described in the supplementary material (SM1).

2.3. Determination of AA metabolites in urine

2.3.1. Urine preparation and analysis

A "dilute-and-shoot" method based on Brisson et al. (2014) was used to analyze AAMA, GAMA3 and AAMA-Sul in the urine samples. Briefly, after allowing the urine samples to thaw at room temperature, an amount of 200 μl of the homogenized sample was transferred into a 1.5-ml injection vial and spiked with 10 μl of the IS working solution and 790 μl of 0.08% acetic acid in water. Then, the mixture was shaken and 20 μl were injected into a high-performance liquid chromatography system coupled to a tandem mass spectrometer (HPLC-MS/MS).

An isocratic elution with 0.08% acetic acid in water with a flow rate of 400 μl min^{-1} was used for the chromatographic separation of the three AA metabolites in a Vanquish HPLC system from Thermo Fisher Scientific Inc. (San Jose, CA, USA), equipped with an Atlantis dC_{18} column (150 mm × 2.1 mm, 2.6 μm, 100 Å) from Waters (Milford, MA, USA) at 40 °C. The detection was performed in a TSQ Altis™ Triple Quadrupole Mass Spectrometer from Thermo Fisher Scientific Inc. The instrument operated with a heated electrospray ionization source (HESI) in negative ion mode, and with selected reaction monitoring. The precursor/product ion pairs, collision energies, and other spectrometric parameters used for determining the different target compounds and

their IS are summarized in Table S1. The ion transfer tube and vaporizer temperatures were set at 325 and 350 °C, respectively; the ion spray voltage was 2500 V; the sheath, auxiliary and sweep gas pressures were set to 50, 10 and 0 arb. Units, respectively; and the collision gas pressure was 1.0 mTorr. The total run time was 10 min. The limit of quantification (LoQ) was 0.25 ng ml^{-1} for all metabolites. Data acquisition and processing was carried out using the Thermo Scientific Trace Finder™ 5.1 software.

2.3.2. Quality assurance and quality control (QA/QC)

The analyses were conducted under a highly consolidated quality system established by UNE-EN ISO/IEC 17025:2017. Quantification of AA metabolites was performed with a calibration curve in water with concentrations between 0.25 and 1000 ng ml^{-1} using the analyte to IS peak area ratio. In order to ensure a proper quantification, each batch included quality controls (QCs) at LoQ (0.25 ng ml^{-1}), medium (50 ng ml^{-1}) and high (1000 ng ml^{-1}) levels as well as a reagent blank to assess for any background contribution during the analysis. The Bioanalytical Method Validation Guidance for Industry was used as a reference for acceptance criteria for QA/QC and method validation (U.S. Department of Health and Human Services, 2018), as it is further described in SM2. Results of the method validation are presented in Table S2. The quantification and confirmation criteria for the HPLC-MS/MS were based on the SANTE/11813/2017 guide (European Commission, 2017b), as it is described in SM2.

Additionally, QA was externally assessed through participation in the German External Quality Assessment Scheme (G-EQUAS) intercomparison programs, and internally by using the SRM 3672 (urine of smokers) and 3673 (urine of non-smokers), resulting in satisfactory results ($|z\text{-scores}| \leq 2$). The quantification of AAMA-Sul was not evaluated externally due to the lack of certified reference materials or interlaboratory assays.

2.4. Determinants of exposure

A complete descriptive analysis of the urinary levels of AAMA, GAMA3 and AAMA-Sul was performed by calculating the most relevant descriptors: detection frequency (DF, %), arithmetic mean (AM), geometric mean (GM), 25^{th}, 50^{th}, 75^{th}, and 95^{th} percentiles (P25, P50, P75, and P95), minimum, maximum, and standard deviation (SD) in ng·ml^{-1} and μg·g^{-1} creatinine.

For data analysis, IBM SPSS 26.0 (2019) was used. A Spearman correlation test was performed to determine the relationship among the three urinary AA metabolites. As recommended by the Human Biomonitoring for Europe initiative (HBM4EU, 2019b), a forward stepwise selection procedure based on the corrected Akaike information criterion (AICc) (Konishi and Kitagawa, 2008) was selected to build a linear regression model. For this purpose, the logarithmic transformation of the sum of urinary AA metabolites (∑UAAM) (in nmol·l^{-1}), all variables from Table 1 and urinary creatinine concentration were used. The quantitative explanatory variables were divided by twice their SD in order to obtain directly-comparable regression coefficients (Gelman, 2008). Confidence intervals at 95% significance were calculated for the regression model coefficients, and a p-value < 0.05 was considered as a statistically significant relationship. Finally, Shapiro-Wilk test (normality of residuals), Durbin-Watson statistic (no autocorrelation), scatter plots (homoscedasticity), and tolerance and condition indexes (no multicollinearity), were used to verify the regression assumptions.

2.5. Risk assessment

HBM data can be put in a risk assessment context in different ways. In the present research, the obtained urinary levels of AA metabolites were interpreted using the two main approaches currently used in HBM studies: reverse and forward dosimetry (Gurusankar et al., 2017), as it is explained in the following sections. Although AA has been shown to have neoplastic effects in mice, only the risk of non-neoplastic effects was evaluated in the present study due to the lack of epidemiological studies demonstrating that AA is a human carcinogen (EFSA, 2015). In addition, it is important to highlight that some authors stated the possibility of the endogenous formation of AA and its metabolites in the human body (Goempel et al., 2017; Mojska et al., 2016; WHO, 2002).

2.5.1. Reverse dosimetry

In the reverse dosimetry approach, urinary levels of AA metabolites are converted to an estimate daily intake (EDI) based on dietary exposure to AA using pharmacokinetic data (Zidek et al., 2017). After that, EDI is compared to the $BMDL_{10}$ for neurotoxicity in rats as a health-based reference value by calculating the margin of exposure (MOE) (EFSA, 2015).

2.5.1.1. Estimation of daily AA intake. EDIs of AA based on the urinary mercapturic acids concentrations were calculated at GM and P95 levels, and were stratified by the smoking habit of the target population (non-smokers, former smokers and smokers). For this purpose, equations (1) and (2) were used to convert ∑UAAM expressed as volume-based (μmol·l^{-1}) and creatinine-based concentration (μmol·g Cre^{-1}), respectively, in the EDI of AA based on dietary ingestion (Heudorf et al., 2009; Mojska et al., 2020), as follows:

$$EDI_{Vol}\left(\frac{\mu g\ AA}{kg\ bw{\cdot}day}\right) = \frac{\sum UAAM_{Vol} \times V_{24h} \times MW_{AA}}{F_{UE} \times BW} \quad (1)$$

$$EDI_{Cre}\left(\frac{\mu g\ AA}{kg\ bw{\cdot}day}\right) = \frac{\sum UAAM_{Cre} \times Cre_{24h} \times MW_{AA}}{F_{UE} \times BW} \quad (2)$$

where ∑UAAM = GM or P95 of the sum of the urinary molar concentrations of AAMA, GAMA3 and AAMA-Sul (μmol·l^{-1} for EDI_{Vol}, or μmol·g Cre^{-1} for EDI_{Cre}); V_{24h} = total urinary volume excreted within 24 h for adults (1.6 l·day^{-1}) (Aylward et al., 2011); Cre_{24h} = estimated daily urinary creatinine excretion normalized by mean body weight (60 kg), age (34 years old) and height (164 cm) of the women under study (1.03 g Cre·day^{-1}), calculated as it is shown in Eq. 3b in Mage et al. (2004); MW_{AA} = molecular weight of AA (71 g mol^{-1}); F_{UE} = fraction of the ingested dose of AA which is eliminated as urinary mercapturic acids (0.5) (Kocadağli and Gökmen, 2016); BW = mean body weight of the participants before pregnancy (60 kg).

2.5.1.2. Calculation of the MOE. The characterization of non-neoplastic risks using EDI values was carried out by comparing them with the $BMDL_{10}$ of 430 μg AA·kg bw^{-1}·day^{-1} derived from the incidence of neurological degeneration in rats (EFSA, 2015). To this end, the MOE approach was used by applying equation (3).

$$MOE\ (dimensionless) = \frac{BMDL_{10}\left(\frac{\mu g\ AA}{kg\ bw{\cdot}day}\right)}{EDI\left(\frac{\mu g\ AA}{kg\ bw{\cdot}day}\right)} \quad (3)$$

According to the EFSA's CONTAM Panel, MOEs above 125 are considered sufficient to conclude that there is no concern for non-genotoxic health effects of AA (EFSA, 2015).

2.5.2. Forward dosimetry

In the present study, the concept of BE was used in the forward dosimetry method. In this approach, no transformation of the HBM data is needed, since the obtained urinary levels of AA can be directly compared to the derived BEs (Gurusankar et al., 2017).

For AA in urine, BEs have been calculated only for AAMA, its major urinary metabolite (Hays and Aylward, 2008). BE values for AA represent the concentrations of urinary AAMA that are consistent with the United States Environmental Protection Agency (US EPA) chronic reference dose (RfD), based on the no-observed-adverse-effect level

(NOAEL) for rats as a non-cancer endpoint, and the pharmacokinetic properties of AA (Hays and Aylward, 2008). This derivation process resulted in two estimated BE levels for AA: i) the BE consistent with the human equivalent point of departure (BE_{POD}), taking into account interspecies extrapolations, but omitting intraspecies uncertainty factors; and, ii) the final BE value, consistent with the selected exposure guidance value, considering both intraspecies and interspecies uncertainty factors (Aylward et al., 2013; Hays and Aylward, 2009). Hays and Aylward (2008) derived BEs and BE_{PODs} for urinary AAMA concentrations based on volume and creatinine levels, with values of 13 and 130 μg l^{-1}; and values of 16 and 160 μg g Cre^{-1}, respectively. In addition, they derived individual BE for different population groups (women, men, adolescents and children) using different parameters, concluding that BEs varied little by age and gender and that a single average value could be chosen for all groups.

In this approach, two complementary strategies were conducted for risk characterization of the obtained urinary levels of AAMA at GM and P95 levels: i) the representation of the AAMA concentrations in prioritization schemes for direct comparison to BEs (Hays and Aylward, 2009); and, ii) the calculation of the hazard quotient (HQ) (Gurusankar et al., 2017). These screening approaches can be useful to set priorities for additional research and strategies to reduce AA exposure, among other risk management activities, but they cannot be used as bright lines that separate "safe" from "unsafe" exposures (Hays and Aylward, 2008, 2009).

2.5.2.1. Construction of the prioritization schemes. Diagrams representing urinary AAMA concentrations (GM and P95) as vertical bars and the existing BEs as horizontal lines were made to provide an initial estimation of whether the observed values were of low, medium, or high priority for further risk assessment follow-up in the target population. The results are interpreted as follows (Hays and Aylward, 2009):

- High priority: urinary AAMA concentrations exceed the human equivalent BE_{POD}.
- Medium priority: urinary AAMA levels are below the BE_{POD} but above the BE.
- Low priority: AAMA levels in urine are below the BE.

2.5.2.2. Calculation of the HQ. The concept of HQ is defined as the ratio of an exposure dose to a reference dose (Gurusankar et al., 2017). In this case, the HQ was calculated as the ratio of the obtained urinary AAMA concentrations (exposure dose) at GM and P95 distribution levels and the derived BEs (reference dose), as follows:

$$HQ_{Vol}(dimensionless) = \frac{C_{AAMA}\ (\mu g/l)}{BE(13\ \mu g/l)} \quad (4)$$

$$HQ_{Cre}(dimensionless) = \frac{C_{AAMA}\ (\mu g/g\ Cre)}{BE\ (16\ \mu g/g\ Cre)} \quad (5)$$

If the calculated HQ is less than one, it is unlikely that there is any risk from AA exposure. Conversely, when HQ is higher than one, it suggests that AA exposure levels may exceed the human health benchmark dose (BE) and a risk could not be discarded. As a result, further research and risk assessment follow-up is necessary (Gurusankar et al., 2017).

3. Results

Urinary mercapturic acids of AA were analyzed in 117 out of the 119 urine samples collected in the BETTERMILK project, since two donors did not provide enough volume of urine to carry out the analysis. In addition, three urine samples were excluded from the study due to an abnormal creatinine concentration (lower than 30 or higher than 300 mg dl^{-1}). In total, 114 urine samples with creatinine levels ranging from 30 to 2400 mg dl^{-1} (mean = 1100 mg dl^{-1}) were included in the present study.

Table 1 summarizes the socio-demographic characteristics and those dietary habits related to AA exposure of the population under study. The mean age of the subjects was 34 years old, and more than 80% of them lived in an urban area and were employed. Of the 114 women, only 9 were active smokers at the time of the sample collection. Potatoes and coffee products intake in the last 72 h were not recorded in the questionnaire of the BETTERMILK project, so the monthly frequency of consumption of these food groups were taken into account instead.

3.1. Urinary levels of AA biomarkers

Descriptive statistics for AAMA, GAMA3 and AAMA-Sul levels found in the urine samples unadjusted and adjusted by creatinine are presented in Table 2. All metabolites were above the LoQ in 100% of the analyzed urines. The highest concentrations were found for AAMA, the major urinary biomarker of AA, with 50th and 95th percentiles of 73 and 266 ng ml^{-1}, respectively. Lower urinary levels were observed for AAMA-Sul and GAMA3, with medians of 36 and 14 ng ml^{-1}, respectively. Very similar concentrations were obtained when adjusted by creatinine. The ratio GAMA3/AAMA, which reflects the rate of oxidative metabolism, was also determined with values ranging from 0.06 to 0.48, with a median of 0.21.

3.2. Determinants of exposure

The Spearman correlation matrix, including AAMA, GAMA3 and AAMA-Sul, is presented in Table S3. According to this table, highly statistically significant correlations were noted among all biomarkers (p-value < 0.01), with Spearman coefficients closer to 1. This fact showed that AA exposure was the parent compound for the three analyzed mercapturic acids in urine.

Fig. 1 presents the results of the log-linear regression analysis obtained for ∑UAAM. As can be seen, creatinine concentration was highly and directly related to ∑UAAM, showing a high dependence of these AA biomarkers on this physiological value. As expected, the smoking habit of the studied women had a significant effect on the internal exposure to AA, since smokers, despite being only 8% of the participants, seemed to have statistically higher ∑UAAM levels than non-smokers. Regarding dietary habits, a positive association was found with the recent consumption of bakery and precooked products. In the same way, a high monthly coffee intake appeared to increase ∑UAAM in urine. Another relevant finding was that the use of sunscreen was directly related to urinary AA metabolites but not at a significant level (p-value > 0.05). On the other hand, lower ∑UAAM concentrations were observed in subjects living in urban areas compared to those living in rural areas. In total, the variables included in the regression model were able to explain 61% of the variability in ∑UAAM.

3.3. Risk assessment

The risk evaluation of the obtained urinary levels of AA metabolites at GM and P95 distribution levels was carried out by two different strategies: reverse and forward dosimetry, based on animal evidence. For smokers, only the GM was considered, since the sample size was too low (n = 9) to calculate the P95.

3.3.1. Reverse dosimetry

For reverse dosimetry, daily intakes of AA were estimated based on molar concentrations of $\sum UAAM_{Vol}$ and $\sum UAAM_{Cre}$ stratified by the smoking habit of the subjects, resulting in EDIs represented in Fig. 2. The mean EDI_{Cre} and EDI_{Vol} were 1.2 and 1.9 μg AA·kg^{-1}·day^{-1}, respectively. In particular, EDIs were between two and three times higher among smoker participants (2.6–3.9 μg AA·kg^{-1}·day^{-1}), compared to those who never smoked (1.0–1.5 μg AA·kg^{-1}·day^{-1}) or were former

Table 2
Descriptive analysis of the urinary AA metabolites (UAAM) in Spanish breastfeeding mothers (n = 114).

Metabolites	LoQ	DF (%)	Min.	P25	P50	AM	GM	P75	P95	Max.	SD	Units
AAMA	0.25	100	10	41	73	99	70	117	266	627	94	$ng{\cdot}ml^{-1}$
AAMA-Sul	0.25	100	3	20	36	44	33	55	109	276	39	
GAMA3	0.25	100	4	9	14	19	15	22	47	112	15	
[a]∑UAAM	–	100	73	297	534	672	500	833	1803	4231	603	$nmol{\cdot}l^{-1}$
Ratio GAMA3/AAMA	–	–	0.06	0.16	0.21	0.22	0.21	0.27	0.39	0.48	0.08	Dimensionless
$AAMA_{Cre}$	–	–	9	45	69	89	71	112	204	586	76	$\mu g{\cdot}g\ Cre^{-1}$
$AAMA\text{-}Sul_{Cre}$	–	–	3	24	32	40	33	50	88	181	28	
$GAMA3_{Cre}$	–	–	4	10	14	17	15	21	32	73	10	
[b]$\sum UAAM_{Cre}$	–	–	67	330	495	608	503	759	1332	3287	460	$nmol{\cdot}g\ Cre^{-1}$

LoQ = Limit of quantification; DF = Detection Frequency (% samples > LoQ); Min. = minimum; P = percentile; AM = Arithmetic mean; GM = Geometric mean; Max. = maximum; SD = Standard deviation.
[a] ∑UAAM = AAMA + AAMA-Sul + GAMA3.
[b] $\sum UAAM_{Cre} = AAMA_{Cre} + AAMA\text{-}Sul_{Cre} + GAMA3_{Cre}$.

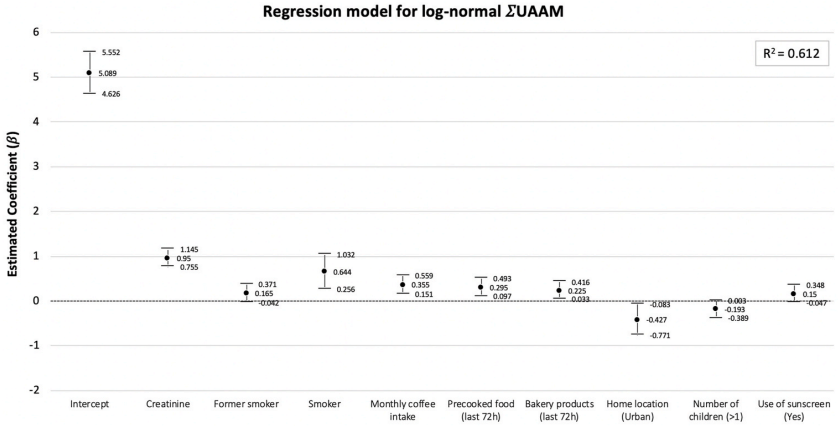

Fig. 1. Standardized coefficients of the linear regression model fitted for the log-transformed urinary concentration of ΣUAAM (n = 91), with their corresponding 95% confidence intervals and coefficient of determination (R^2).

smokers (1.4–2.1 μg $AA{\cdot}kg^{-1}{\cdot}day^{-1}$).

Fig. 3a shows the interpretation of the obtained EDIs adjusted by creatinine in a risk context using the MOE concept. The same data, but using EDIs adjusted by volume, are presented in Fig. S1a. In this approach, EDIs were compared to the $BMDL_{10}$ obtaining mean MOEs from 423 (non-smokers) to 167 (smokers) (Fig. 3a). Only a MOE lower than 125 was found in former smokers at a P95 distribution level. Regarding MOEs based on volume, values lower than 125 were observed at the 95th percentile, ranging from 98 to 47, and at GM level for smokers (MOE = 109) (Fig. S1a).

3.3.2. Forward dosimetry

In the forward dosimetry strategy, a direct comparison of the obtained urinary concentrations of AAMA to the BEs was made. For this purpose, prioritization schemes representing BE and BE_{POD} values, and HQs based on BEs, were used as complementary alternatives to estimate the risk associated to AA internal exposure (see Fig. 3b and S1b).

Concerning the prioritization diagrams, mean AAMA levels found in the BETTERMILK project were higher than the reference BE, showing HQs from 4.4 to 5.5, but lower than the BE_{POD} for non-cancer risks. Consequently, the exposure to AA in the assessed Spanish population may be considered a medium priority for risk assessment follow-up. On the other hand, taking the 95th percentile into account, the observed AAMA concentrations were about twice the BE_{POD} values, with HQs between 10.5 and 22.1, exceeding the high priority line. Regarding the smoking status of the participants, non-smokers showed HQs between 3.5 and 4.5, while smokers had AAMA urinary concentrations similar to the BE_{POD}, resulting in HQs ranging from 10 to 12.

3.3.3. Comparison between risk assessment strategies

The rationale behind the existing approaches to assess AA exposure, previously described in section 2.5, and differences between them, as well as the risk assessment results obtained in each case, are represented in Fig. 4. As it can be observed, forward dosimetry showed that AA exposure is a health concern for non-cancer risks at both distribution levels (GM and P95), since all calculated HQs were above 1. On the contrary, reverse dosimetry only indicated a concern with respect to non-neoplastic effects in the 95th percentile, where some MOEs were below 125.

4. Discussion

Urinary levels of AA metabolites have been reported in other international HBM studies. Table 3 summarizes the main data of recent studies on HBM of AA in adults.

In general, AAMA is the most commonly analyzed AA metabolite in urine, being detected in more than 98% of the samples worldwide, although some differences were observed among studies when comparing the median urinary levels of this metabolite. Firstly, most of the international studies reported AAMA levels up to three times lower than in the present study, showing a higher exposure in the Spanish population. On the other hand, taking into account median levels stratified by the smoking habit, it can be observed that non-smokers from Taiwan (Huang et al, 2007, 2015,; Li et al., 2005) and USA

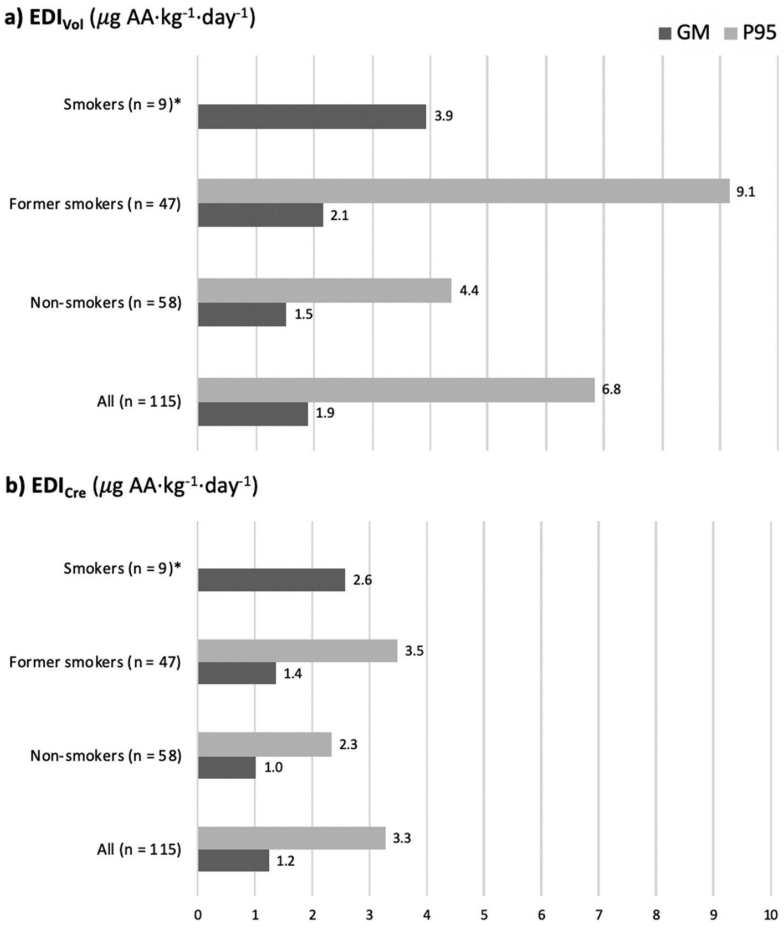

Fig. 2. Estimated daily intakes of AA (EDI, in ng AA·kg^{-1}·day^{-1}) calculated from (a) ΣUAAM concentrations based on volume (EDI_{Vol}) and (b) ΣUAAM concentrations adjusted by creatinine (EDI_{Cre}), stratified by the smoking habit of the participants. *Number of smoker women was too low to calculate the EDI at P95 level.

(Alwis et al., 2012; Keith et al., 2020; Smith et al., 2020) seemed to be more exposed to AA than non-smoker mothers from the BETTERMILK project. In addition, smokers from Poland (women) (Mojska et al., 2016), Norway (Bjellaas et al., 2007), Germany (Kellert et al., 2006; Schettgen et al., 2008) and USA (Alwis et al., 2012; Keith et al., 2020) presented higher urinary levels of AAMA than the studied smoker Spanish women, although the number of samples of smokers in our study was too low to be statistically comparable. Regarding GAMA3, higher urinary levels were found in the U.S. population (Alwis et al., 2012; Keith et al., 2020), non-smoker women from Germany (Hartmann et al., 2008), and Taiwanese adults (Chiang et al., 2015). The rest of the studies had similar or lower levels of urinary GAMA3 as/than in the present survey.

Due to the fact that GA is the genotoxic agent in AA exposure (Besaratinia and Pfeifer, 2004), the ratio GAMA3/AAMA was determined in the women under study with a median of 0.21. As some HBM studies did not report this value, it was calculated using the median concentrations reported for GAMA3 and AAMA in each study (Table 3). As it can be observed, similar ratios were found in the German population (0.15–0.23) (Urban et al., 2006) and U.S. adults (0.18–0.23) (Goniewicz et al., 2018; Keith et al., 2020). However, there are other studies showing ratios higher than 0.30 (Alwis et al., 2012; Hartmann et al., 2008; Mojska et al., 2016; Pluym et al., 2015), and others showing lower ratio values, ranging from 0.04 to 0.16 (Bjellaas et al., 2007; Boettcher and Angerer, 2005; Frigerio et al., 2020; Kellert et al., 2006; B. Wang et al., 2020b, 2020a) (see Table 3). These considerable variations among populations were probably due to differences in the metabolism of individuals, in the exposure dose, or in the type of urine sample used in each study (Bjellaas et al., 2005; Skog and Alexander, 2006). For smokers, GAMA3/AAMA ratios were lower than those calculated for non-smokers in the present study (0.16 vs 0.22), which is in line with the results reported by Mojska et al. (2016) and Urban et al. (2006), and ratios calculated for other studies (see Table 3). This might be explained by a possible inhibition of the cytochrome P450 2E1 activity by nicotine from cigarette smoke (Bjellaas et al., 2005). Concerning AAMA-Sul, no other studies have been found reporting the urinary levels of this metabolite in adults.

Among the food groups studied by regression analysis, the consumption of coffee was observed to have the highest influence on ∑UAAM levels. This was not an unexpected finding, since previous HBM studies have identified the intake of coffee as an important predictor of AA levels in urine (Bjellaas et al., 2007; Choi et al., 2019; Goerke et al., 2019), being responsible for approximately 11% of the AA intake in coffee drinkers (Goerke et al., 2019). Furthermore, EFSA showed that the highest AA levels among many food commodities are found in coffee, since it can be formed during the roasting process (EFSA, 2015). Therefore, coffee intake is considered to be a major contributor to AA dietary exposure in adults and the elderly (EFSA,

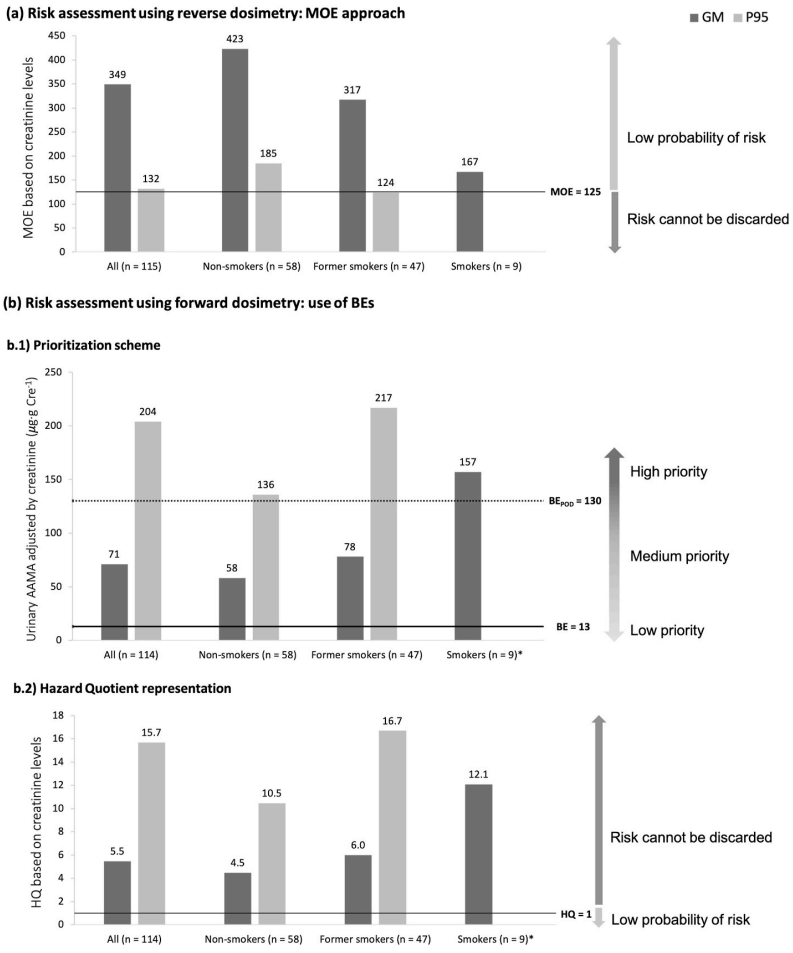

Fig. 3. Interpretation of biomonitoring data of exposure to AA (adjusted by creatinine and stratified by the smoking habit of the target population) obtained in the present study in a risk context using different screening tools: (a) Reverse dosimetry: representation of margin of exposure (MOE) values; and (b) Forward dosimetry by two different strategies: a prioritization diagram (b.1), and a hazard quotient (HQ) representation (b.2) using biomonitoring equivalents (BEs). *Number of smoker women was too low to calculate the risk at P95 level.

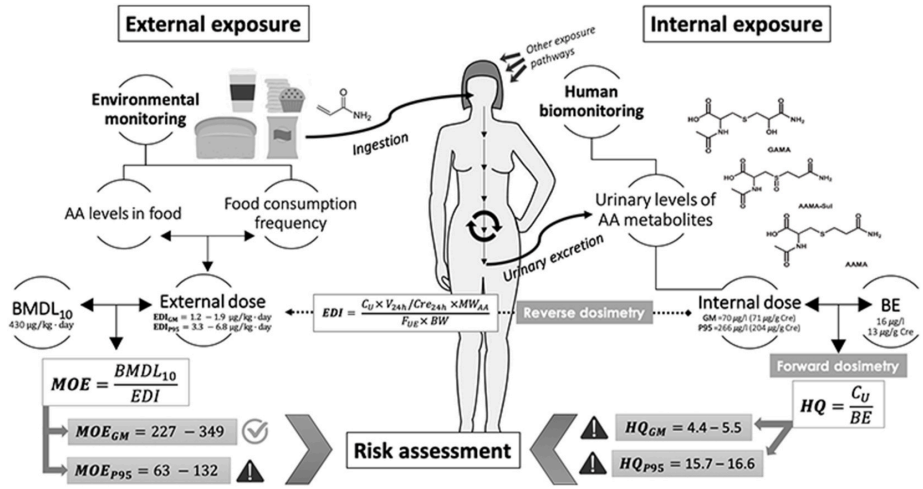

Fig. 4. Comparison of existing approaches for evaluating human exposure to AA in a risk assessment context (environmental monitoring vs human biomonitoring; and, reverse vs forward dosimetry), and results obtained in the population under study.

