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Programa de Doctorado con Mención a la Excelencia en Ciencias de la Alimentación

VALORIZACIÓN DE SUBPRODUCTOS DE PESCADO MEDIANTE LA EXTRACCIÓN DE COMPUESTOS ASISTIDA POR MICROONDAS Y LÍQUIDOS PRESURIZADOS. PROPIEDADES NUTRICIONALES Y BIOLÓGICAS.

FISH SIDE STREAMS VALORIZATION THROUGH THE EXTRACTION OF COMPOUNDS ASSISTED BY MICROWAVES AND PRESSURIZED LIQUIDS. NUTRITIONAL AND BIOLOGICAL PROPERTIES

TESIS DOCTORAL INTERNACIONAL

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Valencia, enero 2022

El Dr. Francisco José Barba Orellana, profesor titular de Universidad del Área de Nutrición y Bromatología y la Dra. Houda Berrada Ramdani, profesora titular de Universidad del Área de Toxicología, del departamento de Medicina Preventiva y Salud Pública, Ciencias de la Alimentación, Toxicología y Medicina Legal, de la Universitat de València, **CERTIFICAN QUE:**

La Graduada en Nutrición Humana y Dietética Dña. Beatriz de la Fuente Miguel ha realizado bajo su dirección el trabajo que lleva por título **"Valorización de subproductos de pescado mediante la extracción de compuestos asistida por microondas y líquidos presurizados. Propiedades nutricionales y biológicas"/** **"Fish side streams valorization through the extraction of compounds assisted by microwaves and pressurized liquids. Nutritional and biological properties"**, y autorizan su presentación para optar al título de Doctora Internacional por la Universitat de València.

Para que así conste, expiden y firman el presente certificado

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La presente Tesis Doctoral ha dado lugar a 6 artículos publicados o que se publicarán en las siguientes revistas:

1. An integrated approach for the valorization of sea bass (*Dicentrarchus labrax*) side streams: Evaluation of contaminants and development of antioxidant protein extracts by pressurized liquid extraction (2021). *Foods,* 10, 546. *Impact factor: 4.121*
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3. Salmon (*Salmo salar*) side streams as a bioresource to obtain potential antioxidant peptides after applying pressurized liquid extraction (PLE) (2021). *Marine drugs,* 19, 323. *Impact factor: 4.762*
4. Nutritional and bioactive oils from salmon (*Salmo salar*) side streams obtained by Soxhlet and optimized microwave-assisted extraction (2022). *Food Chemistry. Under review. Impact factor: 7.514*
5. Sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) head oils recovered by microwave-assisted extraction: nutritional and biological properties (2022). *Food Chemistry. Under review. Impact factor: 7.514*
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"Para lo que algunos es basura, para otros es un tesoro"

Refrán popular / Lema por el reciclaje

A todas las niñas y mujeres

valientes y valiosas

A las mujeres más
importantes de mi vida

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List of abbreviations

A	Alanina
ALA	Ácido α-linolénico
AGI	Ácidos grasos insaturados
AGMI	Ácidos grasos monoinsaturados
AGPI	Ácidos grasos poliinsaturados
AGS	Ácidos grasos saturados
ANOVA	Análisis de la varianza
As	Arséncio
AUC	Área bajo la curva
CAA	Actividad antioxidante celular
CCD	Diseño de compuesto central
CCRD	Diseño de compuesto central giratorio
CE	Comisión Europea
Cd	Cadmio
CFU	Unidades formadoras de colonias
DHA	Ácido docosahexaenoico
DON	Deoxinivalenol
ECACC	Colección Europea de Cultivos Celulares Autenticados
EFSA	Agenca Europea de Seguridad Alimentaria
EPA	Ácido eicosahexaenoico
EU	Unión Europea
FAO	Organización de las Naciones Unidas para la Agricultura y la Alimentación
G	Glicina
GC-FID	Cromatografía de gases con detector de llama
HSD	Diferencia honestamente significativa
Hg	Mercurio
ICP-MS	Espectrometría de plasma acoplada inductivamente a detector de masas
ISP	Solubilización/precipitación isoeléctrica
kDa	Kilodalton
LA	Ácido linoleico
LC-ESI-qTOF-MS	Cromatografía líquida acoplada a espectrometría de masas de alta resolución TOF
LC-MS/MS	Cromatografía líquida acoplada a espectrometría de masas
LPS	Lipopolisacárido
LOD	Límite de detección
LOQ	Límite de cuantificación

MAE	Extracción asistida por microondas
MBC	Concentración mínima bactericida
MFC	Concentración mínima fungicida
MIC	Concentración mínima inhibitoria
Mpa	Megapascal
NO	Óxido de nitrógeno
ODS	Objetivos de desarrollo sostenible
ONU	Organización de las Naciones Unidas
ORAC	Capacidad de absorción de radicales de oxígeno
OTA	Ocratoxina A
P	Prolina
PAGE	Electroforesis en gel de poliacrilamida
PEF	Pulsos eléctricos de alta intensidad
PLE	Extracción mediante líquidos presurizados
Pb	Plomo
PI	Punto isoeléctrico
Psi	Libras por pulgada cuadrada
R^2	Coeficiente de determinación
R^2_{adj}	Coeficiente de determinación ajustado
ROO•	Radicales de tipo peróxido
ROS	Especies reactivas de oxígeno
RNS	Especies reactivas de nitrógeno
RSM	Metodología de superficie de respuesta
SDS	Dodecil sulfato sódico
SE	Extracción Soxhlet
SFE	Extracción mediante fluidos supercríticos
TEAC	Capacidad antioxidante equivalente al Trolox
UAE	Extracción asistida por ultrasonidos
UV	Ultravioleta
W	Vatios

RESUMEN

En la presente Tesis Doctoral se ha utilizado la extracción mediante líquidos presurizados (PLE) para obtener extractos acuosos proteicos con potencial antioxidante a partir de subproductos de lubina, dorada y salmón. Las condiciones óptimas de extracción fueron pH 7, 20 ºC, 5 min para restos de músculo, pH 4, 60 ºC, 15 min para cabezas, pH 7, 50 ºC, 15 min para vísceras, pH 7, 55 ºC, 5 min para piel y pH 7, 60 ºC, 15 min para colas. Los extractos óptimos presentaron mayor porcentaje de recuperación de proteínas y mejores valores de capacidad antioxidante en comparación con los extractos obtenidos por agitación convencional (control). Los mejores resultados se observaron en los extractos óptimos de vísceras de salmón, con un 92% de recuperación proteica y valores TEAC y ORAC de 3700 y 7800 equivalentes de Trolox μM, respectivamente.

El estudio de la distribución del peso molecular de la fracción proteica de los extractos se llevó a cabo mediante electroforesis SDS-PAGE. Las imágenes de los perfiles electroforéticos revelaron que los extractos óptimos contenían más cantidad de fragmentos proteicos de menor peso molecular que los extractos control. Adicionalmente, se realizó el análisis peptídico de los extractos de vísceras de salmón mediante espectrometría de masas (nanoESI qTOF), identificando 137 péptidos en los extractos óptimos y 67 en los extractos control. A través de un estudio bioinformático, GPP y GAA resultaron ser las secuencias de aminoácidos con potencial antioxidante más repetidas encriptadas en las cadenas peptídicas de los extractos de vísceras de salmón obtenidos mediante PLE.

Así mismo, se ha empleado la extracción asistida por microondas (MAE) para obtener aceite con propiedades nutricionales y bioactivas a partir de tres

subproductos de salmón y uno de lubina y dorada. Las condiciones óptimas de extracción fueron 14.6 min, 291.9 W, 80.1 g/L para esqueletos, 10.8 min, 50.0 W, 80.0 g/L para cabezas y 14.3 min, 960.6 W, 99.5 g/L para vísceras. El mejor rendimiento de aceite se observó en las vísceras de salmón (92% del contenido total de lípidos). Mediante cromatografía de gases con detector de ionización de llama (GC-FID) se analizó la composición de ácidos grasos de los aceites extraídos por MAE y por el método Soxlhet, sin observar diferencias en el perfil lipídico entre ambas técnicas. Los principales ácidos grasos identificados en todos los aceites fueron ácido oleico (36-38%), ácido linoleico (14-18%), ácido palmítico (10-15%), ácido docosahexaenoico (DHA, 7-13%) y ácido eicosapentaenoico (EPA, 4-6%). Los aceites de las cabezas de lubina y dorada presentaron niveles más altos de DHA que los aceites procedentes de los subproductos de salmón. El porcentaje de ácidos grasos insaturados (AGI) fue mayor que el de ácidos grasos saturados (AGS) en todas las muestras.

Se utilizaron ensayos con cultivos celulares para evaluar la citotoxicidad de los aceites frente a células tumorales de estómago (AGS), colon (Caco-2), pecho (MCF-7) y pulmón (NCI-H460). La línea celular de cáncer de pecho fue la más susceptible al efecto de los aceites de las cabezas de lubina y dorada (GI_{50}= 38-93 μg/mL) mientras que la línea celular de cáncer de pulmón fue más sensible a la acción del aceite de esqueletos de salmón (GI_{50}= 76-142 μg/mL). Se llevaron a cabo ensayos celulares con macrófagos para estimar el potencial antioxidante y antiinflamatorio de los aceites. Los aceites de vísceras de salmón redujeron más de un 75% la reacción de oxidación generada en las células. Todos los aceites exhibieron efecto antiinflamatorio, disminuyendo los niveles del NO producido en los macrófagos con concentraciones inferiores a 65 μg/mL. También se realizaron ensayos de actividad antimicrobiana, donde los aceites de las cabezas

de lubina y dorada obtenidos por MAE mostraron mayor capacidad antibacteriana y antifúngica que los extraídos por Soxhlet.

Por otra parte, la presencia de metales pesados y micotoxinas en restos de músculo, cabezas, vísceras, piel y colas de lubina, dorada y salmón fue investigada. Las concentraciones de arsénico, mercurio, cadmio y plomo analizadas mediante espectrometría de plasma acoplada inductivamente a detector de masa (ICP-MS) se encontraron por debajo de los límites establecidos por las autoridades para productos pesqueros comercializados, excepto para el cadmio (0.068 μg/g) en las vísceras de dorada. A través de cromatografía líquida acoplada a espectrometría de masa de alta resolución TOF (LC-ESI-qTOF-MS) se identificó la micotoxina deoxivalenol (DON) en niveles traza (<0.5 ppb) en las vísceras de lubina.

Teniendo en cuenta los resultados obtenidos, los subproductos de pescado de cultivo contienen compuestos con propiedades nutricionales y/o bioactivas que los convierten en una materia prima valiosa, principalmente para las industrias alimentaria y farmaceútica. La extracción de estas sustancias mediante tecnologías no convencionales de forma sostenible puede considerarse como una opción interesante para la valorización de subproductos de pescado desde un enfoque de economía circular.

ABSTRACT

In this Doctoral Thesis, pressurized liquid extraction (PLE) has been used to obtain aqueous protein extracts with antioxidant potential from sea bass, sea bream, and salmon side streams. The optimal extraction conditions were pH 7, 20 °C, 5 min for muscle leftovers, pH 4, 60 °C, 15 min for heads, pH 7, 50 °C, 15 min for viscera, pH 7, 55 °C, 5 min for skin, and pH 7, 60 °C, 15 min for tailfins. The optimal extracts presented a higher percentage of protein recovery and better antioxidant capacity values compared to extracts obtained by conventional shaking (control). The best results were observed in the optimal extracts of salmon viscera, with 92% protein recovery and TEAC and ORAC values of 3700 and 7800 of μM Trolox equivalents, respectively.

The study of the molecular weight distribution of the extracts' protein fraction was carried out by SDS-PAGE electrophoresis. The images of the electrophoretic profiles revealed that the optimal extracts contained a higher amount of protein fragments of lower molecular weight than the control extracts. In addition, peptide analysis of salmon viscera extracts was performed by mass spectrometry (nanoESI qTOF), being 137 peptides identified in the optimal extracts and 67 in the control extracts. Through a bioinformatic study, GPP and GAA were found to be the most repeated amino acid sequences with antioxidant potential encripted into the peptide chains of salmon viscera extracts obtained by PLE.

Likewise, microwave-assisted extraction (MAE) was used to obtain oils with nutritional and bioactive properties from three side streams of salmon and one of sea bass and sea bream. The optimal extraction conditions were 14.6 min, 291.9 W, 80.1 g/L for backbones, 10.8 min, 50.0 W, 80.0 g/L for heads, and 14.3 min, 960.6 W, 99.5 g/L for viscera. The best oil yield was observed in

salmon viscera (92% of the total lipid content). The fatty acid composition of the oils extracted by MAE and the Soxlhet method was analyzed by gas chromatography with a flame detector (GC-FID), and no differences were observed in the lipid profile between both techniques. The main fatty acids identified in all oils were oleic acid (36-38%), linoleic acid (14-18%), palmitic acid (10-15%), docosahexaenoic acid (DHA, 7-13%), and eicosapentaenoic acid (EPA, 4-6%). Sea bass and sea bream heads oils had higher levels of DHA than salmon side streams oils. The percentage of unsaturated fatty acids (UFA) was higher than that of saturated fatty acids (SFA) for all samples.

Cell culture assays were used to evaluate the cytotoxicity of the oils against stomach (AGS), colon (Caco-2), breast (MCF-7), and lung (NCI-H460) tumor cells. The breast cancer cell line was the most susceptible to the effect of the sea bass and sea bream heads oils (GI50 = 38-93 μg/mL) while the lung cancer cell line was more sensitive to the action of salmon backbones oils (GI50 = 76-142 μg/mL). Macrophage cell assays were carried out to estimate the antioxidant and anti-inflammatory potential of the oils. Salmon viscera oils reduced the oxidation reaction generated in the cells by more than 75%. All oils exhibited anti-inflammatory effect by decreasing the levels of NO produced in macrophages at concentrations below 65 μg/mL. Antimicrobial activity tests were also performed, where the sea bass and sea bream heads oils obtained by MAE showed a greater antibacterial and antifungal capacity than those extracted by Soxhlet.

On the other hand, the presence of heavy metals and mycotoxins in muscle remains, heads, viscera, skin, and tailfins of sea bass, sea bream, and salmon was investigated. The concentrations of arsenic, mercury, cadmium, and lead analized by inductively coupled plasma spectrometry mass detector (ICP-MS)

were found below the limits established by the authorities for commercialized fish products, except for cadmium (0.068 μg/g) in sea bream viscera. Through liquid chromatography coupled with high-resolution mass spectrometry TOF (LC-ESI-qTOF-MS), the mycotoxin deoxyvalenol (DON) was identified at trace levels (<0.5 ppb) in sea bass viscera.

Considering the results obtained, farmed fish side streams contain compounds with nutritional and/or bioactive properties that make them a valuable raw material, mainly for the food and pharmaceutical industries. The extraction of these substances using non-conventional technologies in a sustainable way can be considered as an interesting option for the valorization of fish side streams under a circular economy approach.

1. INTRODUCTION

1. INTRODUCCIÓN

1.1. Sistema alimentario actual

Actualmente, la población mundial continúa en pleno crecimiento y se espera que para 2050 haya más de 9 mil millones de personas en el planeta (FAO, 2020). Para cubrir la futura demanda de alimentos, se necesitará producir hasta un 60% más de lo que se produce en estos momentos. Esta situación contribuye a la necesidad ya existente de modificar tanto la manera de producir alimentos como los hábitos de consumo. La producción de alimentos actual depende de un sistema alimentario globalizado y complejo. De acuerdo con la Organización de las Naciones Unidas para la Agricultura y la Alimentación (FAO, *Food and Agriculture Organization*), el sistema alimentario está formado por todas las personas y actividades relacionadas con la producción, transformación, distribución, consumo y eliminación de alimentos. El sistema alimentario abarca todos los productos alimentarios originados a partir de la agricultura, ganadería, pesca, acuicultura y silvicultura, así como los entornos económicos, sociales y naturales en los que está integrado. Debido a su gran alcance y necesidades, el sistema alimentario interactúa con otros sistemas importantes (comerciales, energéticos, de suministro de insumos, de salud y de gestión de residuos) (**Figura 1**) (FAO, 2018a).

El sistema alimentario tiene una relación única con el medio ambiente ya que los recursos naturales son esenciales para su desarrollo al mismo tiempo que las actividades que lleva a cabo influyen de forma negativa en el entorno del que dependen. El sistema alimentario depende de los combustibles fósiles para conseguir el aporte energético necesario para realizar sus actividades, lo que supone un 25% de todas las emisiones de gases de efecto invernadero. Además,

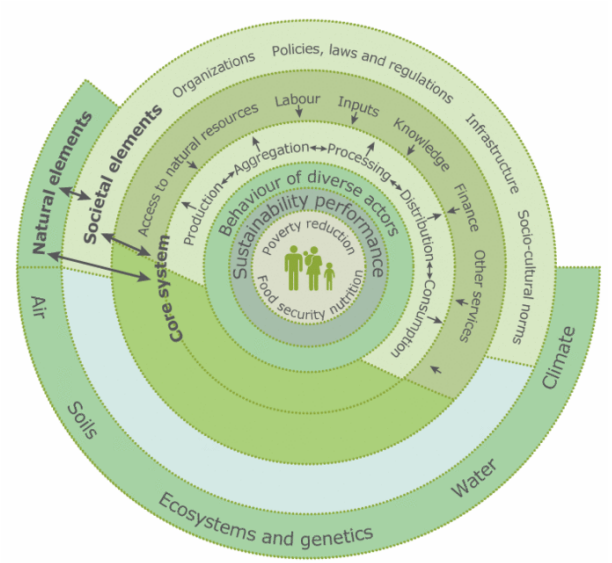

Figura 1. Rueda del sistema alimentario de la FAO: elementos e interrelaciones (FAO, 2018a).

es responsable del 80% de la pérdida de biodiversidad, el 80% de la deforestación y el 70% del consumo total de agua dulce. La producción de alimentos en particular, genera aproximadamente el 32% de la acidificación de la tierra y el 78% de la eutrofización (Grigoriadis, Nugent, & Brereton, 2021).

1.1.1. Pérdida y desperdicio de alimentos

Una de las causas que contribuyen al impacto ambiental provocado por el actual sistema alimentario y que se produce a lo largo de toda la cadena alimentaria, es la pérdida y el desperdicio de alimentos. Ambos conceptos hacen referencia a la disminución en la cantidad o en la calidad de los alimentos. Sin embargo, la pérdida de alimentos suele asociarse con las primeras fases de la cadena alimentaria (producción, elaboración, almacenamiento, transporte) mientras que el desperdicio se vincula con las etapas finales (servicios de

alimentos y consumidor) (FAO, 2019). Esta situación no es homogénea, en los países en vías de desarrollo la gran mayoría de las pérdidas (80%) se producen en los eslabones iniciales de la cadena alimentaria, mientras que en los países industrializados los consumidores finales son responsables de más del 50% de los desperdicios (Valavanidis et al, 2021). Los motivos para los descartes alimentarios son muy diversos, dependiendo del tipo de alimento y del momento en el que se encuentre dentro de la cadena alimentaria. Así, puede tratarse de excedentes de cultivo, subproductos generados durante el procesado, exigencias estéticas para su comercialización, deterioro de la materia prima (poscosecha y almacenamiento) o del producto final (comercio y consumidor), fallos en el embalaje, etc. (FAO, 2019). Según datos de la FAO, un tercio de todos los alimentos producidos en el mundo para consumo humano se pierden cada año, lo que equivale a 1,3 billones de toneladas de alimentos (FAO, 2013). En cuanto a los distintos grupos alimentarios, se ha estimado que la pérdida y el desperdicio a nivel global es de aproximadamente un 40-50% para tubérculos, frutas y verduras, 35% para el pescado y productos del mar, 30% para cereales y 20% para semillas oleaginosas, carne y productos lácteos (FAO, 2013). Cabe señalar que no solo se pierden los alimentos, sino todos los recursos utilizados para su obtención.

En lo que respecta a los residuos generados por parte de la industria agroalimentaria, éstos han sido tradicionalmente incinerados o eliminados en vertederos con la consiguiente contaminación del aire, agua, suelos y alimentos (Socas-Rodríguez, Álvarez-Rivera, Valdés, Ibáñez, & Cifuentes, 2021). Además del coste ambiental, este tipo de tratamientos conlleva un elevado coste económico.

1.1.2. Gestión de residuos alimentarios

En el año 2015, los Estados miembros de la Organización de las Naciones Unidas (ONU) aprobaron la Agenda 2030 para el Desarrollo Sostenible. Se trata de un plan de acción universal constituido por 17 Objetivos de Desarrollo Sostenible (ODS) de carácter social, económico y ambiental. La Agenda 2030 responde a la necesidad de establecer un marco internacional común para afrontar retos globales. Uno de sus ejes centrales es la protección del planeta a través de la producción, consumo y gestión de los recursos de forma sostenible (ODS 12). En este sentido, la Comisión Europea (CE) ha hecho una apuesta clara en materia de gestión de residuos con el objetivo de proteger el medio ambiente y la salud humana mediante la prevención y la reducción de la generación de residuos. Para ello, se ha aprobado un paquete de medidas dirigidas a mejorar la eficiencia de los recursos como elemento clave hacia la transición a una economía circular (CE DIRECTIVA (UE) 2018/851).

Actualmente, la legislación europea en materia de residuos se basa en la Directiva 2018/851/CE, que establece un marco jurídico para el tratamiento de los residuos (incluidos los residuos alimentarios) y enfatiza la importancia de utilizar técnicas adecuadas de gestión, recuperación y reciclado de residuos para reducir la presión sobre los recursos y mejorar su uso. Como puntos destacables, esta Directiva distingue entre los conceptos de residuo y subproducto e incluye una jerarquía para priorizar su utilización (prevención, reutilización, reciclado, recuperación y eliminación). De acuerdo con esta normativa, se considera residuo a cualquier sustancia u objeto que su poseedor deseche o tenga intención u obligación de desechar, mientras que el término subproducto es definido como una sustancia u objeto resultante de un proceso de producción cuya finalidad primaria no sea la producción de esa sustancia u objeto. Adicionalmente, para

que esa sustancia adquiera la denominación de subproducto debe cumplir con una serie de condiciones para que su posterior utilización sea legal y segura para las personas y el medio ambiente. Por lo tanto, si una sustancia que se genera durante un proceso de producción puede tener una segunda vida útil en otro proceso productivo se convierte en un subproducto. Si la sustancia tiene que ser eliminada definitivamente, entonces será un residuo.

En cuanto a la jerarquía de desperdicios alimentarios, se trata de una herramienta para facilitar la toma de decisiones en materia de gestión. Como muestra la **Figura 2**, esta jerarquía en forma de pirámide es un esquema que ordena por prioridad las opciones de utilización de excedentes, subproductos y residuos alimentarios. En este sentido, como primera opción, en lo más alto de la pirámide se sitúa la prevención de la generación de residuos para evitar tanto excedentes como pérdidas y desperdicios de alimentos. A continuación, se encontraría la reutilización, concepto basado en utilizar los alimentos sin ser transformados en otros productos. Esta opción está enfocada principalmente a mantener los productos en la cadena alimentaria para consumo humano a través de redes de distribución o bancos de alimentos. Si no es posible, su reutilización sería como alimento animal. La siguiente opción en la pirámide sería el reciclaje, término que implica la transformación de los subproductos alimentarios en otro tipo de productos. En el reciclaje se incluye la obtención de productos de alto y de bajo valor añadido a partir de subproductos generados en la producción y transformación de alimentos. Como última elección se encuentra la utilización de subproductos alimentarios para la recuperación de energía.

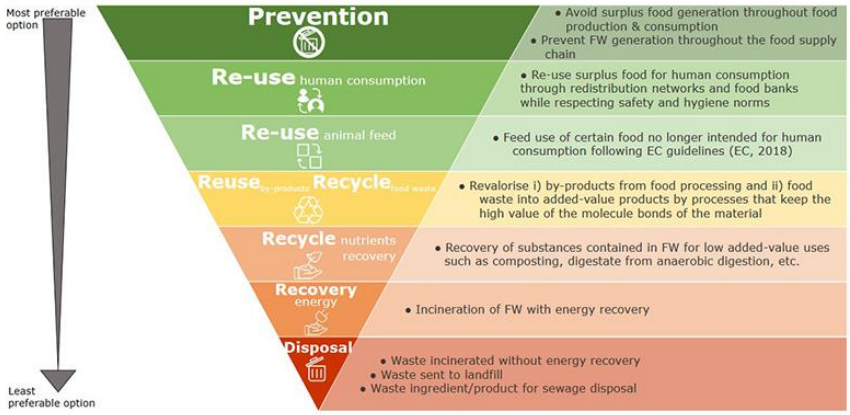

Figura 2. Jerarquía de priorización de excedentes, subproductos y residuos alimentarios (ECJRC, 2020).