Table 3
Comparison of urinary concentrations of AA biomarkers in different countries.

Continent	Country	Study population	Age range (years)	Sampling period	Type of urine sample	Subgroup	N	Statistic value	AAMA	AAMA-Sul	GAMA3	Units	Ratio GAMA3/AAMA	Reference
Europe	Eastern Spain	Lactating women	20–45	2015	First-morning urine	All	114	P50	73 (69)	36 (32)	14 (14)	ng·ml^{-1} (μg·g Cre^{-1})	0.21	This study
						Non-smokers	58		59 (59)	29 (27)	12 (12)		0.22	
						Former smokers	47		80 (74)	43 (38)	17 (13)		0.20	
						Smokers	9		155 (161)	67 (53)	21 (25)		0.16	
								DF%	100	100	100		–	
								LoQ	0.25	0.25	0.25		–	
	Norway	Adults	30–58	NR	24 h-urine	All	53	P50	31	NA	3	ng·ml^{-1}	0.06	Bjellaas et al. (2007)
						Non-smokers	47		32	NA	3		0.06	
						Smokers	6		184	NA	10		0.07	
								DF%	100	NA	50		–	
								LoQ	2	NA	5		–	
	Nothern Italy	Exposed workers	27–62	2017	Spot urine (end of the shift)	Non-smokers	38	P50	65 (48)	NA	3 (2)	ng·ml^{-1} (μg·g Cre^{-1})	0.05[b]	Frigerio et al. (2020)
						Electronic cigarrete users	7		84 (56)	NA	4 (4)		0.04[b]	
						Tobacco users	22		113 (115)	NA	8 (5)		0.07[b]	
								DF%	100	NA	84–100		–	
								LoQ	3.2	NA	1		–	
	Poland	Women (after delivery)	20–40	2012	First-morning urine	All	93	P50	21 (32)	NA	9 (12)	ng·ml^{-1} (μg·g Cre^{-1})	0.37	Mojska et al. (2016)
						Non-smokers	78		19 (31)	NA	7 (12)		0.37	
						Passive smokers	10		22 (25)	NA	9 (10)		0.41	
						Active smokers	5		168 (165)	NA	27 (47)		0.18	
								DF%	NR	NA	NR		–	
								LoQ	1	NA	1		–	
	Germany	General population	6–80	NR	Spot urine	Non-smoker adults	91	P50	29 (30)	NA	7 (10)	ng·ml^{-1} (μg·g Cre^{-1})	0.3	Hartmann et al. (2008)
						Non-smoker women	46		22 (30)		34 (30)		0.3	
						Non-smoker men	45		5 (10)		9 (19)		0.3	
								DF%	98.9	NA	75		–	
								LoD	1.5	NA	1.5		–	
			NR	NR	24 h-urine	Non-smokers	25	P50	11	NA	4	μg·g Cre^{-1}	0.33[b]	Pluym et al. (2015)
						Light smokers (<10 cig/day)	12		65	NA	12		0.18[b]	
						Smokers (>10 cig/day)	13		68	NA	13		0.18[b]	
								DF%	NR	NA	NR		–	
								LoQ	10	NA	1		–	
		Adults	26–41	NR	Spot urine	All	29	P50	60	NA	8	ng·ml^{-1}	0.12[b]	Boettcher and Angerer (2005)
						Non-smokers	16		29	NA	5		0.16[b]	
						Smokers	13		127	NA	19		0.14[b]	
								DF%	100	NA	97		–	
								LoQ	5	NA	5		–	
			23–53	NR	Spot urine	Non-smokers	14	P50	53 (55)	NA	NA	ng·ml^{-1} (μg·g Cre^{-1})	–	Schettgen et al. (2008)
						Smokers	14		243 (179)	NA	NA		–	
								DF%	100	NA	NA		–	
								LoD	2.5	NA	NA		–	
		NR	NR	NR	NR	All	38	P50	58 (69)	NA	8 (7)	ng·ml^{-1} (μg·g Cre^{-1})	0.13[b]	Kellert et al. (2006)
						Non-smokers	13		26 (37)	NA	3 (4)		0.11[b]	
							12		56 (41)	NA	9 (7)		0.15[b]	

(*continued on next page*)

Table 3 (*continued*)

Continent	Country	Study population	Age range (years)	Sampling period	Type of urine sample	Subgroup	N	Statistic value	AAMA	AAMA-Sul	GAMA3	Units	Ratio GAMA3/AAMA	Reference
						Occasional smokers								
						Smokers	13		283 (163)	NA	20 (12)		0.07[b]	
								DF%	100	NA	100		–	
								LoQ	0.5	NA	1		–	
America	USA	General population	>3	2015–2016	NR	Adults (>20 years)	1788	P50	52 (51)	NA	<LoD	ng·ml^{-1} (μg·g Cre^{-1})	–	CDC (2021a,b)
						Females	1516		42 (56)	NA	<LoD		–	
						Non-smoker females	802		36 (48)	NA	<LoD		–	
						Smoker females	340		109 (123)	NA	13 (18)		–	
								DF%	NR	NA	NR		–	
								LoD	2.2	NA	9.4		–	
			>12	NR	NR	Non-smokers	1203	Mean	82	NA	28	ng·ml^{-1}	0.32[b]	Alwis et al. (2012)
						Smokers	347		196	NA	57		0.27[b]	
								DF%	NR	NA	NR		–	
								LoD	2.2	NA	9.4		–	
		Adults	21–45	2014–2016	NR	All	371	Mean	157	NA	39	μg·g Cre^{-1}	0.23[b]	Keith et al. (2020)
						Non-smokers	87		68	NA	25		0.35[b]	
						Electronic cigarrete users	17		88	NA	36		0.38[b]	
						Tobacco users	237		192	NA	44		0.22[b]	
						Both users	30		182	NA	39		0.20[b]	
								DF%	NR	NR	NR		–	
								LoD	NR	NR	NR		–	
			35–54	2013–2014	Full-void spot urine	Non-smokers	1655	GM	47	NA	9	μg·g Cre^{-1}	0.18[b]	Goniewicz et al. (2018)
						Electronic cigarrete users	247		56	NA	10		0.16[b]	
						Tobacco users	2411		136	NA	17		0.12[b]	
						Both users	792		144	NA	18		0.12[b]	
								DF%	100	NA	41–75		–	
								LoD	2.2	NA	9.4		–	
			>18	2013–2014	Full-void spot urine	Non-smokers	1736	GM	64	NA	NA	ng·ml^{-1}	–	Smith et al. (2020)
						Electronic cigarrete users	181		59	NA	NA		–	
						Tobacco users	2412		125	NA	NA		–	
						Both users	792		124	NA	NA		–	
								DF%	NR	NA	NA		–	
								LoD	2.2	NA	NA		–	
Asia	Korea	Adults	18–69	2009	Spot urine	All	1873	P50	29 (27)	NA	NA	ng·ml^{-1} (μg·g Cre^{-1})	–	Lee et al. (2013, 2019)
						Males	805		43 (33)	NA	NA		–	
						Females	1068		22 (23)	NA	NA		–	
								DF% (>LoD)	99.4	NA	NA		–	
								LoQ	10	NA	NA		–	
	Taiwan	Adult males	20–38	NR	Spot urine	Non-smokers	33	P50	112 (68)	NA	NA	ng·ml^{-1} (μg·g Cre^{-1})	–	Huang et al. (2007, 2015)
						Smokers	30		152 (99)	NA	NA		–	
								DF%	NR	NA	NA		–	
								LoD	5	NA	NA		–	

(*continued on next page*)

Table 3 (*continued*)

Continent	Country	Study population	Age range (years)	Sampling period	Type of urine sample	Subgroup	N	Statistic value	AAMA	AAMA-Sul	GAMA3	Units	Ratio GAMA3/AAMA	Reference
			20–48	NR	NR	Non-smokers	33	Mean	113 (76)	NA	NA	ng·ml^{-1} (μg·g Cre^{-1})	–	Li et al. (2005)
						Smokers	37		159 (135)	NA	NA		–	
								DF%	NR	NA	NA		–	
								LoD	5	NA	NA		–	
			≈30 - 50	NR	Morning urine	Non-smokers	55	Mean	NA	NA	29	ng·ml^{-1}	–	Chiang et al. (2015)
						Smokers	55		NA	NA	54		–	
								DF%	NA	NA	NR		–	
								LoD	NA	NA	0.014		–	
	China	Adults	18–80	2012	Morning urine	All	2997	P50[a]	29	NA	4	μg·g Cre^{-1}	0.14[b]	Wang et al. (2020a,b)
						Non-smokers	2551		27	NA	4		0.15[b]	
						Smokers	446		46	NA	5		0.11[b]	
								DF%	NR	NA	NR		–	
								LoD	0.6	NA	1.5			

LoQ: limit of quantification; LoD: limit of detection; P50: 50th percentile; DF%: detection frequency; NA: not analyzed; NR: not reported.
[a] Converted from μg·mmol Cre^{-1} to μg·g Cre^{-1} (Molecular weight of Cre = 113.12 g mol^{-1}).
[b] Ratio calculated using the provided statistic values of GAMA3 and AAMA in molar concentrations.

2015). By contrast, according to Molina Périz et al. (2016), coffee consumption is responsible for only 3% of the daily intake of AA in the Spanish population. Another relevant contributor to AA exposure observed in the present study was the intake of bakery products, as also previously reported by some HBM (Brantsæter et al., 2008; Goerke et al., 2019; Heudorf et al., 2009) and dietary studies (EFSA, 2015; Molina Périz et al., 2016). The recent consumption of pre-prepared food was also significantly associated with high ∑UAAM concentrations. This is in line with two studies on pre-cooked products (Michalak et al., 2017; Paleologos and Kontominas, 2007), which found high AA concentrations in these foodstuffs even before heat treatment at home, due to their favorable environment for AA formation during processing and storage (Michalak et al., 2017; Paleologos and Kontominas, 2007). However, more research is needed on the occurrence of AA in this kind of food. On the other hand, although potatoes and cereal consumption were reported to be the main source of dietary exposure to AA by many authors (EFSA, 2015; Heudorf et al., 2009; Schwedler et al., 2021), no relationship with ∑UAAM levels was observed in the present study. This could be due to differences in home-cooking habits of the participants (EFSA, 2015) and the lack of data about the recent consumption of potato-based products in the present study, since AA metabolites are rapidly excreted in urine a few hours after dietary exposure (Kocadağli and Gökmen, 2016).

Apart from diet, tobacco smoke seemed to be a strong contributor of AA internal exposure in smoker women, as it was observed in many recent studies (Choi et al., 2019; Huang et al., 2015; Lee et al., 2013; Mojska et al., 2016; Schwedler et al., 2021). However, in our study, this subgroup sample size was too small to draw conclusive findings. Another significant socio-demographic characteristic contributing to AA exposure was the place of residence, showing higher ∑UAAM concentrations in those women who lived in rural areas compared to those living in urban areas. Nevertheless, although similar results were obtained in a study on Korean children (Choi et al., 2019), no explanation was found in the literature to interpret this observation. A positive association was also found between the use of sunscreen and ∑UAAM levels, but not at a significant level, probably because it is a minor source of exposure to AA as a residual monomer in cosmetic preparations (Dybing et al., 2005).

In general, the risk characterization of AA exposure is carried out through dietary studies, taking into account only the external exposure to this pollutant by food consumption (EFSA, 2015). A different perspective was used in the present study, since the internal exposure to AA was considered in the risk evaluation by using reverse and forward dosimetries.

According to the reverse dosimetry approach, the mean EDIs (1.2–1.9 μg AA·kg bw^{-1}·day^{-1}) obtained in the present study were in the range of those observed in the European population (0.4–1.9 μg AA·kg bw^{-1}·day^{-1}) (EFSA, 2015), and higher than the EDIs derived from diet studies carried out in Spain (0.83 μg AA·kg bw^{-1}·day^{-1}) and, specifically, in adult women of the Valencian Region (0.54 μg AA·kg bw^{-1}·day^{-1}) (Molina Périz et al., 2016). Moreover, the EDIs derived from the 95th percentile (3.3–6.8 μg AA·kg bw^{-1}·day^{-1}) were higher than those reported by EFSA (0.6–3.4 μg AA·kg bw^{-1}·day^{-1}) (EFSA, 2015). These differences could be probably due to the fact that in the present study not only dietary exposure was considered, but also other sources of exposure that contributed to the total AA exposure, such as tobacco smoke. In other HBM studies, the EDIs ranged from 0.15 to 1.04 μg AA·kg bw^{-1}·day^{-1} (Brantsæter et al., 2008; Hartmann et al., 2008; Heudorf et al., 2009; Ji et al., 2013; Mojska et al., 2016), so the EDIs obtained here were the highest reported so far, showing a higher exposure in the Spanish women under study. However, these discrepancies could also be due to the different assumptions made in each study to estimate the daily intake of AA based on HBM data (see Table S4).

In terms of risk, MOEs obtained in the present study (423–109) were between 3 and 7 times lower than MOE values reported in European adults (1075–717) (EFSA, 2015), showing values lower than or close to

125, and therefore, a possible health concern for neurotoxicity in the population under study. Additionally, the risk assessment based on the forward dosimetry showed that AAMA concentrations were substantially higher than the BE, with HQs much higher than 1, concluding that AA exposure should be considered a medium priority for risk assessment follow-up, since non-carcinogenic risks could not be discarded. Similar results were found in HBM studies in Germany (Schwedler et al., 2021), USA (Aylward et al., 2013) and Canada (Brisson et al., 2014; Faure et al., 2020). With respect to the different risk assessment strategies used in HBM studies, it should be noted that there is a need to harmonize methodologies for the risk evaluation criteria of HBM data in a global context.

In summary, both risk assessment strategies showed that AA exposure might indicate a health concern with respect to its non-carcinogenic toxicity in the Spanish population. Therefore, it is concluded that there is a need to reduce this exposure and to follow-up the risk assessment in this country.

5. Study limitations

I.The risk assessment was calculated taking into account only the dietary exposure to AA, although inhalation has also been shown to be a relevant route of exposure to this substance, mainly in smokers.
II.The percentage of smokers was too low to draw conclusive findings for this population subgroup.

6. Conclusions

This research work is the first study reporting data on AA internal exposure in a Spanish population. The obtained results showed that 100% of the Spanish women were exposed to AA, with mean EDIs of 1.2–1.9 μg AA·kg bw^{-1}·day^{-1}. The intake of coffee, bakery and precooked products were strongly associated with high urinary levels of AA metabolites.

The risk assessment showed mean HQs ranging from 4.5 (all participants) to 12 (smokers), and MOEs between 349 (all participants) and 167 (smokers). This confirms that AA exposure presents a concern with respect to its non-neoplastic effects in the target population, showing that further regulation and risk management activities, such as a risk assessment follow-up, are necessary to minimize AA exposure in Spain.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2021.111832.

Author statement

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6.2 BIOMONITORING OF ACRYLAMIDE IN CHILDREN: URINARY LEVELS, PREDICTORS OF EXPOSURE AND RISK ASSESSMENT

6.2.1 RESULTS AND DISCUSSION

A total of 612 urine samples with creatinine levels between 33 and 266 mg·dl^{-1} (mean = 105 mg·dl^{-1}) were included in this study, as the urine volumes of 49 children were not sufficient to determine AA metabolites and creatinine concentrations in 5 urines were beyond the 30 – 300 mg·dl^{-1} range. The analytical method to analyze AAMA, AAMA-Sul and GAMA3 was successfully validated as shown in Section 6.1.1. A summary of the socio-demographic characteristics and food consumption habits of the children under study is presented in Table 23.

As urinary AAMA, GAMA3 and AAMA-Sul are short-term biomarkers of exposure to AA[203,210], the information of the 72 h-recall questionnaire was used to characterize and identify potential dietary determinants of recent AA exposure. Based on EFSA's opinion, the following food commodities were selected (Table 23): fried potato products (such as French fries, chips, and other snacks), cereals, bread, precooked products, biscuits and plastic-packed products, including processed and plastic-wrapped foods, which can contain polyacrylamides used in their food packaging[181]. The last two mentioned items were considered as 'frequency of intake' in grams per month, since they were not included in the 72 h-recall questionnaire.

Table 23. Socio-demographic characteristics, lifestyle and food consumption habits of the children of the BIOVAL study included in the analysis of acrylamide metabolites in urine (n = 612).

Parents' characteristics	n (%)
Country of birth	
Both parents were Spanish	527 (86 %)
At least one of the parents was foreign	67 (11 %)
Missing data	18 (3 %)
Maximum level of education	
Without studies or primary studies	67 (11 %)
Secondary studies	185 (30 %)
University studies	360 (59 %)
Employment situation	
Both unemployed	39 (6 %)
Only one parent employed	181 (30 %)
Both employed	392 (64 %)

Children's characteristics	n (%)	Mean (minimum – maximum)
BMI ($kg \cdot m^{-2}$)		**18 (12 – 45)**
Missing data	19 (3 %)	
Age (years)	-	**8 (5 – 12)**
Province		-
Alicante	225 (37 %)	-
Castellón	134 (22 %)	-
Valencia	253 (41 %)	-
Place of residence		-
Urban area	482 (79 %)	-
Rural area and others	129 (21 %)	-
Missing data	1 (0.2 %)	-
Sex		-
Male	311 (51 %)	-
Female	301 (49 %)	-
Country of birth		-

Children's characteristics	n (%)	Mean (minimum – maximum)
Spain	600 (98 %)	-
Foreign	9 (1.5 %)	-
Missing data	3 (0.5 %)	-
Physical activity		-
Frequently (3 or more days/week)	320 (52 %)	-
Occasionally (1 or 2 days/week)	225 (37 %)	-
Never	67 (11 %)	-

Food consumption habits related to AA exposure in children	n (%)	Mean (minimum – maximum)
Potato fried products (French fries, chips and other snacks) in the last 72 h (g)	-	26 (0 – 210)
Cereals in the last 72 h (g)	-	29 (0 – 270)
Bread in the last 72 h (g)	-	80 (0 – 225)
Precooked products in the last 72 h (g)	-	3 (0 – 90)
Missing data	1 (0.2 %)	-
Monthly consumption of biscuits ($g \cdot month^{-1}$)	-	1000 (25 – 4500)
Missing data	7 (1 %)	-
Monthly consumption of plastic-packed food (number of products$\cdot month^{-1}$)	-	-
Missing data	7 (1 %)	6 (0 – 21)

6.2.1.1 URINARY LEVELS OF ACRYLAMIDE METABOLITES

Herein, the urinary levels of AAMA, GAMA3, and AAMA-Sul were measured in children living in the Valencian Region (Spain). Due to the representativeness of the sample, these data may be used as reference values for this subpopulation group in the Eastern Spanish region. Statistical descriptors of urinary levels of AAMA, AAMA-Sul, and GAMA3 ($ng \cdot ml^{-1}$ and $mg \cdot g^{-1}$ Cre) for the children of the BIOVAL study are presented in Table 24.

Table 24. Descriptive statistics of the concentrations of urinary acrylamide metabolites in children of the BIOVAL program (n = 612).

Metabolite	LoQ	DF (%)	P25	P50
AAMA	0.25	100	46	78
AAMA-Sul	0.25	100	18	29
GAMA3	0.25	100	11	18
[a]∑UAAM	-	100	321	528
Ratio GAMA3/AAMA	-	-	0.16	0.24
$AAMA_{Cre}$	-	-	51	76
$AAMA\text{-}Sul_{Cre}$	-	-	20	28
$GAMA3_{Cre}$	-	-	13	18
[b]$\sum UAAM_{Cre}$	-	-	366	519

LoQ: Limit of quantification; DF: Detection Frequency (% samples > LoQ); P: percentile; GM: Geometric mean; Min. = minimum; Max. = maximum; SD: Standard deviation; Cre: creatinine

[a]∑UAAM = AAMA + AAMA-Sul + GAMA3

[b]$\sum UAAM_{Cre} = AAMA_{Cre} + AAMA\text{-}Sul_{Cre} + GAMA3_{Cre}$

The three AA metabolites were quantified above the LoQ in all urine samples. These high detection frequencies (100 %) were similar to those reported by other authors[190,220,221,223,224,229,411] indicating a widespread AA exposure in children living in different parts of the world. The GM levels of the mercapturic acid metabolites of AA in urine were in the following order: AAMA (79 $ng{\cdot}ml^{-1}$) > AAMA-Sul (28 $ng{\cdot}ml^{-1}$) > GAMA3 (18 $ng{\cdot}ml^{-1}$). This was in accordance with the metabolic and toxicokinetic properties of AA in humans[203], and the abundances observed in section 6.1 and in a similar HBM study conducted by Brisson et al. (2014)[229]. The mean oxidative conversion of AA to GA (GAMA3/AAMA) was estimated to be 23 %, which was similar to the ratio calculated in the BETTERMILK study. However, the AAMA and GAMA3 concentrations observed here were slightly lower than those reported for adult women of the previous study (Section 6.1).

Only few studies have been reported on the urinary levels of AA biomarkers in children. Figure 34 shows a comparison between the P50 levels of urinary AAMA, AAMA-Sul, and GAMA3 reported in ten studies conducted in children from different countries.

Median AAMA levels in children involved in the BIOVAL study (5 – 12 years old) were comparable to those in children and adolescents from Canada (10 – 17 years old)[229] and Korea (10 – 13 years old)[221], whereas considerably lower AAMA concentrations were observed in studies conducted in Germany (3 – 18 years old)[223,224,226], USA (6 – 11 years old)[400], China (0 – 11 years old)[190,411] and in the Korean National Human Biomonitoring Survey (3 – 18 years old)[203] (Figure 34).

GM	P75	P95	Range (Min – Max.)	SD	Units
79	124	290	11 – 796	97	$ng \cdot ml^{-1}$
28	45	78	5 – 140	22	
18	27	49	2 – 125	14	
535	818	1719	88 – 4013	532	$nmol \cdot l^{-1}$
0.23	0.32	0.46	0.06 – 0.92	0.12	Dimensionless
81	121	270	10 – 742	84	$\mu g \cdot g\ Cre^{-1}$
29	43	72	5 – 170	20	
18	27	46	4 – 135	14	
549	796	1503	78 – 3664	463	$nmol \cdot g\ Cre^{-1}$

Regarding GAMA3, the urinary concentrations obtained in the present research were similar to those observed in children living in Germany[223,224,226], Canada[229], the USA[400], Korea[220,221], and China[190]. The lowest urinary levels of GAMA3 (3 $\mu g \cdot l^{-1}$, adjusted by specific gravity) were observed in young Chinese children (0 – 7 years old)[411]. Only one study reporting urinary levels of AAMA-Sul in Canadian adolescents (10 – 17 years old)[229] was found, which were higher than the AAMA-Sul concentrations obtained herein. Note that the variability observed in the urinary levels of AA metabolites among children from different nationalities could be related to differences in not only their lifestyles, genetic factors, secondhand smoke exposure, dietary patterns, and cooking traditions, but also the urine sample type, collection year, age range, and the analytical methods used in each study[220] .

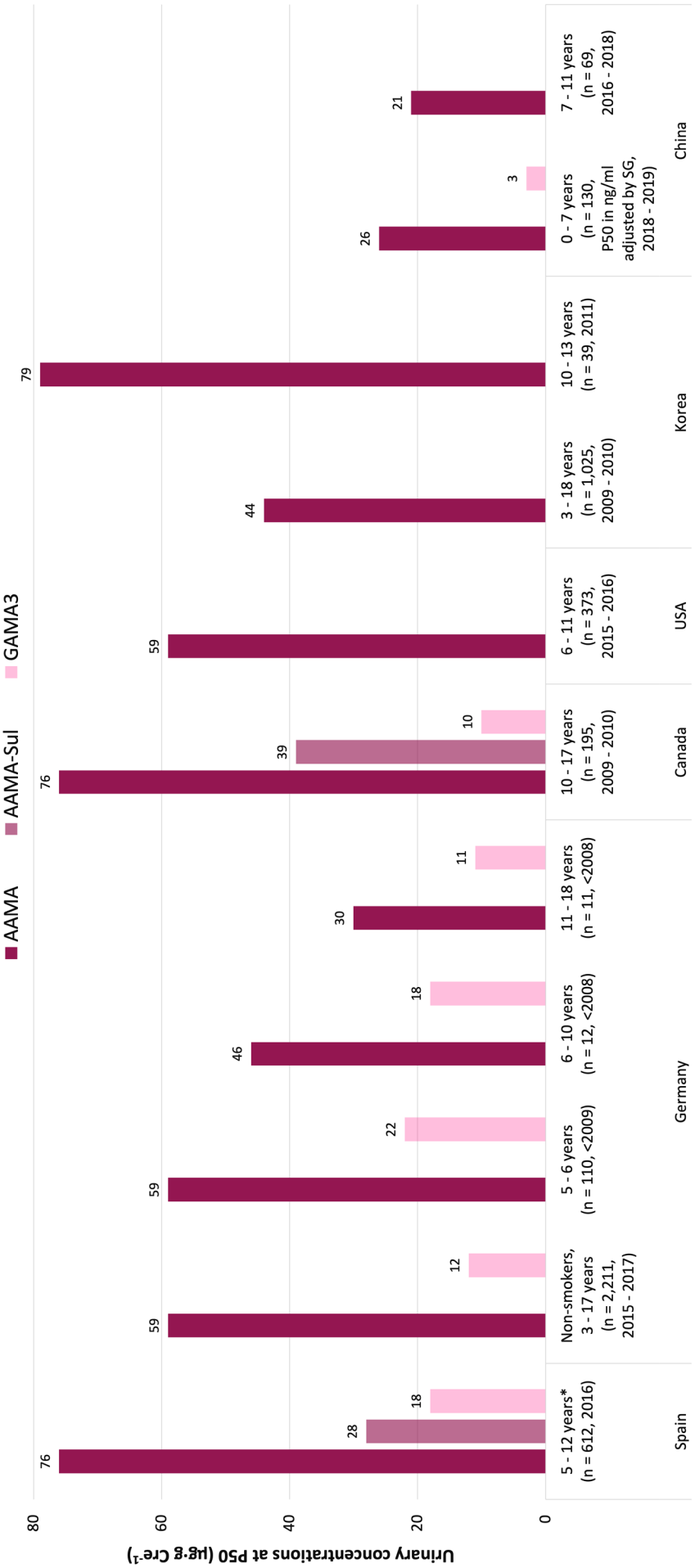

Figure 34. Comparison of existing HBM data of urinary AAMA, AAMA-Sul and GAMA3 (µg·g Cre^{-1}) in children of different ages (number of samples and sample collection years within parenthesis) across the following countries: Spain (*Present study), Germany[223,224,226], Canada[229], USA[400], Korea[220,221], and China[190,411]. *P50: 50th percentile; cre: creatinine; SG: specific gravity.*

6.2.1.2 STATISTICAL ANALYSIS

The Spearman correlation coefficients for AAMA, GAMA3, and AAMA-Sul ranged from 0.719 to 0.856, showing high correlations among them (p-values < 0.01) and corroborating that AA is the common parent compound of these mercapturic acids, as it was also observed in the BETTERMILK study.

Identifying the pathways of AA exposure in humans is crucial for the risk assessment of AA exposure and the development of mitigation and control strategies, specifically for food safety. Therefore, associations between ∑UAAM and some characteristics and dietary habits of the target population were evaluated. A schematic showing the results of the prediction model for log-transformed ∑UAAM is depicted in Figure 35. The obtained model explained the 27.5 % of the data variability.

Figure 35. Linear regression model fitted for the log-transformed ∑UAAM concentrations of the children participating in the BIOVAL study (n = 560, only donors with a completed questionnaire were considered), with its corresponding regression coefficients, 95 % confidence intervals and coefficient of determination (R^2).

According to the regression analysis, the effect of diet on AA exposure was confirmed. Children who consumed higher quantities of potato fried products (including French fries, chips, and other snacks) in the last 72 h, as well as those who reported a higher monthly intake of biscuits had significantly higher ∑UAAM in their urines than those from children who consumed less quantity of these food items. In fact, EFSA claims that 51 and 25 % of the dietary AA exposure in infants, toddlers, and other children is due to the ingestion of fried potato products and biscuits (together with other bakery products), respectively[181]. Similar tendencies were observed in other HBM studies, in which fried potatoes[220,223,226] and bakery products (including biscuits)[226,402] were found to be relevant contributors to the internal exposure to AA in this population group.

With respect to socio-demographic characteristics, an inverse association between age and AA internal exposure was observed, since higher ∑UAAM was obtained for children in lower age ranges. This finding agreed with the observed mean levels of ∑UAAM, which were of 723, 685, 629, and 593 nmol·g Cre^{-1} for 5 – 6-, 7 – 8-, 9 – 10-, and 11 – 12-year-old children, respectively. Similar results were obtained in other studies[223,224], confirming that young children are more vulnerable to AA exposure as they consume more food, inhale more air, and drink more water per kg-bw than older children[225]. Gender-related differences were also noticed, since males had higher urinary levels of AA metabolites compared to females. However, the relationship between internal AA burden and sex is controversial, as some authors claim that urinary creatinine levels may distort this relationship due to the lower creatinine excretion observed in females compared to males[221,224,230]. In fact, when comparing creatinine-adjusted concentrations, only slight differences were found between mean ∑UAAM levels of males (694 nmol·g Cre^{-1}) and females (621 nmol·g Cre^{-1}).

6.2.1.3 RISK ASSESSMENT

This is the first study on the estimation of AA intake among Spanish children based on HBM data. The mean EDIs calculated based on creatinine levels (1.2 µg AA·kg-bw^{-1}·day^{-1}) and on volume (1.5 µg AA·kg-bw^{-1}·day^{-1}) derived herein were in the range of those EDIs calculated by EFSA for infants, toddlers, and other children in Europe (0.5 – 1.9 µg AA·kg-bw^{-1}·day^{-1}). By contrast, EDIs at P95, ranging from 3.2 to 4.9 µg AA·kg-bw^{-1}·day^{-1} (adjusted by creatinine and by volume, respectively) were close to or exceeded the maximum upper limit reported by EFSA (3.4 µg AA·kg-bw^{-1}·day^{-1})[181]. In a previous diet study based on the food consumption habits of Spanish children (5 – 14 years old) in 2013, lower EDIs were also observed (≈0.90 µg AA·kg-bw^{-1}·day^{-1})[405]. These differences might be due to the fact that HBM data takes into account not only ingestion of AA, but also other routes of AA exposure, such as inhalation of secondhand smoke[190] and dermal contact with consumer products[194]. Additionally, the potential contribution of endogenous generation of AA in the human body to the total internal exposure to AA should also be taken into account[412]. Similar EDIs were observed in the

children of the BIOVAL study compared to the women of the BETTERMILK project (see section 6.1.1.3). Nevertheless, other HBM studies focusing on children from Korea[221] and Germany[224,226] showed lower mean EDIs (1.04 and ≤1.13 μg AA·kg-bw^{-1}·day^{-1}, respectively) than those found in the population under study. Note that none of these studies included the urinary levels of AAMA-Sul (both AAMA-Sul and GAMA3 were not considered in the Korean study) in the daily intake estimations, which could lead to a lower robustness of the EDI calculation.

To evaluate the obtained EDIs in a risk assessment context, the MOE concept was applied. Figure 36a depicts the MOEs obtained using the EDIs adjusted by volume and creatinine levels. According to the EFSA's CONTAM Panel, current levels of dietary AA exposure in infants, toddlers, and other children are not of concern with respect to the non-neoplastic effects of AA as MOEs were estimated at mean levels ranging from 860 to 226 and from 307 to 126 in the P95[181]. However, although the MOEs at GM level acquired herein were in the same safe range (283 – 364), MOEs at P95 were close to or below 125 (88 – 133), revealing a potential health risk of AA exposure in children living in Spain.

Regarding the forward dosimetry approach, risk prioritization schemes were used to interpret AAMA urinary levels (Figure 36b). The mean concentrations of AAMA were up to six orders of magnitude higher than the BE, although substantially lower than BE_{POD}, indicating a medium-priority risk of AA exposure in the children under study. This finding was also observed for children living in the abovementioned countries (see P50 of AAMA in Figure 34). In addition, the high-priority cut-off value (BE_{POD}) was exceeded when P95 was considered.

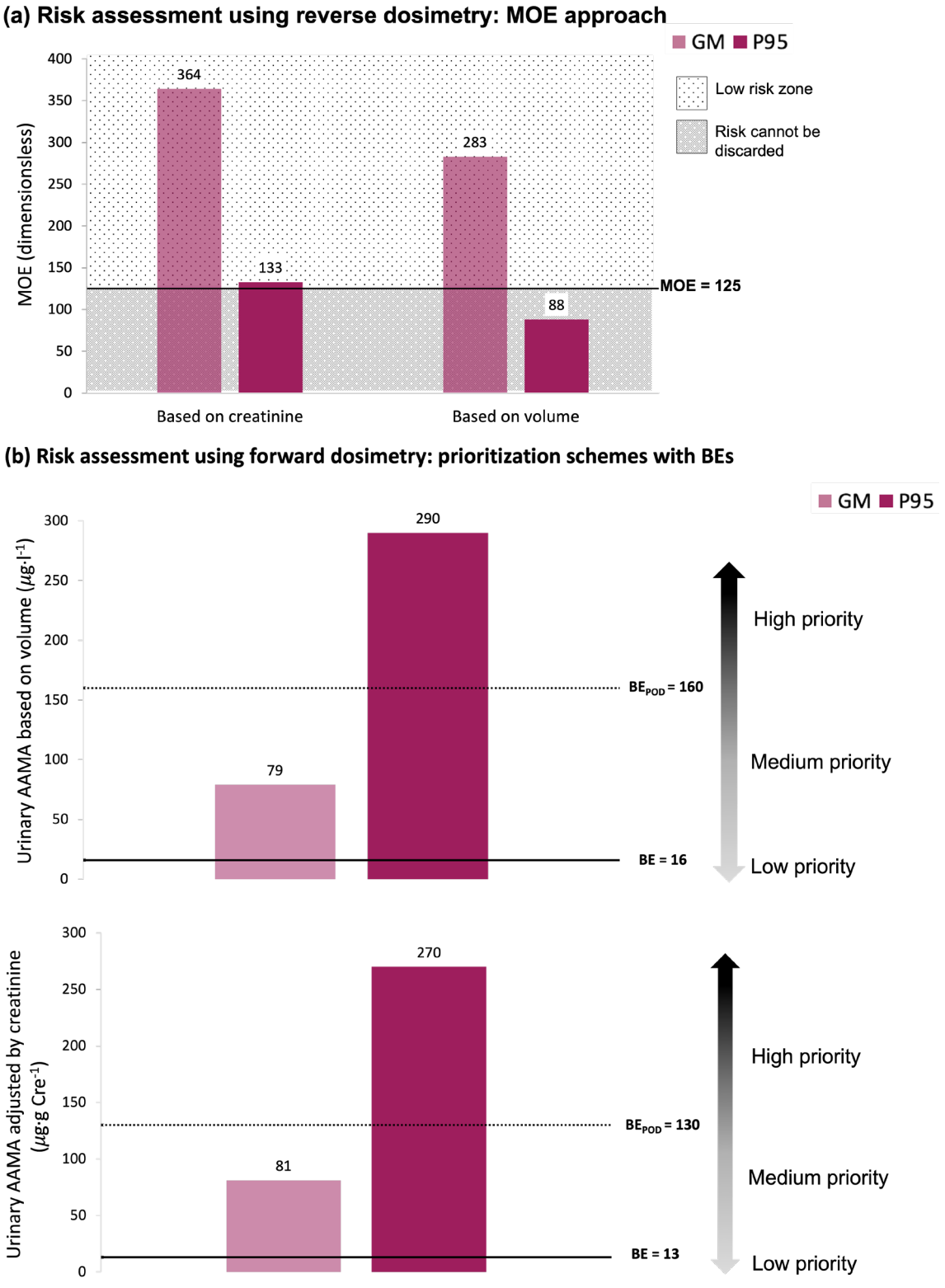

Figure 36. Results of the risk assessment for children of the BIOVAL program using (a) Reverse dosimetry: representation of MOEs at GM and P95 distribution levels adjusted by volume and creatinine; and (b) Forward dosimetry: prioritization diagrams based on BE at GM and P95 distribution levels adjusted by volume and creatinine. *MOE: margin of exposure; GM: geometric mean; P95: 95th percentile; BE: biomonitoring equivalent; BE_{POD}: biomonitoring equivalent directly derived from a point of departure (POD) without considering intraspecies uncertainty factors.*

6.2.2 CONCLUSIONS

Herein, the urinary concentrations of AA metabolites in children between 5 and 12 years old living in Spain (n = 612) were analyzed for the first time. Mean levels of AAMA, AAMA-Sul and GAMA in urine were 79, 28 and 18 ng·ml^{-1}, respectively, resulting in EDIs ranging from 1.2 to 1.5 µg AA·kg-bw^{-1}·day^{-1}. The consumption of fried potato products and biscuits were important contributors to internal AA exposure in this population group. Given that the observed concentrations of AA metabolites were considerably higher than the BE, and considering the high frequency of detection of AA biomarkers in urine, as well as the potential negative health effects related to AA exposure, it is concluded that AA may pose a health risk to children and should be listed as a priority for risk assessment and management activities in Spain and other countries.

In conclusion, further HBM studies on internal body burden of AA are necessary to characterize and monitor the associated risks, as well as to identify potential determinants of AA exposure in children. Thus, HBM data provide scientific evidence to guide food safety authorities in the development of mitigation measures to reduce dietary exposure to AA in this population group. In fact, the need to reduce the consumption of foodstuffs with a high content of AA, such as fried potato products, in the Spanish population has been evidenced and should be communicated to consumers more effectively.

6.2.3 ARTICLE III.2. *RISK ASSESSMENT OF EXPOSURE TO ACRYLAMIDE IN SPANISH CHILDREN USING HUMAN BIOMONITORING*

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Risk assessment of the exposure of Spanish children to acrylamide using human biomonitoring☆

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ABSTRACT

Acrylamide (AA) is an organic contaminant that naturally forms in starchy foods during high-temperature cooking under low-moisture conditions. It is mainly produced from the sugars and amino acids present in food by the Maillard reaction. When humans are exposed to AA, AA is eliminated in the urine as mercapturic acid conjugates, primarily including N-acetyl-S-(2-carbamoylethyl)-L-cysteine (AAMA), N-acetyl-S-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA3), and N-acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine (AAMA-Sul), which are used as exposure biomarkers of AA in human biomonitoring studies. Although the carcinogenic effects of AA on humans have not been demonstrated yet, some studies have shown that AA may negatively affect children's health.

The main objective of this study was to evaluate the exposure of Spanish children (n = 612) to AA. For this purpose, the levels of AAMA, AAMA-Sul, and GAMA3 in first-morning urine samples were analyzed by "dilute and shoot" and liquid chromatography coupled to tandem mass spectrometry. The three metabolites were detected in all the children involved in this study in the following order (geometric mean (GM)): AAMA (79 ng ml^{-1}) > AAMA-Sul (28 ng ml^{-1}) > GAMA3 (18 ng ml^{-1}). Statistical analysis suggested that the intake of fried potato products and biscuits could be associated with higher levels of AA metabolites in urine. Estimated daily intakes of AA in the children under study were in the range of 1.2–1.5 μg AA·kg-body $weight^{-1}$·day^{-1} (GM). Risk assessment calculations indicate that the health risk of AA exposure cannot be overlooked and the exposure of Spanish children to AA should be closely monitored.

1. Introduction

Acrylamide (AA) is a volatile contaminant with various industrial applications and has been classified as a "probable human carcinogen" (Group 2A) by International Agency for Research on Cancer (IARC) (HBM4EU, 2019a; IARC, 1994).

In 2002, the discovery of the formation of AA in carbohydrate-rich foods at temperatures above 120 °C and under low-moisture conditions (for example, during frying, roasting, and baking) via the Maillard reaction had attracted significant attention of the scientific society and public health authorities (WHO, 2002). In fact, the general population is exposed to AA mainly through the consumption of potato crisps, French fries, bakery products (bread and biscuits), and coffee (EFSA, 2015) because of their composition, processing conditions, or high consumption frequency (Rifai and Saleh, 2020).

Although there are some non-dietary sources, such as tobacco smoke, diet is speculated to be the main source of AA exposure in the non-smoking population (EFSA, 2015; Rifai and Saleh, 2020). Consequently, national food authorities in the European Union countries are continuously monitoring the presence of AA in certain food commodities following the Recommendation 2007/331 of the European Commission (European Commission, 2007). In 2015, based on the abovementioned

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data, the Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA) published a scientific opinion assessing the risk associated with the exposure of European citizens to AA (EFSA, 2015). However, this approach has two primary disadvantages: i) it only considers dietary intake and does not include other possible routes or sources of AA exposure and ii) the presence of AA in food may not reflect the actual uptake of AA by the human body, since it depends not only on individual eating habits in terms of cooking methods, portion size and frequency of consumption (Timmermann et al., 2021), but also on the protein content of each individual diet (Schabacker et al., 2004). A complementary and more suitable alternative for evaluating human exposure to AA and the risk associated with it could be human biomonitoring (HBM). This approach facilitates an accurate calculation of the internal dose of AA and considers all pathways (namely, inhalation, ingestion, and dermal contact) of AA exposure by measuring the levels of AA metabolites in biological matrices such as urine and blood (Louro et al., 2019).

After the absorption of AA by the human body, AA is distributed to all tissues and rapidly metabolized via one of the following two pathways: i) it may react with glutathione and produce the mercapturic acid *N*-acetyl-*S*-(2-carbamoylethyl)-L-cysteine (AAMA) or ii) it may be oxidized to glycidamide (GA) by cytochrome P450 (Kocadağli and Gökmen, 2016; Rifai and Saleh, 2020). GA can be further hydrolyzed to glyceramide (2,3-dihydroxypropionamide) by an epoxide hydrolase (Albiach-Delgado et al., 2022; Hartmann et al., 2011), and can also conjugate with glutathione to form *N*-acetyl-*S*-(2-carbamoyl-2-hydroxyethyl)-L-cysteine (GAMA3), which are then excreted in the urine (Kocadağli and Gökmen, 2016; Rifai and Saleh, 2020). Additionally, AAMA can be oxidized to its corresponding sulfoxide, generating acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine (AAMA-Sul).

The three mercapturic acids are the major urinary metabolites of AA, which have been proposed as suitable biomarkers of short-term exposure to AA in HBM studies (Hays and Aylward, 2008; Kocadağli and Gökmen, 2016).

Generally, very few HBM studies have been reported on internal AA exposure in humans, specifically, those belonging to vulnerable groups, including infants and young children, which are of special concern as they need more food, water, and air per kilogram of body weight (kg-bw) as compared to the cases of adults, resulting in a higher exposure of these groups to AA (Hartmann et al., 2008; Mielech et al., 2021). Although there is no evidence of neoplastic effects of AA on humans (EFSA, 2015), two studies conducted in several European populations have shown that AA intake may negatively affect the development of children (Duarte-Salles et al., 2013; Pederson et al., 2012). Other health conditions, such as cardiovascular diseases (Hsu et al., 2020) and oxidative stress damage (Kuang et al., 2021; Qian et al., 2021), have also been associated with the exposure of this population group to AA.

To date, among the European countries, only Germany has reported quantifiable data on the urinary levels of AAMA and GAMA3 in children and adolescents (Hartmann et al., 2008; Heudorf et al., 2009; Schwedler et al., 2021). Indeed, HBM for Europe (HBM4EU) initiative included AA in its 2nd list of priority substances (2018) because of not only the existing gap in its potential adverse effects on humans, but also the lack of data on internal AA exposure in the European population (HBM4EU, 2018). Therefore, considering the potential carcinogenic activity of AA and the scarcity of the HBM studies focused on the exposure of children to AA, obtaining data on the internal exposure of this vulnerable group to AA is of substantial interest.

Herein, data on the internal exposure of Spanish children to AA are presented and the corresponding risk is assessed for the first time. Moreover, potential exposure factors were identified by regression analysis.

2. Materials and methods

2.1. Study design: recruitment, data, and sample collection

This study was performed in the framework of BIOVAL program, a HBM cross-sectional study promoted by the Health Department of the Regional Government of Valencia (Spain) in 2016. The main objective of this program was to more comprehensively characterize the exposure profiles of children (5–12 years old) from the Valencian Region (Spain) to food contaminants. A complete description of the different fieldwork stages (that is, logistics, recruitment, sampling, and data collection) of the BIOVAL program can be found in the literature (Pérez et al., 2017).

Briefly, a total of 666 children from different primary schools throughout the three provinces (Castelló, València, and Alacant) of the Valencian Region participated in the BIOVAL study. The selection requirements were as follows: i) age between 5 and 12 years; ii) no diseases related to the urinary system; and iii) home residence located in the Valencian Region. Before sampling and data collection, informed consent was obtained from the parents/guardians of each volunteer.

The families were provided with all the necessary materials and detailed instructions for sample collection. First-morning urine samples were acquired by all volunteers in 100-ml polypropylene containers using disposable gloves. All urine samples were transported by the BIOVAL staff and refrigerated (4 °C) until storage in 10-ml aliquots at −80 °C in the Biobank for Biomedical Research and Public Health of the Valencian Community (IBSP-CV Biobank, Spain; PT13/0010/0064). Additionally, sociodemographic information and food consumption habits (monthly consumption frequency and 72-h recall) of the children were obtained via a self-administered questionnaire.

The BIOVAL program was approved by the Ethics Committee for Clinical Research of the Directorate General of Public Health and the Centre for Advanced Research in Public Health of the Valencian Government. The participants were notified that the information would be published only for scientific purposes and would not include any personal details. The collection, handling, transfer, and analysis of personal data were conducted by secure settings. Moreover, these data were destroyed after final processing, which was carried out in compliance with ethics and legal aspects at the national and European level, particularly the General Data Protection Regulation.

2.2. Chemical analysis

Methods for the preparation and analysis of samples and details of the standards, reagents, analysis conditions, and quality assurance/quality control criteria used to examine the levels of AAMA, AAMA-Sul, and GAMA3 in the urine samples have been comprehensively described in the literature (Fernández et al., 2022).

Briefly, the homogenized sample (200 μl) was diluted with the internal standard solution and water (0.01% acetic acid) and then injected into a Vanquish high-performance liquid chromatography (Thermo Fisher Scientific Inc.; San Jose, CA, USA) equipped with an Atlantis dC_{18} column (150 mm × 2.1 mm, 2.6 μm, 100 Å) (Waters, Milford, MA, USA) and coupled to an Altis™ triple quadrupole mass spectrometer (Thermo Fisher Scientific Inc.; San Jose, CA, USA); the spectrometer was operated using an electrospray ionization source in the negative-ion mode. All metabolites presented a limit of quantification (LOQ) of 0.25 ng ml^{-1}. Chromatograms of different concentration levels (LOQ, 50 and 1000 ppb) in the calibration curve and of a real urine sample, showing the chromatographic separation of AAMA-Sul, GAMA3 and AAMA, are presented in Fig. S1. During the analysis of the BIOVAL urine samples, the external quality assurance of the results was evaluated through participation in the 66th German External Quality Assessment Scheme (G-EQUAS). As a result, |Z| (absolute Z-scores) between 0.15 and 1.35 for AAMA, and between 0.32 and 0.59 for GAMA3 were obtained, which were classified as satisfactory results ($|Z| \leq 2$). Quantification of AAMA-Sul was not assessed since it was not included in the G-EQUAS 66.

Creatinine (Cre) analysis was performed using the Jaffé's reaction at the University Hospital Doctor Peset (València, Spain) for two purposes: i) to correct urine dilution differences among samples and ii) to exclude those urine samples with abnormal Cre concentrations. As a 'normal' urinary Cre range for children has not been described yet in the literature, the 'normal' values for adults (30–300 mg dl^{-1}) were considered (Barr et al., 2005).

2.3. Statistical analysis

Initially, the urinary levels of AA metabolites adjusted by volume ($ng{\cdot}ml^{-1}$) and Cre ($\mu g{\cdot}g^{-1}$ Cre) were summarized using the following descriptive statistics: detection frequency (DF, percentage of samples above LOQ), geometric mean (GM), 25th, 50th, 75th, and 95th percentiles (P25, P50, P75, and P95, respectively), minimum and maximum values, and standard deviation (SD).

Sociodemographic characteristics of the participants and dietary habits related to AA exposure (namely, exposure variables) were extracted from the questionnaires and described by absolute frequencies and percentages for qualitative variables (with two or more categories) and median, minimum, and maximum values for the quantitative variables.

SPSS statistics (version 26.0, 2019) was used to conduct statistical analysis of the data. A Spearman correlation matrix was employed to investigate the correlations among AAMA, AAMA-Sul, and GAMA3. Urinary concentrations of these AA biomarkers were converted to $nmol{\cdot}l^{-1}$ and added together to obtain the sum of urinary AA metabolite levels ($\sum$UAAM). Due to the non-normal distribution of the data, the logarithm of $\sum$UAAM was used as a dependent variable in the construction of a regression model based on the corrected Akaike information criterion (Konishi and Kitagawa, 2008) to explore associations between $\sum$UAAM and the exposure variables, including Cre as an independent variable (HBM4EU, 2019b). Qualitative variables having categories with frequencies higher than 90% were not included in the exposure variables. A p-value of 0.05 was set as a cutoff for significance. In the final model, the regression coefficients and their 95% confidence intervals for each significant variable reflected the change in the logarithm of $\sum$UAAM of the target population.

To check the assumptions of normality, non-autocorrelation, homoscedasticity, and non-multicollinearity, the Shapiro–Wilk test, the Durbin–Watson statistic, scatter plots, and tolerance and condition indices, respectively, were employed.

2.4. Calculation of estimated daily intake (EDI) of AA

Daily intake of AA based on dietary ingestion was evaluated using the HBM data acquired herein and by applying the methodology previously described in the literature (Fernández et al., 2022). Briefly, GM and P95 of $\sum$UAAM (both adjusted by volume and Cre) in the children under study were transformed to EDIs of AA via the following equations (Heudorf et al., 2009; Mojska et al., 2020):

$$EDI_{Vol}\left(\frac{\mu g\ AA}{kg-bw{\cdot}day}\right) = \frac{\sum UAAM_{Vol} \times V_{24h} \times MW_{AA}}{F_{UE} \times BW} \quad (1)$$

$$EDI_{Cre}\left(\frac{\mu g\ AA}{kg-bw{\cdot}day}\right) = \frac{\sum UAAM_{Cre} \times Cre_{24h} \times MW_{AA}}{F_{UE} \times BW} \quad (2)$$

where $\sum$UAAM = sum of the molar concentrations of AAMA, GAMA3, and AAMA-Sul in urine ($nmol{\cdot}l^{-1}$ for EDI_{Vol} or $nmol{\cdot}g\ Cre^{-1}$ for EDI_{Cre}); V_{24h} = daily volume of urine normally excreted by children ($0.66\ l{\cdot}day^{-1}$) (Aylward et al., 2011); Cre_{24h} = daily urinary creatinine excreted by children ($0.50\ g\ Cre{\cdot}day^{-1}$) (Aylward et al., 2011); MW_{AA} = molecular weight of AA ($71\ g\ mol^{-1}$); F_{UE} = urinary excretion factor of ingested acrylamide which is eliminated as $\sum$UAAM (0.5) (Kocadağli and Gökmen, 2016); and BW = mean body weight of the children under study (33 kg).

2.5. Risk assessment

Interpretation of the risks associated with the AA urinary levels based on the non-neoplastic effects of AA on the target population was performed using two different approaches: reverse and forward dosimetry (Gurusankar et al., 2017). The risk associated with the neoplastic effects of AA was not estimated due to there is no clear evidence of its carcinogenicity in humans (EFSA, 2015).

2.5.1. Reverse dosimetry

In this strategy, the risk was estimated considering only ingestion as the main exposure route as the predominant source of AA exposure in children was food (EFSA, 2015). For this purpose, the margin of exposure (MOE) was calculated as the ratio of the benchmark dose lower confidence limit ($BMDL_{10}$) for neurotoxicity in rats ($430\ \mu g\ AA{\cdot}kg{\cdot}bw^{-1}{\cdot}day^{-1}$) (EFSA, 2015) and EDI (equation (3)).

$$MOE\ (dimensionless) = \frac{BMDL_{10}\left(\frac{\mu g\ AA}{kg-bw{\cdot}day}\right)}{EDI\left(\frac{\mu g\ AA}{kg-bw{\cdot}day}\right)} \quad (3)$$

According to the EFSA's estimation, MOEs lower than 125 may indicate a human health concern because of the non-genotoxic effects of AA on human health (EFSA, 2015).

2.5.2. Forward dosimetry

In the forward dosimetry approach, transformation of the HBM data is not required as the urinary levels of AAMA are directly compared with the non-cancer Biomonitoring Equivalent (BE) and the BE based on the human equivalent point of departure (BE_{POD}) derived for AA (Hays and Aylward, 2008). Consequently, BE and BE_{POD} for urinary AAMA concentrations based on volume (13 and 130 $\mu g\ l^{-1}$, respectively) and Cre levels (16 and 160 $\mu g\ g\ Cre^{-1}$, respectively) were characterized in prioritization schemes for direct comparison with GM and P95 of the obtained AAMA urinary levels (Hays and Aylward, 2009). These schemes provide an initial estimation of whether the exposure to AA is of low (below BE), medium (between BE and BE_{POD}), or high (above BE_{POD}) concern and priority for further monitoring of risk assessment in the study population group. However, they cannot be used to distinguish "safe" exposures from "unsafe" exposures (Hays and Aylward, 2009, 2008).

3. Results

A total of 612 urine samples were included in this study as the urine volumes of some children (n = 49) were not sufficient to determine AA metabolites and the urinary Cre concentrations in 5 children were abnormal (namely, beyond the 30–300 mg dl^{-1} range) (Barr et al., 2005).

A summary of the sociodemographic characteristics and food consumption habits of the children under study is presented in Table 1. As the urinary biomarkers of AA represent short-term exposure to AA (Hays and Aylward, 2008; Kocadağli and Gökmen, 2016), the information of the 72 h-recall questionnaire was used to characterize and identify potential sources of AA exposure. This included the following food commodities: fried potato products (such as French fries, chips, and other snacks), cereals, bread, and precooked products (EFSA, 2015). According to EFSA, biscuits are also a relevant source of exposure to AA in children (EFSA, 2015), and due to the employment of AA in polymers used in food packaging, the consumption of plastic-packed products was also considered a potential exposure source in the present study (EFSA, 2015). However, information on the consumption of these foods was only included in the food frequency questionnaire and not in the 72 h-recall. Therefore, the intake of biscuits and plastic-packed products

Table 1
Sociodemographic characteristics and food consumption habits of the children participating in the BIOVAL study (n = 612).

Children's characteristics	n (%)	Mean (minimum – maximum)
Body Mass Index (BMI, kg·m^{-2})		18 (12–45)
Missing data	19 (3%)	
Age (years)		8 (5–12)
Province		
Alacant	225 (37%)	
Castelló de la Plana	134 (22%)	
València	253 (41%)	
Home location		
Urban	482 (79%)	
Other	129 (21%)	
Missing data	1 (0.2%)	
Sex		
Male	311 (51%)	
Female	301 (49%)	
Country of birth		
Spain	600 (98%)	
Foreign	9 (1.5%)	
Missing data	3 (0.5%)	
Physical activity		
3 or more days per week	320 (52%)	
1 or 2 days per week	225 (37%)	
Occasionally or never	67 (11%)	
Parents' characteristics	**n (%)**	
Country of birth		
Both parents were Spanish	527 (86%)	
At least one of the parents was foreign	67 (11%)	
Missing data	18 (3%)	
Maximum level of education		
Without studies or primary studies	67 (11%)	
Secondary studies	185 (30%)	
University studies	360 (59%)	
Employment situation		
Both unemployed	39 (6%)	
Only one parent employed	181 (30%)	
Both employed	392 (64%)	
Children's food consumption habits related to AA exposure	**n (%)**	**Mean (minimum – maximum)**
Potato fried products in the last 72 h (g)[a]		26 (0–210)
Cereals in the last 72 h (g)		29 (0–270)
Bread in the last 72 h (g)		80 (0–225)
Precooked products in the last 72 h (g)		3 (0–90)
Missing data	1 (0.2%)	
Monthly consumption of biscuits (g·month^{-1})		1000 (25–4500)
Missing data	7 (1%)	
Monthly consumption of plastic-packed food (number of products·month^{-1})		
Missing data	7 (1%)	6 (0–21)

[a] Including French fries, chips and other snacks.

(including processed and plastic-wrapped foods) in grams per month were considered (Table 1). The complete consumption frequency questionnaire (grams of food group per month) is presented in Table S1.

The study population was a representative sample of 5–12-year-old children living in the Valencian Region (Spain) because the percentage of the children recruited by province was in accordance with the geographical distribution of the Valencian population (Pérez et al., 2017), and both sexes were equally represented (51% males and 49% females). Nevertheless, exposure to secondhand smoke was not recorded in the questionnaire; therefore, its influences on the levels of AA metabolites were not analyzed.

3.1. Urinary concentrations of AA biomarkers

Statistical descriptors of urinary levels of AAMA, AAMA-Sul, and GAMA3 (ng·ml^{-1} and μg·g^{-1} Cre) for the children of the BIOVAL study are presented in Table 2.

Urinary Cre levels were between 33 and 266 mg dl^{-1}, with a mean of 105 mg dl^{-1}. The three AA metabolites were quantified above the LOQ in all urine samples, exhibiting wide AA exposure in children living in the Valencian Region. The major AA biomarker, AAMA, was detected at the highest concentrations, 11–796 ng ml^{-1} (GM = 79 ng ml^{-1}), the levels of its sulfoxide derivative, AAMA-Sul, ranged from 5 to 140 ng ml^{-1} (GM = 28 ng ml^{-1}), and the concentrations of the mercapturic acid of GA (GAMA3) were the lowest, ranging from 2 to 125 ng ml^{-1} (GM = 18 ng ml^{-1}). The mean oxidative conversion of AA to GA (GAMA3/AAMA) was estimated to be 23%.

3.2. Statistical analysis

The Spearman correlation matrix provided in Table S2 shows a high correlation among AAMA, GAMA3, and AAMA-Sul (p-value < 0.01), corroborating that AA is the common source of these mercapturic acids.

A schematic showing the results of the prediction model for log-transformed ∑UAAM is depicted in Fig. 1. The thickness of the lines demonstrates the influence of each significant variable on ∑UAAM. The obtained model explained the 27.5% of the data variability. It should be considered that only children with a completed questionnaire (n = 560) were included in the construction of the prediction model.

According to the regression analysis, Cre concentration had the strongest positive influence on AA metabolite levels. Furthermore, the consumption of fried potato products (including French fries, chips, and other snacks) in the last 72 h and the monthly intake of biscuits were related to higher ∑UAAM.

Regarding the sociodemographic characteristics of the participants, sex was considerably associated with internal exposure to AA; however, only slight differences were found between the mean ∑UAAM of males (694 nmol g Cre^{-1}) and females (621 nmol g Cre $^{-1}$). Another relevant predictor was the age of the children, which showed a negative correlation with AA exposure.

3.3. EDIs and risk assessment

The EDIs calculated based on volume and Cre levels are shown in Fig. 2. Similar EDIs were acquired at GM concentration levels, ranging from 1.2 to 1.5 μg AA·kg-bw^{-1}·day^{-1}. In contrast, substantially higher EDIs were obtained at the P95 distribution levels for volume-based concentrations (4.9 μg AA·kg-bw^{-1}·day^{-1}) as compared to those acquired using Cre levels (3.2 μg AA·kg-bw^{-1}·day^{-1}).

To evaluate these EDIs in a risk assessment context, the MOE concept was applied. Fig. 3 depicts the MOEs obtained using the EDIs adjusted by volume and Cre levels. The mean MOEs ranged from 283 to 364. Only the MOE for P95 based on volume was lower than 125.

Regarding the forward dosimetry approach, risk prioritization schemes were used to interpret the AAMA urinary levels (Fig. 4). The mean concentrations of AAMA were significantly higher than BE,

Table 2
Descriptive statistics of the urinary AA metabolites (UAAM) concentrations in Spanish children (n = 612).

Metabolites	LOQ	DF (%)	Minimum	P25	P50	GM	P75	P95	Maximum	SD	Units
AAMA	0.25	100	11	46	78	79	124	290	796	97	ng·ml^{-1}
AAMA-Sul	0.25	100	5	18	29	28	45	78	140	22	
GAMA3	0.25	100	2	11	18	18	27	49	125	14	
[a]∑UAAM	–	100	88	321	528	535	818	1719	4013	532	nmol·l^{-1}
Ratio GAMA3/AAMA	–	–	0.06	0.16	0.24	0.23	0.32	0.46	0.92	0.12	Dimensionless
$AAMA_{Cre}$	–	–	10	51	76	81	121	270	742	84	μg·g Cre^{-1}
$AAMA\text{-}Sul_{Cre}$	–	–	5	20	28	29	43	72	170	20	
$GAMA3_{Cre}$	–	–	4	13	18	18	27	46	135	14	
[b]$\sum UAAM_{Cre}$	–	–	78	366	519	549	796	1503	3664	463	nmol·g Cre^{-1}

LOQ: Limit of quantification; DF: Detection Frequency (% samples > LoQ); P: percentile; GM: Geometric mean; SD: Standard deviation; Cre: creatinine.
[a] $\sum UAAM = AAMA + AAMA\text{-}Sul + GAMA3$.
[b] $\sum UAAM_{Cre} = AAMA_{Cre} + AAMA\text{-}Sul_{Cre} + GAMA3_{Cre}$.