Para finalizar, en el punto más bajo de la pirámide se coloca la eliminación de los residuos que no han podido ser aprovechados como subproductos. La eliminación de residuos no se considera como una opción de la jerarquía de desperdicios alimentarios.

La última actualización de la Directiva 2018/851/CE establece la obligación de realizar una transición hacia una economía circular en materia de residuos a través de una gestión sostenible para garantizar la utilización prudente, eficiente y racional de los recursos naturales, mejorar el uso de la energía renovable, aumentar la eficiencia energética, crear nuevas oportunidades económicas y contribuir a la competitividad a largo plazo. La revisión de la Directiva requiere que los Estados miembros reduzcan las pérdidas de alimentos a lo largo de las cadenas de suministro y de producción, fomentando la aplicación de la jerarquía de desperdicios en consonancia con los ODS y la economía circular.

La economía circular es un nuevo modelo de producción y consumo basado en compartir, reutilizar, reparar, renovar y reciclar los materiales y productos

existentes el mayor tiempo posible, alargando su ciclo de vida y reduciendo al mínimo la generación de residuos (Brandão, Gonçalves, & Santos, 2021). Este concepto debe sustituir al modelo económico lineal basado en extraer, producir, usar y eliminar que ha supuesto la sobreexplotación de los recursos naturales en los últimos 50 años. Teóricamente, el cambio de modelo debería aportar beneficios ambientales y socioeconómicos (FAO, 2018a). Las industrias de transformación de materia prima en productos alimentarios comerciales son piezas clave para implementar estrategias como la valorización de subproductos, encaminadas a cumplir con el enfoque de economía circular.

1.2. Sector acuícola

Según los últimos datos recopilados por la FAO, la producción acuícola mundial alcanzó los 114,5 millones de toneladas en 2018. La producción total consistió en 82,1 millones de toneladas de animales acuáticos, 32,4 millones de toneladas de algas y 26.000 toneladas de productos no alimentarios. Además, el 66% por de los animales cultivados fueron peces comestibles (FAO, 2020). Desde 2016 la cantidad de pescado procedente de acuicultura representa la mitad del pescado destinado a consumo humano en todo el mundo (FAO, 2018b). Aunque la utilización y los métodos de procesamiento del pescado varían de un país a otro, se ha estimado que alrededor del 88% de la producción total de pescado se destina al consumo humano directo (45% como producto vivo o fresco y 31% como productos congelados). El 12% restante es utilizado principalmente para la obtención de harina y aceite de pescado (FAO, 2020). A partir de los años 60 el consumo de pescado ha ido en aumento, superando al de otros productos de origen animal en cuanto al contenido de proteínas aportado en la dieta (Lopes, Antelo, Franco-Uría, Alonso, & Pérez-Martín,

2015). La demanda de nuevos formatos de comercialización de un número creciente de especies también ha contribuido al incremento en la elaboración de productos derivados de pescado. En esta línea, los procesos de fileteado, salazón, ahumado y conservas generan 4,67 millones de toneladas de subproductos en Europa cada año (Lopes et al., 2015). Por lo tanto, una gestión adecuada es esencial para optimizar el valor de los subproductos y reducir su impacto ambiental. De acuerdo con Regueiro et al. (2021), deben explorarse nuevos modelos de acuicultura basados en la economía circular para ofrecer a largo plazo la rentabilidad y la sostenibilidad del sector acuícola a través de la valorización de subproductos, incluyendo las tecnologías necesarias para su consecución.

1.2.1. Subproductos del procesado de pescado

1.2.1.1. Tipos de subproductos

Los subproductos de pescado se definen generalmente como restos de material de pescado que se generan tras el procesamiento llevado a cabo para obtener el producto principal (He, Franco, & Zhang, 2013). Se trata de descartes heterogéneos en términos de cantidad y calidad debido a factores como el tamaño, la alimentación, la estacionalidad, el tipo de cultivo y la transformación del pescado de cara a su finalidad comercial (Olsen, Toppe, & Karunasagar, 2014; Pędziwiatr, Zawadzki, & Michalska, 2017). Mientras que para el pescado congelado entero solo se retiran las vísceras, para la preparación de filetes todas las partes del cuerpo del pescado son descartadas, incluyendo recortes de la carne de los filetes elaborados. Dependiendo de la especie y el nivel de procesado, los subproductos pueden representar entre el 50 y el 70% del pescado transformado (Nawaz et al., 2020). En general, cabezas, vísceras, espinas, piel, colas y restos de

carne son las fracciones sólidas del pescado descartadas para consumo humano, exclusivamente por motivos comerciales.

1.2.1.2. Composición nutricional

El primer paso para poder llevar a cabo una valorización de los subproductos de pescado es la caracterización de su composición nutricional (Kandyliari, Mallouchos, et al., 2020). En este sentido, se han analizado los componentes nutricionales de los subproductos de las principales especies de pescado utilizadas para la elaboración de filetes en diferentes países. Por ejemplo, una amplia variedad de subproductos de salmón, bacalao, sardina, corvina, abadejo, lubina y dorada han sido caracterizados (Bechtel & Johnson, 2004; He, Franco, & Zhang, 2011; Jafarpour et al., 2020; Kandyliari, Mallouchos, et al., 2020; Munekata et al., 2020; Pateiro et al., 2020; Tengku-Rozaina, Jeng, & Amiza, 2018). Los resultados muestran que los principales subproductos de pescado contienen proteínas (10-25 %), lípidos (10-50 %) y minerales.

La evaluación de la composición de aminoácidos y ácidos grasos pone de manifiesto que estos macronutrientes son de alta calidad nutricional. En función de la especie y del tipo de subproducto, existe un porcentaje relevante de aminoácidos esenciales respecto al total de aminoácidos. En cuanto al perfil de ácidos grasos, el porcentaje total de ácidos grasos insaturados (AGI) supera al de ácidos grasos saturados (AGS), confirmando el carácter saludable de la fracción grasa de los diferentes subproductos. Además, los lípidos de los subproductos de pescado contienen tanto los dos ácidos grasos esenciales para que el organismo humano funcione correctamente, ácido linoleico (LA) y ácido α-linolénico (ALA), como los dos AGPI más importantes dentro de la familia de los omega-3, el ácido eicosapentaenoico (EPA) y el ácido docosahexaenoico

(DHA). En cuanto al contenido de minerales, los principales macroelementos (calcio, sodio, potasio, magnesio, fósforo) y oligoelementos (hierro, zinc, cobre) han sido identificados. Normalmente, los subproductos de origen inorgánico como las espinas y otras partes del pescado que contienen estructuras óseas son ricas en calcio y en fósforo (Ahuja, Dauksas, Remme, Richardsen, & Løes, 2020). En general, las vísceras presentan mayor concentración de magnesio, hierro, zinc y cobre respecto a otros tejidos orgánicos, mientras que el zinc es el principal mineral en la piel. El potasio, sin embargo, parece tener una distribución bastante uniforme entre los diferentes tipos de subproductos. También se ha observado la presencia de manganeso en subproductos de bacalao (Bechtel & Johnson, 2004). En cuanto al contenido de vitaminas, existe información relativa a las vitaminas liposolubles A y E en el aceite del pescado procedente de hígado de bacalao y de tiburón (Moovendhan, Kavisri, Vairamani, & Shanmugam, 2021). Sin embargo, no hay estudios sobre la determinación de vitaminas en otros subproductos de pescado.

1.2.1.3. Compuestos bioactivos

Además de los constituyentes nutricionales, hoy en día se presta una atención especial al contenido de compuestos biológicamente activos en los alimentos. Aunque no hay una definición establecida, está aceptado que los compuestos bioactivos se caracterizan por aportar beneficios para la salud (Granato et al., 2020). En lo que respecta a los subproductos de pescado, los péptidos y los ácidos grasos EPA y DHA son las moléculas más estudiadas como sustancias bioactivas. Los péptidos son cadenas cortas de 2 a 20 aminoácidos que permanecen inactivas en la secuencia de la proteína original y necesitan ser liberados para llevar a cabo su función (Sila & Bougatef, 2016). Diferentes

péptidos obtenidos a partir de una amplia variedad de subproductos, especies de pescado y métodos de extracción, con efectos beneficiosos frente a importantes enfermedades no concomitantes han sido descritos (Ishak & Sarbon, 2018; Kemp & Kwon, 2021; Le Gouic, Harnedy, & FitzGerald, 2018; Mutalipassi et al., 2021; Neves, Harnedy, O'Keeffe, Alashi, et al., 2017; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017; Sila & Bougatef, 2016; Ucak et al., 2021; Zamora-Sillero, Gharsallaoui, & Prentice, 2018). Por ejemplo, se han identificado péptidos antihipertensivos en la piel, recortes de músculo y aletas de salmón, así como en el esqueleto de bonito y en la piel de bacalao y pez roca. Se han estudiado los mecanismos de acción de péptidos extraídos de huesos y aletas de salmón, escamas de pez choclo y vísceras de anchoa como tratamientos alternativos a terapias agresivas en inflamación crónica. También se ha observado actividad antimicrobiana frente a bacterias Gram-positivo y Gram-negativo en péptidos procedentes de vísceras de pescados como trucha, caballa y tilapia. Distintos métodos *in vitro* han confirmado la capacidad antioxidante de péptidos extraídos de la piel de salmón, bacalao, lubina, siluro y atún rojo; de esqueletos de abadejo, bonito y hoki; de vísceras de sardina, palometa y caballa; y de cabezas de sardina y atún rojo. Otras actividades biológicas como la reducción de la glucosa sanguínea, el efecto antiproliferativo de células cancerígenas, la absorción de minerales o la capacidad anticoagulante han sido atribuidas a péptidos de diferentes orígenes, incluyendo aquellos obtenidos de subproductos de pescado.

En cuanto a compuestos bioactivos de origen lipídico, los ácidos grasos EPA y DHA son reconocidos principalmente como moléculas clave en la prevención de factores de riesgo asociados con el desarrollo de enfermedades cardiovasculares, pero también con factores de riesgo relacionados con

trastornos neurológicos e hipertensivos, procesos inflamatorios, diferentes tipos de cáncer, problemas de obesidad y osteoartritis (Chen & Liu, 2020; Durmuş, 2019; Oppedisano et al., 2020). Diferentes AGPI de la familia omega-3 extraídos de distintas especies de pescado han mostrado tener efectos citotóxicos o antiproliferativos en una amplia variedad de líneas celulares de cáncer humano sin afectar a las células normales (Jameel, Agarwal, Arshad, & Serajuddin, 2019). También se ha descrito actividad antibacteriana relacionada con compuestos específicos como EPA, DHA y ALA (Chanda et al., 2018; Inguglia, Chiaramonte, Stefano, et al., 2020).

1.2.1.4. Posibles contaminantes

Cualquier materia prima que vaya a ser utilizada para la elaboración de productos destinados al consumo humano debe estar libre de sustancias potencialmente perjudiciales para la salud. El aumento de la producción de pescado en el sector acuícola ha provocado un cambio en la dieta de los peces de cultivo. La harina y el aceite de pescado de los piensos empleados para su alimentación, han sido parcialmente sustituidos por distintos ingredientes de origen vegetal, lo que puede contribuir a la transferencia de micotoxinas y sus metabolitos desde los piensos contaminados hasta los tejidos de los animales acuáticos (Tolosa, Barba, Pallarés, & Ferrer, 2020; Tolosa, Font, Mañes, & Ferrer, 2014).

Para algunas especies de pescado como el salmón, el incremento de la productividad se ha alcanzado en paralelo al aumento en la utilización de antibióticos, por lo que los niveles de estas sustancias veterinarias también podrían acumularse en el pescado, especialmente en los tejidos grasos (Inguglia, Chiaramonte, Stefano, et al., 2020). Otro grupo de contaminantes relevantes en

el pescado de cultivo son los metales tóxicos como el arsénico, el mercurio, el cadmio y el plomo, ya que pueden encontrarse en piensos, agua y partículas en suspensión del entorno acuícola (Adamse, Van der Fels-Klerx, & de Jong, 2017; Kalantzi et al., 2016). Debido a que los subproductos de pescado suelen ser tratados para su eliminación o para producir pienso, no hay mucha información en bibliografía respecto a la presencia de contaminantes perjudiciales para la salud de las personas.

A través de un estudio toxicocinético de las micotoxinas ocratoxina A (OTA) y deoxivalenol (DON) en salmón de cultivo alimentado durante dos meses con pienso contaminado, se observó la acumulación de OTA en hígado y riñón mientras que DON fue distribuido de forma homogénea en músculo, cerebro, piel, hígado y riñón (Bernhoft et al., 2017). El análisis de 53 antibióticos en el aceite de cabezas y de tejidos blandos de salmón mostró la presencia simultánea de niveles traza de cuatro tipos de antibióticos (quinolónicos, β-lactámicos, macrólidos y sulfonamidas), mientras que los antibióticos fluoroquinolónicos y sulfamídicos solo se encontraron en el aceite procedente de las cabezas (Inguglia, Chiaramonte, Stefano, et al., 2020). También se han determinado niveles traza de arsénico, mercurio, plomo y cadmio en cabezas, vísceras, huesos, piel y músculo de lubina, dorada y corvina (Kalantzi et al., 2016; Kandyliari, Karavoltsos, et al., 2020). A pesar de que las concentraciones de micotoxinas, antiobióticos y metales pesados en los subproductos de pescado estuvieron por debajo de los límites establecidos para el pescado comercializado, la presencia de estos contaminantes pone de manifiesto la necesidad de confirmar la calidad y seguridad de los subproductos de pescado antes de ser incorporados en un proceso de producción dentro de la cadena alimentaria.

1.2.2. Valorización de subproductos de pescado

Tanto en el sector pesquero como en el acuícola, los descartes obtenidos desde la producción hasta el consumidor final han sido tradicionalmente eliminados, utilizados directamente como alimento para animales acuáticos y terrestres o empleados en ensilado y fertilizantes (FAO, 2020). La transformación de esos descartes en harinas proteicas y aceites de pescado para alimentación animal supuso un primer paso hacia el aprovechamiento de los residuos. A pesar de seguir siendo el uso principal de los subproductos pesqueros, la harina y el aceite de pescado se consideran productos de bajo valor económico para el sector.

Las particularidades de los subproductos de pescado, los colocan directamente en el tercer escalón de la pirámide de jerarquía de excedentes, subproductos y residuos alimentarios mencionada previamente (Figura 2). El conocimiento cada vez mayor de las propiedades nutricionales y bioactivas de los componentes de los subproductos de pescado, ha desembocado en nuevas opciones para su transformación en productos de mayor valor económico. Las posibilidades de valorización de los subproductos de pescado se han centrado en investigar las fracciones grasa y proteica. Entre los principales productos de valor añadido se encontrarían los ácidos grasos esenciales omega-3, aceite de pescado, concentrados de proteínas, hidrolizados de proteínas, péptidos bioactivos, colágeno y gelatina.

Ácidos grasos omega-3

Probablemente los ácidos grasos omega-3 como producto nutracéutico es el mejor ejemplo de la posibilidad de valorización de los subproductos de pescado. Además de ser comercializadas en forma de cápsulas, las moléculas EPA y DHA

también se han utilizado para fortificar una gran variedad de alimentos como productos lácteos, productos cárnicos, huevos, pan y bebidas (Jamshidi, Cao, Xiao, & Simal-Gandara, 2020).

Aceite de pescado

El aceite de pescado es una fuente rica en AGPI, principalmente EPA y DHA. Se sabe que una dieta rica en omega-3 conlleva beneficios para la salud, especialmente para prevenir enfermedades cardiovasculares (Kapoor et al., 2021). Debido a sus propiedades saludables, el aceite de pescado ha sido incorporado en diferentes productos alimentarios como una estrategia de fortificación de alimentos (Jamshidi et al., 2020). En la misma línea, también ha sido empleado para mejorar el perfil lipídico de piensos (Vidanarachchi et al., 2014). En cuanto a la valorización del aceite para obtener energía, el aceite de pescado puede reemplazar a los destilados de combustibles de petróleo (Adeoti & Hawboldt, 2014).

Concentrados de proteínas de pescado

Los concentrados proteicos de pescado son productos en forma de polvo seco y estable con una concentración final de proteínas más alta que en el material de partida (Shaviklo, 2013). Estos concentrados pueden obtenerse de una amplia variedad de pescados enteros o subproductos mediante diferentes métodos en los que se eliminan la fracción grasa y los huesos para aumentar el contenido de proteínas y disminuir el de cenizas (Khan et al., 2020; Shaviklo, 2013). Al ser considerados una buena fuente de aminoácidos, estos concentrados proteicos se han utilizado para mejorar el perfil nutricional de varios alimentos (pan, cereales, snacks, productos listos para consumir) y para suplementar la

dieta de niños en China (Shaviklo, 2013). Debido a sus características fisicoquímicas, los concentrados proteicos de subproductos de pescado también pueden incorporarse como aglutinantes, emulsionantes y dispersantes en la preparación de productos reestructurados (Khan et al., 2020).

Hidrolizados y péptidos bioactivos

Los hidrolizados de proteínas son el resultado de la fragmentación de las proteínas en cadenas peptídicas de diferentes tamaños (Chalamaiah, Dinesh Kumar, Hemalatha, & Jyothirmayi, 2012). Cuando el fragmento proteico obtenido contiene entre 2 y 20 aminoácidos se denomina péptido (Sila & Bougatef, 2016). En general, está aceptado que los péptidos de menor tamaño son los responsables de la actividad biológica exhibida (Ucak et al., 2021). Varios estudios han descrito actividades antioxidantes, antimicrobianas, antihipertensivas, antiproliferativas e inmunomoduladoras de hidrolizados y péptidos obtenidos a partir de diferentes subproductos de pescado (Chalamaiah et al., 2012; Neves et al., 2017; Sae-leaw, O'Callaghan, Benjakul, & O'Brien, 2016; Sila & Bougatef, 2016; Ucak et al., 2021; Villamil, Váquiro, & Solanilla, 2017; Zamora-Sillero et al., 2018). Este amplio abanico de bioactividades es especialmente interesante para las industrias alimentaria, farmacéutica y nutracéutica. Actualmente, existen algunos productos comercializados basados en hidrolizados y péptidos de pescado (Ishak & Sarbon, 2018).

Colágeno

El colágeno es la proteína estructural más abundante en la piel y en los huesos de los animales, constituyendo entre el 30% y el 60% del contenido proteico total (Pal & Suresh, 2016). El colágeno procedente de pescado ha ganado un

gran interés como alternativa al colágeno bovino y porcino debido a las actuales tendencias nutricionales, creencias religiosas y problemas de salud pública como la encefalopatía espongiforme bovina (Bai, Wei, & Ren, 2017; Liu, Nikoo, Boran, Zhou, & Regenstein, 2015). Tanto el colágeno como los péptidos de colágeno se utilizan en el tratamiento de la artritis y la osteoporosis, en cremas cosméticas para el antienvejecimiento de la piel y en la reconstrucción de huesos o dientes (Liu et al., 2015; Mutalipassi et al., 2021).

Gelatina

La gelatina es un biopolímero obtenido tras la hidrólisis del colágeno y ha sido ampliamente utilizada en las industrias alimentaria, farmacéutica, cosmética, fotográfica y de materiales (Vidanarachchi et al., 2014). La gelatina de pescado tiene propiedades estabilizantes y emulsionantes para productos como mermeladas, sopas y helados (Lv et al., 2019). Si procede de pescado de agua fría, la gelatina es útil en la encapsulación de compuestos sensibles al calor. Además, la gelatina derivada de diferentes subproductos de pescado e invertebrados se ha incorporado con éxito en formulaciones de recubrimientos comestibles (Domínguez et al., 2018). Al igual que en el caso del colágeno, la gelatina pueden ser hidrolizada para obtener péptidos con propiedades saludables de interés para las industrias alimentaria, farmacéutica y nutracéutica (Liu et al., 2015; Mutalipassi et al., 2021; Pal & Suresh, 2016).

De acuerdo con Chemat et al. (2020), el concepto de valorización no solo se refiere a la elaboración de productos de alto valor económico a partir de recursos naturales infrautilizados como los subproductos alimentarios, sino también a su producción de una forma respetuosa con el medio ambiente. En este contexto, se han investigado diferentes técnicas no convencionales para extraer

compuestos nutricionales y bioactivos de subproductos de origen marino, como alternativa a las limitaciones de los métodos tradicionales de extracción (Bruno, Ekorong, Karkal, Cathrine, & Kudre, 2019; Khawli et al., 2019).

1.3. Tecnologías de extracción

1.3.1. Tecnologías de extracción convencionales

1.3.1.1. Extracción mediante solventes

La extracción con disolventes se basa en la utilización de determinados tipos de disolventes con afinidad hacia la molécula de interés. En el caso de recuperación de proteínas de pescado, el etanol y el propanol han sido los principales solventes de tipo alcohólico empleados para eliminar agua, grasa y componentes con sabor a pescado de la materia prima (Khan et al., 2020; Shaviklo, 2013). Dependiendo del material de partida y de las condiciones de extracción, normalmente se obtienen diferentes tipos de concentrados de proteínas de pescado en función del contenido final de grasa (0.75 y 3%) y de la presencia o ausencia de olor y sabor a pescado. El mayor factor limitante para la aplicación de la extracción mediante solventes es la pérdida de funcionalidad de las proteínas (Khan et al., 2020; Shaviklo, 2013).

En cuanto a la recuperación de la fracción grasa, las técnicas tradicionales de extracción del contenido total de lípidos de los alimentos utilizan solventes orgánicos como hexano, éter de petróleo, metanol o cloroformo, así como sus mezclas (Ozogul et al., 2018). Los métodos Bligh and Dyer, Folch y Soxlhet son los más empleados a escala de laboratorio, normalmente con una finalidad analítica y comparativa frente a otros métodos (Alfio, Manzo, & Micillo, 2021; Ozogul et al., 2018). En este sentido, se han aplicado diferentes técnicas

convencionales para la extracción lipídica en distintas especies de pescado (Ozogul et al., 2018; Rincón-Cervera, Villarreal-Rubio, Valenzuela, & Valenzuela, 2017) y subproductos de pescado (Franklin, Haq, Roy, Park, & Chun, 2020; Inguglia, Chiaramonte, Stefano, et al., 2020; Kuvendziev, Lisichkov, Zeković, Marinkovski, & Musliu, 2018).

1.3.1.2. Solubilización/precipitación isoeléctrica

En el método de solubilización/precipitación isoeléctrica (ISP, *isoelectric solubilitation/precipitation*) se aplican cambios de pH para inducir la solubilidad en agua de las proteínas musculares de la carne y posteriormente conseguir su precipitación (Tahergorabi & Jaczynski, 2014). El primer paso es la homogeneización de la muestra en medio acuoso. A continuación, la etapa de solubilización se basa en el principio de que las condiciones ácidas o alcalinas del medio provocan fuertes cambios positivos o negativos respectivamente, en la carga de las proteínas miofibrilares y citoesqueléticas, separándolas por repulsión y permitiendo su interacción con las moléculas de agua, es decir, su solubilización (Nolsøe & Undeland, 2009). Una vez que las proteínas están disueltas en el medio acuoso, se realiza una centrifugación para separar las proteínas solubilizadas de la fracción lipídica y de los materiales sólidos insolubles como huesos, piel y escamas. A partir de la fase acuosa obtenida, la precipitación proteica se lleva a cabo modificando de nuevo el pH del medio. Esta etapa de precipitación se basa en el punto isoeléctrico (PI) de las proteínas, que es el pH al cual las proteínas no tienen carga. El PI de las proteínas musculares del pescado es 5.5, por lo que ajustando el pH a este valor se consigue que las proteínas precipiten. Tras una nueva centrifugación o por filtración, las proteínas aisladas pueden mezclarse con crioprotectores y congelarse como surimi o

pescado triturado, o pueden secarse para dar lugar a polvo de concentrado de proteínas de pescado (Abdollahi & Undeland, 2018; Khan et al., 2020).