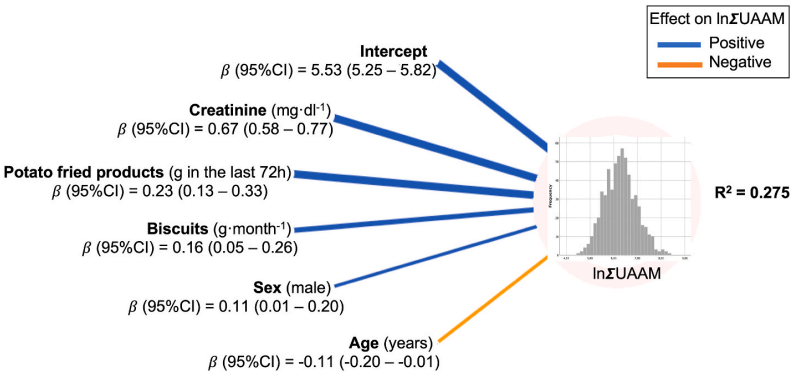

Fig. 1. Linear regression model fitted for the log-transformed ∑UAAM (n = 560, only participants with a completed questionnaire were considered), with its corresponding regression coefficients, 95% confidence intervals and coefficient of determination (R^2). The thickness of the lines represents the influence of each significant variable on ∑UAAM.

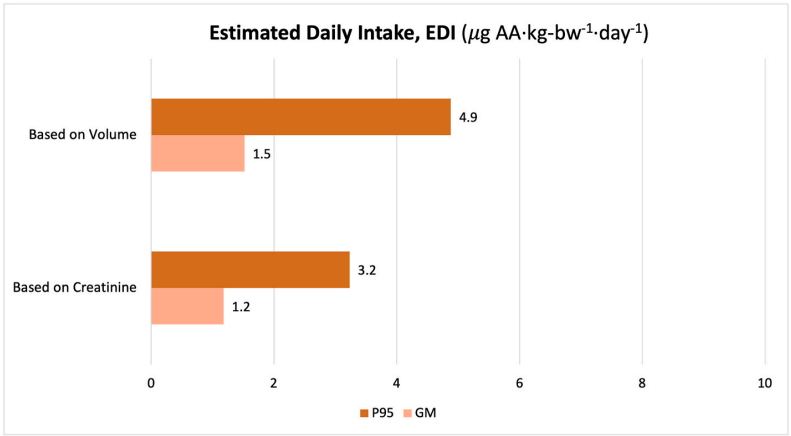

Fig. 2. Estimated daily intakes of AA (EDI, in μg AA·kg-bw^{-1}·day^{-1}) calculated from ∑UAAM adjusted by volume and by creatinine at GM and P95 distribution levels.

whereas they were substantially lower than BE_{POD}, indicating a medium-priority risk of AA exposure in the children under study. However, the high-priority cut-off value exceeded when P95 was considered.

It is important to note that the use of biomarkers to estimate exposure to food contaminants, such as acrylamide, has advantages and disadvantages. First, biomarkers of exposure provide us a confirmation of the absorption of contaminants by the human body and give information on aggregate and cumulative exposure to the parent compound (Kromerova and Bencko, 2018; WHO, 2011). In addition, the evaluation of biomarker levels helps to understand their toxicokinetic, as well as to identity exposure trends and evaluate public health interventions

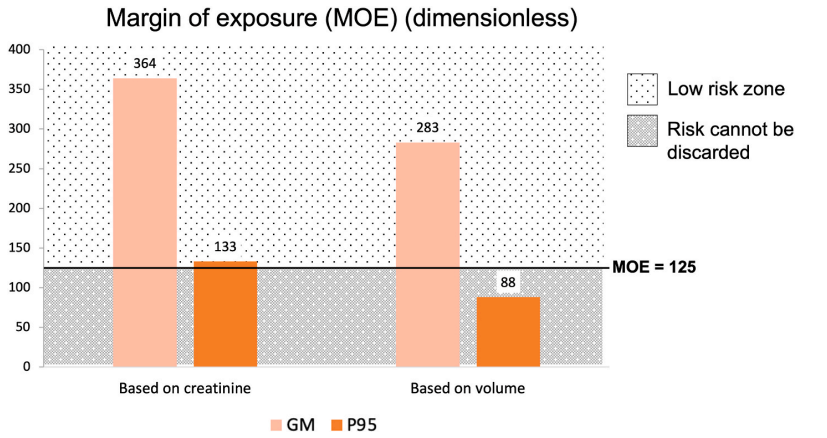

Fig. 3. Results of the risk assessment using reverse dosimetry: representation of the margin of exposure (MOE) at GM and P95 distribution levels adjusted by volume and creatinine.

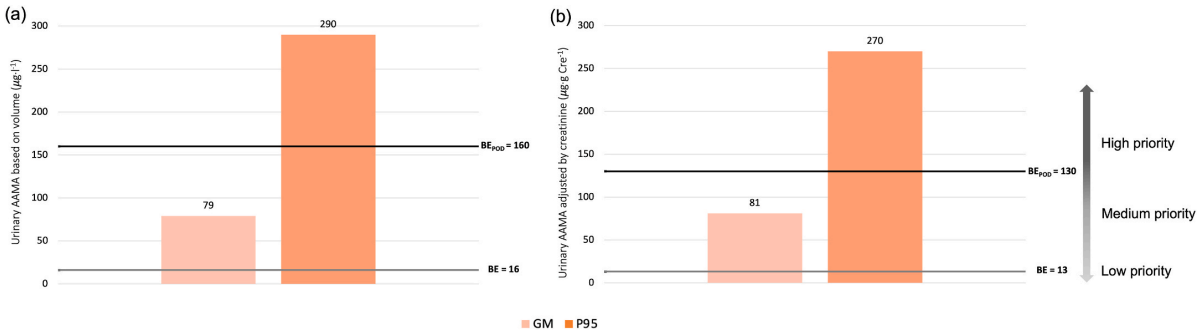

Fig. 4. Results of the risk assessment using forward dosimetry: prioritization diagrams based on biomonitoring equivalents (BEs) at GM and P95 distribution levels adjusted by volume (a) and creatinine (b).

(Kromerova and Bencko, 2018; WHO, 2011). However, biomarkers cannot distinguish among sources, pathways or different durations of exposure (WHO, 2011). Furthermore, health-based reference values in HBM, such as BEs or HBM-I and –II, are still lacking for most food contaminants, and they do not represent diagnostic criteria (safe vs unsafe exposure). In the case of acrylamide, BEs were derived with a "medium" confidence (Hays and Aylward, 2008).

4. Discussion

Herein, the urinary levels of AAMA, GAMA3, and AAMA-Sul were measured in children living in the Valencian Region (Spain) to evaluate the levels of AA exposure and the risk associated with AA exposure. Due to the representativeness of the sample, these data may be used as reference values for this subpopulation group in the Eastern Spanish region.

The levels of the mercapturic acid metabolites of AA in urine were in the following order: AAMA > AAMA-Sul > GAMA3. This is in accordance with the metabolic and toxicokinetic properties of AA in humans (Kocadağli and Gökmen, 2016) and the abundances observed in two similar HBM studies (Brisson et al., 2014; Fernández et al., 2022). Additionally, the high detection frequencies acquired in this study (100%) are similar to those reported by other authors (Brisson et al., 2014; Choi et al., 2019; Hartmann et al., 2008; Ji et al., 2013; Kuang et al., 2021; Schwedler et al., 2021; Song et al., 2022), indicating widespread AA exposure in children from different parts of the world.

Only few studies have been reported on the urinary levels of AA biomarkers in children. As many of these studies do not provide GM values, P50 of urinary AA levels was used to compare among populations. Fig. 5 shows a comparison between the P50 levels of urinary AAMA, AAMA-Sul, and GAMA3 acquired in ten studies focused on the population group investigated herein. A more detailed description of each study is provided in Table S3. It should be noted that AAMA-Sul is not usually analyzed in many HBM studies, probably because is a minor metabolite of AA. Most authors only report urinary levels of AAMA (major metabolite of AA) and GAMA3 (major metabolite of GA) (see Table S3).

The median AAMA levels in children involved in the BIOVAL study (76 μg g Cre^{-1}) were comparable to those in children and adolescents from Canada (76 μg g Cre^{-1}) (Brisson et al., 2014) and Korea (79 μg g Cre^{-1}) (Ji et al., 2013), whereas they were considerably higher than those in the rest of the studies (21–59 μg g Cre^{-1}) (Fig. 5). In contrast, the urinary concentrations of GAMA3 were very similar to those observed in children living in Germany (Hartmann et al., 2008; Heudorf et al., 2009; Schwedler et al., 2021), Canada (Brisson et al., 2014), the USA (CDC, 2021), Korea (Choi et al., 2019; Ji et al., 2013), and China (Kuang et al., 2021); nevertheless, the urinary levels of GAMA3 in young Chinese children (0–7 years old) were the lowest (3 μg l^{-1}, adjusted by specific gravity) (Song et al., 2022). In addition to the present study, only one study has been reported on the urinary levels of AAMA-Sul in Canadian adolescents (10–17 years old) (39 μg g Cre^{-1}) (Brisson et al., 2014), which are higher than the concentrations obtained herein (29 μg

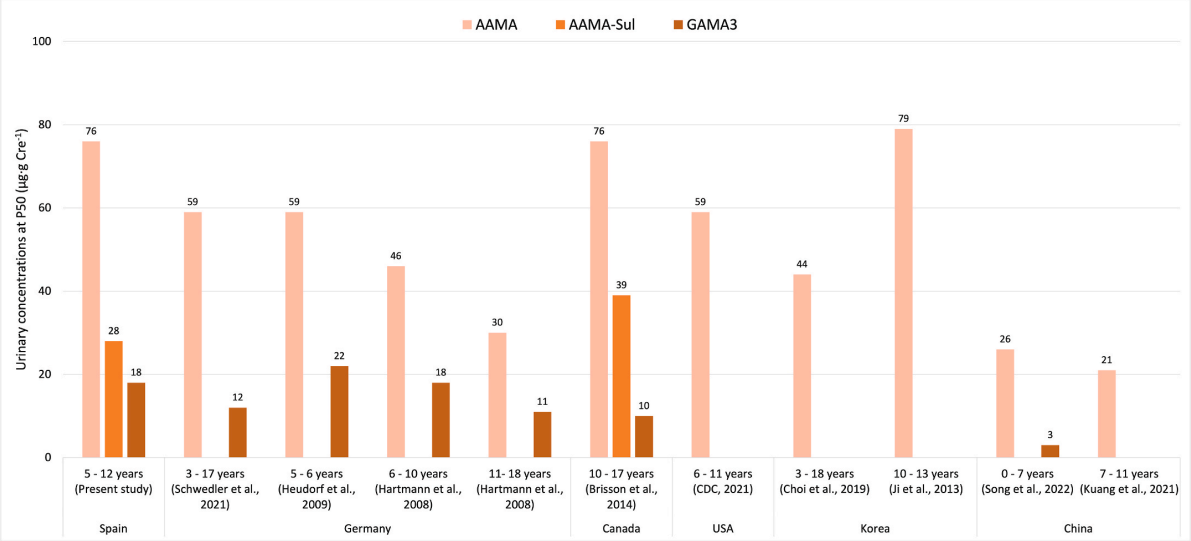

Fig. 5. Comparison of existing HBM data of urinary AA metabolites in children and adolescents from different countries. A detailed description of each study can be found in Table S2.

g Cre^{-1}). The AAMA and GAMA3 concentrations acquired herein for the evaluated children were slightly lower than those recently reported for adult women living in the Valencian Region (Fernández et al., 2022).

Note that this variability in the urinary levels of AA metabolites among children from different nationalities could be related to differences in not only their lifestyles, genetic factors, secondhand smoke exposure, dietary patterns, and cooking traditions, but also the urine sample type, collection year, age range, and the analytical methods used (Choi et al., 2019).

Identifying the sources of AA exposure in humans is crucial for the risk assessment of AA exposure and the development of mitigation and control strategies, specifically for food safety. Therefore, associations between ∑UAAM and some characteristics and dietary habits of the target population were evaluated. Consequently, the effect of diet on AA exposure was confirmed. The urine samples of children who more regularly consumed higher quantities of fried potato products in the last 72 h and biscuits had significantly higher ∑UAAM than those in the case of the children who consumed less quantity of these food items. In fact, EFSA estimates that 51 and 25% of the dietary AA exposure in infants, toddlers, and other children are owing to the ingestion of fried potato products and biscuits (together with other bakery products), respectively (EFSA, 2015). Similar tendencies have been observed in other HBM studies, in which fried potatoes (Choi et al., 2019; Heudorf et al., 2009; Schwedler et al., 2021) and bakery products (including biscuits) (Heudorf et al., 2009; Lee et al., 2013) were found to be relevant contributors to the internal exposure of this population group to AA.

With respect to sociodemographic characteristics, higher ∑UAAM was obtained for children in lower age ranges, implying an association between age and AA internal exposure. This finding was in agreement with the observed mean ∑UAAM of 723, 685, 629, and 593 nmol g Cre^{-1} for 5–6-, 7–8-, 9–10-, and 11–12-year-old children, respectively. Similar findings have been obtained in other studies (Hartmann et al., 2008; Schwedler et al., 2021), confirming that younger children are more vulnerable to AA exposure as they consume more food, inhale more air, and drink more water per kg-bw than those in the cases of older children (Mielech et al., 2021). Gender-related differences were also noticed: males demonstrated higher urinary levels of AA metabolites as compared to those of females. However, the relationship between internal AA burden and sex is controversial as some authors have claimed that urinary Çre levels may distort this relationship due to the lower Cre excretion of females comparing to males (Barr et al., 2005; Hartmann et al., 2008; Ji et al., 2013).

To the best of our knowledge, this is the first study on the estimation of AA intake among Spanish children based on HBM data. The mean EDIs derived herein are in the range of those calculated by EFSA for infants, toddlers, and other children in Europe (0.5–1.9 µg AA·kg-bw^{-1}·day^{-1}). In contrast, when P95 was considered, EDIs were close to or exceeded the maximum upper limit reported by EFSA (3.4 µg AA·kg-bw^{-1}·day^{-1}) (EFSA, 2015). In a diet study based on the food consumption habits of Spanish children (5–14 years old) in 2013, lower EDIs were acquired (≈0.90 µg AA·kg-bw^{-1}·day^{-1}) (Molina Périz et al., 2016). These differences might be due to the fact that the HBM data take into account not only ingestion of AA, but also other pathways of AA exposure, such as inhalation of secondhand smoke (Kuang et al., 2021) and dermal contact with consumer products (WHO, 2002). Additionally, endogenous generation of AA in the human body can be a potential contributor to internal AA exposure (Rietjens et al., 2018). Compared to our previous study, herein, comparable EDIs were obtained for women living in the same region (Fernández et al., 2022). Nevertheless, other HBM studies focused on children from Korea (Ji et al., 2013) and Germany (Hartmann et al., 2008; Heudorf et al., 2009) demonstrated lower mean EDIs (1.04 and ≤ 1.13 µg AA·kg-bw^{-1}·day^{-1}, respectively) than those found for the population under study. Note that these studies did not include the urinary levels of AAMA-Sul (both AAMA-Sul and GAMA3 were not considered in the Korean study) in the daily intake calculations, which could lead to lower robustness of the EDI estimations.

According to the EFSA CONTAM Panel, current levels of dietary AA exposure in infants, toddlers, and other children are not of concern with respect to the non-neoplastic effects of AA as MOEs were estimated at mean levels ranging from 860 to 226 and from 307 to 126 in P95 (EFSA, 2015). However, although the MOEs at GM level acquired herein were in the same safe range, MOEs at P95 were close to or below 125, revealing a potential health risk of AA in children living in Spain. Additionally, the urinary AAMA levels found in the BIOVAL study were up to six orders of magnitude higher than the corresponding BE, indicating that risk assessment of AA should be performed. This finding was also acquired for children living in the other abovementioned countries (Fig. 5).

5. Limitations and strengths of the study

The major limitation of the study was the lack of information about exposure to second-hand smoke, consumption of specific foods, such as breaded food, and culinary practices of the study population, which may have a big influence on urinary levels of AA metabolites. In addition, the inter-individual variability in the metabolism of children was considered as their creatinine concentration in urine, which may not be the best surrogate estimator since its excretion is influenced by physiological and exogenous factors. Moreover, spot urine samples were used for the exposure estimation, which give us a less certain estimate of long-term exposure than 24-h samples.

On the other hand, a major strength of the study was that it was conducted using a representative sample of the infant population living in the Valencian Region. Thus, the sample size of the study together with the high detection frequencies observed for the three main metabolites of AA led to a high statistical power of the results. Additionally, the first HBM data on AA exposure in Spanish children were presented, highly contributing to the knowledge of AA exposure in this country.

6. Conclusions

Herein, the urinary concentrations of AA metabolites in children (5–12 years old) living in Spain were analyzed for the first time. The consumption of fried potato products and biscuits was an important contributor to internal AA exposure in this population group. Considering the potential negative effects of AA, its high frequency of detection in urine, and the higher concentrations of AA metabolites than the BE, it is concluded that AA may pose a health risk for children and should be listed as a priority for risk assessment and management activities in Spain and other countries.

In conclusion, further HBM studies on the internal body burden of AA are necessary to characterize and monitor the potential risks and sources of AA in children and to provide more data to food safety authorities to facilitate mitigation measures for reducing dietary exposure to AA. In addition, the need of reducing the consumption of foodstuffs with a high content of AA, such as fried potato products, in Spain has been evidenced and should be recommended to consumers by Spanish public health authorities with more effectiveness.

Author statement

Sandra F. Fernández: Methodology, Validation, Investigation, Writing – original draft, Visualization, Software, Formal analysis, Data curation; **Olga Pardo:** Writing – review & editing, Supervision, Funding acquisition; **Clara Coscollà;:** Supervision, Project administration; **Vicent Yusà:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2022.119319.

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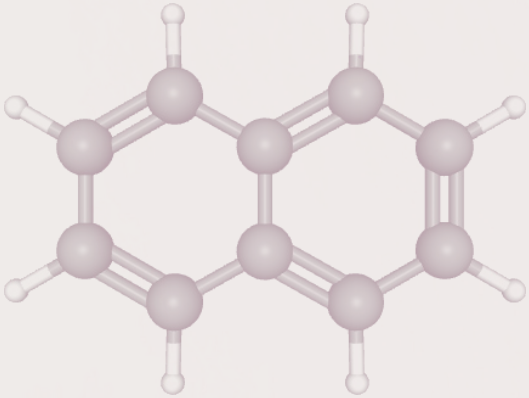
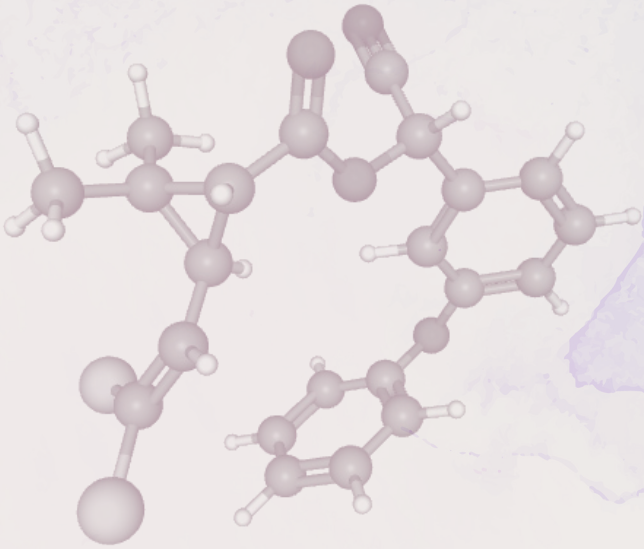
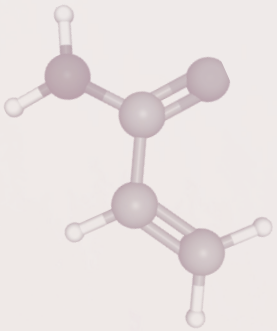
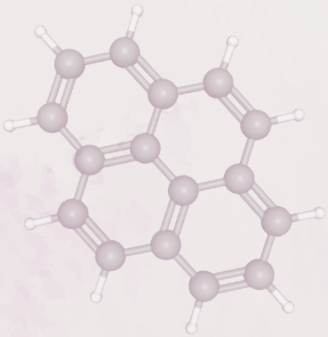
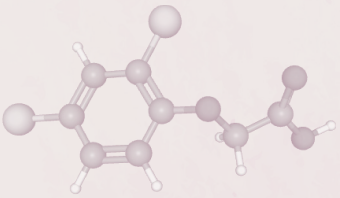
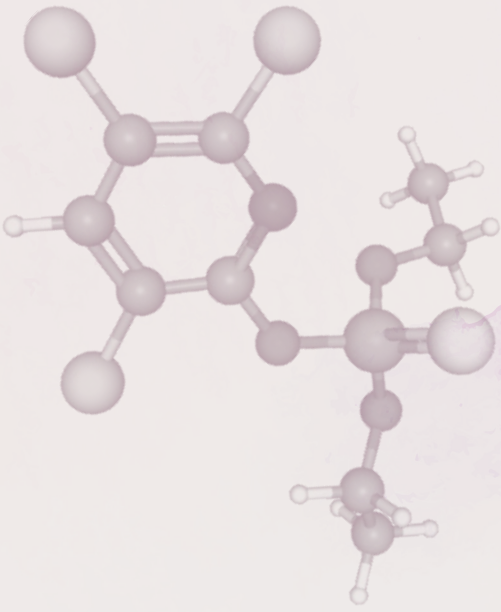
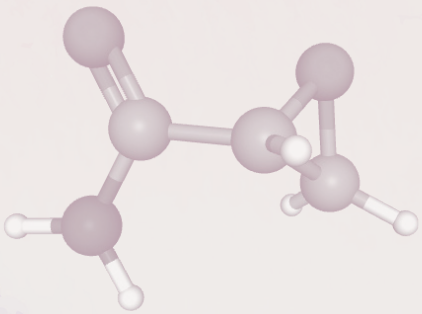

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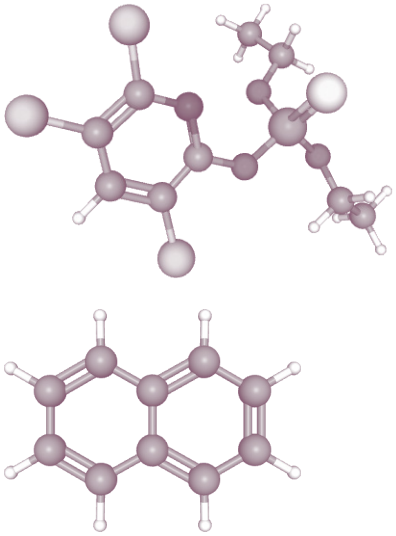
OVERALL CONCLUSIONS



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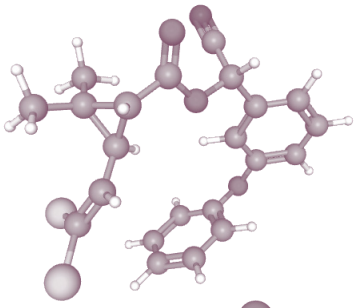

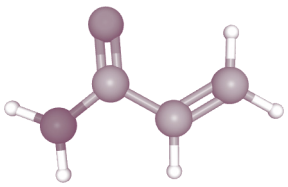

OVERALL CONCLUSIONS

The main conclusions of the present thesis are presented under the following subsections.

7.1 ANALYTICAL METHODOLOGIES

- The target analytical methodologies developed here were successfully applied to determine urinary concentrations of non-persistent pesticides, PAHs and acrylamide metabolites. This shows the utility of target strategies to determine internal exposure to these organic substances by urine analysis. In the case of benzo(a)pyrene, other matrices, such as feces or blood, should be used to evaluate internal exposure to this contaminant.

- Some analytical aspects, such as the lack of blank urine samples and the influence of the enzymatic deconjugation conditions of the studied biomarkers during sample treatment, could have affected the obtained concentrations. In addition to this, potential sources of variability, such as possible bias of stratification of the study population, seasonal exposure differences, collection, transport and storage conditions of urine, as well as other urinary correction factors different from creatinine (e.g., specific gravity and osmolality), may influence the comparability and representativeness of urinary results. All these limitations should be carefully considered in future HBM projects, highlighting the need to harmonize sampling and QA/QC strategies in this field.

- Additionally, a global tendency towards multianalyte methods comprising different chemical groups, as well as suspect and non-target screening strategies, is increasing and should be also implemented in HBM projects, since these methodologies are promising and fast approaches for the simultaneous detection of a large number of substances, e.g., CECs, which are of growing interest in current HBM studies.

7.2 EXPOSURE LEVELS

- In general, the results and conclusions obtained in this thesis highly contribute to the knowledge of exposure to non-persistent pesticides, PAHs, and acrylamide in Spain, being among the first data on internal exposure to these chemicals ever published in this country. The wide exposure of Spanish children and mothers to these organic chemicals, mainly to organophosphate and pyrethroid pesticides, naphthalene and acrylamide, was evidenced.

- Although the results of the BETTERMILK study should not be extrapolated to the entire population, since a limited geographic location was covered and the sample population was small, results and conclusions achieved here could be used as starting points for the design and development of future and larger HBM studies. By contrast, the representativeness of the BIOVAL study allowed to use urinary levels of chemicals obtained here as reference values of children in the Valencia Region.

7.3 DETERMINANTS OF EXPOSURE

- Potential determinants of exposure were identified by the developed HBM strategies, being those related to food consumption the most relevant ones, followed by smoking habits and environmental factors. In general, young children were observed to be more exposed to the assessed chemicals, so they are more vulnerable due to their possible higher dietary intake-body weight ratio.

- The statistical power of these results was not high enough to achieve final conclusions due to the limited information available in the questionnaires of the BETTERMILK and BIOVAL studies. This evidenced the need of an appropriate statistical analysis plan to be able to address exposure-related research questions specifically defined for each chemical group. To this end, the accurate design of detailed and harmonized questionnaires should be encouraged to cover a broader range of exposure pathways, such as second-hand smoke, culinary practices and consumption of organic food, which may have a strong influence on urinary levels of the evaluated biomarkers. Moreover, the most suitable statistical tests, uncertainty analyses, complete data descriptions, and relevant visualizations should be evaluated in each study. By doing this, HBM studies not only provide a confirmation of the absorption of chemicals by the human body, give information on individual and cumulative exposure, and help to understand chemicals' toxicokinetic, but also can be used to identity time trends, geographical comparisons, exposure determinants, and to link exposure with health effects in epidemiological studies based on HBM data.

7.4 RISK ASSESSMENT

- In terms of risk, the estimations performed in the present thesis showed a relatively low health risk evidence due to pesticides and PAH exposure. On the contrary, more attention should be paid to acrylamide since it could pose a concern for a significant percentage of the Spanish population. This fact highlighted that acrylamide exposure should be a priority for risk assessment-following up in further HBM studies and more mitigation activities in public health are necessary.

- Some of the drawbacks observed during risk estimation in the present thesis were that urinary exposure biomarkers are highly influenced by inter-individual and intra-individual variability in human metabolism, so risk assessment results should be interpreted with caution. In addition, endogenous formation of chemical's metabolites in the human body and other potential parent compounds, as well as mixture effects, must be also into consideration when assessing exposure using HBM data.

- Some uncertainties and gaps in the risk assessment of human exposure to chemicals based on HBM were identified, such as the low availability of HBGV for most chemicals to properly interpret HBM data in a risk context, and the lack of harmonization in methodologies used for estimating the risk of individual, cumulative and mixture exposure. Thus, the need for further research to fully understand these phenomena is evident. Fortunately, the HBM4EU initiative made significant progress in this sense, and some of these issues will be further investigated in the upcoming PARC project.

7.5 COMMUNICATION AND DISSEMINATION IN HBM STUDIES

- As a final conclusion, if an effective interpretation and dissemination of HBM data is performed, HBM studies might be powerful complementary tools to external monitoring programs of environmental compartments, being extremely useful to guide and evaluate strategies for prevention, intervention, and reduction of chemical exposure in the context of human health and environmental policies. In this sense, healthy habits and a balance diet are the key to substantially reduce human exposure to harmful substances, such as in the case of acrylamide, and the development of chronic diseases, so communication of dietary recommendations and promotion of healthy lifestyles to the public should be highly encouraged.

8

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8 REFERENCES

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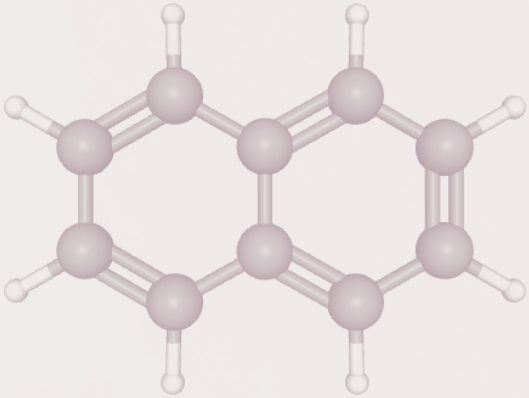
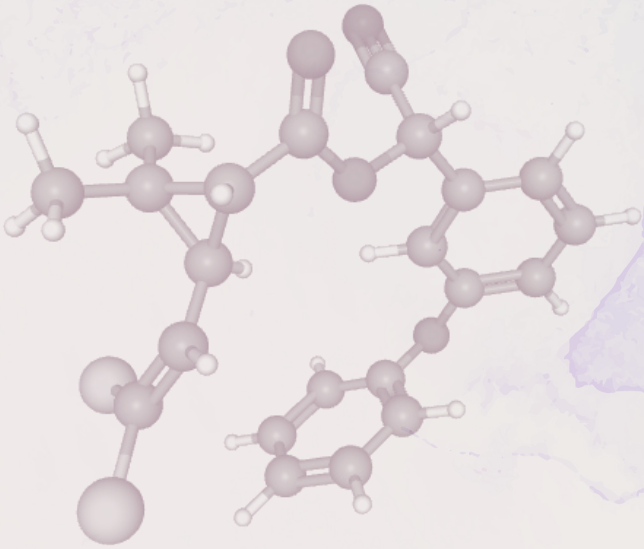
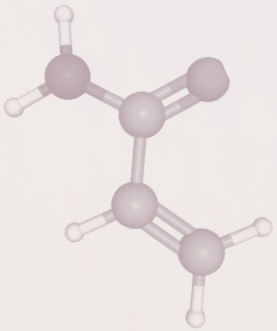

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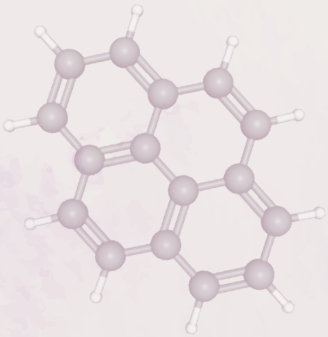
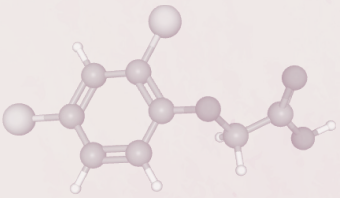
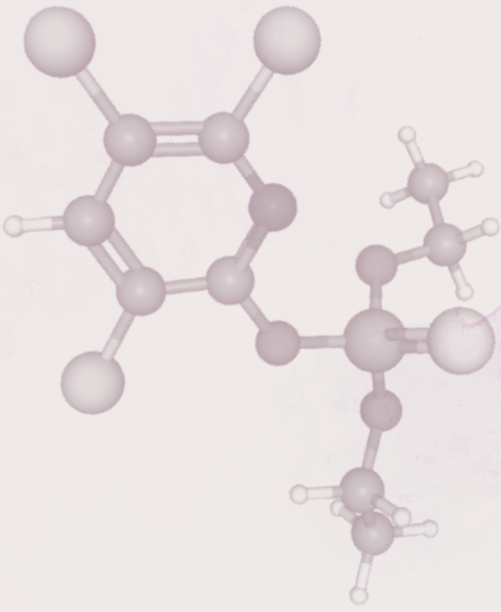
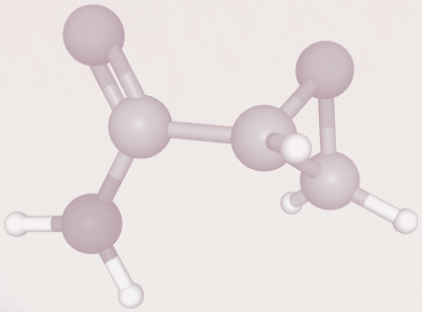
ANNEXES

9.1 ANNEXES TO CHAPTER I

9.1.1 SUPPLEMENTARY INFORMATION OF ARTICLE I.1

SUPPLEMENTARY DATA FOR:

Development of a novel methodology for determination of dialkyl phosphates in human urine using liquid chromatography-tandem mass spectrometry.