El método ISP se desarrolló como alternativa al proceso de obtención de surimi debido a que no es necesaria la eliminación previa de huesos y piel, se pueden recuperar también las proteínas sarcoplásmicas y se evita el consumo excesivo de agua durante el procesado de surimi (Nolsøe & Undeland, 2009). Por estos motivos, el método ISP se ha utilizado tanto en pescados enteros de bajo valor comercial como en subproductos de pescado (Tahergorabi & Jaczynski, 2014). Cabezas, colas y esqueletos de arenque, bacalao y salmón se han utilizado para investigar las propiedades nutricionales, funcionales y sensoriales de las proteínas aisladas de estos subproductos tras aplicar el método ISP (Abdollahi & Undeland, 2018). Las diferencias entre los concentrados de proteínas obtenidos dependieron en gran medida de la especie utilizada y fueron considerados como ingredientes potenciales en la industria alimentaria. Independientemente de la materia prima, el método ISP fue diseñado para la recuperación específica de proteínas musculares completas que mantengan sus propiedades funcionales (gelificante, emulsionante, espumante, aglutinante, etc.) para ser incorporadas como ingrediente alimentario en una gran variedad de productos reestructurados (Abdollahi & Undeland, 2018; Tahergorabi & Jaczynski, 2014). La selectividad proteica del proceso de extracción evita problemas de oxidación lipídica en el concentrado de proteínas final (Nguyen, Heimann, & Zhang, 2020).

1.3.1.3. Hidrólisis

Además de proteínas intactas, se pueden obtener fragmentos proteicos de distintos tamaños y diversas propiedades mediante la hidrólisis de las proteínas

presentes en los subproductos de pescado. Hoy en día los métodos más utilizados para la producción de hidrolizados de proteínas de pescado a escala industrial son la hidrólisis química y la biológica (Zamora-Sillero et al., 2018).

En la hidrólisis química la fragmentación de los enlaces peptídicos se produce en condiciones ácidas o alcalinas (Nguyen et al., 2020; Sanmartín, Arboleya, & Moreno, 2009). La hidrólisis ácida de los subproductos de pescado se lleva a cabo mediante la adición de ácido clorhídrico o ácido sulfúrico a alta temperatura (128-131 °C) y, en algunos casos, a alta presión (220-310 MPa) (Nguyen et al., 2020; Ozogul et al., 2021). Tras la reacción de hidrólisis, es necesario neutralizar los hidrolizados antes de ser secados para obtener el concentrado proteico final en forma de polvo. De la misma manera, reactivos alcalinos como el hidróxido sódico se utilizan en la hidrólisis básica. Aunque ambos procedimientos son rápidos y relativamente económicos, la reacción es inespecífica y difícil de controlar. Como resultado, los hidrolizados obtenidos son heterogéneos, de sabor amargo y con una calidad nutricional y funcional reducida debido a la destrucción de algunos aminoácidos como cisteína, serina y treonina (Ozogul et al., 2021). Actualmente, los hidrolizados proteicos obtenidos de subproductos de pescado mediante hidrólisis química se destinan principalmente para la elaboración de fertilizantes (Nguyen et al., 2020; Zamora-Sillero et al., 2018).

La hidrólisis biológica se basa en la utilización de enzimas para conseguir la fragmentación de las proteínas. Dependiendo del origen de los enzimas empleados, existen dos clases de hidrólisis biológica, la autolítica o fermentación y la enzimática. La fermentación consiste en la acción de los enzimas proteolíticos del pescado sobre sus propios tejidos. El pescado contiene enzimas como tripsina, quimotripsina y diferentes proteasas en los órganos del tracto digestivo, mientras que proteasas lisosomales y enzimas catépticos se encuentran

en el músculo (Nguyen et al., 2020). Debido a que cada enzima actúa bajo unas condiciones de temperatura y pH específicas, los hidrolizados de pescado obtenidos mediante enzimas endógenos no son lo suficientemente homogéneos para producir péptidos con propiedades bioactivas similares (Nguyen et al., 2020; Zamora-Sillero et al., 2018). El hecho de que estos enzimas varíen en función de la especie, el sexo, la edad o la alimentación, también dificulta la estandarización del proceso (Ozogul et al., 2021). Sin embargo, este tipo de fermentación sí se utiliza para la elaboración de dos productos, salsa de pescado comercial a partir de especies infrautilizadas y ensilado para alimentación animal a partir de pescado entero o subproductos (Nguyen et al., 2020; Ozogul et al., 2021).

La hidrólisis enzimática se realiza con enzimas exógenos seleccionados, lo que permite un mayor control sobre el proceso y, por tanto, sobre los hidrolizados resultantes. Estos enzimas pueden proceder de animales (pepsina y tripsina), plantas (papaína y bromelaína) y microorganismos. Normalmente, enzimas o mezcla de enzimas comerciales (Alcalase®, Flavourzyme® y Protamex®) son utilizados para producir hidrolizados proteicos y péptidos a partir de subproductos de pescado (Zamora-Sillero et al., 2018). Actualmente, se considera que la hidrólisis enzimática es la técnica más eficaz para obtener hidrolizados proteicos con propiedades bioactivas. La relación enzima-sustrato, el pH, la temperatura y el tiempo de la reacción son las variables del proceso de hidrólisis que pueden ser optimizadas para conseguir los hidrolizados proteicos con las características deseadas (Nguyen et al., 2020; Zamora-Sillero et al., 2018). Los subproductos de pescado triturados se mezclan con una cantidad de agua o tampón apropiada antes de ser introducidos en un reactor donde se alcanza la temperatura adecuada. A continuación, se añade la mezcla de enzimas

seleccionados y se ajusta el pH para iniciar la reacción de hidrólisis proteica. Una vez finalizado el tiempo de hidrólisis, es necesario inactivar los enzimas por choque térmico o modificación del pH del medio para conseguir parar la reacción. La mezcla resultante se separa entonces en tres fases: sólida, líquida (hidrolizados solubles) y aceite (Ozogul et al., 2021). A escala industrial, la tecnología de membranas puede incluirse en el proceso de producción de hidrolizados de proteínas, reduciendo el coste asociado con la inactivación final de enzimas (Zamora-Sillero et al., 2018). La hidrólisis catalizada por enzimas exógenos produce hidrolizados proteicos con mejores cualidades nutricionales y funcionales que los obtenidos a través de enzimas endógenos (Nguyen et al., 2020).

Además de la obtención de hidrolizados proteicos, la hidrólisis enzimática también se realiza con el objetivo de extraer el aceite de pescado o subproductos de pescado. En este caso, normalmente recibe el nombre de extracción enzimática. La extracción de aceite de pescado asistida por enzimas se realiza en condiciones moderadas de temperatura, utilizando proteasas y una proporción adecuada de agua/pescado para conseguir la máxima eficiencia de la extracción (Marsol-Vall, Aitta, Guo, & Yang, 2020). Los enzimas se utilizan para romper las proteínas de los tejidos del pescado, lo que permite la extracción del aceite. Subproductos de salmón, arenque, atún, sardinas o bacalao han sido utilizados para la extracción de aceite mediante la acción de enzimas proteolíticos (Aitta, Marsol-Vall, Damerau, & Yang, 2021; Głowacz-Różyńska et al., 2016; Liu, Ramakrishnan, & Dave, 2021).

1.3.1.4. Prensado húmedo

A nivel industrial, el principal método empleado y globalmente aceptado para la extracción de aceite de pescado es el prensado húmedo (Marsol-Vall et al., 2020). El pescado o las vísceras de pescado se mezclan con agua y se mantienen a 85-90 °C durante aproximadamente 30 minutos con el objetivo de coagular las proteínas para liberar el aceite. Los siguientes pasos son prensado y centrifugación para separar las tres fases formadas: sólida, líquida y aceite. A pesar de que la utilización de agua es una forma barata, segura y fácil de trabajar, con el método de prensado húmedo no se produce un alto rendimiento de aceite, se consume gran cantidad de energía, puede afectar negativamente a los componentes termolábiles y provocar la oxidación del aceite (Marsol-Vall et al., 2020; Melgosa, Sanz, & Beltrán, 2021). Para evitar los inconvenientes de la aplicación de altas temperaturas, el mismo proceso se ha llevado a cabo a temperaturas inferiores a 15 °C, lo que se conoce como prensado en frío. Subproductos de salmón como cabezas, piel y esqueletos han sido sometidos a prensado en frío, obteniéndose mejor rendimiento y calidad del aceite en comparación con el obtenido mediante prensado húmedo (Głowacz-Różyńska et al., 2016). De acuerdo con Ozogul et al. (2021), la mayor parte del aceite producido mediante prensado húmedo se utiliza en acuicultura mientras que un pequeño porcentaje se refina y se trata para obtener aceite de alta calidad que puede destinarse para consumo humano.

Los métodos de extracción suelen centrarse en obtener una fracción concreta (proteica o lipídica) con la finalidad de optimizar sus características y proporcionar un valor añadido al producto final. Como consecuencia, la otra fracción o fracciones obtenidas se consideran secundarias (Aitta et al., 2021). Sin embargo, a pesar de no ser el objetivo del proceso de extracción, todas pueden

ser aprovechadas. Dependiendo de cómo afecten las condiciones del método empleado a su estructura o composición, los materiales sólidos, constituidos principalmente por huesos de las cabezas o esqueletos del pescado y piel, pueden servir para obtener minerales y colágeno; las proteínas pueden transformarse en harina de pescado para alimentación animal; y el aceite también puede ser incluido en piensos, mejorando su perfil lipídico.

En general, la utilización de grandes cantidades de solventes, la larga duración del proceso de extracción y el alto consumo de energía son los principales inconvenientes de los métodos tradicionales de extracción que han llevado tanto a la industria como a los investigadores a la búsqueda de tecnologías alternativas de extracción de nutrientes y compuestos bioactivos a partir de recursos naturales de origen alimentario, incluyendo los subproductos de pescado.

1.3.2. Tecnologías de extracción no convencionales

En los últimos años se han investigado tecnologías innovadoras que permitan una buena recuperación de las sustancias de interés de una forma lo más rápida, económica y sostenible posible. A este grupo de técnicas de extracción no convencionales pertenecen la extracción mediante líquidos presurizados (PLE, *pressurized liquid extraction*), también conocida como extracción acelerada con solventes (ASE, *accelerated solvent extraction*), la extracción mediante fluidos supercríticos (SFE, *supercritical fluid extraction*), la extracción mediante pulsos eléctricos (PEF, *pulsed electric fields*), la extracción asistida por microondas (MAE, *microwave-assisted extraction*) y la extracción asistida por ultrasonidos (UAE, *ultrasound-assisted extraction*). Entre ellas, PLE y MAE cuentan con una amplia aceptación en diversos campos, incluidos aquellos relacionados con los alimentos y la salud.

1.3.2.1. Extracción mediante líquidos presurizados (PLE)

La técnica PLE se basa en la utilización de solventes a alta presión y temperatura para favorecer la extracción de los compuestos de interés, normalmente a partir de material sólido (Alvarez-Rivera, Bueno, Ballesteros-Vivas, Mendiola, & Ibañez, 2019; Andreu & Picó, 2019; Sun, Ge, Lv, & Wang, 2012). La presión elevada (500 - 300 psi) mantiene el solvente en estado líquido por debajo de su punto de ebullición durante todo el proceso de extracción. Además, podría ayudar a la penetración del solvente en la estructura de la matriz, aumentando el contacto entre extractante y compuesto para facilitar su recuperación. Recientemente se ha considerado que la presión tiene un efecto limitado en la eficiencia de la extracción, por lo que se suele fijar un valor durante todo el proceso, siendo 1500 psi la presión más comúnmente empleada (Andreu & Picó, 2019; He, Du, & Xu, 2018; Poveda, Loarce, Alarcón, Díaz-Maroto, & Alañón, 2018; Repajić et al., 2020; Toubane, Rezzoug, Besombes, & Daoud, 2017).

La temperatura elevada debilita las interacciones entre los compuestos de interés y otros componentes de la matriz al mismo tiempo que disminuye la viscosidad y la tensión superficial del solvente (Sun et al., 2012). De esta forma, se produce un incremento de la ratio de difusión, lo que supone un aumento de la transferencia de masa y de la solubilidad de los compuestos en el solvente. Sin embargo, las altas temperaturas también podrían solubilizar otros constituyentes de la matriz, así como degradar compuestos termolábiles (Andreu & Picó, 2019). En general, las extracciones con esta técnica suelen realizarse desde temperatura ambiente hasta los 200 °C. El tiempo de extracción en la PLE hace referencia al tiempo que el solvente está en contacto con la matriz a unos valores de presión, temperatura y flujo preestablecidos (Alvarez-Rivera et al., 2019). El tiempo de

extracción está relacionado con otros parámetros del equipo como el modo de extracción o los ciclos de extracción.

La PLE puede llevarse a cabo de modo dinámico o estático. La forma dinámica aplica un flujo continuo del solvente, pero no suele utilizarse por requerir un mayor consumo de solvente (Sun et al., 2012). La forma estática permite seleccionar entre uno y cinco ciclos de extracción estáticos. Un mismo volumen se utiliza para una sola extracción o se divide en varias extracciones secuenciales. Normalmente, en los métodos de extracción utilizados en la PLE se emplean entre 1 y 3 ciclos (Andreu & Picó, 2019).

Se considera que los tiempos de extracción largos junto con elevadas temperaturas son los responsables de la degradación de compuestos. La aplicación de períodos cortos de extracción es una de las ventajas más destacadas de la técnica PLE. El tipo de solvente o mezcla de solventes es crucial para obtener una recuperación de compuestos eficiente. Tanto las propiedades fisicoquímicas como la toxicidad deben tenerse en cuenta a la hora de seleccionar el solvente. En la PLE se pueden utilizar una amplia variedad de solventes, excluyendo ácidos y bases fuertes, así como líquidos inflamables a la temperatura empleada en el proceso de extracción (Sun et al., 2012).

La adición de aditivos o la modificación del pH pueden ser de utilidad para facilitar la liberación y solubilidad de algunos compuestos (Alvarez-Rivera et al., 2019). A pesar de que la extracción con PLE no es una técnica selectiva, la polaridad del solvente es importante para intentar lograr un equilibrio entre maximizar la extracción de los compuestos deseados y minimizar la de otros componentes de la matriz (Andreu & Picó, 2019). La utilización de agua como solvente para recuperar compuestos hidrosolubles es recomendable desde un punto de vista respetuoso con el medio ambiente. Además, algunos estudios han

mostrado cómo a determinados valores de presión y temperatura, la polaridad del agua puede modificarse hasta ser similar a la del alcohol. Esto conlleva la posibilidad de disolver en agua un amplio rango de compuestos de polaridad media y baja (Sun et al., 2012). Previo al proceso de extracción, la muestra se mezcla con material inerte como tierra de diatomeas o diferentes tipos de arena que actúan como agentes dispersantes para reducir la aglomeración de partículas en la muestra y facilitar el contacto entre el solvente y la matriz (Andreu & Picó, 2019; Sun et al., 2012).

La eficiencia de la extracción está influenciada principalmente por la naturaleza de la matriz y del compuesto a extraer (Sun et al., 2012). Las diferentes variables que componen el proceso de extracción (presión, temperatura, tiempo de extracción, ciclos de extracción, tipo de solvente, tipo de dispersante), pueden afectar en mayor o menor medida al rendimiento del proceso de extracción (Alvarez-Rivera et al., 2019; Andreu & Picó, 2019; Sun et al., 2012).

Debido a la diversidad de compuestos y matrices posibles, la optimización de varios de estos factores es considerada como la mejor forma de obtener recuperaciones aceptables. Para llevar a cabo la PLE lo más común es utilizar equipos comerciales que permiten la automatización del proceso de extracción y el control de las condiciones de presión, temperatura y tiempo de extracción a través de un panel de control manual o digital. De forma simplificada, la instrumentación consiste en al menos un depósito para el disolvente, una bomba de alta presión, un horno, celdas de acero inoxidable resistentes a la presión, viales colectores, dos carruseles (uno para colocar las celdas y otro para los viales), válvulas y limitadores de presión. El proceso completo de extracción consta de varios pasos secuenciales. La muestra, previamente mezclada con el agente disgregante, se introduce en la celda de extracción que contiene un filtro

en su parte final. La celda cerrada se coloca en el carrusel y de forma automática es transportada al horno. A continuación, el sistema incorpora el solvente y seguidamente comienza el incremento de presión y temperatura dentro de la celda. Cuando se alcanzan los valores de presión y temperatura preestablecidos, comienza a contar el tiempo de extracción. Tras este periodo, el solvente con los compuestos extraídos es automáticamente descargado en el vial colector correspondiente con ayuda de nitrógeno. Este proceso se repite tantas veces como ciclos de extracción hayan sido seleccionados para cada muestra. El circuito de nitrógeno también se emplea para purgar las celdas y los conductos del equipo una vez finalizada la extracción. La **Figura 3** muestra una imagen y un esquema básico de un equipo de PLE.

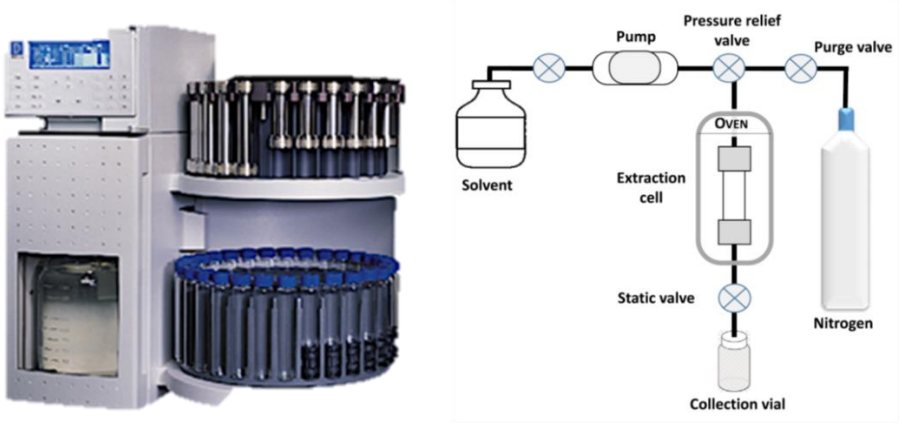

Figura 3. Imagen de un extractor modelo ASE 200 y esquema básico de un equipo de extracción mediante líquidos presurizados.

Tradicionalmente, la técnica PLE se ha utilizado para la preparación de muestras (Sun et al., 2012). Sin embargo, se ha convertido en una herramienta alternativa a las extracciones tradicionales basadas en solventes. En los últimos años, la PLE ha llegado a establecerse como una de las principales técnicas de extracción de compuestos orgánicos contaminantes presentes en matrices ambientales y alimentarias (Andreu & Picó, 2019). La posibilidad de utilizar disolventes no tóxicos y tiempos cortos de extracción ha fomentado su uso para investigar la recuperación de nutrientes y moléculas con actividad biológica a partir de alimentos y subproductos generados en la industria agroalimentaria. En este sentido, las algas han sido una de las principales fuentes naturales estudiadas. A través de la utilización de la PLE se han obtenido proteínas, lípidos, carbohidratos, polifenoles, pigmentos, ácidos grasos, fucoidan, alginato, etc. a partir de macroalgas y microalgas (Harrysson et al., 2018; Muñóz-Almagro et al., 2020; Otero, Quintana, Reglero, Fornari, & García-Risco, 2018; Saravana, Cho, Park, Woo, & Chun, 2016; Sumampouw, Jacobsen, & Getachew, 2021).

En cuanto a subproductos alimentarios, recientemente se han publicado varios estudios centrados en la aplicación de la PLE para extraer diferentes compuestos fenólicos contenidos en piel de granada (García et al., 2021), piel y pulpa de ciruela (Sabino, Filho, Fernandes, de Brito, & Júnior, 2021) y orujos de uva, manzana y arándano (da Silva et al., 2020; Huamán-Castilla et al., 2021; Tamkutė, Liepuoniūtė, Pukalskienė, & Venskutonis, 2020). Mediante PLE también se han obtenido ácidos grasos de semillas de rábano y de cáscaras de nuez (Ferreira de Mello, Stevanato, Filho, & da Silva, 2021; Herrera et al., 2020), así como compuestos reductores de colesterol a partir de semillas de aceituna (Vásquez-Villanueva, Plaza, García, & Marina, 2020). Por otra parte, hay pocos ejemplos en bibliografía sobre el uso de la PLE en subproductos de origen

animal. Como ejemplos, el aceite del hígado de atún y el carotenoide astaxantina de las cabezas de camarón han sido extraídos con éxito utilizando la PLE (Bruno et al., 2019).

1.3.2.2. Extracción asistida por microondas (MAE)

La técnica de extracción asistida por microondas (MAE) se basa en la utilización de energía de microondas para mejorar la extracción de compuestos contenidos en matrices sólidas en contacto con solventes. La MAE ha sido descrita como el resultado de la combinación sinérgica de un proceso de transferencia de energía y un proceso de transferencia de masa en la misma dirección (Bruno et al., 2019). Las microondas son un tipo de radiación electromagnética con una frecuencia que oscila de 0.3 a 300 GHz. Cuando se utilizan para procesos de extracción, se aplican siempre a 2.45 GHz (Kapoore, Butler, Pandhal, & Vaidyanathan, 2018). Debido a su baja energía, son consideradas ondas no ionizantes, por lo que no afectan directamente a la estructura de las moléculas.

Las microondas están formadas por un campo eléctrico y un campo magnético perpendiculares entre sí y se propagan de forma sinusoidal. El material sobre el que inciden absorbe parte de la energía electromagnética y la transforma en calor a través de dos mecanismos simultáneos, la conducción iónica y la rotación de dipolos. La conducción iónica hace referencia al movimiento de los iones bajo la influencia del campo eléctrico. La resistencia que ofrece el material a la migración de los iones produce fricción, generando calor. La rotación de dipolos indica el proceso de alineación de los iones en el campo eléctrico cambiante, provocando colisiones entre ellos y entre moléculas circundantes e iones, lo que también contribuye a la generación de calor

(Llompart, Garcia-Jares, Celeiro, & Dagnac, 2018; Mandal, Mohan, & Hemalatha, 2007).

En la MAE, estos fenómenos se producen al mismo tiempo en la muestra y en el solvente, por lo que el calentamiento es mucho más rápido que en métodos tradicionales donde el calor se transfiere por conducción y parte de la energía térmica se pierde en el medio ambiente (Mandal et al., 2007). El aumento de temperatura en la muestra conlleva un aumento de presión en el interior de las células hasta su ruptura. Esta disrupción celular supone una mayor porosidad de la matriz, la cual, junto con la temperatura y la presión generados, permite la liberación de compuestos y su transferencia hacia el solvente (Llompart et al., 2018; Mandal et al., 2007). Tras el tiempo de extracción, la mezcla debe ser filtrada para separar la matriz del solvente con los compuestos extraídos.

La eficiencia del proceso de extracción mediante MAE está influenciada por la naturaleza de la muestra, las propiedades dieléctricas del solvente, la potencia de las microondas, el tiempo de extracción y la relación masa/volumen de la mezcla (Bruno et al., 2019; Llompart et al., 2018). La rapidez del sistema para transformar la radiación electromagnética en energía térmica es la principal ventaja de la técnica MAE, permitiendo reducir el tiempo de extracción. Los componentes básicos de un aparato de microondas son cuatro: un generador de microondas (magnetrón), un dispositivo para propagar las ondas desde el magnetrón hacia la muestra (guía de ondas), un dispositivo para permitir que las ondas avancen sin volver al generador (circulador) y una cámara donde se colocan uno o varios recipientes conteniendo la muestra y el solvente (Llompart et al., 201; Mandal et al., 2007).

Los equipos empleados para la MAE se clasifican según la manera en que las ondas se aplican a la muestra. En el sistema multimodo, la radiación se dispersa

de forma aleatoria por toda la cámara, incidiendo también en la muestra. En el sistema monomodo o focalizado, las ondas se dirigen directamente hacia el área donde se encuentra la muestra (Llompart y col 2018). Por otra parte, las extracciones pueden llevarse a cabo en recipientes cerrados o abiertos. En general, los recipientes cerrados se utilizaban para procesos de extracción a elevada presión y temperatura mientras que los abiertos se empleaban en extracciones a presión atmosférica. Hoy en día existen extractores comerciales que permiten la combinación de ambos tipos de recipientes con radiaciones tanto multimodo como focalizadas. Elementos adicionales como refrigerante externo, agitador magnético o sensores de presión y temperatura pueden ser incorporados. Los modelos más modernos de MAE incluyen una cámara externa para visualizar la muestra a tiempo real, un panel de control de las condiciones experimentales y dispositivos electrónicos de seguridad. La **Figura 4** muestra una imagen y un esquema básico de un equipo de MAE.

La primera utilidad que se dio a las microondas fue para la preparación de muestras en química analítica. En este campo, se aplicaron con el objetivo de digerir las muestras biológicas antes de su análisis elemental (Llompart et al., 2018). Con el desarrollo de la MAE y la aparición de equipos comerciales, la nueva técnica comenzó a utilizarse para la extracción de contaminantes orgánicos ambientales, extendiéndose su uso de forma rápida hacia la extracción de compuestos naturales (Llompart, Celeiro, & Dagnac, 2019). Actualmente, la MAE es una tecnología ampliamente aceptada tanto para la extracción de contaminantes en matrices ambientales y alimentarias (Llompart et al., 2019; Moret, Conchione, Srbinovska, & Lucci, 2019) como para la recuperación de compuestos bioactivos a partir de plantas herbales complejas (Bagade & Patil, 2019).

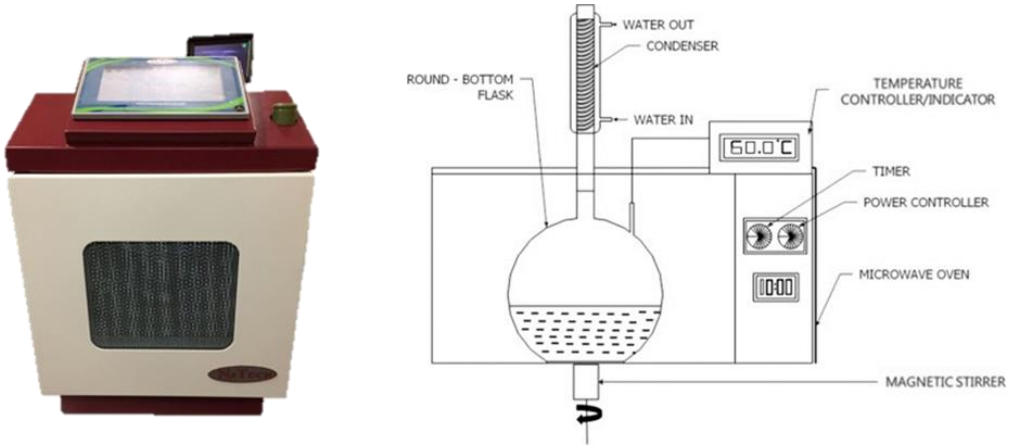

Figura 4. Imagen de un sistema de extracción por microondas modelo NμWave-Uno y esquema básico de un equipo de extracción asistida por microondas.

Recientemente, la técnica MAE se ha utilizado para la obtención de componentes activos de vegetales y subproductos relacionados (Carpentieri, Soltanipour, Ferrari, Pataro, & Donsì, 2021; Zia et al., 2020). La eficaz disrupción de la pared celular por parte de las microondas también ocurre en algas, por lo que lípidos, proteínas, carbohidratos, pigmentos y vitaminas han sido extraídos de estas plantas acuáticas mediante MAE (Kapoor et al., 2018). Debido a sus ventajas con respecto al método tradicional Soxhlet, la técnica MAE se ha empleado para la extracción de aceite de fuentes naturales vegetales y animales. Existen algunos estudios sobre aceite obtenido de semillas oleaginosas (Nde & Anuanwen, 2020), así como de partes comestibles de diversas especies de pescado (Costa & Bragagnolo, 2017; Ozogul et al., 2018). En cuanto a la aplicación de la MAE en subproductos generados por la industria agroalimentaria de origen animal, se ha descrito que el polímero quitosano fue aislado a partir de los caparazones de dos especies de langostinos (Bruno et al., 2019).

1.4. Ensayos de bioactividad

Las proteínas y los lípidos extraídos de subproductos de pescado son considerados compuestos de calidad desde el punto de vista nutricional, por lo que su utilización como ingredientes en la elaboración de alimentos destinados tanto a consumo humano como animal está aceptada. Sin embargo, el conocimiento de la composición de aminoácidos y de ácidos grasos no es suficiente para considerar a los constituyentes de los subproductos alimentarios como compuestos bioactivos. Las sustancias biológicamente activas son aquellas que aportan algún beneficio para la salud, más allá de sus propiedades nutricionales (Granato et al., 2020). El aumento de patologías crónicas ha supuesto un creciente interés por parte de la sociedad acerca de productos con propiedades saludables reales, lo que implica la demostración científica de sus efectos beneficiosos. A pesar de que los ensayos clínicos son la evidencia científica más consistente, tanto los ensayos *in vivo* como los ensayos con cultivos celulares son ampliamente utilizados para evaluar los efectos biológicos de los compuestos alimentarios.

Péptidos de origen marino han mostrado una actividad antihipertensiva elevada en ratas hipertensas, existiendo actualmente suplementos dietéticos con péptidos de bonito para mejorar la presión arterial (Ishak & Sarbon, 2018). Hidrolizados y péptidos obtenidos de subproductos de pescado y crustáceos han provocado respuesta antiinflamatoria en células de macrófagos (Kemp & Kwon, 2021). Péptidos de gelatina y de colágeno de piel de pescado han demostrado efectos protectores sobre el fotoenvejecimiento inducido por radiación UV en la piel de ratones (Sila & Bougatef, 2016). Aunque la mayoría de los ensayos de capacidad antioxidante de compuestos alimentarios se realizan a través de métodos químicos *in vitro*, la actividad antioxidante de péptidos de gelatina de

piel de pescado se ha evaluado en células endoteliales humanas (Ngo, Ryu, & Kim, 2014). Tanto compuestos proteicos como lipídicos obtenidos de subproductos de pescado han presentado propiedades antimicrobianas frente a patógenos clínicos y alimentarios (Chanda et al., 2018; Inguglia, Chiaramonte, Di Stefano, et al., 2020; Ucak et al., 2021; Zamora-Sillero et al., 2018). Así mismo, algunos péptidos y ácidos grasos obtenidos de subproductos de pescado también han exhibido actividad antiproliferativa frente a diversas líneas celulares cancerígenas (Ishak & Sarbon, 2018; Jameel et al., 2019; Jiang et al., 2019; Yaghoubzadeh, Peyravii Ghadikolaii, Kaboosi, Safari, & Fattahi, 2020). En este sentido, hay una gran variedad de organismos marinos que han atraído un gran interés por parte de los investigadores en respuesta a la creciente demanda de compuestos naturales con actividad anticancerígena. Actualmente, algunas moléculas de origen marino están siendo evaluadas en ensayos clínicos o han sido aprobadas para tratamientos contra el cáncer (Wang, Sorolla, Gopal Krishnan, & Sorolla, 2020).

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2. OBJECTIVES

OBJETIVOS

El objetivo general de la presente Tesis Doctoral es la investigación de las tecnologías no convencionales, extracción mediante líquidos presurizados (PLE) y extracción asistida por microondas (MAE), como herramientas para una valorización sostenible de los subproductos del procesado de pescado de cultivo. Para ello, se han planteado los siguientes objetivos específicos:

1. Optimizar las condiciones de extracción de la técnica PLE para obtener extractos proteicos con potencial antioxidante, utilizando el método de superficie de respuesta y cinco subproductos de lubina.
2. Aplicar las condiciones óptimas de extracción (PLE) a los subproductos de lubina, dorada y salmón para obtener extractos proteicos con potencial antioxidante.
3. Caracterizar los extractos obtenidos en base al contenido proteico total, la distribución del peso molecular de la fracción proteica y la capacidad antioxidante total.
4. Identificar péptidos con actividad antioxidante en los extractos de vísceras de salmón.
5. Optimizar las condiciones de extracción de la técnica MAE para recuperar aceite con propiedades nutricionales y bioactivas, utilizando el método de superficie de respuesta y tres subproductos de salmón.
6. Aplicar las condiciones óptimas de extracción (MAE) a los subproductos de salmón, dorada y lubina para obtener aceites con propiedades nutricionales y bioactivas.
7. Determinar la composición de ácidos grasos de los aceites extraídos.

8. Evaluar el efecto citotóxico de los aceites en diferentes líneas celulares tumorales y no tumorales.
9. Evaluar la capacidad antioxidante y antiinflamatoria de los aceites utilizando macrófagos.
10. Investigar la actividad antibacteriana y antifúngica de los aceites frente a patógenos alimentarios.
11. Examinar la presencia de metales pesados y micotoxinas en subproductos de lubina, dorada y salmón de cultivo.

OBJECTIVES

The main objective of this Doctoral Thesis is the investigation of non-conventional technologies, pressurized liquid extraction (PLE) and microwave-assisted extraction (MAE), as tools for a sustainable valorization of farmed fish processing side streams. For this, the following specific objectives have been established:

1. To optimize the extraction conditions of the PLE technique to obtain protein extracts with antioxidant potential, using the response surface method and five sea bass side streams.
2. To apply the optimal PLE extraction conditions to sea bass, sea bream, and salmon side streams in order to obtain protein extracts with antioxidant potential.
3. To characterize the obtained extracts in terms on total protein content, molecular weight distribution of the protein fraction, and total antioxidant capacity.
4. To identify peptides with antioxidant activity in salmon viscera extracts.
5. To optimize the extraction conditions of the MAE technique to recover oil with nutritional and bioactive properties, using the response surface method and three salmon side streams.
6. To apply the MAE optimal extraction conditions to salmon, sea bream, and sea bass side streams in order to obtain oils with nutritional and bioactive properties.
7. To determine the fatty acid composition of the extracted oils.

8. To evaluate the cytotoxic effect of the oils in different tumor and non-tumor cell lines.
9. To evaluate the antioxidant and anti-inflammatory capacity of the oils using macrophages.
10. To investigate the antibacterial and antifungal activity of the oils against food pathogens.
11. To examine the presence of heavy metals and mycotoxins in farmed sea bass, sea bream, and salmon side streams.

3. RESULTS

3.1. An integrated approach for the valorization of sea bass (*Dicentrarchus labrax*) side streams: Evaluation of contaminants and development of antioxidant protein extracts by pressurized liquid extraction

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An integrated approach for the valorization of sea bass (*Dicentrarchus labrax*) side streams: Evaluation of contaminants and development of antioxidant protein extracts by Pressurized Liquid Extraction

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Abstract

In this study, the presence of As, Hg, Cd, Pb and mycotoxins in sea bass side streams (muscle, head, viscera, skin, and tailfin) was evaluated as a preliminary step to assess the effect of an innovative extraction technique (Pressurized Liquid Extraction; PLE) to obtain antioxidant protein extracts. Then, a response surface methodology-central composite design was used to evaluate and optimize the PLE extraction factors (pH, temperature, and extraction time) in terms of total protein content and total antioxidant capacity (TEAC and ORAC). Heavy metals were found in all samples while DON mycotoxin only in viscera, both far below the safe limits established by authorities for fish muscle tissue and fish feed, respectively. The selected optimal PLE extraction conditions were pH 7, 20 °C, 5 min for muscle, pH 4, 60 °C, 15 min for heads, pH 7, 50 °C, 15 min for viscera, pH 7, 55 °C, 5 min for skin, and pH 7, 60 °C, 15 min for tailfins. Optimal PLE conditions allowed increasing protein content (1.2-4.5 fold) and antioxidant capacity (1-5 fold) of sea bass side stream extracts compared to controls (conventional extraction). The highest amount of protein was extracted from muscle while the highest protein recovery percentage was found in viscera. Muscle, head, and viscera extracts showed higher antioxidant capacity than skin and tailfin extracts. Moreover, different SDS-PAGE pattern were observed among samples and a greater quantity of protein fragments of lower molecular weight were found in optimal than control extracts.

Keywords: pressurized liquid extraction; sea bass; side streams; protein; SDS-PAGE; antioxidant capacity; mycotoxins; heavy metals.

1. Introduction

A large amount of side streams is generated by food industry during the transformation of raw material into the final commercial product. Particularly, for fish processing industry, fillets are the main product while heads, viscera, skin, fins, trimmings, roes, backbones, etc. are the resulting discards, accounting more than 60% of the total biomass [1]. In addition, the increased demand for fish for human consumption over the last years indicates that the amount of these fish side streams will continue to increase as well as their negative economic and environmental impact [2]. In general, most of the marine rest raw materials (including fish side streams) are considered non-food products and are transformed into animal feed, silage, and fertilizers. However, they contain a considerable amount of high nutritional quality proteins and lipids as well as valuable compounds, which makes them a natural resource to be explored and used, if possible, before being discarded [3–5].

European sea bass (*Dicentrarchus labrax*) is one of the most consumed fish in Mediterranean countries, being therefore one of the main species farmed at the European Union (EU). Preventive measures adopted by the EU to reduce the catch of wild sea bass has resulted in an increase in cultured sea bass [6]. In 2017 the production of sea bass from aquaculture accounted for 15% of the total farmed fish worldwide [7]. On the other hand, the growing development of both convenience food items and active packaging has led to a change in the commercialization of fishery products. In this sense, sea bass, which have traditionally been marketed as a complete piece, can be currently found as fillets or eviscerated fish. As a result, there will be an increase in sea bass side streams in the upcoming years.

The nutritional characterization of several sea bass side streams has recently been evaluated, showing a wide variety of healthy compounds such as unsaturated fatty acids, calcium, phosphorus, manganese, proteins, and amino acids [8]. In addition, Valcarcel et al. [7] developed fish protein hydrolysates from viscera, frames, and trimmings of sea bass and sea bream with *in vitro* antioxidant and antihypertensive activities, suggesting their use as food additives. Therefore, these studies considered the potential use of sea bass side streams as a raw material to obtain fish compounds to be incorporated into the food industry, thus complying with the circular bioeconomy proposed by the EU [9].

Due to the great interest in the exploitation of fish side streams to obtain high-added-value compounds for the food industry, not only their nutritional composition must be considered, but also their safety. The increase in fish production from the aquaculture sector has also led to a change in the diet of farmed fish. Fish meal and oil have been replaced by different plant-based ingredients, thus contributing to the presence of feed-borne mycotoxins in farmed fish [10,11]. Both marine and vegetable ingredients are used for the formulation of European seabass feedstuffs [12], which may cause the transfer of mycotoxins from the plant fraction of the feed to sea bass tissues. Other group of relevant contaminants within the food chain to be considered in farmed fish are toxic metals such as arsenic, mercury, cadmium, and lead, as these contaminants are usually found in feed, water and particulate matter in the aquaculture environment [13,14]. Although these contaminants have been traditionally investigated in edible fish tissues, the presence of toxic metals in different side streams of farmed sea bass, sea bream, and meagre have been recently reported [14,15]. Therefore, the safety of any fish side stream should be ensured in order to retain the quality of the product.

It should be noted that the valorization concept is not only based on producing value-added products from unconventional biological resources but it is also related to a sustainable and environmentally friendly approach. This involves finding suitable green technologies to recover valuable compounds from food side streams [16]. In this line, several green extraction techniques such as pulsed electric fields, microwaves, ultrasounds, high pressures, supercritical fluids, pressurised liquid extraction, subcritical water extraction, extrusion assisted extraction, membrane filtration, fermentative extraction, and enzymatic assisted extraction have been considered interesting extraction processes in terms of safety and efficiency to recover valuable compounds from marine discards [17–19].

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE) has become an alternative technique to extract organic compounds, mainly contaminant substances from environmental, biological, and food materials [20]. Furthermore, the possibility of using non-toxic solvents has promoted the use of PLE as an interesting tool to recover high-added-value compounds from different food matrices and side streams. In addition to this, PLE is appreciated as a fast, easy, and automated extraction process, which makes profit of pressure and temperature to improve the extraction efficiency [21]. According to these advantageous characteristics, different valuable compounds have been extracted from different matrices using PLE. Although most studies have been focused on obtaining bioactive compounds from terrestrial vegetable food and related by-products, macro and microalgae have also been used to recover bioactive phytochemicals [22], fatty acids [23] and polysaccharides [24]. Regarding marine discards, the carotenoid astaxanthin and fish oil were successfully extracted by PLE from shrimp head and carapace as

well as tuna liver, respectively [19]. However, only red peppers and seaweeds data in relation to protein extraction by PLE were found [4,25].

Therefore, this study aims to explore, for the first time, the use of the green PLE technique to obtain antioxidant protein extracts from sea bass processing side streams, in order to give added value to these underutilized residues. For this purpose, muscle left over, heads, viscera, skin, and tailfins were selected after simulating sea bass filleting. The presence of possible farmed fish contaminants for human health (heavy metals and mycotoxins) in these rest raw materials will be also evaluated. Then, the optimal pH-temperature-time combination for PLE-assisted extraction will be determined using the response surface methodology in terms of protein content and antioxidant capacity. The extracts obtained at optimal conditions will be analysed according to their protein fraction (total content and SDS-PAGE pattern for molecular weight distribution) as well as total antioxidant capacity.

2. Material and Methods

2.1. *Reagents*

Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), ABTS (2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)), DTT (DL-Dithiothreitol), Trizma® base, fluorescein sodium salt, diatomaceous earth (Hyflo® Super Cel®), and formic acid (reagent grade ≥ 95%) were provided by Sigma-Aldrich (Steinheim, Germany). Potassium dihydrogen phosphate, sodium phosphate dibasic, potassium sulphate, sodium chloride, ortho-boric acid, TRIS (ultrapure), AAPH (2,2'-azobis (2-amidinopropane)) (Acros Organics), glycine (proteomics grade), and methanol (HPLC LC-MS grade) were purchased from VWR International Eurolab S.L. (Barcelona, Spain). Sodium hydroxide, glacial acetic acid, and sulphuric acid were supplied by Fisher Scientific (Madrid, Spain).

SDS (sodium dodecyl sulfate, purissimum-CODEX) was obtained from Panreac (Barcelona, Spain). Acetonitrile (HPLC grade), acetone, glycerol, and bromophenol blue indicator (ACS reagent) were supplied by Merck (Darmstadt, Germany). Anhydrous magnesium sulfate (99.5% min powder) was provided by Alfa Aesar (Karlsruhe, Germany). Octadecyl C18 sorbent was from Phenomenex (Madrid, Spain) while absolute ethanol was from J.T. Baker (Deventer, The Netherlands). Deionized water (resistivity >18 MΩ cm-1) was obtained through a Milli-Q SP® Reagent Water System (Millipore Corporation Bedford, USA).

2.2. *Raw Material and Sample Preparation*

Whole sea bass fishes (*Dicentrarchus labrax*) were purchased in a local market in Valencia (Spain) during different days of February 2019. According to the commercial label, they were farmed in Burriana (Valencia, Spain). Immediately, they were transported from the market to the University of Valencia under refrigerated conditions.

For sample preparation (**Figure 1**), each individual sea bass was dissected and different side streams were separated as a simulation of fish processing for human consumption. Then, muscle (white and dark), heads (including gills), viscera, skin, and tailfins were selected and weighed inside aluminium containers before freezing (-80 °C). Frozen samples were lyophilized (LABCONCO, 2.5. FREE ZONE, USA) for 72 h and maintained in a desiccator until constant weight in order to determine their moisture percentage. The moisture values (%) were 65.66±1.55, 51.28±5.08, 21.92±2.28, 34.40±2.37, and 36.44±0.69 for muscle, heads, viscera, skin, and tailfins respectively), which were in accordance to the values reported by [8]. Next, samples were ground as well as possible in an analytical mill (A11 basic IKA® WERKE, Germany). Finally, a pool was made

to homogenize each fish side stream before storage at -25 °C until the extraction process and subsequent experiments.

Figure 1. Scheme of sea bass side streams sample preparation.

2.3. *Analysis of Heavy Metals in Sea Bass Side Streams*

The presence and content of As, Hg, Cd, and Pb in lyophilized muscle, heads, viscera, skin, and tailfins of sea bass was evaluated. A microwave accelerated reaction system (MARS, CEM, Vertex, Spain) was used for the acid mineralization of samples. According to the side stream, between 0.20 and 0.40 g of sample were placed in a Teflon vessel. Next, 1 mL of H_2O_2 (30% v/v) and mL of HNO_3 concentrated (64% v/v) were added to the samples and the digestion was carried out in the microwave system at 800 W and 180 °C for 15 min. After cooling and eliminating the nitrogen vapours, the digested samples were filtered through Whatman No. 1 filter paper and made up to volume with distilled water. Then, an inductively coupled plasma spectrometer mass detector (ICP-MS, Agilent model 7900) was employed to identify and quantify the heavy metals. The operating conditions were as follows: Ar plasma gas flow (15.0 L/min), carrier gas (1.07 L/min), reaction gas (He), nebulizer pump speed (0.10 rps), RF power (1550 W), and RF matching (1.80 V). Internal standard solutions of ^{72}Ge, ^{103}Rh, and ^{193}Ir (ISC Science) at 20 μg/g were used to correct matrix induced signal fluctuations and instrumental drift.

A standard calibration curve with concentrations ranging from 0-1000 μg/L was used for the quantification of As, Cd, and Pb and while a standard calibration curve between 0 and 100 μg/L was used for Hg. Limits of detection (LOD) were calculated according to the following equation: LOD = 3sB/a where, 3sB is 3 times the standard deviation at zero concentration and a is the slope of the calibration curve. LOD values (μg/L) for each element were As = 0.012, Hg = 0.0015, Cd = 0.0015, and Pb = 0.004. Distilled water was used as a blank and the metal concentrations in the digested blank were subtracted from the sample values. The results were expressed as μg of each element/g of side stream in wet weight. In addition, fish protein powder (Certified Reference Material for Trace Metals DORM-3) was used to confirm the accuracy of the method. It was prepared and analysed using the same procedure as that followed for the sea bass side streams. The recovery percentages were 98%, 86%, 76%, and 77% for As, Hg, Cd, and Pb, respectively.

2.4. *Analysis of Mycotoxins in Sea Bass Rest Raw Material*

Mycotoxins analysis was conducted by High Performance Liquid Chromatography coupled with Electrospray Ionization-Quadrupole-Time of flight-mass spectrometry (LC-ESI-qTOF-MS). An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a vacuum degasser, binary pump, and autosampler as well as a Gemini® column NX-C18 (3 μM, 150×2 mm ID) (Phenomenex) were employed for the chromatographic determinations. The mobile phases consisted of water (A) and acetonitrile (B), both with 0.1% of formic acid. The gradient program was 50% B (0 - 6 min); 100% B (7 - 12 min); 50% B (13 - 20 min). The injection volume was fixed at 5 μL and the flow rate at 0.2 mL/min. Mass spectrometry (MS) analysis was performed using a 6540 Agilent Ultra-High-Definition-Accurate-Mass-q-TOF-

MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in positive and negative ionization modes. The analysis conditions were as follows: nitrogen drying gas flow (12.0 L min $^{-1}$); nebulizer pressure (50 psi); drying gas temperature (370 ºC); capillary voltage (3500 V); fragmenter voltage (160 V); and scan range (m/z 50 - 1500). Automatic MS/MS experiments were carried out under the following collision energy values: m/z 100, 30 eV; m/z 500, 35 eV; m/z 1000, 40 eV; and m/z 1500, 45 eV. Mass Hunter Workstation software was used for data acquisition and integration.

The extraction of mycotoxins from the freeze-dried side streams was carried out using the QuEChERS procedure according to Pallarés et al. [26] with some modifications. Depending on the sample, between 2 and 4 g were mixed with 30 mL of acidified water (2% formic acid) and stirred for 30 min in an orbital shaker (IKA KS 260). Next, 10 mL of acetonitrile were added and an additional 30 min shaking was performed. Then, 2 g of NaCl and 8 g of $MgSO_4$ were added and vortexed for 30 s before centrifugation at 4000*x*rpm for 10 min. Afterward, 2 mL of supernatant were transferred into a 15 mL tube containing 0.3 g of $MgSO_4$ and 0.1 g of Octadecyl C18 sorbent. The mixture was shaken and centrifuged under the same previous conditions and the supernatant was filtered (13 mm/0.22 μm nylon filter). Finally, 20 μL were injected into the LC-ESI-qTOF-MS system.