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Table S1. Comparison of bibliographic methods based on chromatography and spectrometry detection to analyze DAPs in urine.

Reference	Urine Volume (mL)	Sample Extraction	Analytical Detection	Run time (min)	Ionization	Calibration Curve	CV%		R%	R^2	LoQs (ng·mL^{-1})					
							Intraday	Interday			DMP	DMTP	DMDTP	DEP	DETP	DEDTP
This study	4	LLE + salting-out	LC-MS/MS	20	ESI (-)	MC	-	3-20	82-117	0.989-0.999	0.5	0.5	0.5	0.5	0.5	0.5
[32]	5 aprox.	SPE	LC-MS/MS	-	(-)	MC	2.4-14.7	3.1-8.5	54.2-101.4	0.93-1.00	1.53	0.09	0.14	1.77	0.02	0.07
[33]	0.3	X-AW96-well plates	UPLC-MS/MS	11	ESI (-)	-	-	6-70	39-112	≥0.991	4	0.66	1.9	1.3	1	0.5
[34]	10	Lyophilize + LLE	LC-MS/MS	35	ESI (-)	MC	-	0.25-10.76	93-107	0.99-1.00	0.074	0.040	0.0287	0.0299	0.49	0.066
[9]	5	QuEChERS	UHPLC-HRMS	20	HESI-II (-)	MC	-	3-28	69-127	≥0.95	10	1.6	1.6	10	3.2	3.2
[35]	1	Double SPE	LC-MS/MS	12	ESI (+/-)	MC	2.7-10.7	4.3-9.6	64-105	0.997-0.999	1.2	1.2	NA	0.6	0.3	NA
[36]	3	LLE	LC-MS/MS	28	ESI (-)	MC	-	-	99-122	-	NA	2	NA	2	1	0.6
[37]	0.6	SPE	LC-MS/MS	10	ESI (-)	MC/NE	-	8.13-21.37	99.99-100	0.9997-0.9999	1.40*	0.20*	0.22*	0.13*	0.33*	4.65*
[38]	5	LLE	LC-MS/MS	22	(-)	MC	1.9-15.8	4.6-23.7	13.2-99.8	-	2	2	2	2	2	2
[39]	0.5	Direct injection	LC-MS/MS	-	ESI (-)	MC	-	-	37-116	≥0.99	1	1	NA	NA	NA	NA
[28]	2.5	LLE + Derivatization	GC-MS	34.8	-	MC	0.97-7.9	2.8-15	92-103	0.9933-0.9998	7.5*	0.75*	0.75*	0.75*	0.75*	0.75*
[3]	4	LLE + Derivatization	GC-MS	30.5	-	MC	-	9.7-19.0	92.9-116.4	-	0.2	NA	NA	0.08	1	0.18
[29]	5	Sorbent + Derivatization	GC-MS	12	-	MC	-	0.9-7.8	50-103	0.9991-1.0000	1.4	0.8	NA	1.2	1.6	NA
[37]	2	LLE + Derivatization	GC-MS/MS	40	-	MC	0.7-5.0	3.1-6.9	88-122	-	0.1	0.1	0.1	0.1	0.1	0.01
[27]	2.5	LLE + Derivatization	GC-MS	22	-	MC	3.6-27.8	7.0-51.3	62.6-102.0	0.973-0.998	0.5	0.2	NA	0.3	0.2	NA
[31]	2	SPE + Derivatization	GC-MS	25	-	MC	-	2-15	93.2-105.1	0.993-1.000	0.3*	0.45*	0.3*	0.3*	0.3*	0.3*

SPE: solid-phase extraction
LLE: liquid-liquid extraction
HESI-II: heat electrospray ionization interface
MC: matrix-matched curve
NE: non-extracted curve
NA: not analyzed
LoD: limit of detection
* LoQ calculated as 3 x LoD (ng·mL^{-1})

Table S2. Different sample preparation methods based on SPE with Strata X-AW tested for DAPs analysis.

SPE Method	Conditioning step	Equilibration step	Sample dilution	Washing 1	Washing 2	Elution
A	10 mL Methanol	10 mL Water (pH = 6.4)	4 mL Sample + 4 mL Water + 80 µL Formic acid (100 mM)	No	No	6 mL Methanol (5% Ammonia)
B				6 mL Ammonium acetate (pH = 6.4)	5 mL Methanol	
C				No	No	6 mL Methanol (5% Formic acid)
D				6 mL Ammonium acetate (pH = 6.4)	5 mL Methanol	
E				6 mL Water (pH = 6.4)	5 mL Acetonitrile	6 mL Acetonitrile (5% Ammonia)
G				6 mL Ammonium acetate (pH = 6.4)		
H				6 mL Water (pH = 6.4)	5 mL Methanol	6 mL Acetone (5% Triethylamine)
I		10 mL Sodium acetate (pH = 4)	4 mL Sample + 4 mL Sodium acetate (pH = 4)	5 mL Sodium acetate (pH = 4)		6 mL Methanol (5% Ammonia)
J	9 mL Methanol	9 mL Sodium acetate (pH = 4)			5 mL Acetonitrile	
K				No	No	
L	10 mL Methanol	10 mL Ammonium acetate (pH = 4)	4 mL Sample + 4 mL Ammonium acetate (pH = 4)	5 mL Ammonium acetate (pH = 4)	5 mL Methanol	
M		10 mL Sodium acetate (pH = 4)	2 mL Sample + 2 mL Sodium acetate (pH = 4)	5 mL Sodium acetate (pH = 4)		

Table S3. Different sample preparation methods based on LLE tested for DAPs analysis.

LLE Method	Extraction solvent	Salts	Sorbents
N	8 mL Acetonitrile	1g NaCl	No, PSA, C*, PSA + C*
O		1g NaCl + 4g anhydrous $MgSO_4$	
P		1g NaCl + 4g $MgSO_4$ + 1g sodium citrate + 0.5g citric acid disodium salt	
Q	4 mL Diethyl ether + 4 mL Ethyl acetate	1g NaCl	
R		1g NaCl + 4g anhydrous $MgSO_4$	
S		1g NaCl + 4g $MgSO_4$ + 1g sodium citrate + 0.5g citric acid disodium salt	

Table S4. Placket-Burman design used for optimization of ESI settings for dialkyl phosphates.

Run	Capillary Temperature (ºC)	Auxilliary Gas (a.u.)	Sheath Gas (psi)	Spray Voltage (V)
1	275	13	23	3500
2	200	5	5	5000
3	350	5	5	2000
4	350	5	40	2000
5	200	20	40	2000
6	200	20	40	5000
7	200	20	5	2000
8	350	20	5	5000
9	350	20	40	2000
10	350	5	40	5000
11	275	13	23	3500
12	350	20	5	5000
13	200	5	5	2000
14	200	5	40	5000
15	275	13	23	3500

Table S5. Plackett- Burman results: estimated effects and p-values (within parenthesis) for DAPs for the selected parameters.

Compound	Capillary Temperature	Auxilliary Gas	Sheath Gas	Spray Voltage
DMP	291745 (0.004)[a]	-50632 (0.520)	346388 (0.001)[a]	-6500 (0.933)
DMTP	4996436 (0.005)[a]	-1781499 (0.220)	10898384 (0.000)	533002 (0.703)
DMDTP	1476188 (0.128)	-576941 (0.529)	6872825 (0.000)[a]	-1452620 (0.134)
DEP	841588 (0.007)[a]	-176220 (0.483)	1215725 (0.001)[a]	68646 (0.782)
DETP	2393253 (0.003)[a]	-557461 (0.368)	5528047 (0.000)[a]	-337217 (0.581)
DEDTP	3677305 (0.022)[a]	-930662 (0.502)	12594782 (0.000)[a]	-1242960 (0.375)

[a] p-value < 0.05

Table S6. CCD design used for optimization of ESI settings for DAPs.

Run	Capillary Temperature (ºC)	Sheath Gas (psi)
1	275	28
2	150	28
3	275	28
4	275	28
5	275	50
6	363	12
7	275	50
8	275	5
9	275	28
10	363	43
11	187	43
12	275	28
13	187	12
14	150	28
15	363	12
16	275	5
17	187	43
18	275	28
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23	275	28
24	363	43
25	275	28
26	275	28

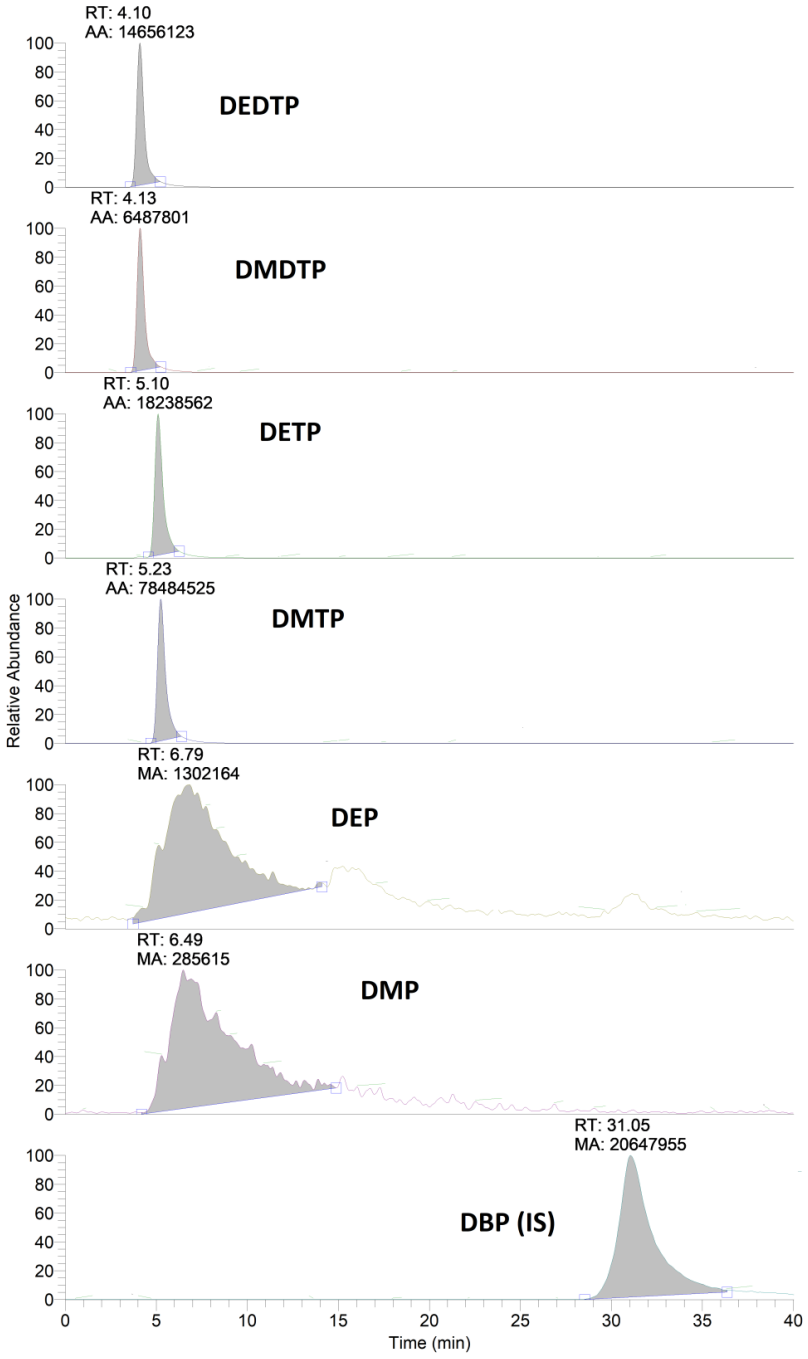

Figure S1. SRM chromatograms of dialkyl phosphates obtained with the KINETEX HILIC column (2.1 × 100 mm, 3 µm) from a standard solution of 100 ng·mL^{-1}.

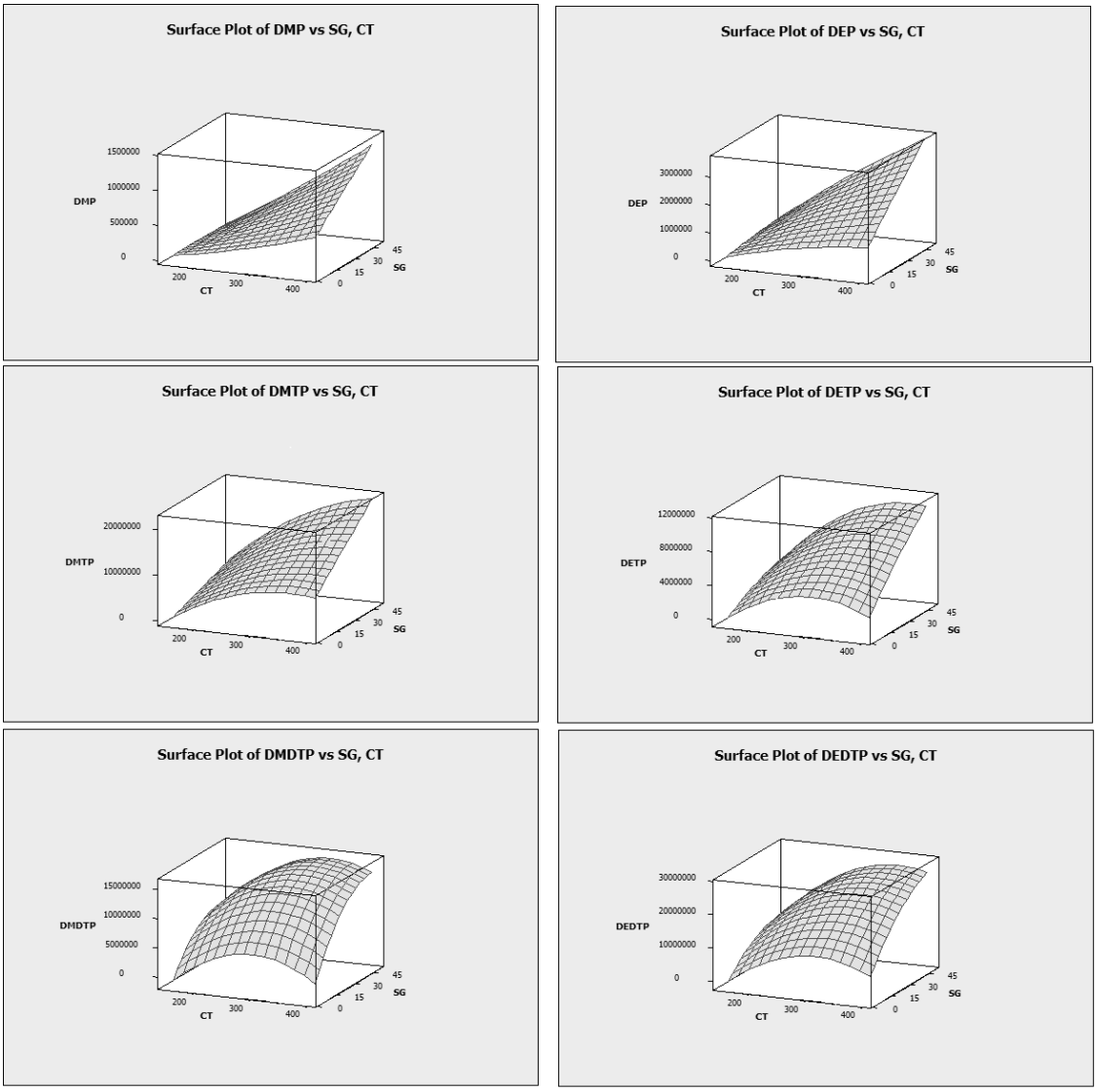

Figure S2. Surfaces Plots of DAPs vs Sheath Gas and Capillary Temperature.

9.1.2 SUPPLEMENTARY INFORMATION OF ARTICLE I.2

SUPPLEMENTARY MATERIAL FOR:

Biomonitoring of non-persistent pesticides in urine from lactating mothers: exposure and risk assessment

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Table S8	Results of multiple log-linear regression models with influencing factors on metabolite concentrations.

[1] BETTERMILK: Gormaz M.; Kuligowski J.; Sánchez A.; Torres E.; Correcher P.; Núñez A.; Parra A. and Ramón A.

SM1. Experimental procedure for analysis of dialkyl phosphates

Dialkyl phosphates extraction was carried out using a methodology developed in the Public Health Laboratory of Valencia. Briefly, 100 µL of dibutyl phosphate (200 ng·ml^{-1}) as internal standard and 800 µL of HCl 6M were added to a 4 ml urine sample. The mixture was centrifuged at 3724 g and 15ºC for 10 min. Then, it was transferred into a 50 mL polypropylene tube for extraction with ethyl acetate (4 ml) and diethyl ether (4 ml). The mixture was cooled down and one ceramic homogeneizer, 4 g anhydrous $MgSO_4$ and 1 g NaCl were added. The tube was mixed by vortex for 1 min and centrifuged at 3724 g and 15ºC for 10 min. The total organic layer was taken and evaporated under a N_2 stream (30º C, 7 bar) until dry. The dry residue was then dissolved in 200 µL of ammonium acetate 10 mM:acetonitrile containing 5% of ammonium acetate 200 mM (95:5), placed into a Millipore 0.2 µm Eppendorf and ultra-centrifuged (11363 g, 2 min and 10ºC). The final extract was transferred into an injection vial and analyzed on a Finnigan Surveyor High-Performance Liquid Chromatography (HPLC) system and Finnigan TSQ Quantum Ultra detector (HPLC-MS/MS) from Thermo Fisher Scientific (Bremen, Germany) equipped with an Hypersil GOLD HILIC column (2.1 x 100 mm, 3 µm) from Phenomenex (California, USA) and ESI (Electrospray ionization) source in negative mode. The mobile phases were (A) an aqueous solution of ammonium acetate 10 mM and (B) acetonitrile containing 5% of an aqueous solution of ammonium acetate 200 mM. The gradient was as follows: 0 min 95% A, 2 min 95% A, 8 min 50% A, 12 min 50% A, 13 min 95% A, 20 min 95% A. The flow rate used was 200 µL·min^{-1} and the injection volume was 10 µL. The mass spectrometer detection settings were: spray voltage, 3000 V; capillary temperature, 400ºC; sheath gas, 50 psi; and auxiliary gas, 10 psi. The procedural limit of quantification (LoQ) established was 0.5 ng·ml^{-1} for every dialkyl phosphate.

SM2. Experimental procedure for analysis of specific metabolites

Specific metabolites extraction was performed following a previously reported QuEChERS methodology (Roca et al., 2014) but with slightly modifications. In brief, 4 ml of urine were mixed with the internal standard solution, acetate buffer and β-glucoronidase aryl sulfatase. The samples were incubated 17h at 37ºC. After the enzymatic treatment, a QuEChERS EN extraction kit and 8 ml of acetonitrile (0.1% HAc) were added to the sample in a 15 ml polypropylene tube and then the mixture was shaken. After centrifugation, the acetonitrile layer was transferred into a glass tube for evaporating in under N_2 stream to dryness. The dry residue was then dissolved in 200 µL of the initial mobile phase, placed into a Millipore 0.2 µm Eppendorf and ultra-centrifuged. The final extract was transferred into an injection vial and analyzed by HPLC-MS/MS equipped with a LUNA® C18 column (2 x 150 mm, 3 µm) from Phenomenex (California, USA) and ESI source. The procedural limits of quantification established for specific metabolites ranged from 0.25 to 2.50 ng·ml^{-1}.

Table S1. Biomarkers of exposure to pesticides analyzed in this study: classification, abbreviation, possible parent compounds (approved and not approved in the EU) and expiration of approval.

Classification		Biomarkers of exposure	Abbreviation	Possible parent compounds[a]		
				Approved in the EU[b]	Expiration of approval (year)	Not approved in the EU[b]
Organophosphate insecticides	Non-specific metabolites	Dimethyl phosphate	DMP	Chlorpyrifos-methyl, dimethoate, malathion, phosmet, primiphos-methyl	2020, 2019, 2022, 2019, 2019	Azinphos-methyl, dichlorvos, dicrotophos, fenitrothion, fenthion, isazofos-methyl, methidathion, methyl-parathion, mevinphos, naled, oxydemeton-methyl, temephos, tetrachlorvinphos, trichlorfon
		Dimethyl thiophosphate	DMTP			
		Dimethyl dithiophosphate	DMDTP			
		Diethyl phosphate	DEP	Chlorpyrifos-ethyl	2020	Chlorethoxyphos, coumaphos, diazinon, disulfoton, ethion, parathion, phorate, phosalone, phoxim, sulfotep, terbufos
		Diethyl thiophosphate	DETP			
		Diethyl dithiophosphate	DEDTP			
	Specific metabolites	3,5,6-Trichloro-2-pyridinol	TCPY	Chlorpyrifos-ethyl, chlorpyrifos-methyl, triclopyr	2020, 2020, 2019	-
		p-Nitrophenol[c]	PNP	-	-	Parathion, methyl-parathion, fluorodifen (herbicide)
		2-Diethylamino-6-methyl-4-pyrimidinol	DEAMPY	Pirimiphos-methyl	2019	-
		2-isopropyl-4-methyl-6-hydroxypyrimidine	IMPY	-	-	Diazinon
		3-chloro-7-hydroxy-4-methylcoumarin	CMHC	-	-	Coumaphos
		3-methyl-4-nitrophenol	MNP	-	-	Fentrothion
		Dimethoate	DIMET	Dimethoate	2019	-
		Omethoate	OMET	Dimethoate	-	Omethoate
		Acephate	ACEP	-	-	Acephate
		Methamidophos	METHA	-	-	Methamidophos, acephate
		Malathion dicarboxilic acid	MDA	Malathion	2022	-
Specific metabolites of herbicides		2,4-Dichlorophenoxyacetic acid	2,4-D	2,4-Dichlorophenoxyacetic acid	2030	-
		2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	-	-	2,4,5-Trichlorophenoxyacetic acid
		Atrazine mercapturate	ATZM	-	-	Atrazine
		Alachlor mercapturate	ALAM	-	-	Alachlor
		Metolachlor mercapturate	METM	-	-	Metolachlor
Pyrethroid insecticides	Non-specific metabolites	3-Phenoxybenzoic acid	PBA	Alpha-Cypermethrin, deltamethrin, beta-cyfluthrin, lambda-Cyhalothrin	2019, 2019, 2019, 2023	Cyhalothrin, cyfluthrin, fenpropathrin, permethrin, tralomethrin
	Specific metabolites	4-Fluoro-3-phenoxybenzoic acid	FPBA	Beta-cyfluthrin	2019, 2019	Cyfluthrin
		2,2,dichlorovinyl-2,2-dimethylcyclopropane-1-carboxilic acid	DCCA	Alpha-Cypermethrin, beta-cyfluthrin	2019, 2019	Permethrin
		2,2,-Dibromovinyl-2,2-dimethylcyclopropane-1-carboxylic acid	DBCA	Deltamethrin	2019	-

[a](Health Canada, 2013; Roca et al., 2014)
[b]Regulation EC No 1107/2009 (European Commission, 2019)
[c]Parents from other origins are possible.

Table S2. Estimation of the food intake frequency (number of portions · month^{-1}).

Questionnaire intake frequency	**Intake frequency (portions · month^{-1})**
Never or less than once a month	0.5
1 – 3 times a month	2
Once a week	4
2 – 4 times a week	12
5 – 6 times a week	22
Once a day	30
2 – 3 times a day	75
4 – 5 times a day	135
More than 6 times a day	195

Table S3. Urinary levels (ng·mL^{-1}) of the least frequently detected (DF < 40%) pesticide metabolites in Spanish mothers (n=116).

Metabolites	LoQ	DF (%)	Percentiles [a]			Maximum
			25th	75th	95th	
Non-specific metabolites of OPs						
DMDTP	0.50	22	<LoQ	<LoQ	1.8	4.2
DEDTP	0.50	3	-	-	-	0.5
Specific metabolites of OPs						
METHA	0.25	28	<LoQ	0.3	0.9	5.1
IMPY	0.25	23	<LoQ	0.3	1.5	4.7
MNP	0.25	22	<LoQ	<LoQ	2.2	11.6
MDA	0.50	15	-	-	-	12.4
DEAMPY	0.25	13	-	-	-	2.6
OMET	0.25	9	-	-	-	1.6
DIMET	0.25	8	-	-	-	6.0
CMHC	0.25	7	-	-	-	1.8
ACEP	0.25	2	-	-	-	19.9
Specific metabolites of herbicides						
2,4-D	0.50	22	<LoQ	<LoQ	0.9	2.0
ALAM	0.25	18	-	-	-	1.1
ATZM	0.25	4	-	-	-	0.5
METM	0.25	3	-	-	-	9.0
2,4,5-T	0.50	0	-	-	-	-
Specific metabolites of pyrethroids						
DBCA	2.50	13	-	-	-	10.6
DCCA	1.25	4	-	-	-	8.6
FPBA	0.50	3	-	-	-	3.0

[a]Percentiles were only calculated in analytes with DF% >20%.
LoQ = Quantification Limit
DF = Detection Frequency (>LoQ)

Table S4. Creatinine-adjusted urinary levels (μg·g^{-1}Cre) of pesticide metabolites in Spanish mothers (n=116).

Metabolites	LoQ	DF (%)	GM (CI)	Median (CI)	Percentiles			Maximum
					25th	75th	95th	
Non-specific metabolites of OPs								
DEP	0.50	91	1.9 (1.5 – 2.4)	2.1 (1.7 – 4.4)	1.9	3.3	7.1	15.7
DMP	0.50	65	0.9 (0.7 – 1.1)	1.0 (0.8 – 2.2)	<LoQ	1.8	3.7	6.3
DETP	0.50	51	0.6 (<LoQ – 1.2)	0.5 (<LoQ – 3.5)	<LoQ	2.9	6.0	24.1
DMTP	0.50	47	<LoQ	0.6 (<LoQ – 2.2)	<LoQ	1.3	4.3	8.7
DMDTP	0.50	22	-	-	<LoQ	<LoQ	2.0	7.2
DEDTP	0.50	3	-	-	-	-	-	0.5
[a]∑DEPs	-	-	21.6 (16.6 – 26.5)	22.7 (17.8 – 49.7)	14.9	36.6	63.0	243.7
[b]∑DMPs	-	-	13.6 (9.8 – 17.4)	15.2 (11.4 – 35.7)	7.8	24.8	56.8	137.6
[c]∑DAPs	-	-	40.1 (33.1 – 46.9)	41.9 (35.0 – 79.2)	28.5	61.8	101.2	267.2
Specific metabolites of OPs								
TCPY	0.25	85	1.5 (0.9 – 2.0)	2.0 (1.4 – 2.5)	0.7	3.2	7.5	18.8
PNP	0.25	84	0.8 (0.4 – 1.2)	0.9 (0.5 – 1.4)	0.4	1.6	3.4	22.6
METHA	0.25	28	-	-	<LoQ	0.3	1.2	3.3
IMPY	0.25	23	-	-	<LoQ	0.3	1.4	5.8
MNP	0.25	22	-	-	<LoQ	<LoQ	2.1	8.3
MDA	0.50	15	-	-	-	-	-	6.2
DEAMPY	0.25	13	-	-	-	-	-	4.1
OMET	0.25	9	-	-	-	-	-	1.5
DIMET	0.25	8	-	-	-	-	-	4.2
CMHC	0.25	7	-	-	-	-	-	2.6
ACEP	0.25	2	-	-	-	-	-	15.2
Specific metabolites of herbicides								
2,4-D	0.50	22	-	-	<LoQ	<LoQ	1.0	1.4
ALAM	0.25	18	-	-	-	-	-	0.8
ATZM	0.25	4	-	-	-	-	-	0.5
METM	0.25	3	-	-	-	-	-	6.1
2,4,5-T	0.50	0	-	-	-	-	-	-
Non-specific metabolites of pyrethroids								
PBA	0.50	65	1.4 (<LoQ – 2.9)	1.5 (<LoQ – 3.1)	<LoQ	4.6	22.8	51.8
Specific metabolites of pyrethroids								
DBCA	2.50	13	-	-	-	-	-	10.4
DCCA	1.25	4	-	-	-	-	-	7.1
FPBA	0.50	3	-	-	-	-	-	2.3

[a]∑DEPs (nmol·g^{-1}) = DEP + DETP + DEDTP
[b]∑DMPs (nmol·g^{-1}) = DMP + DMTP + DMDTP
[c]∑DAPs (nmol·g^{-1}) = ∑DMPs + ∑DEPs
LoQ = Quantification Limit
DF = Detection Frequency (>LoQ)
GM= Geometric mean
CI= Confidence interval 95%

Table S5. Spearman correlations between the most frequently detected pesticide metabolites.

	∑DEPs	PNP	TCPY	PBA
∑DMPs	0.179	0.201*	0.150	0.057
∑DEPs	-	-0.056	0.238*	0.092
∑DAPs	-	0.079	0.253**	0.156
PNP	-	-	-0.161	0.197*
TCPY	-	-	-	0.275**

*p<0.05

**p<0.01

Table S6. Correlations among urinary levels of the most frequently detected pesticide metabolites (nmol·L^{-1}) and lifestyle, sociodemographic characteristics and use of pesticides of the target population.

Lifestyle and sociodemographic variables	PNP		TCPY		PBA		ΣDAPs	
	Median (Min – max)[a] (nmol·L^{-1})	p-value	Median (Min – max)[a] (nmol·L^{-1})	p-value	Median (Min – max)[a] (nmol·L^{-1})	p-value	Median (Min – max)[a] (nmol·L^{-1})	p-value
Mother:								
Creatinine Concentration (g·L^{-1})	**0.417**	**<0.001**	**0.240**	**<0.001**	**0.252**	**<0.001**	**0.287**	**<0.001**
Number of children		0.771		0.159		0.664		0.654
Primiparous	7.05 (0.14 – 52.42)		11.31 (0.35 – 47.22)		8.22 (0.09 – 255.17)		38.33 (6.74 – 178.18)	
Multiparous	5.50 (0.36 – 172.13)		6.73 (0.50 – 84.82)		8.03 (0.14 – 339.65)		44.66 (10.11 – 179.93)	
Age (years)	-0.004	0.967	-0.161	0.086	**-0.194**	**0.038**	-0.080	0.395
Weight before pregnancy (kg)	0.036	0.705	**-0.301**	**<0.001**	-0.179	0.057	-0.177	0.059
Height (cm)	-0.026	0.780	-0.031	0.743	-0.123	0.194	-0.122	0.195
BMI before pregnancy (kg·m^{-2})	0.008	0.930	**-0.281**	**<0.001**	-0.143	0.126	-0.154	0.099
Diet during pregnancy		0.571		0.117		0.380		0.458
Yes	7.48 (0.36 – 41.34)		7.26 (0.50 – 21.92)		12.75 (0.19 – 255.17)		37.27 (15.99 – 127.76)	
No	5.79 (0.14 – 172.13)		10.28 (0.35 – 84.82)		7.78 (0.09 – 339.65)		38.82 (6.74 – 179.93)	
Country of birth		0.420		0.531		0.080		0.175
Spain	6.00 (0.14 – 172.13)		10.28 (0.35 – 68.90)		7.26 (0.09 – 255.17)		38.33 (6.74 – 178.18)	
Foreign	7.95 (0.93 – 38.90)		8.57 (0.71 – 84.82)		20.95 (0.14 – 339.65)		49.12 (21.84 – 179.93)	
Place of residence		0.539		**0.032**		0.618		0.334
Urban	6.83 (0.14 – 172.13)		8.57 (0.50 – 84.82)		8.34 (0.09 – 339.65)		38.53 (6.74 – 179.93)	
Rural	5.75 (0.50 – 68.31)		13.15 (0.50 – 68.90)		7.29 (0.19 – 255.17)		48.41 (15.22 – 127.76)	
Education level		0.590		0.400		0.846		0.106
Only primary school	8.41 (0.93 – 21.57)		12.35 (1.06 – 34.17)		8.87 (0.09 – 339.65)		46.41 (21.84 – 178.18)	
Secondary school	8.09 (0.29 – 68.31)		6.22 (0.50 – 68.90)		13.01 (0.14 – 168.68)		50.54 (11.03 – 104.69)	
University degree	5.68 (0.14 – 172.13)		10.28 (0.35 – 84.82)		7.80 (0.23 – 255.17)		37.36 (6.74 – 179.93)	
Occupational Status		0.957		0.477		0.557		0.120
Employed	7.33 (0.14 – 68.31)		10.23 (0.35 – 84.82)		7.82 (0.09 – 339.65)		37.60 (10.11 – 179.93)	
Unemployed	4.21 (0.72 – 172.13)		9.63 (0.50 – 34.17)		10.53 (0.19 – 186.15)		49.34 (6.74 – 136.00)	
Physical exercise		0.278		0.814		0.369		0.834
Frequently	4.39 (0.14 – 52.42)		11.09 (0.40 – 47.22)		5.70 (0.14 – 144.54)		37.84 (15.22 – 178.18)	
Occasionally	7.91 (0.29 – 39.04)		8.52 (0.35 – 46.37)		9.15 (0.28 – 339.65)		43.41 (6.74 – 136.00)	
Never	4.24 (0.72 – 172.13)		10.28 (0.50 – 68.90)		9.48 (0.09 – 62.67)		38.45 (11.03 – 116.45)	
Smoker		0.405		0.565		0.851		0.614
Yes	7.48 (0.29 – 52.42)		5.75 (0.35 – 27.62)		3.97 (0.37 – 68.93)		51.63 (14.38 – 178.18)	
Exsmoker	7.30 (0.36 – 68.31)		9.70 (0.96 – 38.20)		13.29 (0.09 – 168.68)		42.26 (11.03 – 116.45)	
Never	5.18 (0.14 – 172.13)		11.34 (0.40 – 84.82)		7.24 (0.14 – 339.65)		38.55 (6.74 – 179.93)	
Time with the windows open during the day (hours)	**0.486**	**<0.001**	-0.099	0.292	0.114	0.224	0.035	0.706
Garden or plants at home		0.964		0.342		**0.022**		**0.028**
Yes	6.29 (0.72 – 172.13)		10.28 (0.35 – 84.82)		15.90 (0.14 – 339.65)		48.39 (14.38 – 179.93)	
No	6.90 (0.14 – 41.34)		9.32 (0.40 – 68.90)		5.58 (0.09 – 255.17)		36.85 (6.74 – 178.18)	
Animal deworming in the last month		0.791		0.814		0.958		0.679
Yes	6.40 (0.72 – 68.31)		8.77 (0.40 – 47.22)		3.32 (0.19 – 168.68)		37.36 (15.88 – 178.18)	
No	6.54 (0.14 – 172.13)		10.18 (0.35 – 84.82)		8.22 (0.09 – 339.65)		41.69 (6.74 – 179.93)	
Closeness to farming activity		**<0.001**		**0.037**		0.889		0.279
Yes (<200m)	3.24 (0.36 – 52.42)		11.09 (0.66 – 68.90)		7.71 (0.23 – 62.67)		46.08 (11.33 – 114.87)	
No (>200m)	8.41 (0.14 – 172.13)		8.27 (0.35 – 84.82)		8.03 (0.09 – 339.65)		37.05 (6.74 – 179.93)	
Use of pesticides at home in the last month		0.201		0.168		0.689		0.705
Yes	7.84 (0.14 – 172.13)		8.52 (0.35 – 68.90)		8.87 (0.09 – 249.70)		38.45 (6.74 – 136.00)	
No	4.89 (0.29 – 52.42)		11.24 (0.50 – 84.82)		7.24 (0.14 – 339.65)		39.03 (11.03 – 179.93)	
Children:								
Gestational age (weeks)	**-0.297**	**<0.001**	-0.002	0.984	-0.166	0.121	-0.139	0.196
Weight (at birth) (g)	-0.165	0.081	-0.061	0.522	-0.129	0.174	-0.026	0.783
Height (at birth) (cm)	-0.120	0.309	-0.108	0.362	-0.052	0.662	-0.047	0.691
Cranial Perimeter (cm)	0.008	0.962	-0.050	0.753	-0.037	0.816	-0.096	0.543

[a]For quantitative variables, Spearman coefficient is shown instead of median.

Table S7. Correlations among urinary levels of the most frequently detected pesticide metabolites (nmol·L^{-1}) and food consumption frequency (by groups).

Food consumption frequency	PNP		TCPY		PBA		ΣDAPs	
	Spearman coefficient	p-value	Spearman coefficient	p-value	Spearman coefficient	p-value	Spearman coefficient	p-value
Dairy products	-0.025	0.798	-0.095	0.323	-0.082	0.395	0.000	0.997
Eggs	-0.006	0.951	0.156	0.104	-0.086	0.371	0.003	0.974
Meat products	0.015	0.875	-0.073	0.446	0.008	0.933	0.015	0.873
Fishery products	**0.286**	**<0.001**	-0.081	0.400	0.130	0.176	0.039	0.688
Vegetables	**0.220**	**0.021**	-0.068	0.479	0.066	0.496	-0.064	0.504
Fruits	**0.278**	**<0.001**	**-0.220**	**0.021**	0.081	0.398	-0.022	0.820
Legumes and cereals	-0.002	0.980	-0.113	0.239	0.004	0.970	0.163	0.089
Oils and fats	-0.064	0.504	0.186	0.052	0.061	0.529	-0.003	0.976
Bakery products	0.044	0.646	0.092	0.341	-0.105	0.273	0.025	0.798
Miscellany	-0.002	0.986	-0.058	0.545	0.088	0.362	0.034	0.727
Drinks	**-0.274**	**<0.001**	-0.098	0.310	-0.162	0.090	**-0.229**	**0.016**

Table S8. Results of multiple log-linear regression models with influencing factors on metabolite concentrations[a].

Metabolites	Variable	Estimated coefficients[b] (95% CI)	Standard error	p-value	R^2	adjR^2
PNP (n= 103)	Intercept	1.7005 (1.5043 – 1.8967)	0.0988	<0.0001	0.4474	0.4234
	Time with the windows open during the day (hours)	1.0819 (0.6827 – 1.4812)	0.2010	<0.0001		
	Creatinine concentration (mg·dL^{-1})	1.1237 (0.7287 – 1.5187)	0.1989	<0.0001		
	Place of residence (rural)	-0.6086 (-1.1193 – -0.0979)	0.2571	0.0200		
	Fruits (g·month^{-1})	0.3857 (-0.0015 – 0.7728)	0.1949	0.0508		
TCPY (n= 115)	Intercept	1.9838 (1.7742 – 2.1935)	0.1058	<0.0001	0.1329	0.1174
	BMI "before pregnancy" (kg·m^{-2})	-0.6850 (-1.1045 – -0.2656)	0.2117	0.0016		
	Closeness to farming activity (Yes: <200 m)	0.5718 (0.1159 – 1.0276)	0.2301	0.0144		
PBA (n= 116)	Intercept	1.8542 (1.5222 – 2.1862)	0.1676	<0.0001	0.0646	0.0564
	Creatinine concentration (mg·dL^{-1})	0.9449 (0.2780 – 1.6117)	0.3366	0.0059		
∑DAPs (n= 116)	Intercept	3.6867 (3.5733 – 3.8000)	0.0572	<0.0001	0.0516	0.0433
	Garden or plants at home (Yes)	0.2894 (0.0592 – 0.5195)	0.1162	0.0142		

[a]Only biomarkers with a detection frequency > 40% were considered in order to build a linear regression model.

[b]Standardized regression coefficients.

9.1.3 SUPPLEMENTARY INFORMATION OF ARTICLE I.3

SUPPLEMENTARY MATERIAL FOR:

Exposure and cumulative risk assessment to non-persistent pesticides in Spanish children using biomonitoring

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SM1. Chemical analysis of dialkyl phosphates

Dialkyl phosphates extraction was carried out using a methodology developed in the Public Health Laboratory of Valencia (Fernández et al., 2019). Briefly, 100 µl of dibutyl phosphate (200 $ng \cdot ml^{-1}$) as internal standard (IS) and 800 µl of HCl 6M were added to a 4 ml urine sample. The mixture was centrifuged for 10 min and transferred into a 50 ml polypropylene tube for extraction with ethyl acetate (4 ml) and diethyl ether (4 ml). The mixture was cooled down and one ceramic homogeneizer, 4 g anhydrous $MgSO_4$ *and* 1 g NaCl were added. The tube was mixed by vortex and centrifuged for 10 min. The total organic layer was taken and evaporated under a N_2 stream until dry. The dry residue was then dissolved in 200 µl of the initial mobile phase, placed into a Millipore 0.2 µm Eppendorf and ultra-centrifuged. The final extract was transferred into an injection vial and analyzed on a Finnigan Surveyor High-Performance Liquid Chromatography (HPLC) system and Finnigan TSQ Quantum Ultra detector (HPLC-MS/MS) from Thermo Fisher Scientific (Bremen, Germany) equipped with an Hypersil GOLD HILIC column (2.1 x 100 mm, 3 µm) from Phenomenex (California, USA) and ESI (Electrospray ionization) source in negative mode. The procedural limit of quantification (LoQ) established was 0.5 $ng \cdot ml^{-1}$ for every dialkyl phosphate.

SM2. Chemical analysis of specific metabolites

Specific metabolites extraction was performed following a previously reported methodology based on QuEChERS extraction (Roca et al., 2014) but with slightly modifications. In brief, 4 ml of urine were mixed with the IS solution, acetate buffer and β-glucoronidase aryl sulfatase. The samples were incubated 17h at 37ºC. After the enzymatic treatment, a QuEChERS EN extraction kit and 8 ml of acetonitrile (0.1% HAc) were added to the sample in a 50 ml polypropylene tube and then the mixture was shaken. After centrifugation, the acetonitrile layer was transferred into a glass tube for evaporating in under N_2 stream to dryness. The dry residue was dissolved in 200 µl of the initial mobile phase, placed into a Millipore 0.2 µm Eppendorf and ultra-centrifuged. The final extract was transferred into an injection vial and analyzed by HPLC-MS/MS equipped with a LUNA® C18 column (2 x 150 mm, 3 µm) from Phenomenex (California, USA) and ESI source. The procedural limits of quantification established for specific metabolites ranged from 0.125 to 5.00 $ng \cdot ml^{-1}$.

Table S1. Urinary metabolites of non-persistent pesticides analyzed in the BIOVAL program: classification, application, possible parent substances and acronym of the metabolites.

Class of pesticide	Application	Possible parent pesticide	Metabolite	Acronym
Organophosphate insecticides	Agriculture (specially fruits), gardens, veterinary practices, etc.	Chlorpyrifos[b], chlorethoxyphos[b], coumaphos[b], diazinon[b], dichlofenthion[b], disulfoton[b], ethion[b], parathion[b], phorate[b], phosalone[b], sulfotep[b], terbufos[b]	Diethyl phosphate[d]	DEP
		Chlorpyrifos[b], chlorethoxyphos[b], coumaphos[b], diazinon[b], disulfoton[b], ethion[b], parathion[b], phorate[b], phosalone[b], sulfotep[b], terbufos[b]	Diethyl thiophosphate[d]	DETP
		Disulfoton[b], phorate[b], phosalone[b], terbufos[b]	Diethyl dithiophosphate[d]	DEDTP
		Azinphos-methyl[b], chlorpyrifos-methyl[b], dichlorvos[b], dicrotophos[b], dimethoate[b], fenitrothion[b], fenthion[b], isazophos-methyl[b], malathion[a], methidathion[b], methyl-parathion[b], naled[b], mevinphos[b], omethoate[b], oxydemeton-methyl[b], phosmet[a], pirimiphos-methyl[a], temephos[b], tetrachlorvinphos[b], trichlorfon[b]	Dimethyl phosphate[d]	DMP
		Azinphos-methyl[b], fenitrothion[b], fenthion[b], isazophos-methyl[b], chlorpyrifos-methyl[b], dimethoate[b], malathion[a], methidathion[b], methyl-parathion[b], omethoate[b], oxydemeton-methyl[b], phosmet[a], pirimiphos-methyl[a], temephos[b]	Dimethyl thiophosphate[d]	DMTP
		Azinphos-methyl[b], dimethoate[b], malathion[a], methidathion[b], phosmet[a]	Dimethyl dithiophosphate[d]	DMDTP
		Chlorpyrifos-ethyl[b], chlorpyrifos-methyl[b], triclopyr[a]	3,5,6-Trichloro-2-pyridinol[c]	TCPy
		Pirimiphos-methyl[a]	2-Diethylamino-6-methyl-4-pyrimidinol[c]	DEAMPY
		Diazinon[b]	2-Isopropyl-4-methyl-6-hydroxypyrimidine[c]	IMPY
		Fluorodifen[b] (herbicide), methyl-parathion[b], parathion[b], other non-pesticides substances	p-Nitrophenol[d]	PNP
		Fenitrothion[b]	3-Methyl-4-nitrophenol[c]	MNP
		Dimethoate[b]	Dimethoate[c]	DIMET
		Dimethoate[b], omethoate[b]	Omethoate[c]	OMET
		Acephate[b]	Acephate[c]	ACEP
		Acephate[b], methamidophos[b]	Methamidophos[c]	METHA
		Coumaphos[b]	3-Chloro-7-hydroxy-4-methylcoumarin[c]	CMHC
		Malathion[a]	Malathion dicarboxilic acid[c]	MDA
Pyrethroid insecticides	Crops protection, parks, gardens, household insecticides and mosquito control	Cyfluthrin[a,e], cyhalothrin[a,e], cypermethrin[a,e], deltamethrin[a], etofenprox[a], fenpropathrin[b], permethrin[b], fluvalinate[a], tralomethrin[b]	3-Phenoxybenzoic acid[d]	PBA
		Cyfluthrin[a,e]	4-Fluoro-3-phenoxybenzoic acid[c]	FPBA
		Cyfluthrin[a,e], cypermethrin[a,e], permethrin[b]	2,2-Dichlorovinyl-2,2-dimethylcyclopropane-1-carboxilic acid[c]	DCCA
		Deltamethrin[a]	2,2-Dibromovinyl-2,2-dimethylcyclopropane-1-carboxylic acid[c]	DBCA
Phenoxy herbicides	Weed control in monocotyledonous crops	2,4-Dichlorophenoxyacetic acid[a]	2,4-Dichlorophenoxyacetic acid[c]	2,4-D
		2,4,5-Trichlorophenoxyacetic acid[b]	2,4,5-Trichlorophenoxyacetic acid[c]	2,4,5-T
Chloroacetanilide herbicides	Grasses and broadleaved weed control	Atrazine[b]	Atrazine mercapturate[c]	ATZM
		Alachlor[b]	Alachlor mercapturate[c]	ALAM
		Metolachlor[a,e]	Metolachlor mercapturate[c]	METM

[a]Currently approved in the EU; [b]Currently not approved in the EU; [c]Specific metabolite; [d]Non-specific metabolite; [e]Only some isomers are approved

Table S2. Results and evaluation of G-EQUAS materials for TCPY, PNP, DBCA, PBA and FPBA in urine.

Compound	G-EQUAS results (ng·ml^{-1})				Calculated |Z-Score|	Internal evaluation[c]	Final evaluation[d]
	Control material	Our result	Ref. value (tolerance range)	Standard deviation (number of participants)			
[a]TCPY	A	4.35	4.59 (3.39 – 5.79)	1.21 (9)	-0.5	Satisfactory	+
	B	18.93	15.24 (11.55 – 18.93)	3.76 (8)	0.7	Satisfactory	
[a]PNP	A	12.9	16.2 (12.6 – 19.8)	1.5 (9)	-2.0	Satisfactory	+
	B	42.6	51.7 (42.1 – 61.3)	4.0 (9)	-2.0	Satisfactory	
[b]DBCA	A	0.940	0.780 (0.579 – 0.981)	0.123 (15)	1.6	Satisfactory	+
	B	1.520	1.909 (1.471 – 2.347)	0.201 (15)	-1.6	Satisfactory	
[b]PBA	A	0.740	0.779 (0.572 – 0.986)	0.113 (23)	-0.6	Satisfactory	+
	B	4.590	4.675 (3.580 – 5.770)	0.461 (23)	-0.5	Satisfactory	
[b]FPBA	A	0.79	0.78 (0.57 – 0.99)	0.16 (15)	-0.1	Satisfactory	+
	B	2.14	2.24 (1.64 – 2.84)	0.48 (15)	-0.4	Satisfactory	

[a]Results of the G-EQUAS 58 intercomparison program

[b]Results of the G-EQUAS 62 intercomparison program

[c]Satisfactory: $|Z\text{-Score}| \leq 2$; Unsatisfactory: $|Z\text{-Score}| > 2$

[d] Symbol "+" means that both values were within tolerance range; Symbol "-" means that both values were not within tolerance range.

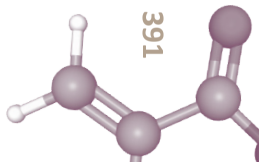

Table S3. Pesticides selected for the risk assessment based on MoA and based on CAGs: selection criteria and parameters for calculation of estimated daily intake, hazard quotient and margin of exposure.

Metabolite[a]	Selected pesticide	Selection criteria	MoA on the nervous system[b]	MW_P ($g \cdot mol^{-1}$)	F_{UE}	ADI[c] ($\mu g \cdot kg\ bw^{-1} \cdot day^{-1}$)	$NOAEL_{motor}$[b,d] ($\mu g \cdot kg\ bw^{-1}$)	$NOAEL_{AChE}$[b,e] ($\mu g \cdot kg\ bw^{-1}$)
∑DEPs	Chlorpyrifos-ethyl	Parent pesticide with the lowest ADI/NOAEL(approved at that time)	AChE inhibitor	350.59	0.50[f]	10	10000	500
∑DMPs	Dimethoate	Parent pesticide with the lowest ADI/NOAEL (approved at that time)	AChE inhibitor	229.26	0.90[g]	2	20000	1000
TCPY	Chlorpyrifos-ethyl	Parent pesticide with the lowest ADI/NOAEL (approved at that time)	AChE inhibitor	350.59	0.50[f]	10	10000	500
PNP	Parathion	Parent pesticide with the lowest ADI/NOAEL (but not approved)	AChE inhibitor	291.30	0.37[h]	4	1750	250
PBA	λ -cyhalothrin	Approved parent pesticide with the lowest ADI/NOAEL	Binding to VGSC	449.50	0.30[i]	2.5	500	-[j]

MoA: mode of action; CAGs: cumulative assessment groups; MW_P: molecular weight of the parent pesticide; F_{UE}: urinary excretion factor of the parent pesticide ; ADI: acceptable daily intake; NOAEL: no observed adverse effect level in mammals; AChE: acetylcholinesterase; VGSC: voltage-gated sodium channel

[a]Only biomarkers with a DF > 40% were included in the risk assessment estimation.

[b](EFSA, 2019)

[c](European Commission, 2019)

[d]NOAEL for alterations on the motor division of the nervous system

[e]NOAEL for brain and/or erythrocyte acetylcholinesterase inhibition

[f](Bouchard et al., 2005)

[g](Katsikantami et al., 2019)

[h](Gosselin et al., 2004)

[i](Khemiri et al., 2017)

[j]This pesticide does not affect the AChE activity, so it does not have a NOAEL associated to this effect.

Table S4. Spearman's correlation coefficients between the most frequently detected pesticide metabolites in urine.

	PBA	PNP	∑DEPs	∑DMPs
TCPY	0.144**	0.099*	-0.004	0.029
PBA	-	0.189**	0.007	0.092*
PNP	-	-	0.095*	0.123*
∑DEPs	-	-	-	0.213**

*p < 0.05

**P< 0.001

9.2 ANNEXES TO CHAPTER II

9.2.1 SUPPLEMENTARY INFORMATION OF ARTICLE II.1

SUPPLEMENTARY MATERIAL FOR:

Biomonitoring of polycyclic aromatic hydrocarbons in the urine of lactating mothers: urinary levels, association with lifestyle factors, and risk assessment

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Figure S2	SRM chromatogram of OHPAHs obtained with the KINETEX Core-shell technology F5 column (150 mm × 2.1 mm, 2.6 µm) from a real urine sample.

[1] BETTERMILK: Vento. M.; Cernada. M.; Gormaz M.; Kuligowski J.; Sánchez A.; Torres E.; Correcher P.; Núñez A.; Parra A. and Ramón A.

SM1. Preparation of the OHPAH stock and working solutions

Individual commercial standards delivered as solids were weighted and dissolved in acetonitrile to obtain a concentration of approximately 400 $\mu g \cdot ml^{-1}$. The stock solutions were stored in the dark at −20 °C for 6 months. Multi-analyte standard solutions containing 1000, 100, 10, and 1 $ng \cdot ml^{-1}$ were prepared by diluting the individual stock solutions with acetonitrile. An internal standard (IS) working solution was also prepared containing all isotopic labelled standards in the following concentrations: 150 $ng \cdot ml^{-1}$ for $^{13}C_6$-2OHFLU, $^{13}C_6$-2OHPHEN and $^{13}C_6$-1OHPYR working solutions; 300 $ng \cdot ml^{-1}$ for $^{13}C_6$-1OHNAP, $^{2}H_7$-2OHNAP and $^{2}H_8$-9OHPHEN working solutions; and, 500 $ng \cdot ml^{-1}$ for $^{2}H_{11}$-3OHBAP working solution. Working solutions were stored in the dark at −20 °C for 3 months. The matrix-matched calibration curve and quality control samples were prepared by spiking synthetic urine with the IS working solution and with the corresponding multi-analyte solution at concentrations between 0.01 and 50 $ng \cdot ml^{-1}$ before the enzymatic hydrolysis.

SM2. Conditions of the HPLC-MS/MS method for the analysis of OHPAHs

Water (A) and methanol (B) containing 0.1% of acetic acid were used as mobile phases. The gradient was as follows: 0 min 90% A, 1 min 45% A, 30 min 10% A, 31 min 90% A, and 35 min 90% A. The flow rate used was 150 $\mu l \cdot min^{-1}$ and the injection volume was 10 µl. The column temperature was maintained at 40 °C. The setting of the MS/MS detector was: spray voltage of 4000 V, sheath gas of 10 a.u., capillary temperature of 400 ºC, and auxiliary gas of 25 a.u. The retention times and transitions of the target analytes are listed in Table S3. The quantification and confirmation criteria for the UHPLC-MS/MS were based on the SANTE/11813/2017 guide: i) the signal-to-noise ratio (S/N) of the peaks had to be ≥ 3; and ii) the retention time (RT) of the OHPAHs in unknown urine samples had to coincide with the RT in the matrix-matched calibration curve with a tolerance of ± 0.1 min in the same analytical set.

SM3. Method validation and QA/QC

The analytical method validation was performed following some points of the Bioanalytical Method Validation Guidance for Industry (U.S. Department of Health and Human Services Food and Drug Administration, 2018). Linearity, accuracy, precision, and specificity were evaluated in five validation runs in different days, as follows:

i) *Linearity.* Linearity was checked using six-point calibration curves, spiking synthetic urine at different concentration levels (from the LoQ to 50 $ng \cdot ml^{-1}$). The deviation of the calculated concentrations of each calibration point from the real concentrations could not differ more than ± 20%. Analysis of Variance (ANOVA) and $R^2 > 0.99$ were also used for evaluating the method's linearity.

ii) *Accuracy and precision.* The accuracy was calculated in terms of relative recovery by spiking the synthetic urine at three levels: LoQ, 0.5 $ng \cdot ml^{-1}$ (intermediate level) and 50 $ng \cdot ml^{-1}$ (high level). Relative recoveries (%R) had to be between 80 and 120% for the LoQ level, and between 85 and 115% for the intermediate and high levels. Mean recoveries of each concentration for all validation days were evaluated by calculating an associated precision as a coefficient of variation (CV%). Precision (CV%) had to be ≤ 20% for the LoQ level, and ≤ 15% for the intermediate and high levels.

iii) *Procedural LoQ.* The LoQ for each OHPAH was the lowest spiked level meeting the aforementioned criteria for accuracy and precision, and with a S/N ratio higher than or equal to 10.

iv) *Specificity.* The response of reagent and sample blanks in each validation batch had to be lower than 30% of the LoQ level area.

The aforementioned criteria were also applied to the QA/QC of the method in each batch of samples.

Table S1. Estimation of the food intake frequency (number of portions · month^{-1}).

Questionnaire intake frequency	Intake frequency (portions · month^{-1})
Never or less than once a month	0.5
1 – 3 times a month	2
Once a week	4
2 – 4 times a week	12
5 – 6 times a week	22
Once a day	30
2 – 3 times a day	75
4 – 5 times a day	135
More than 6 times a day	195

Table S2. Criteria used for the Mediterranean Diet Quality Index (Med-DQI) calculation.

Score	SFA (% energy)	Cholesterol (mg·day^{-1})	Meat (g·day^{-1})	Olive oil (ml·day^{-1})	Fish (g·day^{-1})	Legumes and cereals (g·day^{-1})	Fruits and vegetables (g·day^{-1})
0	<10	<300	<25	>15	>60	>300	>700
1	10 - 13	300 - 400	25 - 125	5 - 15	30 - 60	100 - 300	400 - 700
2	>13	>400	>125	<5	<30	<100	<400

SFA: Saturated fatty acids

Table S3. Parent compound, name, acronym, CAS number, elemental composition, transitions and retention time of OHPAHs and their internal standards.

Parent compound	Metabolite	Acronym	CAS Number	Elemental composition	Transitions (m/z)		RT (min)
					Precursor	Fragment	
Naphthalene, carbaryl[a]	1-hydroxynaphthalene	1OHNAP	90-15-3	$C_{10}H_8O$	143	115	9.88
Naphthalene	2-hydroxynaphthalene	2OHNAP	135-19-3				10.49
Fluorene	2-hydroxyfluorene	2OHFLU	2443-58-5	$C_{13}H_{10}O$	181	180	13.14
	3-hydroxyfluorene	3OHFLU	6344-67-8		181	153	12.97
Phenanthrene	1-hydroxyphenanthrene	1OHPHEN	2433-56-9	$C_{14}H_{10}O$	193	165	15.90
	2-hydroxyphenanthrene	2OHPHEN	605-55-0				14.86
	3-hydroxyphenanthrene	3OHPHEN	605-87-8				15.16
	4-hydroxyphenanthrene	4OHPHEN	7651-86-7				16.70
	9-hydroxyphenanthrene	9OHPHEN	484-17-3				16.02
Pyrene	1-hydroxypyrene	1OHPYR	5315-79-7	$C_{16}H_{10}O$	217	189	19.79
Benzo(a)pyrene	3-hydroxybenzo(a)pyrene	3OHBAP	13345-21-6	$C_{20}H_{12}O$	267	239	25.22
Internal standards	1-hydroxynaphthalene-$^{13}C_6$	$^{13}C_6$-1OHNAP	-	$^{13}C_6{}^{12}C_4H_8O$	149	121	9.88
	2-hydroxynaphthalene-$^{2}H_7$	$^{2}H_7$-2OHNAP	-	$C_{10}D_7HO$	150	128	10.49
	2-hydroxyfluorene-$^{13}C_6$	$^{13}C_6$-2OHFLU	-	$^{13}C_6{}^{12}C_7H_{10}O$	187	186	13.14
	2-hydroxyphenanthrene-$^{13}C_6$	$^{13}C_6$-2OHPHEN	-	$^{13}C_6{}^{12}C_8H_{10}O$	199	171	14.86
	9-hydroxyphenanthrene-$^{2}H_8$	$^{2}H_8$-9OHPHEN	-	$C_{14}D_8H_2O$	201	173	16.02
	1-hydroxypyrene-$^{13}C_6$	$^{13}C_6$-1OHPYR	-	$^{13}C_6{}^{12}C_{10}H_{10}O$	223	195	19.79
	3-hydroxybenzo(a)pyrene-$^{2}H_{11}$	$^{2}H_{11}$-3OHBAP	-	$C_{20}D_{11}H_1O$	278	250	25.22

[a]It is not a PAH, but a pesticide compound

Table S4. Results of the parameters evaluated in the validation of the method for the analysis of 11 OHPAHs in urine: linearity, accuracy, precision and specificity.