2.5. *Protein Determination*

The total nitrogen content was evaluated in sea bass side streams as well as control and PLE extracts using the Kjeldahl method [27]. The protein-nitrogen conversation factor (6.25) used for fish and side streams was applied in order to obtain the total protein content.

2.6. *Evaluation of Total Antioxidant Capacity*

2.6.1. Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The TEAC assay measures the reduction of the radical cation $ABTS^+$ by antioxidant compounds. The spectrophotometric method proposed by Barba et al. [28] was used. The ABTS+ radical cation stock solution was generated by chemical reaction with 7 mM ABTS and 140 mM $K_2S_2O_8$ overnight in darkness at room temperature. Next, it was diluted in ethanol until an absorbance of 0.700 ± 0.020 at 734 nm and 30 °C to obtain the ABTS+ working solution. The optimization of the adequate dilution of the samples to obtain a percentage of absorbance inhibition of approximately 50% was required. Trolox standard solutions were prepared in a range of 0 to 300 μM. The absorbance of 2 mL of $ABTS^+$ working solution was considered the initial point of reaction (A_0). Then, 100 μL of diluted samples or Trolox standards were added immediately. The absorbance after 3 minutes of reaction was considered the final point (A_f). All readings were carried out in a thermostatized UV–vis spectrophotometer. The percentages of absorbance inhibition were calculated from the following equation: $1 - (A_f / A_0) \times 100$ and were compared to Trolox standard curve to express the results as μM Trolox Equivalents.

2.6.2. Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC assay measures the capacity of the antioxidant compounds to scavenge peroxyl radicals. The fluorimetric method described by Barba et al. [28] was applied. The reaction was carried out at 37 °C in a Multilabel Plate Counter VICTOR3 1420 (PerkinElmer, Turku, Finland) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Sodium fluorescein and AAPH solutions were used at a final concentration of 0.015 and 120 mg/mL respectively. Trolox (100 μM) was used as antioxidant standard and

samples were properly diluted. All of them were prepared with phosphate buffer (75 mM, pH 7). The final reaction consisted of 50 μL of diluted sample, Trolox standard or phosphate buffer (blank), 50 μL of fluorescein, and 25 μL of AAPH. The fluorescence was recorded every 5 min over 60 min (until the fluorescence in the assay was less than 5% of the initial value). The results were calculated considering the differences of areas under the fluorescence decay curve (AUC) between the blank and the sample over time and were expressed as μM Trolox Equivalents.

2.7. *Molecular Weight Distribution of Protein Fragments*

The molecular weight distribution of protein in both control (stirring) and optimal (PLE) aqueous extracts from sea bass side streams were investigated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Fish extracts were mixed with cold acetone (1:4, *v*/*v* ratio) and centrifuged at 11,000*x*rpm, 4 °C and 10 min in order to precipitate fish protein. Then, the supernatant was removed and the pellet was dissolved in distilled water assisted by ultrasound (10 min). Next, equal volumes of protein solution and SDS-PAGE sample buffer solution (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 0.01% bromophenol blue, and 50 mM dithiothreitol) were mixed and heated at 95 °C for 5 min. After denaturalization, 10 μL of mixture were loaded on the 8-16% Mini-PROTEAN® TGX™ Precast gels (Bio-Rad) and subjected to electrophoresis using a Mini-PROTEAN® tetra cell (Bio-Rad). The running buffer consisted of Trizma® base (25 mM), glycine (192 mM) and SDS (0.1%). The protein fragments separation was performed at a constant voltage of 80 V for 120 min. Finally, electrophoresed gels were stained in 0.125 % Coomassie brilliant blue R-250 and destained in 20 % methanol and 10 % acetic acid until the background was clear. A standard molecular weight of protein bands from 5

to 250 KDa (Precision Plus Protein™, Bio-Rad) was used to estimate the molecular weight of protein bands. The images of the electrophoretic gels were analysed using the ImageJ® software, a public domain digital image processing program developed at the National Institutes of Health (NIH).

2.8. *Pressurize Liquid Extraction (PLE) Optimization*

2.8.1. PLE Extraction Process

The accelerated solvent extractor ASE 200 Dionex (Sunnyvale, CA, USA) equipped with a solvent controller was used for the extraction of water soluble compounds (protein fraction and antioxidants) from sea bass side streams. Nitrogen (145 psi) was applied to assist the pneumatic system and to purge the cells. Distilled water was used as extracting solvent. The standard operating conditions were as follows: preheating period (1 min), heating period (5 min), flush volume (60%), nitrogen purge (60 s), and extraction pressure (1500 psi). The variable extraction conditions consisted of different ranges of pH (4 - 10), temperature (20 - 60 °C), and time (5 - 15 min).

All samples were mixed with diatomaceous earth (DE) before the extraction process. Both, the ratio (sample:DE) and the total amount of mixture were previously studied for each side stream. The extractions were performed in 22 mL pressure-resistant stainless steel cells with a glass fiber filter placed in the end part. Each aqueous extract obtained was homogenized, divided into several replicates and stored at -25 °C for subsequent analyses.

2.8.2. Experimental Design and Optimization of Extraction Conditions

The response surface methodology (RSM) was used to evaluate the effect of selected independent variables (pH, temperature, time) and determine the optimal conditions for the extraction of water-soluble proteins and antioxidant

compounds. For this purpose, a central composite design (CCD) was used for the optimization of the extraction conditions. This statistical model provided a total of 16 experiments which were conducted in a randomized order (Table 1). According to the CCD, one of the 16 pH-temperature-time extraction combinations was performed in duplicate in order to check the reproducibility and stability of the results. For the rotatable model, 3 to 5 central points are recommended, while for the centered face model used in this study, 1 to 2 central points are considered sufficient (Statgraphics Centurion XVI.I). In this way, some authors have previously used the same conditions for the CCD model [29,30]. Total protein content and total antioxidant capacity in fish extracts were the responses (dependent variables). The desirability method was used to find a common value for the dependent variables. Surface plots were generated by assigning constant value to one of the three variables studied. In addition, the effect of each independent variable on each of the responses was also studied and the corresponding graphics were created. The analysis was carried out through the Statgraphics Centurion XVI.I.

After acquiring the theoretical optimal conditions and knowing the impact of single variables on the responses, final PLE extraction conditions were selected and new extracts were obtained. At the same time, conventional extraction (control) was carried out under stirring (30 min) with distilled water at room temperature. Control samples were performed in parallel for all sea bass processing side streams. Then, extra experiments were carried out to investigate the protein fraction (protein content and protein molecular weight distribution) and the total antioxidant capacity in both control and optimal PLE extracts.

Table 1. Central Composite Experiment

Run	pH (X_1)	T[a] (°C) (X_2)	Time (min) (X_3)
1	4	60	5
2 [a]	7	40	10
3	4	20	15
4	10	40	10
5	7	60	10
6	10	60	5
7	10	60	15
8[a]	7	40	10
9	4	20	5
10	7	20	10
11	10	20	5
12	7	40	5
13	7	40	15
14	4	60	15
15	4	40	10
16	10	20	15

[a]central point

2.9. *Statistical Analysis*

Experimental data were subjected to one-way analysis of variance (ANOVA) to determine the significant differences among samples. Tukey HSD (Honestly Significant Difference) multiple range test, at a significance level of $P<0.05$ was applied. Statistical analyses were performed with the software Statgraphics Centurion XVI.I.

3. Results and Discussion

3.1. *Determination of Heavy Metals and Mycotoxins in Sea Bass Side Streams*

The concentration of heavy metals in sea bass muscle left over, heads, viscera, skin, and tailfins are reported in **Table 2**. As can be seen in the table, the concentration ranges expressed as μg/g of wet weight (ww) were 0.346-1.867,

0.015-0.106, 0.001-0.028, and 0.027-0.063 for As, Hg, Cd, and Pb, respectively. For all rest raw materials, the most abundant element was As. However, the order of Hg, Cd, and Pb differed according to each side stream. Hg ranked second for muscle and tailfins while Pb ranked second for heads, viscera, and skin. In general, the information available in the literature regarding metal contamination in fish tissues other than those considered edible is scarce. This is because the presence of heavy metals in fish has been only considered as a risk to human health when fish meat was the target sample. In this sense, Renieri et al. [31] determined the levels of Hg, Cd, and Pb in muscle tissue of sea bass and sea bream from different aquaculture sites and fisheries. With respect to sea bass, similar results (μg/g, ww) were reported for Cd (0.001), Pb (0.007-0.138), and Hg (0.022-0.113), with all values far below the safe limits for consumption established by authorities [32]. Regarding different sea bass side streams, Kalantzi et al. [14] investigated the accumulation of metals and trace elements in muscle, liver, gills, bones and intestines of farmed seabass as well as its correlation with the environmental conditions at the farming sites. It should be noted that the sum of the intestine and liver values would be equivalent to the values of the viscera sample of this study. Likewise, the gills values would correspond to those of the head samples. Thus, the authors found a higher mean arsenic content (μg/g, ww) in muscle (0.867), gills (0.455), and viscera (2.202).

Table 2. Concentration (μg/g) of As, Hg, Pb and Cd in sea bass side streams.

Sea Bass Side Streams	Heavy metals (μg/g of wet weight)			
	As	**Hg**	**Cd**	**Pb**
Muscle	0.687 ± 0.004	0.106 ± 0.001	0.001 ± 0.00001	0.027 ± 0.0002
Head	0.346 ± 0.003	0.034 ± 0.0004	0.003 ± 0.0001	0.063 ± 0.010
Viscera	1.86 7± 0.0005	0.014 ± 0.0003	0.028 ± 0.0003	0.046 ± 0.0004
Skin	0.387 ± 0.004	0.026 ± 0.0006	0.004 ± 0.0002	0.040 ± 0.0004
Tailfin	0.388 ± 0.006	0.042 ± 0.0006	0.001 ± 0.0002	0.033 ± 0.0004
[Legislation*]	< 13,5	< 0,50	< 0,05	< 0,30

*values referred to fish muscle tissue ([15,31,32])

On the contrary, the average of Hg concentration was lower for all side streams, being 0.062, 0.001, and 0.040 μg/g (ww) for muscle, gills, and viscera, respectively. Pb was not detected in muscle but the mean values in gills and viscera were 0.035 μg/g (ww) and 0.240 μg/g (ww), respectively. Similarly, there was no presence of Cd in muscle or gills, while the values in viscera were 0.322 μg/g (ww). Although there is no literature data regarding the concentration of metals in sea bass skin and tailfins, a recent study determined several trace elements in different sea bream samples (a specie closely related to sea bass) side streams, including skin [15]. Since the limits for toxic metals in fish side streams are not currently legislated, their assessment could be carried out according to those established for fish muscle. In this sense, the existing regulatory limits for As, Hg, Cd, and Pb are 13.5, 0.5, 0.05, and 0.30 μg/g of wet tissue [15,31], values much higher than those obtained in this study. Therefore, all sea bass side streams analysed could be considered as safe to be used for the food industry under the circular economy point of view, in terms of As, Hg, Cd, and Pb content.

Regarding mycotoxins, a home-made spectral library containing of 223 mycotoxins and a non-targeted screening approach were applied to investigate the presence of mycotoxins in sea bass tissues. Deoxynivalenol (DON) was identified in viscera sample. LOD and LOQ were 0.1ppb and 0.5 ppb respectively and a standard calibration curve from 0.5 to 1000 ppb was used for DON quantification with regression coefficients higher than 0.9990. Recoveries assays at 5 and 25 ppb were above 85% very similar to those obtained previously [26]. The positive sample showed traces of DON with levels ranging from LOD and LOQ.

DON is a mycotoxin primarily produced by *Fusarium* fungi, occurring mainly in cereal grains, which are used to elaborate feeding for fish. As a result, DON is also known for its high prevalence and incidence in both feed ingredients and feed end-products in Europe [33]. For instance, high concentration of DON in sea bream feeds due to wheat ingredient has been reported [34]. The maximum level of DON allowed by the European Commission in feed material is 12 mg/kg in maize by-products, 8 mg/kg for other cereals, and up to 5 mg/kg for complete and complementary feedstuff [35]. The intake of DON-contaminated feeds can affect not only fish health but also that of the final consumer of the food chain.

DON is considered to be rapidly metabolized and excreted by fish, thus producing low retention in tissues [33]. However, DON was evenly distributed in muscle, liver, kidney, skin, and brain of Atlantic salmon fed with contaminated feed for 2 months [36]. On the other hand, the estimated mean dietary concentration of DON in different farmed fish species concluded that adverse effects in human health were not expected [37]. Despite this, limit values for mycotoxins in fish tissues should be established by the authorities.

3.2. *PLE Optimization*

The influence of pH, temperature, and time on the development of protein extracts with antioxidant capacity of several sea bass side streams (muscle, heads, viscera, skin, and tailfins) obtained by PLE was studied using a response surface methodology. The experimental values for each independent variable provided from the central composite design and the responses obtained are shown in **Table 3**. Due to the lack of information on the behaviour of the proteins present in fish side streams as well as on their different tissues, the selection criteria for the extraction conditions were based on data from fish muscle proteins and the search for the most sustainable extraction process. It is known that the solubilization of fish muscle proteins depends on the protonation of amino acid residues of the protein side chains, with a mean pH value of 4.0 for aspartyl and glutamyl and 9.9 for lysyl, tyrosyl, and cysteinyl [38]. According to this, acidic (4), neutral (7), and basic (10) pH were selected for all side streams. Since high temperature may affect thermo-labile compounds and proteins from aquatic animals are more sensitive to heat than those from land animals, mild temperatures (up to 60 ºC) were chosen [20,38]. In addition, static extraction cycles of 5 min are required for the ASE equipment used. In order to achieve shorter extraction time, between 1 and 3 extraction cycles were selected.

Table 3. Results of total protein content and total antioxidant capacity (TEAC and ORAC) in sea bass side stream extracts obtained by Pressurized Liquid Extraction according to the response surface methodology-central composite design.

RSM				Muscle			Head			Viscera			Skin			Tailfin		
Run	pH	T[a] (°C)	Time (min)	Protein mg	TEAC μM Trolox Eq	ORAC	Protein mg	TEAC μM Trolox Eq	ORAC	Protein mg	TEAC μM Trolox Eq	ORAC	Protein mg	TEAC μM Trolox Eq	ORAC	Protein mg	TEAC μM Trolox Eq	ORAC
1	7	20	10	456	2079	4572	151	514	911	101	240	978	90	323	525	98	323	605
2	7	40	5	504	1407	2779	156	539	983	111	479	1212	153	376	881	130	399	660
3	7	40	10	549	1356	3058	161	622	1342	117	507	1440	153	403	686	130	422	918
4	7	40	10	540	1222	2837	189	650	1379	115	474	1241	166	381	749	134	345	723
5	7	40	15	510	2111	3060	201	861	1949	115	472	1290	184	159	817	145	592	1179
6	7	60	10	386	1166	1665	245	365	1210	125	464	1333	353	nd	1517	231	512	1177
7	4	20	5	454	1166	2428	149	503	1781	69	220	436	74	298	264	121	357	597
8	4	20	15	226	1273	1465	162	594	1129	68	156	401	79	372	362	105	400	829
9	4	40	10	285	981	1933	192	986	1576	107	376	1209	155	185	685	146	454	806
10	4	60	5	242	1140	918	213	531	952	101	432	1195	214	129	1124	181	392	816
11	4	60	15	312	859	1016	289	986	1794	124	434	1320	267	nd	1204	264	601	1253
12	10	20	5	301	955	2241	132	531	571	78	267	520	92	413	717	102	315	662
13	10	20	15	135	1593	1647	130	469	1700	87	357	968	92	336	487	103	363	663
14	10	40	10	207	1003	941	172	335	1005	103	450	1260	149	346	691	116	357	749
15	10	60	5	301	621	705	253	605	839	108	392	981	252	nd	1228	168	445	914
16	10	60	15	178	688	512	342	537	1540	116	430	1050	249	nd	1407	298	608	1501

RSM: Response Surface Methodology; TEAC: Trolox Equivalent Antioxidant Capacity; ORAC: Oxygen Radical Absorbance Capacity; nd: not detected

In addition to the factors of pH, temperature, and extraction time evaluated, other parameters such as pressure and solvent were also studied. The pressure is mainly responsible for maintaining the solvent in a liquid state, and has a limited impact on the extraction efficiency [39,40]. For the extractions carried out with different ASE Dionex models like the one employed in this study, 1500 psi is the constant pressure typically used [21,41–43]. Since authorized green solvents are recommended to recover food compounds by PLE, water was used in order to perform an extraction as much sustainable as possible for a future application in the food industry.

3.2.1. Protein Content

The results of total protein content in sea bass side streams extracts are shown in **Table 3**. The amount of protein in muscle extracts ranged from 134 mg (pH 10/20 °C/15 min) to 549 mg (pH 7/40 °C/10 min). For head extracts, the results varied from 130 to 342 mg of protein in extract, which corresponded to a combination of factors of pH 10/20 °C/15 min and pH 10/60 °C/15 min respectively. Regarding viscera extracts, the protein values obtained with the different extraction methods were found between 68 and 124 mg for pH 4/20 °C/15 min and pH 7/60 °C/10 min, respectively. As for the skin extracts, the lowest protein content was 73 mg (pH 4/20 °C/5 min) while the highest was 353 mg (pH 7/60°C/10 min). With regard to tailfins, the amount of protein in the extracts ranged from 98 to 298 mg with extraction conditions of pH 7/20 °C /10 min and pH 10/60 °C /15 min. According to these results, the influence of the combination of pH, temperature, and time on protein content in the extracts depended on the fish matrix.

In addition to the extracts, total protein content was also determined in freeze-dried samples in order to know the protein recovery from the sea bass raw materials to the solvent (water) after applying the PLE technique. The percentage of protein in sea bass side streams (dry weight, dw) was 79.18±0.36, 48.08±0.55, 18.24±1.07, 52.14±5.32, and 49.87±0.89 for muscle, heads, viscera, skin, and tailfins, respectively. These results are in close agreement with the values recently reported by Munekata et al. [8] and Valcarcel et al. [7] (except for tailfins that have not been considered in those studies). Regarding protein recovery from fish side streams samples, the percentage was calculated by applying the following equation: (amount of protein in extract/amount of protein in lyophilized sample) x 100. The ranges of protein recovery after applying the different pH-temperature-time combination methods were around 7-28% (muscle), 11-28% (heads), 26-48% (viscera), 7-34% (skin), and 10-30% (tailfins). It should be noted that the best values of protein recover were observed for viscera extracts, despite being the sea bass side stream with the lowest amount of proteins. In addition to the main factors, there are other important parameters such as the particle size of the sample that can influence the efficiency of the PLE extraction process [39]. In this sense, the skin and tailfin tissues could not be completely grinded and homogenized during sample preparation, so the contact surface for the aqueous extraction was smaller compared to muscle and viscera samples, thus being able to decrease the amount of the protein extracted.

Effect of Individual pH, Temperature, and Time on Protein Extraction

The behaviour of each single variable (pH, temperature, and extraction time) on total protein content of each sea bass side stream extract is shown in **Figure 2**. As can be seen in the figures 2a-o, the pH value mainly influenced protein

extraction from muscle (Fig 2a) and viscera (Fig 2g), while it was not a determining factor for heads, skin and tails (Fig 2d,j,m). Protein extraction is clearly improved by increasing the temperature independently of the sample, except for muscle. On the other hand, a longer extraction time enhanced the amount of proteins obtained from heads, viscera, and tails. However, the time factor was not decisive for skin and muscle samples. In general, temperature and pH were more relevant parameters than extraction time for protein response.

3.2.2. Total Antioxidant Capacity

To evaluate the quality of the obtained extracts, the total antioxidant capacity (TEAC and ORAC assays) was determined (Table 3). A wide variety of antioxidant activity values were observed among the different fish extracts. The values ranged from not detected to 2111 and from 401 to 4572 μM Trolox Eq for TEAC and ORAC assays, respectively. For muscle samples, the antiradical activity ranged from 620 to 2111 μM Trolox Eq (TEAC) and from 511 to 4572 μM Trolox Eq (ORAC). Regardless of the extraction time, the lowest values were found at 60 °C for all pH values studied. The antioxidant capacity in head extracts varied from 335 to 986 μM Trolox Eq (TEAC) and from 571 to 1949 μM Trolox Eq (ORAC). Regarding the remaining sea bass side streams (viscera, skin, and tails), the antioxidant capacity values were approximately below 600 and 1500 μM Trolox Eq for TEAC and ORAC tests, respectively. The highest values of antioxidant capacity were found in muscle sample, which seem to be related to the solvent neutral pH. It should be noted that for head, viscera, and tailfin extracts, both mechanisms of antioxidant action showed a similar behavior between the different combinations of pH, temperature, and extraction time used. However, with regard to the skin sample, the extraction method applied at 60 °C negatively affected the antioxidant capacity of the extract

components regardless of pH and extraction time. There is a great variety of phytochemicals with in vitro antioxidant capacity extracted by PLE from plant products and by-products [21,44,45]. However, as far as we know, there are no examples in the literature on the application of PLE to recover food compounds from food of animal origin and related side streams.

Effect of Individual pH, Temperature, and Time on Antioxidant Capacity

The behaviour of each single variable (pH, temperature, and extraction time) on total antioxidant capacity (TEAC and ORAC) of sea bass side stream extracts is shown in **Figure 2**. Although these assays measure the antioxidant capacity of compounds through different mechanisms of action, no differences were observed in the pH influence on the antioxidant activity for each side stream and method used. Moreover, the effect of temperature on the antioxidant capacity (TEAC and ORAC) was similar for each muscle (Fig 2b,c), head (Fig 2e,f), viscera (Fig 2h,i), and tailfin (Fig 2n,o) extracts. Regarding skin, the increase in temperature caused both the decrease of TEAC (Figure 2k) values and the increase of ORAC (Figure 2l) values of the extracts. Differences in the response of antioxidant capacity based on temperature factor have also been observed in extracts of chestnut shell and bean [46,47]. The extraction time had a similar impact on the antioxidant capacity (TEAC and ORAC) of fish extracts for head (Figure 2e,f), skin (Figure 2k,f), and tailfin (Figure 2n,o) samples. However, different behaviour was observed for muscle (Figure 2b,c), and viscera (Figure 2h,i). For instance, increased extraction time augmented TEAC and slightly decreased the ORAC values. Moreover, in general, it was found that the *in vitro* antioxidant properties of the fish extracts were less influenced by pH factor.

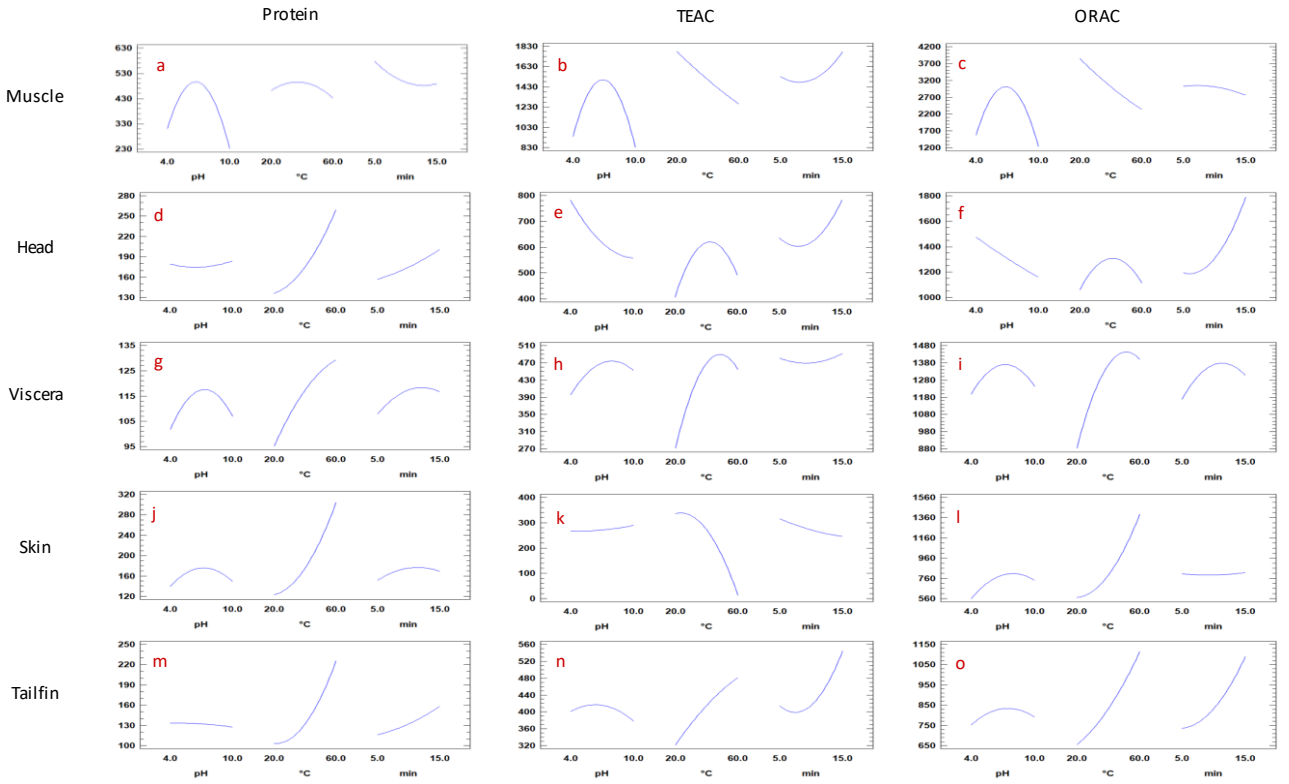

Figure 2. Main effects of each independent variable (pH, temperature, time) of Pressurized Liquid Extraction for each response (total protein content and total antioxidant activity (TEAC and ORAC)) in extracts from seabass muscle, head, viscera, skin, and tailfins.