Analytes	Procedural LoQ ($ng \cdot ml^{-1}$)	Linearity		Accuracy (R%)			Precision (CV%)			Specificity (%)
		R^2	Residuals	LoQ level	Intermediate level	High level	LoQ level	Intermediate level	High level	
1OHNAP	0.01	0.985 - 0.999	0.2 - 18.0	102	101	97	14.8	6.6	3.1	12.7
2OHNAP	0.01	0.972 - 0.998	0.9 - 17.6	108	92	106	17.9	7.5	6.4	11.0
2OHFLU	0.01	0.983 - 0.999	0.0 - 16.9	108	99	103	13.0	2.6	2.0	8.6
3OHFLU	0.05	0.984 - 0.996	0.1 - 16.7	82	95	103	16.6	6.6	2.9	10.7
1OHPHEN	0.01	0.981 - 0.997	0.8 - 13.9	100	103	102	7.1	3.6	2.1	11.3
2OHPHEN	0.01	0.987 - 0.998	0.1 - 16.6	100	97	107	9.9	5.8	4.7	11.4
3OHPHEN	0.01	0.989 - 0.998	0.1 - 19.4	100	98	104	7.1	4.1	4.3	7.4
4OHPHEN	0.01	0.990 - 0.999	0.0 - 17.8	100	105	95	8.2	3.0	9.6	6.9
9OHPHEN	0.01	0.987 - 0.997	0.3 - 15.9	102	96	104	14.8	5.0	8.7	17.3
1OHPYR	0.01	0.99 - 1.00	0.1 - 14.8	110	103	109	8.4	2.0	5.6	11.8
3OHBAP	0.05	0.979 - 0.993	0.0 - 19.4	100	101	97	9.9	8.0	9.0	13.0

LoQ = limit of quantification; R% = relative recovery (%); CV%: coefficient of variation (%); Intermediate level = 0.5 $ng \cdot ml^{-1}$ in urine; High level = 50 $ng \cdot ml^{-1}$ in urine

Table S5. Characteristics and parameters of the PAH compounds selected for the calculation of EDI and estimation of non-cancer risk.

Parent compound	IARC classification[a]	MW (g·mol^{-1})	RfD (µg·kg^{-1}·day^{-1})	F_{UE}[b]	Urinary metabolites
Naphthalene	Group 2B	128.17	20[c]	1	∑OHNAPs
Fluorene	Group 3	166.22	40[d]	0.78	∑OHFLUs
Phenanthrene	Group 3	178.23	30[e]	0.09	∑OHPHENs
Pyrene	Group 3	202.26	30[f]	0.08	OHPYR

EDI = estimated daily intake; ∑OHNAPs = 1OHNAP + 2OHNAP; ∑OHFLUs = 2OHFLU + 3 OHFLU; ∑OHPHENs = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN; IARC = International Agency for Research on Cancer; MW = molecular weight; RfD = reference dose; F_{UE} = urinary excretion factor

[a]Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans

[b]Mean values of the F_{UE}s reported by Li et al., 2012 and Motorykin et al., 2015; except for naphthalene, in which the maximum possible F_{UE} value (100%) was selected.

[c]Basis: decreased mean terminal body weight in male rates (US EPA, 1998)

[d]Basis: decreased red blood cells, packed cell volume and hemoglobin (US EPA, 1990a)

[e]Calculated based on pyrene as a chemical surrogate (TCEQ, 2017)

[f]Basis: kidney effects, such as renal tubular pathology and decreased kidney weights (US EPA, 1990b)

Table S6. Spearman correlations among urinary levels of the OHPAHs.

Biomarker	∑OHFLUs	∑OHPHENs	1OHPYR
∑OHNAPs	0.506**	0.468**	0.590**
∑OHFLUs	-	0.497**	0.519**
∑OHPHENs	-	-	0.625**

*p<0.05
**p<0.01

Table S7. Results of linear regression model with influencing factors on the log-transformed PAH metabolite concentrations including subdivisions of the food groups: Fishery products and Drinks (n = 104).

Metabolite	Variable	Standardized regression coefficients (95% CI)	Standard error	p-value	R^2	adjR^2
ΣOHNAPs	Intercept	3.194 (2.735 – 3.653)	0.231	**<0.001**	0.392	0.355
	Creatinine (g·l^{-1})	0.871 (0.614 – 1.128)	0.129	**<0.001**		
	Smoking habit (Former smoker)	0.124 (-0.142 – 0.390)	0.134	0.358		
	Smoking habit (Currently smokes)	0.803 (0.313 – 1.293)	0.247	**0.002**		
	Smoked fish (g·month^{-1})	0.329 (0.068 – 0.591)	0.132	**0.014**		
	Vegetables (g·month^{-1})	-0.289 (-0.545 – -0.033)	0.129	**0.027**		
	Bakery products	-0.217 (-0.476 – 0.041)	0.130	0.099		
ΣOHFLUs	Intercept	-0.811 (-1.391 – -0.231)	0.292	**0.007**	0.399	0.355
	Smoking habit (Former smoker)	0.273 (-0.018 – 0.564)	0.147	0.066		
	Smoking habit (Currently smokes)	1.329 (0.788 – 1.870)	0.273	**<0.001**		
	Creatinine (g·l^{-1})	0.574 (0.289 – 0.859)	0.144	**<0.001**		
	Coffee (ml·month^{-1})	0.404 (0.122 – 0.686)	0.142	**0.006**		
	Cereals (g·month^{-1})	0.345 (0.061 – 0.630)	0.143	**0.018**		
	Time with the windows open during the day (hours)	-0.261 (-0.540 – 0.018)	0.141	0.066		
	Saturated fatty acids energy (% SFA)	0.251 (-0.040 – 0.541)	0.146	0.090		
ΣOHPHENs	Intercept	0.148 (-0.167 – 0.462)	0.158	0.353	0.293	0.265
	Creatinine (g·l^{-1})	0.524 (0.298 – 0.749)	0.114	**<0.001**		
	Smoking habit (Former smoker)	-0.071 (-0.299 – 0.158)	0.115	0.541		
	Smoking habit (Currently smokes)	0.632 (0.212 – 1.053)	0.212	**0.004**		
	Saturated fatty acids energy (% SFA)	0.227 (0.001 – 0.453)	0.030	**0.049**		
1OHPYR	Intercept	-0.376 (-1.297 – 0.544)	0.464	0.419	0.386	0.348
	Creatinine (g·l^{-1})	0.523 (0.305 – 0.741)	0.110	**<0.001**		
	Smoking habit (Former smoker)	0.098 (-0.122 – 0.318)	0.111	0.379		
	Smoking habit (Currently smokes)	0.825 (0.424 – 1.227)	0.202	**<0.001**		
	Age (years)	-0.398 (-0.669 – -0.127)	0.137	**0.004**		
	Smoked fish (g·month^{-1})	0.266 (0.053 – 0.479)	0.107	**0.015**		
	Saturated fatty acids energy (% SFA)	0.203 (-0.013 – 0.418)	0.109	0.066		

Table S8. Descriptive analysis resulting from the sensitivity analysis of the handling of data below LoQ by different methodologies.[a]

Metabolite	Method	AM	SD	GM	P50	P25	P75	P95
1OHNAP	"Discard"	2.06	4.07	0.91	0.78	0.37	2.05	5.76
	"LoQ/2"	1.99	4.02	0.76	0.74	0.32	1.70	5.70
	"LoQ/√2"	1.99	4.02	0.77	0.74	0.32	1.70	5.70
	"Lower-bound"	1.99	4.02	-	0.74	0.32	1.70	5.70
	"Upper-bound"	1.99	4.02	0.78	0.74	0.32	1.70	5.70
	"MLE 1"	1.99	4.02	0.77	0.74	0.32	1.70	5.70
	"MLE 2"	1.99	4.02	0.77	0.74	0.32	1.70	5.70
	"MLE 3"	1.99	4.02	0.75	0.74	0.32	1.70	5.70
	"MLE 4"	1.99	4.02	0.77	0.74	0.32	1.70	5.70
	"MLE 5"	1.99	4.02	0.77	0.74	0.32	1.70	5.70
2OHFLU	"Discard"	0.31	0.40	0.18	0.16	0.10	0.31	1.15
	"LoQ/2"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
	"LoQ/√2"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
	"Lower-bound"	0.30	0.40	-	0.15	0.10	0.31	1.15
	"Upper-bound"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
	"MLE 1"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
	"MLE 2"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
	"MLE 3"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
	"MLE 4"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
	"MLE 5"	0.30	0.40	0.17	0.15	0.10	0.31	1.15
3OHFLU	"Discard"	0.38	0.41	0.26	0.23	0.13	0.50	1.12
	"LoQ/2"	0.27	0.37	0.13	0.14	0.03	0.35	1.01
	"LoQ/√2"	0.27	0.37	0.14	0.14	0.04	0.35	1.01
	"Lower-bound"	0.26	0.38	-	0.14	0.00	0.35	1.01
	"Upper-bound"	0.28	0.37	0.16	0.14	0.05	0.35	1.01
	"MLE 1"	0.27	0.37	0.13	0.14	0.05	0.35	1.01
	"MLE 2"	0.27	0.37	0.12	0.14	0.04	0.35	1.01
	"MLE 3"	0.27	0.37	0.12	0.14	0.04	0.35	1.01
	"MLE 4"	0.27	0.37	0.13	0.14	0.04	0.35	1.01
	"MLE 5"	0.27	0.38	0.13	0.14	0.04	0.35	1.02
1OHPHEN	"Discard"	0.23	0.22	0.15	0.16	0.08	0.24	0.70
	"LoQ/2"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
	"LoQ/√2"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
	"Lower-bound"	0.22	0.22	-	0.15	0.08	0.24	0.70
	"Upper-bound"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
	"MLE 1"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
	"MLE 2"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
	"MLE 3"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
	"MLE 4"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
	"MLE 5"	0.22	0.22	0.14	0.15	0.08	0.24	0.70
2OHPHEN	"Discard"	0.09	0.06	0.07	0.07	0.04	0.11	0.20

Table S8. Descriptive analysis resulting from the sensitivity analysis of the handling of data below LoQ by different methodologies.[a]

Metabolite	Method	AM	SD	GM	P50	P25	P75	P95
	"LoQ/2"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
	"LoQ/√2"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
	"Lower-bound"	0.08	0.06	-	0.07	0.04	0.10	0.20
	"Upper-bound"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
	"MLE 1"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
	"MLE 2"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
	"MLE 3"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
	"MLE 4"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
	"MLE 5"	0.08	0.06	0.06	0.07	0.04	0.10	0.20
3OHPHEN	"Discard"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"LoQ/2"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"LoQ/√2"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"Lower-bound"	0.12	0.10	-	0.10	0.06	0.16	0.35
	"Upper-bound"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"MLE 1"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"MLE 2"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"MLE 3"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"MLE 4"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
	"MLE 5"	0.12	0.10	0.09	0.10	0.06	0.16	0.35
4OHPHEN	"Discard"	0.06	0.05	0.04	0.04	0.02	0.08	0.16
	"LoQ/2"	0.05	0.05	0.04	0.04	0.02	0.07	0.16
	"LoQ/√2"	0.05	0.05	0.04	0.04	0.02	0.07	0.16
	"Lower-bound"	0.05	0.05	-	0.04	0.02	0.07	0.16
	"Upper-bound"	0.06	0.05	0.04	0.04	0.02	0.07	0.16
	"MLE 1"	0.05	0.05	0.04	0.04	0.02	0.07	0.16
	"MLE 2"	0.05	0.05	0.04	0.04	0.02	0.07	0.16
	"MLE 3"	0.05	0.05	0.04	0.04	0.02	0.07	0.16
	"MLE 4"	0.05	0.05	0.04	0.04	0.02	0.07	0.16
	"MLE 5"	0.05	0.05	0.04	0.04	0.02	0.07	0.16
9OHPHEN	"Discard"	0.16	0.15	0.11	0.12	0.07	0.17	0.44
	"LoQ/2"	0.14	0.15	0.08	0.11	0.05	0.16	0.44
	"LoQ/√2"	0.14	0.15	0.08	0.11	0.05	0.16	0.44
	"Lower-bound"	0.14	0.15	-	0.11	0.05	0.16	0.44
	"Upper-bound"	0.14	0.15	0.09	0.11	0.05	0.16	0.44
	"MLE 1"	0.14	0.15	0.08	0.11	0.05	0.16	0.44
	"MLE 2"	0.14	0.15	0.08	0.11	0.05	0.16	0.44
	"MLE 3"	0.14	0.15	0.08	0.11	0.05	0.16	0.44
	"MLE 4"	0.14	0.15	0.08	0.11	0.05	0.16	0.44
	"MLE 5"	0.14	0.15	0.08	0.11	0.05	0.16	0.44
∑OHNAPs	"Discard"	83	84	58	61	31	90	250
	"LoQ/2"	83	84	58	61	31	90	250

Table S8. Descriptive analysis resulting from the sensitivity analysis of the handling of data below LoQ by different methodologies.[a]

Metabolite	Method	AM	SD	GM	P50	P25	P75	P95
	"LoQ/v2"	83	84	58	61	31	90	250
	"Lower-bound"	83	84	58	61	31	90	250
	"Upper-bound"	83	84	58	61	31	90	250
	"MLE 1"	83	84	58	61	31	90	250
	"MLE 2"	83	84	58	61	31	90	250
	"MLE 3"	83	84	58	61	31	90	250
	"MLE 4"	83	84	58	61	31	90	250
	"MLE 5"	83	84	58	61	31	90	250
∑OHFLUs	"Discard"	3.1	3.9	1.9	2.0	1.0	3.6	10.9
	"LoQ/2"	3.1	3.9	2.0	2.0	1.0	3.6	10.9
	"LoQ/v2"	3.2	3.9	2.0	2.0	1.0	3.6	10.9
	"Lower-bound"	3.1	3.9	1.9	2.0	1.0	3.6	10.9
	"Upper-bound"	3.2	3.9	2.1	2.0	1.0	3.6	10.9
	"MLE 1"	3.1	3.9	2.0	2.0	1.0	3.6	10.9
	"MLE 2"	3.1	3.9	2.0	2.0	1.0	3.6	10.9
	"MLE 3"	3.1	3.9	2.0	2.0	1.0	3.6	10.9
	"MLE 4"	3.2	3.9	2.0	2.0	1.0	3.6	10.9
	"MLE 5"	3.1	3.9	2.0	2.0	1.0	3.6	10.9
∑OHPHENs	"Discard"	3.2	2.4	2.6	2.5	1.7	4.3	8.3
	"LoQ/2"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
	"LoQ/v2"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
	"Lower-bound"	3.2	2.4	2.6	2.5	1.7	4.3	8.3
	"Upper-bound"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
	"MLE 1"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
	"MLE 2"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
	"MLE 3"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
	"MLE 4"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
	"MLE 5"	3.2	2.4	2.6	2.5	1.7	4.3	8.4
∑OHPAHs	"Discard"	90	89	65	66	35	97	259
	"LoQ/2"	90	89	65	66	36	97	259
	"LoQ/v2"	90	89	65	66	36	97	259
	"Lower-bound"	90	89	65	66	35	97	259
	"Upper-bound"	90	89	65	66	36	97	259
	"MLE 1"	90	89	65	66	36	97	259
	"MLE 2"	90	89	65	66	36	97	259
	"MLE 3"	90	89	65	66	36	97	259
	"MLE 4"	90	89	65	66	36	97	259
	"MLE 5"	90	89	65	66	36	97	259

AM = Arithmetic mean; GM= Geometric mean; SD = Standard deviation; P = percentile

[a]The sensitivity analysis was only conducted for PAH metabolites with detection frequencies ranging from 40 to 100%.

Table S9. Linear regression models resulting from the sensitivity analysis of the handling of data below LoQ by different methodologies (n = 104).[a]

Metabolite	Variable	Regression coefficient	p-value	adjR²
ΣOHNAPs[b]	Intercept	3.013	**<0.001**	0.345
	Creatinine (g·l^{-1})	0.807	**<0.001**	
	Smoking habit (Former smoker)	0.082	0.549	
	Smoking habit (Currently smokes)	0.826	**0.001**	
	Vegetables (g·month^{-1})	-0.279	**0.037**	
	Oils and fats (g·month^{-1})	0.306	**0.026**	
	Adherence to the Mediterranean diet (Good)	-0.213	0.115	
ΣOHFLUs[c]	Intercept	-1.452	**0.002**	0.347
	Smoking habit (Former smoker)	0.277	0.051	
	Smoking habit (Currently smokes)	1.360	**<0.001**	
	Creatinine (g·l^{-1})	0.597	**<0.001**	
	Drinks (ml·month^{-1})	0.410	**0.007**	
	Cereals (g·month^{-1})	0.318	**0.024**	
	Saturated fatty acids energy (% SFA)	0.333	**0.029**	
	Time with the windows open during the day (hours)	-0.212	0.124	
ΣOHFLUs[d]	Intercept	-0.972	0.251	0.367
	Smoking habit (Former smoker)	0.327	**0.040**	
	Smoking habit (Currently smokes)	1.606	**<0.001**	
	Creatinine (g·l^{-1})	0.661	**<0.001**	
	Drinks (ml·month^{-1})	0.534	**0.002**	
	Saturated fatty acids energy (% SFA)	0.426	**0.012**	
	Cereals (g·month^{-1})	0.378	**0.016**	
	Primipara (No)	0.341	**0.046**	
	Age (years)	-0.379	0.074	
	Time with the windows open during the day (hours)	-0.254	0.104	
ΣOHPHENs[b]	Intercept	0.151	0.342	0.265
	Creatinine (g·l^{-1})	0.522	**<0.001**	
	Smoking habit (Former smoker)	-0.069	0.550	
	Smoking habit (Currently smokes)	0.631	**0.004**	
	Saturated fatty acids energy (% SFA)	0.227	**0.048**	

[a]The sensitivity analysis was only conducted for PAH metabolites with detection frequencies ranging from 40 to 100%.
[b]All methods for handling data below LoQ included these variables in their fitted models, but with different regression coefficients, p-values and R^2. Only the regression model fitted with the "Upper-bound" method is presented.
[c]The "Upper-bound", "LoQ/2", "LoQ/√2" and "MLE" methods included these variables in their fitted models, but with different regression coefficients, p-values and R^2. Only the regression model fitted with the "Upper-bound" method is presented.
[d]The "Lower-bound" and "Discard" methods included these variables in their fitted models, but with different regression coefficients, p-values and R^2. Only the regression model fitted with the "Lower-bound" method is presented.

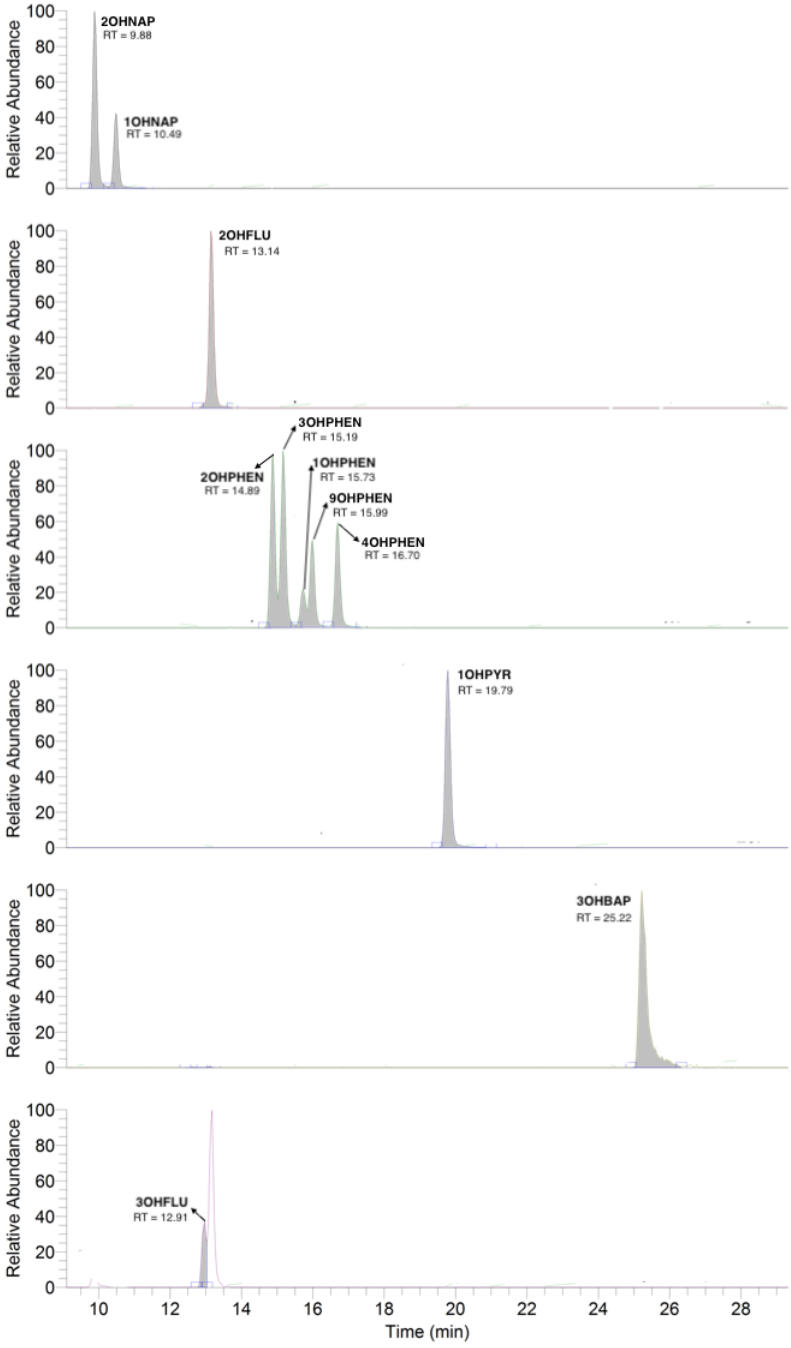

Figure S1. SRM chromatogram of OHPAHs obtained with the KINETEX Core-shell technology F5 column (150 mm × 2.1 mm, 2.6 µm) from the highest level of the matrix-matched calibration curve (50 ng·ml^{-1}).

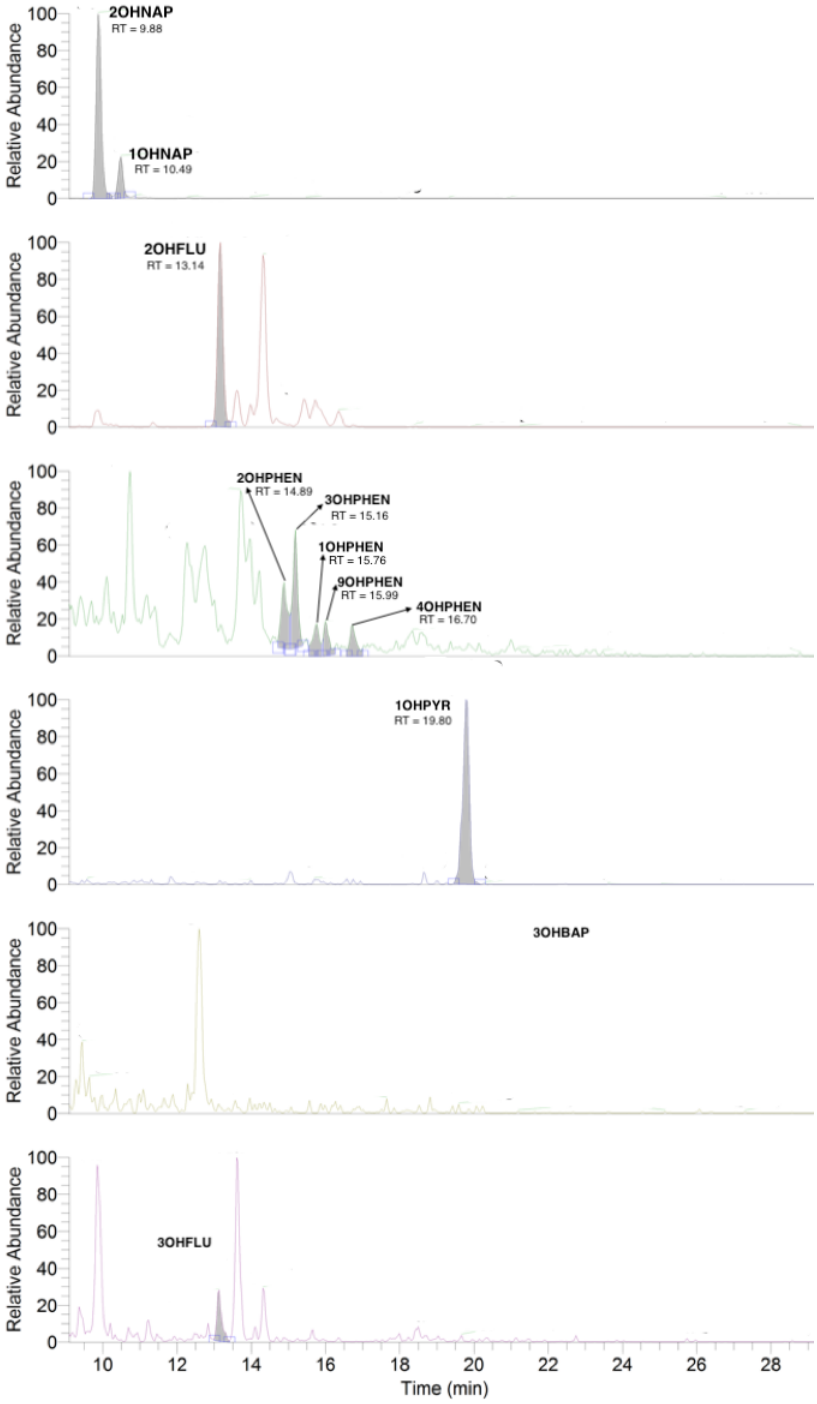

Figure S2. SRM chromatogram of OHPAHs obtained with the KINETEX Core-shell technology F5 column (150 mm × 2.1 mm, 2.6 µm) from a real urine sample.

9.2.2 SUPPLEMENTARY INFORMATION OF ARTICLE II.2

SUPPLEMENTARY MATERIAL FOR:

Children's exposure to polycyclic aromatic hydrocarbons in the Valencian Region (Spain): urinary levels, predictors of exposure and risk assessment

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Table S1. Parent compound, name, acronym, CAS number, elemental composition, transitions and retention time of OHPAHs and their internal standards.

Parent compound	Metabolite	Acronym	CAS Number	Elemental composition	Transitions (m/z)		RT (min)
					Precursor	Fragment	
Naphthalene[a]	1-hydroxynaphthalene	1OHNAP	90-15-3	$C_{10}H_8O$	143	115	9.88
	2-hydroxynaphthalene	2OHNAP	135-19-3				10.49
Fluorene	2-hydroxyfluorene	2OHFLU	2443-58-5	$C_{13}H_{10}O$	181	180	13.14
	3-hydroxyfluorene	3OHFLU	6344-67-8		181	153	12.97
Phenanthrene	1-hydroxyphenanthrene	1OHPHEN	2433-56-9	$C_{14}H_{10}O$	193	165	15.90
	2-hydroxyphenanthrene	2OHPHEN	605-55-0				14.86
	3-hydroxyphenanthrene	3OHPHEN	605-87-8				15.16
	4-hydroxyphenanthrene	4OHPHEN	7651-86-7				16.70
	9-hydroxyphenanthrene	9OHPHEN	484-17-3				16.02
Pyrene	1-hydroxypyrene	1OHPYR	5315-79-7	$C_{16}H_{10}O$	217	189	19.79
Benzo(a)pyrene	3-hydroxybenzo(a)pyrene	3OHBAP	13345-21-6	$C_{20}H_{12}O$	267	239	25.22
Internal standards	1-hydroxynaphthalene-$^{13}C_6$	$^{13}C_6$-1OHNAP	-	$^{13}C_6{}^{12}C_4H_8O$	149	121	9.88
	2-hydroxynaphthalene-$^2H_7$	$^2H_7$-2OHNAP	-	$C_{10}D_7HO$	150	128	10.49
	2-hydroxyfluorene-$^{13}C_6$	$^{13}C_6$-2OHFLU	-	$^{13}C_6{}^{12}C_7H_{10}O$	187	186	13.14
	2-hydroxyphenanthrene-$^{13}C_6$	$^{13}C_6$-2OHPHEN	-	$^{13}C_6{}^{12}C_8H_{10}O$	199	171	14.86
	9-hydroxyphenanthrene-$^2H_8$	$^2H_8$-9OHPHEN	-	$C_{14}D_8H_2O$	201	173	16.02
	1-hydroxypyrene-$^{13}C_6$	$^{13}C_6$-1OHPYR	-	$^{13}C_6{}^{12}C_{10}H_{10}O$	223	195	19.79
	3-hydroxybenzo(a)pyrene-$^2H_{11}$	$^2H_{11}$-3OHBAP	-	$C_{20}D_{11}H_1O$	278	250	25.22

[a]Other substances might be parent compounds of 1OHNAP (e.g. carabaryl).

Table S2. Characteristics and parameters of the PAH compounds selected for the calculation of EDI and estimation of the non-cancer risk.

Parent compound	IARC classification[a]	MW (g·mol^{-1})	RfD (µg·kg-bw^{-1}·day^{-1})	F_{UE}[b]	Urinary metabolites
Naphthalene	Group 2B	128.17	20[c]	1	∑OHNAPs
Fluorene	Group 3	166.22	40[d]	0.78	∑OHFLUs
Phenanthrene	Group 3	178.23	30[e]	0.09	∑OHPHENs
Pyrene	Group 3	202.26	30[f]	0.08	OHPYR

EDI = estimated daily intake; ∑OHNAPs = 1OHNAP + 2OHNAP; ∑OHFLUs = 2OHFLU + 3 OHFLU; ∑OHPHENs = 1OHPHEN + 2OHPHEN + 3OHPHEN + 4OHPHEN + 9OHPHEN; IARC = International Agency for Research on Cancer; MW = molecular weight; RfD = reference dose; F_{UE} = urinary excretion factor

[a]Group 2B: Possibly carcinogenic to humans; Group 3: Not classifiable as to its carcinogenicity to humans

[b]Mean values of the F_{UE}s reported by Li et al., 2012 and Motorykin et al., 2015; except for naphthalene, in which the maximum possible F_{UE} value (100%) was selected.

[c]Basis: decreased mean terminal body weight in male rates (US EPA, 1998)

[d]Basis: decreased red blood cells, packed cell volume and hemoglobin (US EPA, 1990a)

[e]Calculated based on pyrene as a chemical surrogate (TCEQ, 2017)

[f]Basis: kidney effects, such as renal tubular pathology and decreased kidney weights (US EPA, 1990b)

Table S3. Spearman correlations among urinary levels of the OHPAHs.

Biomarker	∑OHNAPs	∑OHFLUs	∑OHPHENs	1OHPYR
∑OHNAPs	-	0.406*	0.393*	0.419*
∑OHFLUs	-	-	0.717*	0.610*
∑OHPHENs	-	-	-	0.737*

*p<0.01

Table S4. Results of the linear regression models with influencing factors on the log-transformed PAH metabolite concentrations (n = 530).

Metabolite	Variable	Standardized regression coefficients (95% CI)	Standard error	p-value	R^2	adjR^2
[a]ΣOHNAPs	Intercept	3.438 (2.877 – 3.998)	0.285	**<0.001**	0.171	0.160
	Creatinine (g·l^{-1})	0.725 (0.557 – 0.893)	0.085	**<0.001**		
	Country of birth of the parents (Foreign)	-0.441 (-0.705 – -0.177)	0.134	**0.001**		
	Age (years)	-0.335 (-0.508 – -0.162)	0.088	**<0.001**		
	BMI (kg·m^{-2})	0.248 (0.078 – 0.419)	0.087	**0.004**		
	Chips (g in the last 72h)	0.169 (0.003 – 0.334)	0.085	**0.046**		
	Eggs (g in the last 72h)	0.167 (-0.001 – 0.335)	0.085	0.051		
	Vegetables (g in the last 72h)	0.120 (-0.048 – 0.287)	0.085	0.161		
[b]ΣOHFLUs	Intercept	-0.452 (-0.829 – -0.076)	0.192	**0.018**	0.174	0.162
	Creatinine (g·l^{-1})	0.602 (0.477 – 0.727)	0.064	**<0.001**		
	Province (Castellón)	0.255 (0.091 – 0.420)	0.084	**0.002**		
	Province (Valencia)	0.279 (0.136 – 0.421)	0.072	**<0.001**		
	Canned food (g·month^{-1})	-0.238 (-0.365 – -0.111)	0.064	**<0.001**		
	Plastic-packed food (g·month^{-1})	0.210 (0.083 – 0.336)	0.064	**0.001**		
	Age (years)	-0.190 (-0.315 – -0.064)	0.064	**0.003**		
	Legumes (g in the last 72h)	0.130 (0.006 – 0.253)	0.063	**0.039**		
	Cereals (g in the last 72h)	0.097 (-0.025 – 0.219)	0.062	0.119		
[c]ΣOHPHENs	Intercept	-0.222 (-0.538 – 0.093)	0.160	0.166	0.227	0.215
	Creatinine (g·l^{-1})	0.635 (0.529 – 0.741)	0.054	**<0.001**		
	Province (Castellón)	0.084 (-0.056 – 0.224)	0.071	0.237		
	Province (Valencia)	0.236 (0.116 – 0.356)	0.061	**<0.001**		
	Canned food (g·month^{-1})	-0.156 (-0.263 – -0.048)	0.055	**0.005**		
	Age (years)	-0.150 (-0.256 – -0.044)	0.054	**0.006**		
	Sex (female)	0.117 (0.014 – 0.220)	0.052	**0.023**		
	Plastic-packed food (g·month^{-1})	0.124 (0.016 – 0.232)	0.055	**0.024**		
	Eggs (g in the last 72h)	0.098 (-0.006 – 0.203)	0.053	0.065		
	Legumes (g in the last 72h)	0.070 (-0.034 – 0.175)	0.053	0.186		
[d]1OHPYR	Intercept	-1.077 (-1.496 – -0.657)	0.2134	**<0.001**	0.214	0.227
	Creatinine (g·l^{-1})	0.676 (0.560 – 0.791)	0.059	**<0.001**		
	Age (years)	-0.162 (-0.277 – -0.047)	0.059	**0.006**		

Table S4. Results of the linear regression models with influencing factors on the log-transformed PAH metabolite concentrations (n = 530).

Metabolite	Variable	Standardized regression coefficients (95% CI)	Standard error	p-value	R^2	adjR^2
	Plastic-packed food (g·month^{-1})	0.155 (0.040 – 0.270)	0.059	**0.008**		
	Sex (female)	0.132 (0.020 – 0.244)	0.057	**0.021**		
	Maximum educational level of the parents (Secondary studies)	-0.098 (-0.301 – 0.104)	0.103	0.342		
	Maximum educational level of the parents (University studies)	-0.209 (-0.399 – -0.019)	0.097	**0.031**		
	Chips (g in the last 72h)	-0.122 (-0.234 – 0.010)	0.057	**0.033**		
	Fruits (g in the last 72h)	-0.113 (-0.228 – 0.001)	0.058	0.053		
	Dairy products (g in the last 72h)	-0.090 (-0.205 – 0.024)	0.058	0.122		
	Eggs (g in the last 72h)	0.088 (-0.026 – 0.202)	0.058	0.132		

[a]Regression formula for predicting OHNAPs concentrations in urine: $$C_{\sum OHNAPs} = \frac{e^{3.438} \times e^{0.725 Creatinine} \times e^{0.248 BMI} \times e^{0.169 Chips72h} \times e^{0.167 Eggs72h} \times e^{0.120 Vegetables72h}}{e^{0.441 CountryParents} \times e^{0.335 Age}}$$

[b]Regression formula for predicting OHNAPs concentrations in urine: $$C_{\sum OHFLUs} = \frac{e^{0.602 Creatinine} \times e^{0.255 ProvinceCas} \times e^{0.279 ProvinceVal} \times e^{0.210 Plastic} \times e^{0.130 Legumes72h} \times e^{0.097 Cereals72h}}{e^{0.452} \times e^{0.238 Canned} \times e^{0.190 Age}}$$

[c]Regression formula for predicting OHPHENs concentrations in urine: $$C_{\sum OHPHENs} = \frac{e^{0.635 Creatinine} \times e^{0.084 ProvinceCas} \times e^{0.236 ProvinceVal} \times e^{0.117 Sex} \times e^{0.124 Plastic} \times e^{0.098 Eggs72h} \times e^{0.070 Legumes72h}}{e^{0.222} \times e^{0.156 Canned} \times e^{0.150 Age}}$$

[d]Regression formula for predicting OHNAPs concentrations in urine: $$C_{\sum 1OHPYR} = \frac{e^{0.676 Creatinine} \times e^{0.155 Plastic} \times e^{0.132 Sex} \times e^{0.088 Eggs72h}}{e^{1.077} \times e^{0.162 Age} \times e^{0.098 Ed.level_Secondary} \times e^{0.209 Ed.Level_University} \times e^{0.122 Chips72h} \times e^{0.113 Fruits72h} \times e^{0.090 Dairy72h}}$$

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9.3 ANNEXES TO CHAPTER III

9.3.1 SUPPLEMENTARY INFORMATION OF ARTICLE III.1

SUPPLEMENTARY MATERIAL FOR:

Exposure assessment of Spanish lactating mothers to acrylamide via human biomonitoring

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SM1. Preparation of the stock and working solutions

Individual stock solutions were prepared by weighing neat compounds (5 - 10 mg) and diluting each in 5 - 10 ml of water HPLC grade to obtain a concentration of approximately 500 $\mu g \cdot ml^{-1}$. The stock solutions were stored in the dark at −20 °C for 1 year. Mixed working solutions containing between 0.25 and 2000 $ng \cdot ml^{-1}$ of each compound were prepared by diluting the individual stock solutions with water HPLC grade. An internal standard (IS) working solution was also prepared containing all isotopic labelled standards in a concentration of 500 $ng \cdot ml^{-1}$. Aliquots of 1.5 ml of the working solutions were made and stored in the dark at −20 °C until use (for up to 6 months). Once an aliquot was thawed and under use, it was stored at 4°C. The calibration curve and quality control samples were prepared with the working solutions in the same way as the real urine samples.

SM3. Method validation and quality control

The analytical method validation was performed following some points of the Bioanalytical Method Validation Guidance for Industry (U.S. Department of Health and Human Services Food and Drug Administration, 2018). Linearity, accuracy, precision, and specificity were evaluated in five validation runs in different days, as follows:

i) *Linearity.* Linearity was checked using ten-point calibration curves from 0.25 to 1000 $ng \cdot ml^{-1}$. The deviation of the calculated concentrations of each calibration point from the real concentrations could not differ more than ± 20%. Analysis of Variance (ANOVA) and $R^2 > 0.98$ were also used for evaluating the method's linearity.

ii) *Accuracy and precision.* The accuracy was calculated in terms of relative recovery at three levels: 0.25 (LoQ), 100 $ng \cdot ml^{-1}$ (medium level) and 1000 $ng \cdot ml^{-1}$ (high level). Relative recoveries (%R) had to be between 80 and 120% for the LoQ level, and between 85 and 115% for the medium and high levels. Mean recoveries of each concentration for all validation days were evaluated by calculating an associated precision as a coefficient

of variation (CV%). Precision (CV%) had to be ≤ 20% for the LoQ level, and ≤ 15% for the medium and high levels.

iii) *Procedural LoQ.* The LoQ was the lowest spiked level meeting the aforementioned criteria for accuracy and precision, and with a signal-to-noise ratio (S/N) higher than or equal to 10.

iv) *Specificity.* The response of sample blanks in each validation run had to be lower than 30% of the LoQ level area.

The aforementioned criteria were also applied to the quality assurance/quality control (QA/QC) of the method in each batch of samples. In addition to this, the laboratory participates in German External Quality Assessment Scheme (G-EQUAS) intercomparison programs and uses the Standard Reference Materials® 3672 and 3673 (NIST, USA), for external QA purposes.

The quantification and confirmation criteria for the HPLC-MS/MS were based on the SANTE/11813/2017 guide (European Commission, 2017), and are listed below:

i) The S/N of the peaks should be ≥ 3.

ii) At least 2 product ions (one for quantification and one for confirmation) are required for identification of each compound, with their analyte peaks completely overlapping.

iii) The RT of the target analytes in unknown urine samples have to coincide with the RT in the calibration curve with a tolerance of ± 0.1 min in the same analytical set. However, larger RT deviations are acceptable when both RTs and peak shapes of the analytes match with those of their respective IS's.

iv) The ion ratio of the 2 product ions from unknown urine samples should be within ±30% of average of calibration standards from same sequence.

Table S1. Parent compound, metabolite name, acronym, CAS number, elemental composition, transitions, collision energy (CE), RF lens and retention time (RT) of the target analytes and their internal standards.

Parent compound	Metabolite	Acronym	CAS Number	Elemental composition	Transitions (m/z)			CE (Q/q) (V)	RF lens (V)	RT (min)
					Precursor	Quantification (Q)	Confirmation (q)			
Acrylamide	N-Acetyl-S-(carbamoylethyl)-L-cysteine	AAMA	81690-92-8	$C_8H_{14}N_2O_4S$	233.038	104.054	162.054	14.0/9.9	51	4.50
Acrylamide/ Gycidamide	N-Acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine	GAMA3	137698-08-9	$C_8H_{14}N_2O_5S$	249.088	120.071	128.071	15.4/10.4	69	2.70
Acrylamide	N-Acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine	AAMA-Sul	861959-88-8	$C_8H_{14}N_2O_5S$	249.088	178.071	116.071	9.2/14.6	69	2.00
Internal standards	N-Acetyl-S-(carbamoylethyl)-L-cysteine-d_3	AAMA-d_3	-	$C_8H_{11}N_2O_4S^2H_3$	236.079	104.054	-	14.0	51	4.50
	N-Acetyl-S-(2-hydroxy-3-propionamide)-L-cysteine-d_3	GAMA3-d_3	-	$C_8H_{11}N_2O_5S^2H_3$	252.073	131.071	-	10.4	69	2.70
	N-Acetyl-3-[(3-amino-3-oxopropyl)sulfinyl]-L-alanine-d_3	AAMA-Sul-d_3	-	$C_8H_{11}N_2O_5S^2H_3$	252.073	181.071	-	9.2	69	2.00

Table S2. Results of the method validation (n=5) for the analysis of the three urinary AA metabolites: linearity, accuracy, precision and specificity.

Analytes	Procedural LoQ ($ng \cdot ml^{-1}$)	Linearity		Accuracy (R%)			Precision (CV%)			Specificity (%)
		R^2	Residuals (%)	LoQ level	Medium level	High level	LoQ level	Medium level	High level	
AAMA	0.25	0.9877 - 0.9904	0.55 - 17.65	105	114	108	3.7	1.3	1.7	28
GAMA3	0.25	0.9935 - 0.9967	0.05 - 14.66	98	102	96	9.7	1.1	1.2	28
AAMA-Sul	0.25	0.9970 - 0.9983	0.01 - 8.17	97	102	107	6.8	1.0	1.6	28

LoQ = limit of quantification; R% = relative recovery (%); CV%: coefficient of variation (%); LoQ level = 0.25 $ng \cdot ml^{-1}$ in urine; Medium level = 50 $ng \cdot ml^{-1}$ in urine; High level = 1000 $ng \cdot ml^{-1}$ in urine

Table S3. Spearman correlations among AA urinary metabolites.

Biomarker	AAMA	GAMA3	AAMA-Sul
AAMA	-	0.869*	0.907*
GAMA3	-	-	0.866*
AAMA-Sul	-	-	-

*Correlation is significant at the 0.01 level

Table S4. Differences in the parameters used for the estimation of the daily intake of AA based on its urinary biomarkers in several HBM studies.

Reference	Type of urine sample	Biomarkers of exposure to AA	F_{UE}	Method correction	V_{24h} selection criteria	Parameters used for Cre_{24h} calculation
This study	First-morning urine	AAMA + GAMA3 + AAMA-Sul	0.50	24h-urine volume and 24h-creatinine	Theoretical value for adults (1.6 l·day^{-1})	BW, gender, height and age
Brantsæter et al., (2008)	24h-urine	AAMA + GAMA3	0.55	24h-urine volume	Experimental value (NR)	-
Hartmann et al., (2008)	NR	AAMA + GAMA3	0.50	24h-creatinine	-	BW
Heudorf et al., (2009)	Spot urine collected at different times (8 am - 1 pm)	AAMA + GAMA3	0.50	24h-urine volume and 24h-creatinine	Theoretical value for children (0.4 l·day^{-1})	Height and gender
Ji et al., (2013)	First-morning urine	AAMA	0.50	24h-creatinine	-	Age and gender
Mojska et al., (2016)	First-morning urine	AAMA + GAMA3	0.50	24h-creatinine	-	Measured creatinine levels, BW and theoretical V_{24h} for adult women (1.4 l·day^{-1})

NR: not reported; F_{UE}: molar fraction of the ingested dose of AA which is eliminated as urinary mercapturic acids; V_{24h}: total urinary volume excreted within 24h; Cre_{24h}: estimated urinary creatinine excreted within 24h

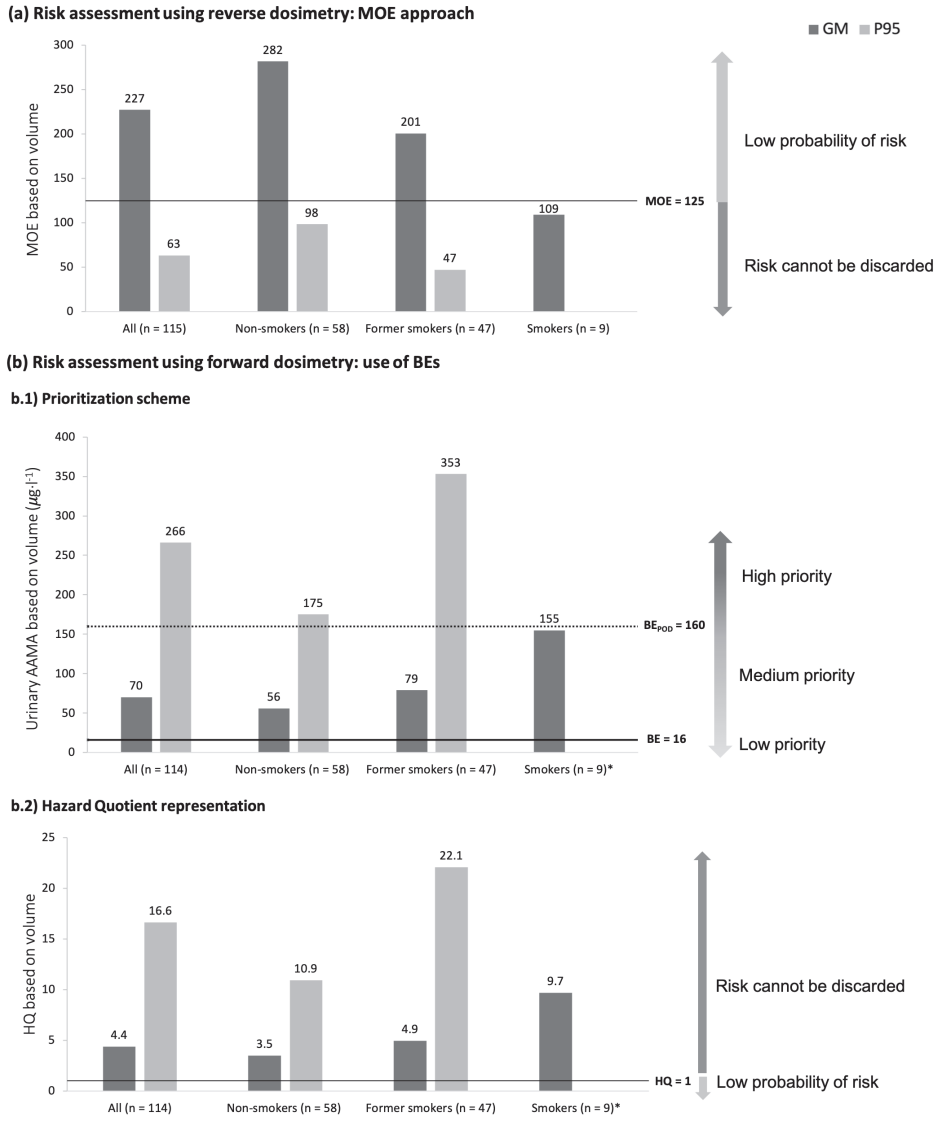

Figure S1. Interpretation of biomonitoring data of exposure to AA (adjusted by volume and stratified by the smoking habit of the target population) obtained in the present study in a risk context using different screening tools: (a) Reverse dosimetry: representation of margin of exposure (MOE) values; and (b) Forward dosimetry by two different strategies: a prioritization diagram (b.1), and a hazard quotient (HQ) representation (b.2) using biomonitoring equivalents (BEs). *Number of smoker women was too low to calculate the risk at P95 level.

9.3.2 SUPPLEMENTARY INFORMATION OF ARTICLE III.2

Risk assessment of the exposure of Spanish children to acrylamide using human biomonitoring

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Figure S1	Chromatograms at a) LOQ (0.25 ng·ml^{-1}), b) medium (50 ng·ml^{-1}) and c) high (1000 ng·ml^{-1}) concentration levels of the calibration curve, and d) of a real urine sample, showing the chromatographic separation of AAMA-Sul, GAMA3 and AAMA. Other peaks with the same Q⇒q transitions as GAMA3 and AAMA-Sul can be observed, which might correspond to different isomers of these metabolites (not included in the method). RT = retention time; Q = quantification transition (m/z); q = confirmation transition (m/z)
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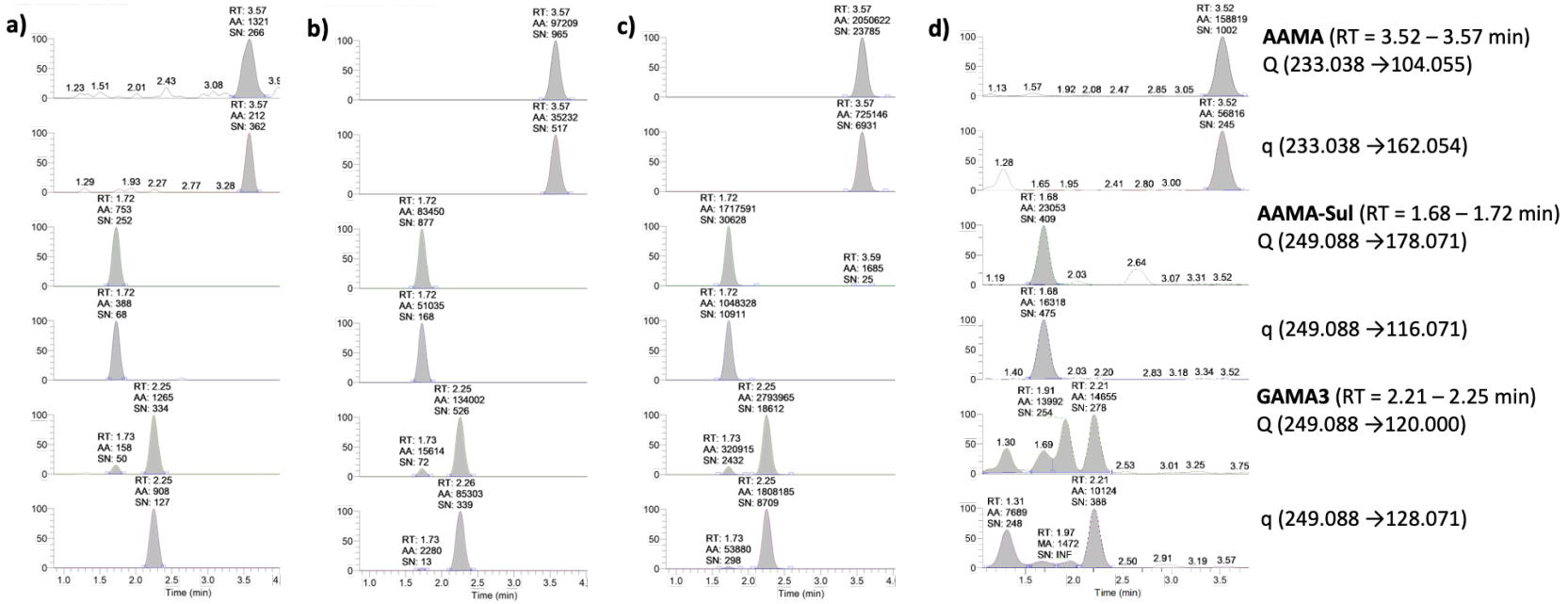


Figure S1. Chromatograms at a) LOQ (0.25 ng·ml^{-1}), b) medium (50 ng·ml^{-1}) and c) high (1000 ng·ml^{-1}) concentration levels of the calibration curve, and d) of a real urine sample, showing the chromatographic separation of AAMA-Sul, GAMA3 and AAMA. Other peaks with the same Q⇒q transitions as GAMA3 and AAMA-Sul can be observed, which might correspond to different isomers of these metabolites (not included in the method). RT = retention time; Q = quantification transition (m/z); q = confirmation transition (m/z)

Table S1. Food consumption frequency of the studied population (n=612).

Food group		n (%)	Mean (minimum – maximum) ($g \cdot month^{-1}$)
Dairy products		-	14184 (1621 - 58406)
Eggs		-	615 (30 - 1800)
	Missing data	4 (0.6%)	
Meat products		-	5327 (670 - 25275)
	Missing data	1 (0.16%)	
Fishery products		-	3219 (150 - 28680)
Vegetables and fruits		-	14027 (1020 - 48600)
Nuts		-	159 (15 - 2250)
	Missing data	7 (1.1%)	
Legumes, potatoes and cereals		-	9190 (1388 - 33325)
Oils and fats		-	649 (25 - 2271)
	Missing data	5 (0.8%)	
Bakery products		-	2042 (101 - 12626)
	Missing data	1 (0.16%)	
Miscellany		-	1471 (60 - 21396)
	Missing data	2 (0.3%)	
Drinks (different from water)		-	4030 (0 - 61731)

Table S2. Spearman correlations among AA urinary metabolites.

Biomarker	AAMA	GAMA3	AAMA-Sul
AAMA	-	0.763*	0.856*
GAMA3	-	-	0.719*
AAMA-Sul	-	-	-

*Correlation significant at the 0.01 level

Table S3. Detailed description of the HBM studies selected for comparison of the urinary levels of AA metabolites in children and adolescents worldwide.

Continent	Country	Study population	Age range (years)	Sampling period	Type of urine sample	Subgroup	N	Statistic value	AAMA	AAMA-Sul	GAMA3	Units	Ratio GAMA3/AAMA	Reference
Europe	Eastern Spain	Children	5 - 12	2016	First-morning urine	-	612	P50	78 (76)	29 (28)	18 (18)	ng·ml^{-1} (µg·g Cre^{-1})	0.23	This study
								DF%	100	100	100		-	
								LOQ	0.25	0.25	0.25		-	
	Germany	Children and adolescents	3 - 17	2015 - 2017	Morning urine	Non-smokers	2211	P50	71 (59)	NA	15 (12)	ng·ml^{-1} (µg·g Cre^{-1})	0.21*	Schwedler et al., (2021)
								DF%	100	NA	100		-	
								LOQ	1	NA	1		-	
		Children	5 - 6	NR	Spot urine	-	110	P50	36 (59)	NA	13 (22)	ng·ml^{-1} (µg·g Cre^{-1})	0.42	Heudorf et al., (2009)
								DF%	NR	NA	NR		-	
								LOD	2	NA	2		-	
		Children and adolescents	6 - 18	NR	Spot urine	6 - 10 years	12	P50	37 (46)	NA	16 (18)	ng·ml^{-1} (µg·g Cre^{-1})	0.50	Hartmann et al., (2008)
								DF%	100	NA	100		-	
						11 - 18 years	11	P50	44 (30)	NA	15 (11)		0.30	
								DF%	100	NA	100		-	
								LOD	1.5	NA	1.5		-	
America	USA	Children	6 - 11	2015 - 2016	NR	-	373	P50	44 (59)	NA	<LOD	ng·ml^{-1} (µg·g Cre^{-1})	-	CDC (2021)
								DF%	NR	NA	NR		-	
								LOD	2.2	NA	9.4		-	
	Canada	Children and adolescents	10 - 17	2009 - 2010	12h-urine	-	195	P50[a]	76	39	10	µg·g Cre^{-1}	0.13*	Brisson et al., (2014)
								DF%	100	100	100		-	
								LOQ	0.32	1.2	1.9	ng·ml^{-1}	-	
Asia	Korea	Children and adolescents	3 - 18	2009 - 2010	Urine collected at different times	-	1025	P50	31 (44)	NA	NA	ng·ml^{-1} (µg·g Cre^{-1})	-	Choi et al., (2019)

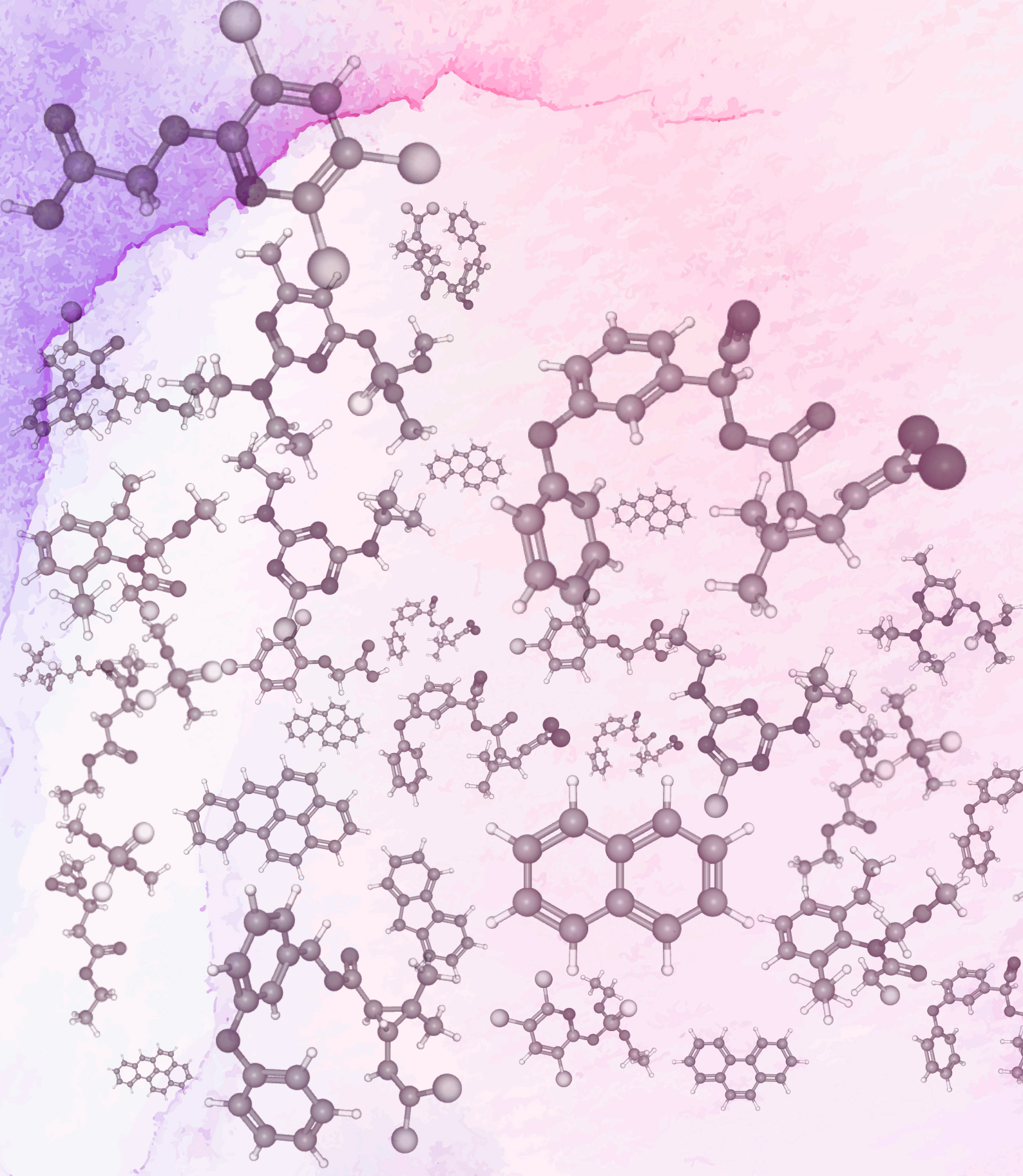
Table S3. Detailed description of the HBM studies selected for comparison of the urinary levels of AA metabolites in children and adolescents worldwide.

Continent	Country	Study population	Age range (years)	Sampling period	Type of urine sample	Subgroup	N	Statistic value	AAMA	AAMA-Sul	GAMA3	Units	Ratio GAMA3/AAMA	Reference
								DF%	99	NA	NA		-	
								LOD	0.6	NA	NA		-	
		Children	10 - 13	2011	First-morning urine	-	39	P50	65 (79)	NA	NA	ng·ml^{-1} (µg·g Cre^{-1})	-	Ji et al., (2013)
								DF%	100	NA	NA		-	
								LOD	5	NA	NA		-	
	China	Young children	0 - 7	2018 - 2019	Urine collected at different times	-	130	P50	26	NA	3	ng·g ml^{-1} (adjusted by specific gravity)	0.12*	Song et al., (2022)
								DF%	100	NA	99.8		-	
								LOD	0.1	NA	0.06	ng·ml^{-1}	-	
		Children	7 - 11	2016 - 2018	Spot urine	Healthy children	69	GM	21	NA	NA	µg·g Cre^{-1}	-	Kuang et al., (2021)
								DF%	100	NA	NA		-	
								LOD	0.8	NA	NA	ng·ml^{-1}	-	

LOQ: limit of quantification; LOD: limit of detection; P50: 50th percentile; DF%: detection frequency; NA: not analyzed; NR: not reported; GM: geometric mean

[a]Converted from µmol·mol Cre^{-1} to µg·g Cre^{-1} (Molecular weight of Cre = 113.12 g·mol^{-1})

*Ratio calculated using the provided statistic values of GAMA3 and AAMA.



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