3.2.3. Effect of the pH-Temperature-Time Combination on Common Response

Graphical analysis in terms of response surfaces was performed in order to visually interpret the effect of the combination of the different PLE extraction variables (pH, temperature, and time) on the different analysed responses (protein content and antioxidant capacity by TEAC and ORAC). It should be noted that the optimization was carried out on the basis of both the protein content values and the antioxidant capacity (TEAC and ORAC) values. For this purpose, the desirability method was used as a common value to the three responses and then the response surface method provided the combination of the experimental factors that simultaneously optimizes several responses (by maximizing the desirability).

According to this, **Figure 3** shows the response surface plots of the effect of the combination of PLE extraction variables on protein content and antioxidant capacity in fish extracts obtained.

The predicted optimal extraction conditions (pH/°C/min) were 6.8/20 °C/5.0 min for muscle left over, 4.0/60 °C/15.0 min for heads, 7.3/49 °C/12.7 min for viscera, 7.7/53 °C/5.0 min for skin, and 9.4/59 °C/15.0 min for tailfins. However, the ASE equipment does not allow entering a temperature value other than an exact number. In the same way, the static extraction mode used by PLE is based on 5 minute cycles, thus reducing the selection of extraction time. Taking into account these technical limitations, the optimal PLE conditions selected were (pH/°C/min): 7/20/5 for muscle, 4/60/15 for heads, 7/50/15 for viscera, 7/55/5 for skin, and 7/60/15 for tailfins. Noteworthy that the optimum extraction time for two of the five side streams studied was 5 minutes, confirming PLE as a fast technique.

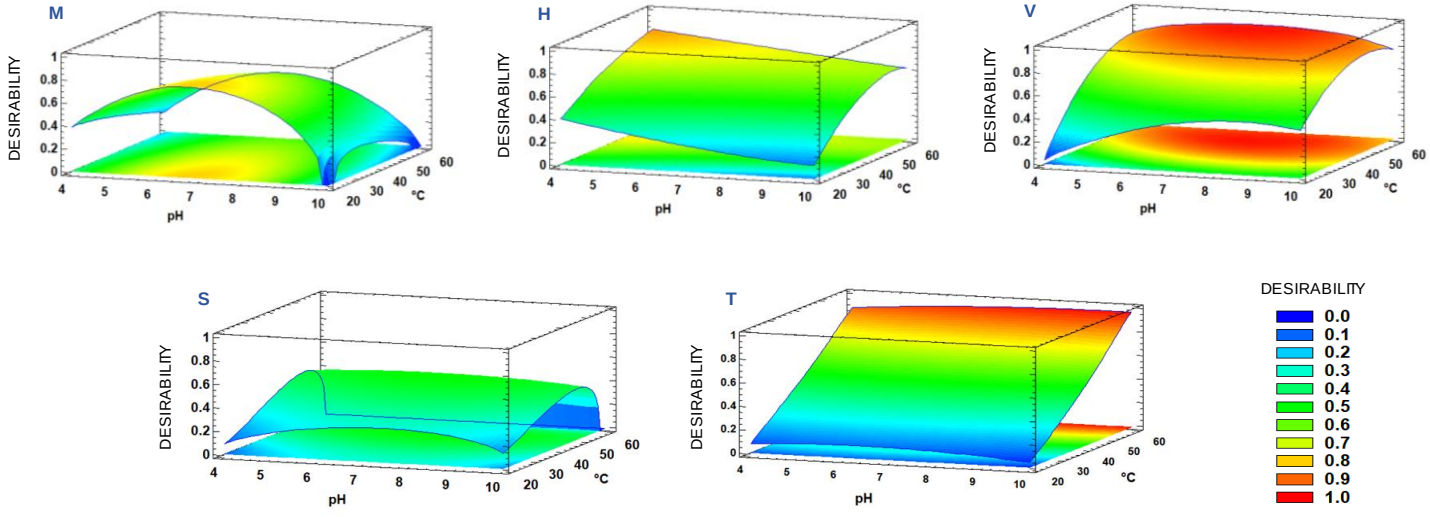

Figure 3. Estimated response surface by plotting desirability versus pH (4-10), temperature (20-60 °C), and 15 minutes of extraction time for each sea bass side stream (M: Muscle, H: Head, V: Viscera, S: Skin, T: Tailfin). Desirability is based on the joint response of the different responses analysed (total protein content and total antioxidant capacity by TEAC and ORAC assays).

After verifying that the pH factor did not significantly influence protein content and antioxidant capacity in tailfin extracts (Figure 2m–o), neutral pH instead of basic pH was selected for this sample. It should be noted that 59 and 60 °C were the optimum temperatures for tailfin and head, respectively. Since the antioxidant capacity (TEAC and ORAC) decreased at 60 °C (Figure 2e,f), a higher temperature could affect even negatively the antioxidant compounds of head extracts. In addition to this, extraction time of 25 min was explored following the trending of 15 min as optimal extraction for some head, viscera, and tailfin samples. However, no higher values were obtained either for protein or antioxidant capacity compared to 15 min extractions (data not shown). Noteworthy that the optimum extraction time for two of the five side streams studied was 5 min, confirming PLE as a fast technique.

3.3. *Evaluation of RSM Mode*

The RSM model provided a regression equation fitted to the experimental data and specific for each response and side stream (a total of 15 equations). The substitution of the pH, temperature, and time values in the equations resulted in a theoretical value for each response according to the CCD model. In general, the predicted and experimental values were similar for all pH-temperature-time combinations and samples. In addition, the reproducibility of the results was verified from the values obtained in the central points of the CCD model. Good coefficients of variation were obtained for protein content, TEAC and ORAC for all side streams, except for the antioxidant capacity values of the tailfin extracts. Experimental and predicted data for the response variables obtained from the CCD for sea bass muscle, head, viscera, skin, and tailfin extracts are reported in Supplementary Material (Tables S1-S5). All the regression equations required for the calculations (Equations (S1)-(S15)) as well as the coefficient of

variation of the central point from the CCD model (Table S6) are also included. Since maximal protein content, TEAC, and ORAC values were achieved individually at different extraction parameter combinations, the RSM method was applied to optimize the PLE conditions for these three responses together. For this purpose, the desirability (d) function approach was used. It is a multiplicative model of individual desirability that provide a desirability scale ranging from 0 to 1 [40]. The ideal optimum value is d = 1 and an acceptable value is 0.6 < d < 0.8. According to the RSM method, desirability values of 0.8 were obtained for muscle and head, 0.9 for viscera and tails, and 0.6 for skin.

3.4. *Optimal PLE Extracts and Comparison to Control Extracts*

Both the extracts obtained by PLE after applying the selected optimal extraction conditions and the extracts obtained by conventional stirring (control) were characterized based on total protein content and molecular weight distribution of protein fragments as well as the total antioxidant capacity.

3.4.1. Total protein content

The results of the amount of protein extracted in optimal and control extracts of all sea bass side streams are shown in **Figure 4**. The milligrams of protein in the optimal extracts of sea bass muscle, head, viscera, skin, and tailfins were 440±4, 285±2, 159±5, 241±6, and 299±6 respectively while they were 351±5, 185±13, 138±7, 113±7, and 67±7 in their corresponding control extracts. Therefore, PLE-assisted extraction improved by 1.2 to 4.5 times (depending on the sample) the protein extraction of sea bass side streams in one sixth and half the time compared to controls (5 and 15 vs 30 min). Since neutral pH water was used for most of the fish side streams in both PLE extraction and traditional stirring, it could be concluded that pressure and temperature played an important

role in the extraction process. The protein recovery percentages were also calculated, being around 22, 18, 61, 18, and 30% for optimal extracts of sea bass muscle, head, viscera, skin, and tailfins respectively whilst 18, 15, 52, 11, and 14% for controls. As far as we know, no studies about protein extraction from fish side streams using PLE technique have been reported. Marine matrices (red, green, and brown seaweeds) were used to compare PLE with different protein extraction methods [4]. The application of a 50% methanol-water mixture at 37 °C resulted in a protein recovery of less than 5%, a lower percentage than in this study. In addition, the optimal protein extraction from red pepper seed meal was 12% after applying PLE technique [25].

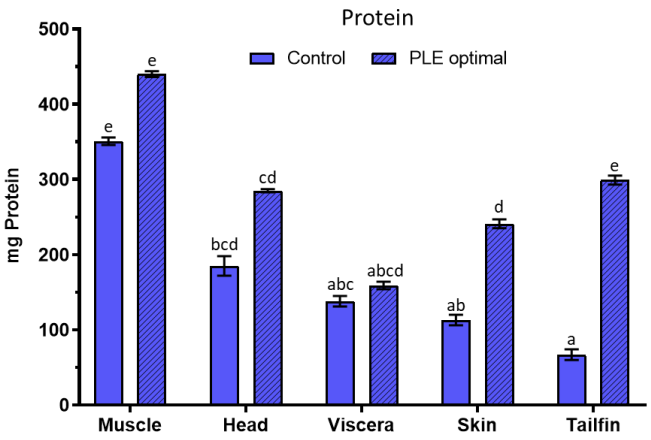

Figure 4. Total protein content in control extracts and optimal PLE extracts from sea bass muscle, head, viscera, skin, and tailfin side streams. PLE: Pressurized Liquid Extraction. Results are expressed as mean ± standard deviation (n = 2). Different lowercase letters in the bars indicate statistically significant differences ($P<0.05$) among samples.

3.4.2. Protein molecular weight distribution

The electrophoretic pattern of sea bass muscle, heads, viscera, skin, and tailfins extracts obtained both by conventional stirring and PLE is shown in **Figure 5 A.** As expected, different protein molecular weight (MW) distribution profiles were observed among the samples due to the different components of each sea bass side stream. In order to achieve a better approximation of the kDa values corresponding to each band compared to the MW standard, the image of the electrophoresis gel was evaluated by the ImageJ Program. In addition, the superposition of the electrophoretic images (standard-sample) allowed grouping in MW intervals the areas of the different bands of each sample (**Figure 5B**). Although visually no great differences were observed in the electrophoretic profiles of controls compared to the optimal ones, the analysis of the images revealed that optimal PLE extracts contained a greater amount of protein fragments of lower MW than control extracts. Therefore, the PLE extraction conditions selected in this study could influence the characteristics of the protein fragments obtained in sea bass side stream extracts.

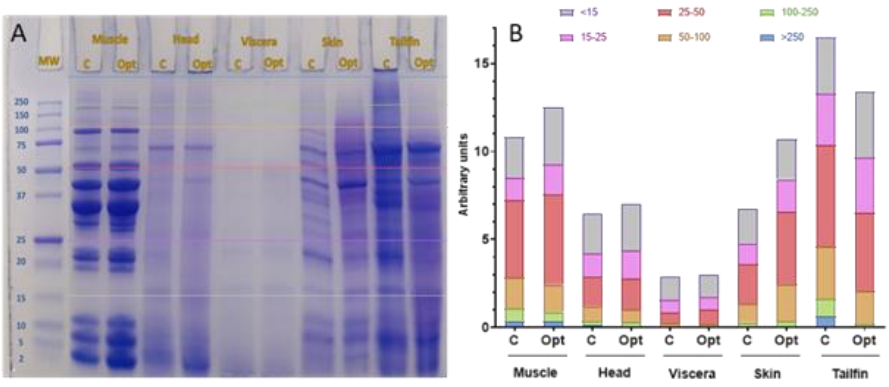

Figure 5. Molecular weight distribution of protein fraction of sea bass side stream extracts. SDS-PAGE electrophoresis patterns (A) and grouping of band areas by molecular weight ranges (B). MW: Molecular Weight Standard. C: Control extract. Opt: Optimal extract.

The muscle proteins (opt) exhibited bands of MW from 9 to 126 kDa while those of the muscle control extract were from 9 to 146 kDa. The clearest bands were found between 25 and 100 kDa in both extracts, in agreement with the electrophoretic profiles of sarcoplasmic proteins from striped catfish and carp meat [49]. Few protein bands ranging from 10 to 85 kDa were observed for both control and optimal head extracts which was in a similar range to that obtained in parrotfish head protein hydrolysates [50]. Poorly defined bands were obtained in viscera samples. The range of MW between 7 and 60 kDa was the same for both extracts. However, a 110 kDa band in the control extract was not found at the optimum. This SDS-PAGE pattern of seabass viscera proteins (8, 18, 27, 49, 60 kDa) was similar to that of undigested cod viscera proteins reported by Aspmo et al. [51]. The presence of protein fragments below 5 kDa could also be observed in both profiles which could be possibly reported as viscera enzymes. Several bands (8-100 kDa) were found both in the controls and in the optimal sea bass skin extracts. The bands corresponding to 85, 46 and 38 kDa of the control extract were not found in the optimal PLE extract, which may be due to the difference in the extraction conditions. The SDS-PAGE technique is usually used to examine collagen or gelatin proteins obtained from fish skin. In this sense, the electrophoretic profiles of hoki and rainbow trout skin gelatin did not correspond to the profiles of the sea bass skin extracts [52,53]. MW of protein fragments of optimal sea bass tailfin extracts ranged between 8 and 80 kDa while those of the controls ranged between 8 and 141 kDa. The two bands with the highest molecular weight (101 and 141 kDa) as well as the 39 kDa band of the control extract did not appear in the electrophoretic profile of the optimal extract, showing again that the extraction parameters could influence the protein fraction.

3.4.3. Total Antioxidant Capacity

The results of total antioxidant capacity in optimal PLE and control extracts of all sea bass side stream extracts are shown in **Figure 6**. Total antioxidant capacity measured by both TEAC and ORAC assays was also higher in optimal samples than control extracts for all fish side streams.

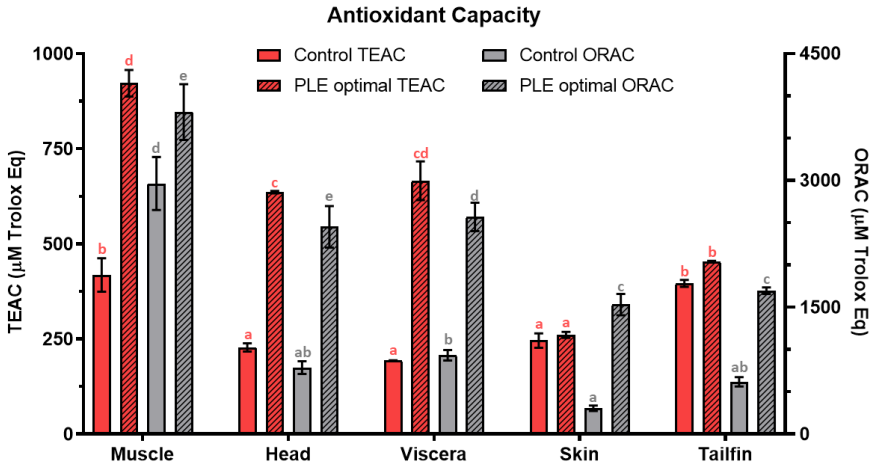

Figure 6. Total antioxidant capacity (TEAC and ORAC) in control extracts and optimal PLE extracts from sea bass muscle, head, viscera, skin, and tailfin side streams. TEAC: Trolox Equivalent Antioxidant Capacity. ORAC: Oxygen Radical Absorbance Capacity. PLE: Pressurized Liquid Extraction. Results are expressed as mean ± standard deviation (n = 3 for TEAC and n = 6 for ORAC). Different lowercase letters in the bars indicate statistically significant differences ($P<0.05$) among samples.

TEAC values were 922±35, 636±37, 666±51, 261±8, and 453±2 µM Trolox Eq for optimal muscle, head, viscera, skin, and tailfins extracts, respectively while values of 418±44, 228±11, 193±1, 246±19, and 396±9 µM Trolox Eq were found for the corresponding controls. Similarly, optimal ORAC values (µM Trolox Eq) were 3808±33 (muscle), 2452±25 (heads), 2569±17 (viscera), 1531±13 (skin), and 1696±39 (tailfins) whereas control ORAC values were

2963±31, 787±76, 934±32, 306±32, and 619±56, respectively. According to these results, the application of PLE improved the antioxidant capacity of all sea bass side stream extracts. Comparing data of control extracts with the optimal ones, the antioxidant capacity increased by 120, 179, 245, 6, and 14% (TEAC) and by 29, 211, 175, 400, and 174% (ORAC) for muscle, heads, viscera, skin, and tailfins, respectively.

Phytochemical compounds such as polyphenols, carotenoids, anthocyanins, etc. from plant foods and their residues are usually considered responsible for their antioxidant capacity. In the case of foods of animal origin and related side streams, the antioxidant properties have been attributed to amino acids, peptides, and proteins [54]. For instance, protein hydrolysates and peptides from several fish processing side streams have shown antioxidant activities and they have been considered as potential substitutes of synthetic antioxidants for the food industry [1,55,56].

Both protein chain size and composition of amino acids are considered key in the antioxidant activity exhibited by protein fragments. In this sense, hydrophobic amino acids and proline, methionine, tyrosine, histidine, lysine and cysteine may improve the efficiency of antioxidant peptides [1]. Recently, the amino acid profile of several side streams from farmed sea bass have been reported [8]. According to the results, lysine, proline, and tyrosine were determined in muscle, head, gills, guts, liver, and skin while methionine was not detected in guts and gills. Histidine was also no detected in guts.

Conclusions

Pressurized Liquid Extraction (PLE) is presented here as an interesting tool to obtain protein extracts with antioxidant capacity from sea bass processing side streams. Since the optimal pH value for four of the five samples was close to 7, the PLE-assisted recovery of high-added-value compounds from sea bass side streams could be performed in a sustainable and sustainable way. Optimal pH-temperature-time combinations allowed to obtain higher total protein content and total antioxidant capacity in PLE extracts. Muscle, head, and viscera optimal extracts showed better total antioxidant capacity (TEAC and ORAC) than skin and tailfin extracts. The highest amount of protein was recovered from sea bass muscle left over while the highest protein recovery percentage was observed in viscera. Furthermore, the SDS-PAGE pattern of each extract revealed a specific protein molecular weight distribution for each sea bass side stream. Recovering proteins from natural unexploited resources in a sustainable way is one of the H2020 challenges. Finally, this is the first step towards a possible sustainable application of PLE technique to obtain antioxidant protein extracts from fish side streams. Further research is encouraged in this direction to convert fish side stream materials into nutritional and bioactive ingredients for food, feed, and other high-value market.

Supplementary Materials: Tables S1-S5: Experimental and predicted data for the response variables obtained from the central composite design for sea bass muscle (S1), head (S2), viscera (S3), skin (S4), and tailfin (S5) extracts; Table S6: Reproducibility of the results according to the coefficient of variation from the values obtained in the central points of the central composite design model for sea bass side stream extracts. Equations S1-S15: Regression equations provided by Response Surface Methodology and Statgraphics Centurion XVI.I for muscle (S1-S3), head (S4-S6), viscera (S7-S9), skin (S10-S12), and tailfin (S12-S15) extracts.

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3.2. Development of antioxidant protein extracts from gilthead sea bream (*Sparus aurata*) side streams assisted by pressurized liquid extraction (PLE)

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Development of Antioxidant Protein Extracts from Gilthead Sea Bream (*Sparus aurata*) Side Streams Assisted by Pressurized Liquid Extraction (PLE)

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Abstract

The pressurized liquid extraction (PLE) technique was used, for the first time, to obtain protein extracts with antioxidant activity from side streams (muscle, heads, viscera, skin, and tailfins) of gilthead sea bream (*Sparus aurata*) in order to give added value to these underutilized matrices. Extraction conditions previously optimized for sea bass (*Dicentrarchus labrax*) side streams were applied. Protein recovery percentages were 22% (muscle), 33% (heads), 78% (viscera), 24% (skin), and 26% (tailfins), which represented an increase of 1.2–4.5-fold compared to control samples (extraction by stirring). The SDS-PAGE profiles revealed that PLE-assisted extraction influenced protein molecular weight distribution of the obtained extracts. PLE conditions also allowed increasing the antioxidant capacity measured by both Trolox equivalent antioxidant capacity (TEAC; 1.3–2.4 fold) and oxygen radical absorbance capacity (ORAC; 1.9–6.4) assays for all fish extracts. Inductively coupled plasma mass spectrometry (ICP-MS) and high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (LC-ESI-qTOF-MS) were used to investigate the presence of toxic metals and mycotoxins in sea bream side streams. The levels of As, Hg, Cd, and Pb were below those established by authorities for fish muscle for human consumption (except for Cd in viscera samples). Through a nontargeted screening approach, no mycotoxins or related metabolites were detected for all sea bream side streams. This study contributes to the research on the valorization of fish processing side streams using environmentally friendly technology.

Keywords: pressurized liquid extraction; gilthead sea bream; side streams; protein; SDS-PAGE; antioxidant capacity; mycotoxins; heavy metals

1. Introduction

The European Union (EU) is the world's second largest trader of fishery and aquaculture products after China [1]. The increasing importance of the European aquaculture sector is due to the increased production of high-value species. For instance, the production of the main commercial species such as salmon, bluefin tuna, sea bass, and sea bream has increased by 11% over the last decade. In terms of value, one of the most significant growths in recent years was obtained for European gilthead sea bream (*Sparus aurata*), reaching 94.936 tons and 485 million EUR in 2017 [2]. This trend is supported by the increased demand, which, together with the consumer growing interest in convenience products, has led to a greater manufacture of gutted and filleted sea bream [3]. In this sense, Pateiro et al. [4] reported that discards accounted for ~60% of the whole sea bream after the filleting process. Therefore, an increase in gilthead sea bream side streams in the upcoming years is expected.

The relevance of fish processing side streams as an alternative source of nutrients and bioactive compounds for the food and feed industries is increasing the research on nutritional characterization and the presence of possible contaminants in these underutilized raw materials [5,6]. In this context, the nutritional composition of several sea bream side streams was recently evaluated [4,5]. The authors concluded that sea bream side streams had a significant protein, fat, and mineral content. The nutritional profiles also showed a percentage of essential amino acids close to 50%, as well as a higher content of mono- and polyunsaturated fatty acids compared to saturated ones. Therefore, they suggested sea bream side stream materials as a promising source of valuable compounds to be exploited for human consumption. In this way, fish protein hydrolysates with a remarkable essential amino-acid profile were produced from

sea bream filleting side streams and were considered as a suitable tool for developing food additives and supplements [3]. Currently, only industrially processed feeds are used to grow gilthead sea bream in aquaculture systems [7]. Ingredients such as corn, wheat, pea, and soybean represent protein sources for farmed sea bream feeding [7,8]. Therefore, evaluating the transfer of feed-borne mycotoxins to different sea bream tissues is recommended. In addition, trace metals may also be transferred from the aquaculture environment to farmed fishes [6,9]. Toxic metals such as As, Hg, Cd, and Pb and mycotoxins have been screened in several side streams of sea bream, sea bass, meager, and salmon, although levels found of these contaminants were below the limits stablished by authorities [6,9–11].

The Horizon 2020 program stands for research and innovation to reach Europe's global competitiveness, encouraging the introduction of sustainable technologies to fisheries and aquaculture [12]. One of the Horizon 2020 challenges is the recovery of proteins from natural underexploited resources in a sustainable way. Fish processing side streams have been considered ideal candidates for protein recovery due to their relevant content protein, wide availability, and low cost [13,14]. Among the different techniques developed to recover and produce proteins from marine organisms and related byproducts, solvent and enzymatic extraction processes are preferred for production of proteins of high nutritional quality with bioactive and functional properties [14]. The application of pressurized liquid extraction (PLE) to obtain protein extracts with antioxidant activity from sea bass processing side streams was recently investigated [10]. The use of water as sustainable solvent and the optimization of extraction conditions resulted in a protein recovery of 18% to 61%,

depending on the fish raw material. The optimal fish extracts obtained also showed in vitro antioxidant capacity.

It should be noted that PLE is currently considered a fast and easy extraction process, as well as an important technology for recovering a great variety of compounds from different food matrices [15,16]. PLE is based on the use of high pressure and temperature to improve the extraction efficiency by increasing the diffusion rate and solubility of analytes [16–18]. However, to achieve this, the optimization of different additional parameters is also required. For instance, dispersing agents are used to reduce particle clumping and solvent channeling in the extraction cell [17]. As the number of extraction cycles and the total extraction time are related to the contact time between matrix and solvent, they should be carefully selected [16]. In addition to the physicochemical properties, the nontoxicity of the solvent is a crucial factor for the sustainable recovery of food compounds, which is why the use of water as a solvent is recommended.

Since sea bass and sea bream are considered to be closely related species, similar results would be expected. Therefore, the main objective of the present study was to apply, for the first time, the PLE technique to obtain antioxidant protein extracts from sea bream processing side streams in a sustainable way. Muscle left over, heads, viscera, skin, and tailfins of gilthead sea bream were selected as a valorization approach for these underutilized raw materials. Protein recovery, protein molecular weight distribution, and antioxidant capacity were evaluated in PLE extracts. In order to provide additional data on potential contaminants, the determination of As, Hg, Cd, and Pb, as well as a multi-mycotoxin screening in sea bream side streams, was also carried out. Overall, this study contributes to the research on the valorization of fish processing side streams using environmentally friendly technology.

2. Results and Discussion

2.1. Protein Recovery Percentage

The results of protein recovery in control and PLE extracts from side streams of gilthead sea bream are shown in Figure 1. The percentage protein recovery in PLE extracts of sea bream muscle, head, viscera, skin, and tailfins were 22.06±0.68, 33.48±0.47, 77.66±3.01, 23.80±1.43, and 26.37±0.48, respectively, while they were 16.52±0.20, 19.09±0.84, 62.46±2.85, 10.67±0.49, and 2.73±0.15 in their corresponding control extracts. Therefore, PLE-assisted extraction improved the protein recovery ($p<0.05$) for all side streams, except for muscle remains. In addition, the protein yield of fish extracts increased from 1.2 (viscera) to 9.6 (tailfin) folds. The best recovery was observed in viscera, in agreement with the protein recoveries previously obtained by PLE from sea bass side streams [10]. Similar protein recovery was observed for muscle and tailfins for both species, while higher percentages of protein recovery were found in sea bream head, viscera, and skin compared to sea bass. The PLE technique was also used to extract proteins from freeze-dried seaweeds and pepper seeds, showing protein recovery percentages of <5% and 52%, respectively [13,14].

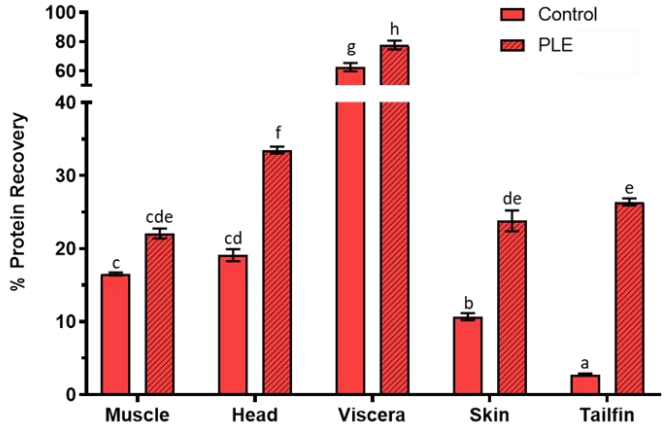

Figure 1. Percentage of protein recovery in control and PLE extracts from muscle, head, viscera, skin, and tailfin of gilthead sea bream. PLE: Pressurized Liquid Extraction. Results are expressed as mean ± standard deviation (n=2). Different lowercase letters in the bars indicate statistically significant differences ($P<0.05$) among samples.

2.2. Protein Molecular Weight Distribution

SDS-PAGE provided the electrophoretic pattern of side stream extracts of gilthead sea bream obtained by both conventional stirring and PLE-assisted extraction (Figure 2A). The gel image was evaluated through the ImageJ Program not only to obtain the molecular weight of each band but also to group the areas of the bands by kDa ranges (Figure 2B). On the one hand, different protein profiles can be observed according to the matrix studied, probably due to the particular protein composition of each side stream. On the other hand, some differences between the bands of PLE extracts with respect to the bands of control extracts were found for all sea bream side streams. Both results are in agreement with those recently reported by de la Fuente et al. [10] for protein extracts of sea bass side streams obtained by PLE.

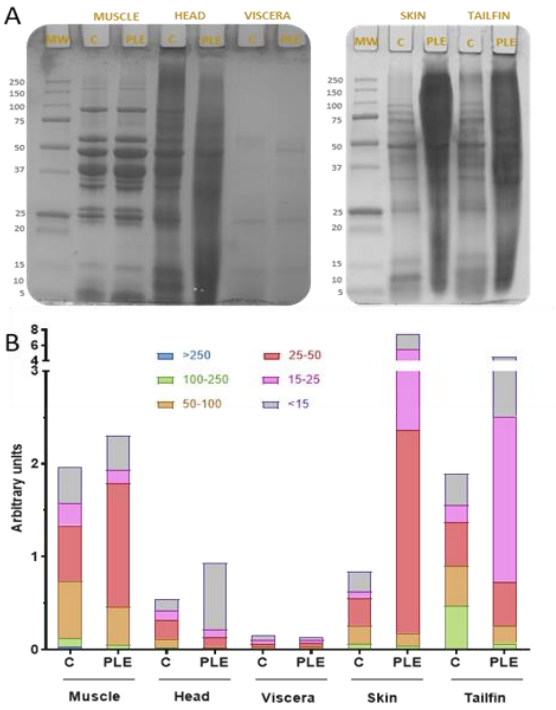

Figure 2. Protein molecular weight distribution of control and PLE extracts from sea bream side streams. SDS-PAGE protein profiles (**A**) and molecular weight ranges for band areas (**B**). MW: molecular weight standard. C: control extract. PLE: extract obtained by pressurized liquid extraction.

Several clear bands (8–166 kDa) were observed for both control and PLE muscle extract profiles. This similarity could be due to the fact that both extractions were carried out at room temperature. However, the differences in the width of the bands revealed that PLE-assisted extraction provided more amount of total protein fragments, mainly in the ranges 25–50 kDa and below 15 kDa. In general, the electrophoretic patterns of sea bream muscle extracts are in agreement with those of sea bass, catfish, carp, and mackerel protein muscle [10,21,22]. The head proteins (PLE) exhibited bands from 8 to 103 kDa while those of head control extract were from 8 to 86 kDa. This range of molecular

weights was equivalent to that obtained in sea bass head PLE extracts (10–85 kDa), as well as similar to parrotfish head hydrolysates subjected to hydrolysis at pH 9 for 24 h (17–76 kDa) [23]. Few differences in protein profiles of sea bream viscera extracts obtained by PLE and conventional stirring were observed. The protein bands higher than 60 kDa in the control extract were not found in the PLE extract.

In addition, protein fragments in sea bream viscera extracts (PLE) ranging from 8 to 61 kDa were also found in sea bass viscera extracts (PLE) and unhydrolyzed cod viscera proteins [10,24]. Regarding skin, there were several bands (10–108 kDa) in control extracts, whereas few (10–62 kDa) were in PLE extracts. These protein molecular weight ranges, especially for control extracts, are in agreement with those found in PLE extracts from sea bass skin [10].

According to the gel image study, extraction of proteins from sea bream skin by PLE resulted in more protein fragments of molecular weight from 15 to 50 kDa compared to conventional extraction. The tailfin extracts showed the same behaviour, which might be due to for the main tissues components and the temperatures applied (55 °C for skin and 60 °C for tailfin). More protein fragments were obtained in PLE extracts compared to controls, highlighting the increase of protein recovery reached by PLE. The SDS-PAGE profiles revealed that PLE-assisted extraction influenced on protein molecular weight distribution of the obtained extracts.

2.3. *Total Antioxidant Capacity*

The results of total antioxidant capacity determined by Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) assays in control and PLE extracts of sea bream side stream extracts are shown in **Figure 3**. Total antioxidant capacity (TEAC and ORAC) was higher in PLE

extracts than control extracts for all samples. TEAC values in PLE extracts were 1739±111, 1184±16, 1030±54, 780±30, and 644±9 μM Trolox Eq for muscle, head, viscera, skin, and tailfins, respectively, while values in the corresponding control extracts were 1345±44, 649±27, 449±9, 410±30, and 268±17 μM Trolox Eq. Similarly, ORAC values (μM Trolox Eq) in PLE extracts were 4445±331 (muscle), 3758±269 (heads), 3069±407 (viscera), 3247±97 (skin), and 1769±382 (tailfins) whereas control ORAC values were 3284±230, 1761±246, 1601±236, 506±66, and 637±77, respectively. It is known that a shorter protein chain results in a greater antioxidant capacity. Therefore, the antioxidant capacity of the sea bream extracts could be related to the protein profile previously analyzed by SDS-PAGE, since the PLE extracts contained more protein fragments of lower molecular weight than the control extracts. The highest antiradical activity was found in muscle followed by head, viscera, skin, and tailfin extracts for both antioxidant methods. According to these results, PLE conditions allowed increasing the antioxidant capacity according to both TEAC (1.3–2.4 fold) and ORAC (1.9–6.4) of sea bream PLE extracts compared to controls. In general, a similar behavior of antiradical activities was obtained from TEAC and ORAC in sea bass side streams extracts [10]. For instance, the highest values were found in muscle, while the lowest were found in tailfins for both fish species. It should be noted that the values obtained (TEAC and ORAC) in PLE extracts of gilthead sea bream were higher than those reported in PLE extracts of sea bass for side streams despite the application of the same extraction conditions.

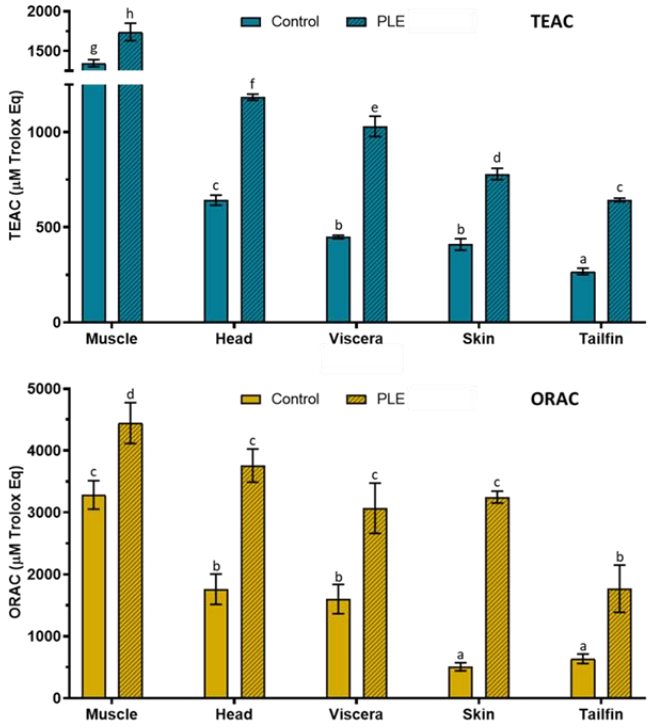

Figure 3. Total antioxidant capacity according to Trolox equivalent antioxidant capacity (TEAC) and oxygen radical absorbance capacity (ORAC) in control and PLE extracts from muscle, head, viscera, skin, and tailfin of gilthead sea bream. Results are expressed as mean ± standard deviation (n=3 for TEAC and n=6 for ORAC). Different lowercase letters in the bars indicate statistically significant differences ($P<0.05$) among samples.

In addition to protein chain size, the presence of some polar amino acids could also contribute to antioxidant capacity [25]. In this sense, tyrosine, histidine, and lysine were recently determined in the muscle, gills, viscera, and skin of gilthead sea bream [4]. Therefore, this study revealed a matrix effect in PLE-assisted extraction as greater protein recovery and antioxidant capacity were obtained for sea bream side streams compared to sea bass. However, for SDS-PAGE profiles, the electrophoretic pattern followed was quite similar.

2.4. Determination of Heavy Metals and Mycotoxins in Side Streams of Gilthead Sea Bream

Heavy-metal concentrations, including As, Hg, Cd, and Pb in muscle, head, viscera, skin, and tailfin of gilthead sea bream are shown in **Table 1**. Mean concentration ranges, expressed as _g/g of wet weight (ww), were 0.4406–2.5865, 0.0422–0.0886, 0.0008–0.0683, and 0.0054–0.0614 for As, Hg, Cd, and Pb, respectively. For muscle and head samples, the most abundant element was As followed by Hg, Pb, and Cd. As for skin and tailfin side streams, the most abundant element was also As, followed by Pb, Hg, and Cd. Regarding viscera, the decreasing order of toxic metals was As>Cd>Hg>Pb. These results are comparable to those previously published about side streams of farmed gilthead sea bream. For instance, Kalantzi et al. [9] reported concentration ranges for As, Hg, Cd, and Pb in muscle, gills, liver, and intestines. Arsenic content was higher in muscle (0.98–2.99 μg/g) and gills (0.93–1.21 μg/g), while it was equivalent in viscera (1.77–2.64 μg/g). The authors found lower Hg levels in gills (0.001 μg/g), as well as similar levels in muscle (0.02–0.10 μg/g) and viscera (0.03–0.05 μg/g). With regard to Cd, the values ranged from 0.12 to 0.26 μg/g for the viscera sample, being higher than those shown in this study. As for Pb concentration, the data for both gills (0.03–0.04 μg/g) and viscera (0.05 μg/g) were also higher. In the same way, the boxplots of toxic metals of muscle, head, gills, viscera, and skin described by Kandyliari et al. [6] showed higher concentrations of As, Cd, and Pb in muscle tissue. They also observed higher levels of As in skin, as well as Cd in viscera and skin. In contrast, the content of As and Pb in viscera was lower, while the results of the three elements examined were similar for head samples. It should be noted that for the purpose of result comparison, data from liver and intestine samples were added and considered equivalent to our viscera

side stream. Similarly, the values of gills were equated to those of whole head sample. On the other hand, the concentration of As, Hg, Cd, and Pb in the same types of side streams (including tailfins) from farmed sea bass was recently published [10]. The results of As content were lower in sea bass (0.346–1.867 μg/g) for all fish samples.

Table 1. Concentration of heavy metals in gilthead sea bream side streams.

Sea Bass Side Streams	Heavy metals (μg/g of wet weight)			
	As	**Hg**	**Cd**	**Pb**
Muscle	0.9381 ± 0.0110	0.0886 ± 0.0014	0.0008 ± 0.0001	0.0054 ± 0.0010
Head	0.8589 ± 0.0370	0.0593 ± 0.0001	0.0019 ± 0.0001	0.0190 ± 0.0002
Viscera	2.5865 ± 0.0233	0.0466 ± 0.0007	0.0683 ± 0.0007	0.0345 ± 0.0046
Skin	0.9694 ± 0.0966	0.0261 ± 0.0030	0.0101 ± 0.0003	0.0614 ± 0.0006
Tailfin	0.4406 ± 0.0055	0.0422 ± 0.0009	0.0079 ± 0.0004	0.0467 ± 0.0007
[Legislation*]	< 13,5	< 0,50	< 0,05	< 0,30

*values referred to fish muscle tissue [6,9,20]

However, differences in the levels of Hg, Cd, and Pb were observed depending on each side stream. Until now, the determination of toxic metals in fish has been carried out mainly in muscle tissue due to the evaluation of risk to human health. In this sense, the content of Hg, Cd, and Pb in muscle of farmed and wild gilthead sea bream was evaluated [27]. Similar results were reported for Cd (<0.001–0.003 μg/g) and Pb (0.010–0.101 μg/g), while lower values were observed for Hg (0.002–0.047 μg/g). The authors concluded that different factors such as location, species, and seasonality influence the accumulation of Hg, Cd, and Pb. Since the limits for heavy metals in fish side streams are not currently regulated, their assessment could be carried out according to those established for edible muscle of fish (13.5 μg/g for As, 0.5 μg/g for Hg, 0.05 μg/g for Cd, and 0.30 μg/g for Pb) [6,9,26]. Considering the heavy-metal

concentrations obtained, all side streams of gilthead sea bream (except for viscera) could be considered as safe raw materials to be used for the food industry according to the circular economy strategy, in terms of As, Hg, Cd, and Pb content.

The possible occurrence of mycotoxins in muscle, head, viscera, skin, and tailfin of gilthead sea bream was also investigated. After applying a nontargeted screening approach against a spectral library containing of 223 mycotoxins and related metabolites, no mycotoxins were identified in any of the sea bream side streams studied. As far as we know, there is no information in the literature on the presence of mycotoxins in sea bream discards. Regarding the edible part of farmed sea bream, different results on mycotoxins content have been reported. There was no transfer of mycotoxins from contaminated plant-based feed to sea bream tissue [8], while emerging Fusarium mycotoxins such as enniantin A1, B, and B1 were identified in the muscle of some farmed fishes such as sea bream [28].

3. Materials and Methods

3.1. Reagents

ABTS (2,2'-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), DTT (DL-Dithiothreitol), diatomaceous earth (Hyflo® Super Cel®), Trizma® base, fluorescein sodium salt, and formic acid (reagent grade ≥ 95%) were purchased from Sigma-Aldrich (Steinheim, Germany). AAPH (2,2'-azobis (2-amidinopropane)) (Acros Organics), TRIS (ultrapure), potassium dihydrogen phosphate, potassium sulphate, sodium phosphate dibasic, sodium chloride, ortho-boric acid, glycine (proteomics grade), and methanol (HPLC LC-MS grade) were provided by VWR International Eurolab S.L. (Barcelona, Spain).

Glacial acetic acid, sulphuric acid, and sodium hydroxide were supplied by Fisher Scientific (Madrid, Spain). SDS (sodium dodecyl sulfate, purissimum-CODEX) and nitric acid (65% p/p) were obtained from Panreac (Barcelona, Spain). Acetonitrile (HPLC grade), acetone, glycerol, and bromophenol blue indicator (ACS reagent) were supplied by Merck (Darmstadt, Germany). Octadecyl C18 sorbent was from Phenomenex (Madrid, Spain) while absolute ethanol was from J.T. Baker (Deventer, The Netherlands). Anhydrous magnesium sulfate (99.5% min powder) was provided by Alfa Aesar (Karlsruhe, Germany). Deionized water (resistivity >18 MΩ cm-1) was obtained through a Milli-Q SP® Reagent Water System (Millipore Corporation Bedford, USA).

3.2. Raw Material and Sample Preparation

Whole gilthead sea bream fishes (*Sparus aurata*) were obtained in a local market in Valencia (Spain) during different days of April 2019. Then, they were immediately transported to the University of Valencia under refrigerated conditions. According to the commercial label, they were farmed in Greece. Individual gilthead sea bream was dissected as a simulation of fish processing for human consumption. Then, muscle remains, complete heads, viscera, flesh-free skin, and tailfins were placed separately inside aluminium containers. Next, they were frozen (-80 °C) for 48 h and freeze-dried (LABCONCO, 2.5. FREE ZONE, USA) for 72 h. Dried samples were maintained in a desiccator until constant weight and their water content was determined. The moisture percentages were 68.77±0.96%, 56.45±0.91%, 32.09±2.37%, 33.99±0.89%, and 37.86±3.85% for muscle, heads, viscera, skin, and tailfins, respectively. Similar values for gilthead sea bream muscle and head as well as higher values for viscera and skin were reported by Pateiro et al. [4] and Kandyliari et al. [5]. Next, the same types of side stream were ground in an analytical mill (A11 basic IKA®

WERKE, Staufen, Germany) and pooled together. The homogenized samples were storage at -25 °C until the extraction process and the analysis of possible contaminants. **Figure 4** shows the different side streams obtained from gilthead sea bream after sample preparation.

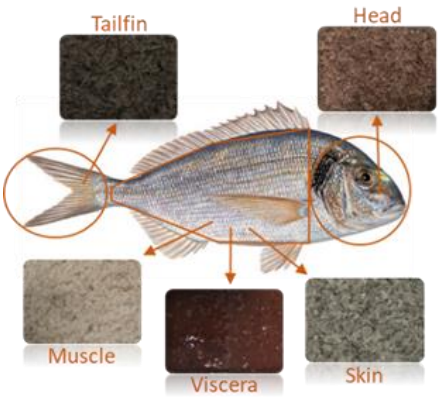

Figure 4. Gilthead sea bream side streams to perform the analysis.

3.3. Pressurized Liquid Extraction (PLE) Process

Extracts were obtained using an accelerated solvent extractor ASE 200 Dionex (Sunnyvale, CA, USA) equipped with a solvent controller. The amount of sample depended on the type of side stream. Thus, 2.5 g for muscle and head, 2 g for skin and tails, and 1.5 for viscera of gilthead sea bream were used. Then, they were mixed with diatomaceous earth and introduced into 22 mL stainless-steel cells with a glass fiber filter placed in the end part. Distilled water was used as extraction solvent, and a static extraction cycle was applied. The standard operations conditions consisted of the preheating period (1 min), heating period (5 min), flush volume (60%), and nitrogen purge (60 s). Nitrogen (145 psi) was applied to assist the pneumatic system and to purge the cells, while extractions

were performed under a pressure of 1500 psi. The extraction conditions were selected according to the recently reported optimal pH–temperature–time combinations for PLE-assisted extraction to obtain antioxidant protein extracts from sea bass side streams [10]: pH 7, 20 °C, 5 min for muscle, pH 4, 60 °C, 15 min for heads, pH 7, 50 °C, 15 min for viscera, pH 7, 55 °C, 5 min for skin, and pH 7, 60 °C, 15 min for tailfins. Extracts obtained by conventional stirring using distilled water without adjusting pH or temperature and for a longer extraction time than that applied by PLE extraction were considered as controls. Then, protein recovery, protein molecular weight distribution, and total antioxidant capacity were analyzed and compared to PLE extracts. Both extraction processes (PLE and stirring) were performed in duplicate. Individual extracts were homogenized, divided into several tubes, and stored at -25 °C for subsequent analyses.

3.4. Determination of Protein Recovery

The total nitrogen content in side streams of gilthead sea bream, as well as in control and PLE extracts, was determined using the Kjeldahl method [29]. Approximately 0.2 g of lyophilized fish raw material or 2 mL of fish extract was used to carry out the acid digestion prior to distillation and titration. Then, total protein content was obtained by applying the protein nitrogen conversation factor (6.25) for fish and fish side streams. In order to calculate the percentage of protein recovery, the following formula was used: (protein in extract/protein in side stream) x 100.

3.5. Molecular Weight Distribution of Protein Fragments

The molecular weight distribution of protein in both control (stirring) and optimal (PLE) extracts from side streams of gilthead sea bream were investigated

by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Since protein concentration in fish extracts was different between samples, 100 μL for muscle extracts and 500 μL for head, viscera, skin, and tailfin extracts were used. For protein precipitation, cold acetone was added to fish extracts (4:1 v/v ratio) and they were mixed and centrifuged (11,000 rpm, 4 °C, 10 min) (Eppendorf 580 R, Thermo Fisher Scientific, Hamburg, Germany). Then, the supernatant was removed, and the pellet was dissolved in distilled water assisted by ultrasound (10 min). Next, equal volumes of SDS-PAGE sample buffer solution (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 0.01% bromophenol blue, and 50 mM dithiothreitol) and protein solution were mixed and heated at 95 °C for 5 min. Afterward, 10 μL of mixture was loaded on 8-16% Mini-PROTEAN® TGX™ Precast gels (Bio-Rad) and subjected to electrophoresis using a Mini-PROTEAN® tetra cell (Bio-Rad). The running buffer consisted of Trizma® base (25 mM), glycine (192 mM), and SDS (0.1%). The distribution of protein fragments was performed at a constant voltage (80 V) for 120 min. After electrophoresis, gels were stained in 0.125% Coomassie brilliant blue R-250 and destained in 20% methanol and 10% acetic acid until the background was as clear as possible. A standard molecular weight of protein bands from 5 to 250 kDa (Precision Plus Protein™, Bio-Rad) was used to estimate the molecular weight of protein bands. The images of the electrophoretic gels were studied using the ImageJ® software, a public domain digital image processing program developed at the National Institutes of Health (NIH). Background subtraction and 8 bit format were selected for image analysis in order to improve band intensities and identify differences across protein fragments between control and PLE extracts.

3.6. Evaluation of Total Antioxidant Capacity

3.6.1. Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The TEAC assay is based on the capacity to reduce the radical cation $ABTS^+$ by antioxidant compounds compared to a reference antioxidant standard (Trolox). The spectrophotometric method described by Barba at al. [24] was applied. The $ABTS^+$ stock solution was generated by chemical reaction with ABTS (7 mM) and $K_2S_2O_8$ (140 mM) overnight in darkness at room temperature. Then, it was diluted in ethanol until an absorbance of 0.700 ± 0.020 at 734 nm and 30 °C to obtain the $ABTS^+$ working solution. An adequate dilution of the extracts to obtain a percentage of absorbance inhibition of approximately 50% was required. Trolox standard solutions were prepared from 0 to 300 μM. The absorbance of 2 mL of $ABTS^+$ working solution was considered the initial point of reaction (A_0). Immediately, 100 μL of diluted extracts or Trolox standards were added and the absorbance after 3 minutes was considered the final point of reaction (A_f). All measures were carried out in a thermostatized UV–vis spectrophotometer. The percentages of absorbance inhibition were calculated from the following equation: $1 - (A_f / A_0) \times 100$ and were compared to Trolox standard curve. The results were expressed as μM Trolox Equivalents.

2.6.2. Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC assay is based on the capacity of the antioxidant compounds to scavenge peroxyl radicals. The fluorimetric method proposed by Barba et al. [24] was used. Sodium fluorescein (0.015 mg/mL), AAPH radical solution (120 mg/mL), and Trolox standard solution (100 μM) were prepared with phosphate buffer (75 mM, pH 7). Properly diluted extracts were required. The final reaction consisted of 50 μL of diluted extract, Trolox standard or phosphate buffer (blank), 50 μL of fluorescein, and 25 μL of AAPH. The reaction was performed

at 37 °C in a Multilabel Plate Counter VICTOR3 1420 (PerkinElmer, Turku, Finland) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence was recorded every 5 min over 60 min (until the fluorescence in the assay was less than 5% of the initial value). The results were calculated considering the differences of areas under the fluorescence decay curve (AUC) between the blank and the sample over time. The results were expressed as μM Trolox Equivalents.

3.7. Analysis of heavy metals in gilthead sea bream side streams

The presence of As, Hg, Cd, and Pb in freeze-dried muscle, head, viscera, skin, and tailfin of gilthead sea bream was evaluated. Microwave oven-assisted digestion (MARS, CEM, Vertex, Spain) was used for the acid mineralization of fish samples. Approximately 0.30 g of side stream was placed in a Teflon reactor vessel and 1 mL of H_2O_2 (30% v/v) and 4 mL of HNO_3 (14M) were added. The digestion was carried out by microwave irradiation at a power setting of 800 W, 180 °C and 15 min. The digested samples were left to cool at room temperature and eliminate the nitrogenous vapour. Then, they were filtered through Whatman No. 1 filter paper and made up to volume with distilled water.

The identification and quantification of As, Hg, Cd, and Pb was conducted by an inductively coupled plasma spectrometer mass detector (ICP-MS, Agilent model 7900). The analytical conditions were as follows: carrier gas (1.07 L/min), Ar gas flow (15.0 L/min), reaction gas (He), RF power (1550 W), nebulizer pump speed (0.10 rps), and RF matching (1.80 V). Internal standard solutions of ^{72}Ge, ^{103}Rh, and ^{193}Ir (ISC Science) at 20 μg/g were used in order to correct matrix induced signal fluctuations and instrumental drift. Standard calibration curves from 0 to 1000 μg/L was used for the quantification of As, Cd, and Pb while a standard calibration curve from 0 to 100 μg/L was used for Hg. Limits

of detection (LOD) were calculated according to the following equation: LOD = 3sB/a where, 3sB is 3 times the standard deviation at zero concentration and a is the slope of the calibration curve. LOD values were 0.0015 μg/L for Hg and Pb, 0.012 μg/L for As, and 0.004 μg/L for Cd. Distilled water was used as a blank and the concentrations of heavy metals in the digested blank were subtracted from the values of fish samples. The results were expressed as μg of element/g of side stream in wet weight. In addition, the Certified Reference Material for Trace Metals DORM-3 (fish protein powder) was used to confirm the accuracy of the method. It was prepared and analysed simultaneously to sea bream side streams. The recovery percentages were 98%, 86%, 76%, and 77% for As, Hg, Cd, and Pb, respectively.

3.8. *Analysis of mycotoxins in gilthead sea bream side streams*

High Performance Liquid Chromatography coupled with Electrospray Ionization-Quadrupole-Time of flight-mass spectrometry (LC-ESI-qTOF-MS) was used to analyse the mycotoxins content in freeze-dried side streams of gilthead sea bream. The chromatographic separations were achieved on an Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Gemini® column NX-C18 (3 μM, 150×2 mm ID) (Phenomenex) and a vacuum degasser, binary pump, and autosampler. The mobile phases consisted of acidified (0.1% of formic acid) water (A) and acetonitrile (B). The gradient program was 50% B (0 - 6 min); 100% B (7 - 12 min); 50% B (13 - 20 min). The injection volume was 5 μL and the flow rate 0.2 mL/min. Mass spectrometry (MS) analysis was performed using a 6540 Agilent Ultra-High-Definition-Accurate-Mass-q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in positive and negative ionization modes. The analytical conditions were as follows: drying gas

temperature (370 °C); nitrogen drying gas flow (12.0 L/min); nebulizer pressure (50 psi); fragmenter voltage (160 V); capillary voltage (3500 V); and scan range (m/z 50 - 1500). Automatic MS/MS experiments were carried out under the following collision energy values: m/z 100, 30 eV; m/z 500, 35 eV; m/z 1000, 40 eV; and m/z 1500, 45 eV. Mass Hunter Workstation software was used for data acquisition and integration.

The QuEChERS procedure described by Pallarés et al. [25] with some modifications was applied for the extraction of mycotoxins from freeze-dried side streams of gilthead sea bream. Approximately 3 g of sample were mixed with 30 mL of acidified water (2% formic acid) in an orbital shaker (IKA KS 260) for 30 min. Next, 10 mL of acetonitrile were added and an additional 30 min stirring was performed. Then, 8 g of $MgSO_4$ and 2 g of NaCl were added to the mixture and vortexed for 30 s before centrifugation at 4000 rpm for 10 min. Afterward, 2 mL of supernatant were transferred into a 15 mL tube containing 0.1 g of Octadecyl C18 sorbent and 0.3 g of $MgSO_4$. The mixture was shaken and centrifuged under the same previous conditions and the supernatant was filtered (13 mm/0.22 μm nylon filter). Finally, 20 μL were injected into the LC-ESI-qTOF-MS system.

3.9. Statistical Analysis

Experimental data were subjected to one-way analysis of variance (ANOVA) to determine the significant differences among samples. Tukey HSD (Honestly Significant Difference) multiple range test, at a significance level of $P<0.05$ was applied. Statistical analyses were performed with the software Statgraphics Centurion XVI.I.

4. Conclusions

PLE was successfully applied for the first time in this present study to obtain protein extracts with antioxidant activity from gilthead sea bream processing side streams in a sustainable way. The highest protein recovery percentage (78%) was found in viscera, while the highest antioxidant capacity was observed in muscle left over. The SDS-PAGE profiles showed differences in protein molecular weight distribution among samples. Both the levels of As, Hg, Cd, and Pb and the absence of mycotoxins in muscle, heads, viscera, skin, and tailfins of gilthead sea bream add to the limited data in the literature about these contaminants in farmed fish. One of the H2020 challenges is the recovery of proteins from natural underexploited resources in a sustainable way, and fish processing side streams may be considered great candidates for this purpose. Further research is required for both the application of sustainable technology and the utilization of fish side stream materials as a source of nutritional and bioactive compounds for the development of commercial food and feed products.

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3.3. Salmon (*Salmo salar*) side streams as a bioresource to obtain potential antioxidant peptides after applying pressurized liquid extraction (PLE)

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Salmon (S*almo salar*) Side Streams as a Bioresource to Obtain Potential Antioxidant Peptides after Applying Pressurized Liquid Extraction (PLE)

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Abstract
The pressurized liquid extraction (PLE) technique was used to obtain protein extracts with antioxidant capacity from salmon muscle remains, heads, viscera, skin, and tailfins. A protein recovery percentage ≈28% was obtained for all samples except for viscera, which was ≈92%. These values represented an increase of 1.5–4.8-fold compared to stirring extraction (control). Different SDS-PAGE profiles in control and PLE extracts revealed that extraction conditions affected the protein molecular weight distribution of the obtained extracts. Both TEAC (Trolox equivalent antioxidant capacity) and ORAC (oxygen radical antioxidant capacity) assays showed an outstanding antioxidant activity for viscera PLE extract. Through liquid chromatography coupled with electrospray ionization triple time-of-flight (nanoESI qQTOF) mass spectrometry, 137 and 67 peptides were identified in control and PLE extracts from salmon viscera, respectively None of these peptides was found among the antioxidant peptides inputted in the BIOPEP-UMP database. However, bioinformatics analysis showed several antioxidant small peptides encrypted in amino acid sequences of viscera extracts, especially GPP (glycine-proline proline) and GAA (glycine-alanine-alanine) for PLE extracts. Further research on the relationship between antioxidant activity and specific peptides from salmon viscera PLE extracts is required. In addition, the salmon side streams studied presented non-toxic levels of As, Hg, Cd, and Pb, as well as the absence of mycotoxins or related metabolites. Overall, these results confirm the feasible use of farmed salmon processing side streams as alternative sources of protein and bioactive compounds for human consumption.

Keywords: pressurized liquid extraction; salmon; side streams; peptides; protein; SDS-PAGE; antioxidant capacity; mycotoxins; heavy metals

1. Introduction

Salmon consumption has tripled since the 1980s, mainly because it is considered a healthy food due to its contents of polyunsaturated fatty acids, quality proteins, vitamins, and minerals [1,2]. The versatility of commercialized salmon products (i.e., fresh, frozen, smoked, fillet, canned, sushi, ready meals) is also related to a wide distribution, as well as an increased interest aroused by consumers and food industry [1,2]. At the same time, the salmon aquaculture sector has grown worldwide. In Europe, Atlantic salmon (*Salmo salar*) is currently the most important farmed species in volume and value, exceeding 1.3 million tons and 5 billion EUR in 2017 [3]. Since salmon has a great fillet yield, it is one of the most highly processed fishes [4]. As a result, 50% of complete fresh salmon has been estimated to correspond to side stream materials [5]. Therefore, a large amount of discards is available to develop high-added-value products, including those intended for human consumption. In this context, the nutritional characterization of several salmon processing side streams revealed that they are rich in protein (10–20%) and fat (20–30%) [5,6], which make them candidate substrates for protein and oil recovery. Salmon side streams also showed relevant levels of essential amino acids (21–35%) as well as oleic acid (39–42%) and omega-3 fatty acids (19–21%) [5,6]. In addition, peptides with functional and bioactive properties are also found in several marine side streams [7–9]. For instance, peptides from salmon trimmings and pectoral fins have exhibited antihypertensive and antioxidant activities [4,10]. Antioxidant peptides from the viscera of sardinella, black pomfret, and mackerel have also been reported [9]. Therefore, salmon side stream materials could be considered a promising source of valuable compounds from the European circular economy point of view [11].

The valorization of seafood discards has been gaining attention over the last years, as their nutritional and bioactive compounds can now be extracted more efficiently using green technologies [12,13]. Pressurized liquid extraction (PLE) is currently considered an environmentally friendly technique to recover bioactive compounds from food matrices, as water is the most preferred solvent for the extraction process [14]. PLE is based on the use of high pressure and temperature to improve the extraction performance [15,16]. The possibility of applying different extraction conditions has made PLE a useful tool to optimize the extraction of high-added-value compounds from a wide variety of matrices, including marine sources and related side streams. For instance, PLE-assisted extraction was recently used to obtain aqueous protein extracts with in vitro antioxidant capacity from several side streams of rainbow trout, sole, sea bass, and sea bream [17–19]. Protein extraction from macro- and micro-algae using PLE has been also investigated [20].

In addition to healthy nutritional properties, any starting material that can be used in the food industry must be free of potentially harmful substances. In this sense, farmed fishes can be exposed to mycotoxins from plant-based feed [21,22], as well as toxic metals from the aquaculture environment [23]. A wide range of ingredients is used in the formulation of Atlantic salmon feed [24]. Because an important protein fraction comes from soy, corn, canola, and pea meals, the occurrence of mycotoxins in fish tissues must be evaluated. In a similar way, heavy metals have been found in several side streams of different fish species [18,19,25]. Therefore, assessing the levels of toxic elements in all fish tissues is advisable.

The main objective of the present study was to apply, for the first time, PLE-assisted extraction as a sustainable technique to obtain antioxidant protein extracts from salmon processing side streams. Muscle remains, heads, viscera, skin, and tailfins of farmed salmon were selected in order to give added value to these underutilized raw materials. Protein recovery, SDS-PAGE profile, and antioxidant capacity were evaluated in extracts obtained from salmon discards. Peptide identification and bioinformatics analysis in terms of potential antioxidant activity were performed for salmon viscera extracts. In order to provide additional data on possible contaminants in farmed fish, the levels of As, Hg, Cd, and Pb, as well as the occurrence of mycotoxins, were also investigated. Overall, this study contributes to the current marine resources valorization approach, focusing on the possibilities of processing side streams from farmed salmon.

2. Results and Discussion

2.1. *Total Antioxidant Capacity*

The results of total antioxidant capacity, determined using the Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC) methods in control and PLE extracts of salmon side streams, are shown in Figure **1**. TEAC values in PLE extracts were 734 ± 38, 472 ± 7, 3739 ± 209, 147 ± 37, and 704 ± 42 μM Trolox Equivalents (Eq) for muscle, head, viscera, skin, and tailfins, respectively, whereas TEAC values in the corresponding control extracts were 776 ± 32, 322 ± 18, 778 ± 26, 206 ± 12, and 324 ± 22 μM Trolox Eq. Regarding the ORAC assay, the values of total antioxidant capacity were higher in PLE extracts than in control extracts for all samples. ORAC values (μM Trolox Eq) in PLE extracts were 4586 ± 241 (muscle), 3567 ± 63

(heads), 7772 ± 1174 (viscera), 1244 ± 94 (skin), and 2620 ± 78 (tailfins), whereas control ORAC values were 3005 ± 217, 797 ± 73, 2451 ± 139, 599 ± 19, and 736 ± 39, respectively. Therefore, PLE-assisted extraction improved the antioxidant capacity (ORAC) compared to conventional extraction for all salmon side streams. The increases were 1.5-, 4.5-, 3.2-, 2-, and 3.6-fold for muscle, head, viscera, skin, and tailfins, respectively.

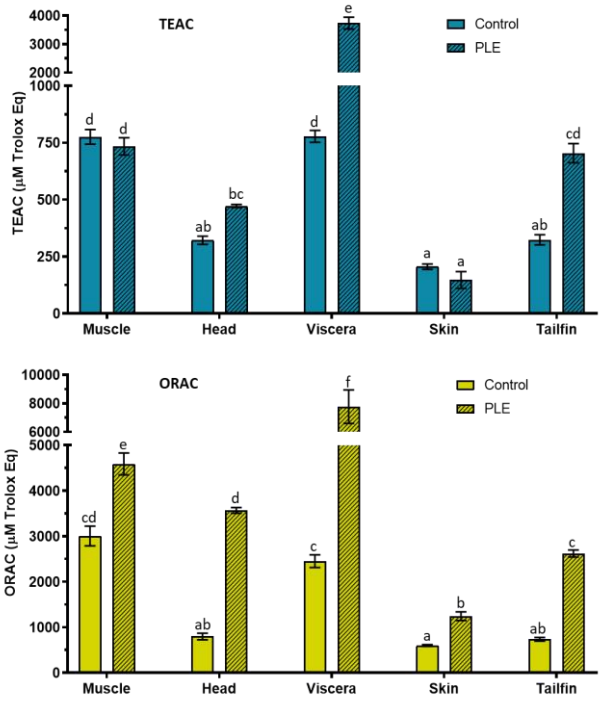

Figure 1. Total antioxidant capacity determined by TEAC and ORAC in control and PLE extracts from salmon muscle, head, viscera, skin, and tailfin. TEAC: trolox equivalent antioxidant capacity. ORAC: oxygen radical absorbance capacity. PLE: pressurized liquid extraction. µM Trolox Eq (micromolar Trolox equivalent). Results of TEAC (n = 3) and ORAC (n =6) are expressed as mean ± standard deviation. Different lowercase letters in the bars indicate statistically significant differences ($p < 0.05$) among samples.

As for TEAC, the antioxidant capacity of PLE extracts also increased compared to the controls for head (1.5), viscera (4.8), and tails (2.2), whereas the muscle and skin values remained without significant changes. The highest antiradical activity was observed in PLE extracts of viscera for both antioxidant assays. These results are slightly different to those obtained for PLE extracts of sea bass and sea bream by-products, in which muscle PLE extracts showed the highest values of antioxidant capacity determined by both TEAC and ORAC methods [18,19]. The antioxidant capacity of viscera PLE extracts from sea bass and sea bream were similar to those of head PLE extracts. These differences may be due to the fact that seabass and sea bream are a more closely related species compared to salmon.

On the other hand, the different antioxidant capacity exhibited by the protein extracts obtained is probably related to both the size and the amino acid composition of the protein fragments of each salmon side stream. Several authors have suggested that hydrophobic this way, glycine and glutamic acid have been reported as the most abundant polar amino acids in salmon heads, skin, and viscera [5]. Hydrophobic amino acids such as alanine, proline, leucine, and valine were also found in relevant quantities. In addition, the molecular weight of fish peptides (0.5–1.5 kDa) has been associated with antioxidant properties [7,26]. According to this, the outstanding antioxidant capacity shown by PLE viscera extracts could mean the presence of bioactive peptides with some of the aforementioned amino acids in their sequence.

2.2. Protein Recovery Percentage

The results of protein recovery in control and PLE extracts from side streams of gilthead sea bream are shown in **Figure 2**. The percentage of protein recovery

in PLE extracts of salmon muscle, head, viscera, skin, and tailfins were 26.65±1.57, 27.50±3.83, 92.03±4.80, 29.39±0.05, and 28.29±3.66, respectively, while their corresponding control extracts were 23.51±0.31, 18.57±1.14, 56.76±1.87, 18.41±0.64, and 5.82±0.63. Therefore, PLE improved the protein recovery for all side streams. The improvement in protein recovery was close to 1.5-fold for heads, viscera, and skin extracts. The tailfin extracts experienced a 5-fold increase with the PLE technique, whereas salmon muscle results were similar for both conventional stirring and PLE extraction. The best protein recovery was observed in viscera, consistent with previously observed protein recoveries in extracts of sea bass and sea bream side streams after applying PLE assisted extraction [18,19]. Few food matrices or related side streams have been used for protein extraction by means of PLE. For instance, different seaweeds, as well as seeds from red pepper, showed protein recovery percentages about 5% and 50%, respectively [20,27].

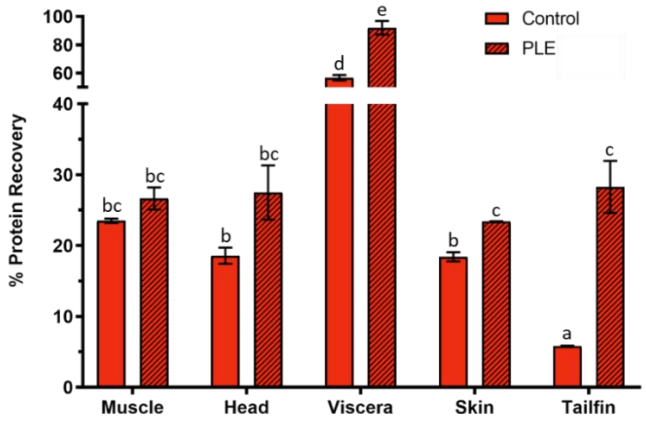

Figure 2. Percentage of protein recovery in control and PLE extracts from salmon muscle, heads, viscera, skin, and tailfin. PLE: pressurized liquid extraction. Results are expressed as mean ± standard deviation (n = 2). Different lowercase letters in bars indicate statistically significant differences ($p < 0.05$) among samples.

2.3. *Protein Molecular Weight Distribution*

The protein molecular weight distribution of salmon side stream extracts, obtained both through conventional stirring and PLE-assisted extraction, was provided by means of SDS-PAGE (Figure 3A). As can be seen in the images, the extracts presented different electrophoretic profiles. In general, these differences appeared to be related to both the type of side stream and the type of extraction process. In order to obtain the molecular weight of each band and also to group the areas of the bands by kDa ranges, the images of the gels were analyzed using ImageJ and GraphPad Prism Programs (Figure 3B). For muscle leftovers, clear bands from 9 to 108 kDa were observed in control and PLE extracts, which could be due to the fact that both extraction process were carried out at room temperature. However, the differences in the width of the bands revealed that PLE extracts presented a greater amount of total protein fragments for all molecular weight groups. This behavior is in agreement to those previously reported for sea bass and sea bream muscle remains subjected to the same PLE and shaking extraction conditions [18,19]. Protein fragments of head control extracts showed several bands from 10 to 108 kDa, whereas the highest protein molecular weight for head PLE extracts was of 96 kDa. In addition, bands of 20–50 kDa in head control extracts were not found in head PLE extracts. In contrast, control and PLE extracts from salmon viscera exhibited the same protein molecular weight distribution (≤7–73 kDa) and few slight bands. The range of values was similar to that shown by sea bass and sea bream viscera extracts (8–61 kDa) [18,19].

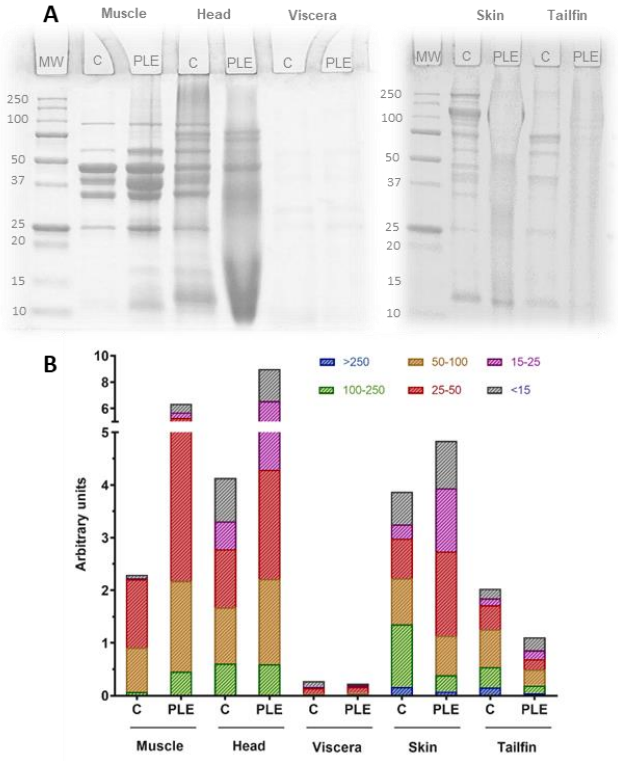

Figure 3. Protein molecular weight distribution of control and PLE extracts from salmon side streams. SDS-PAGE protein profiles (**A**) and molecular weight ranges for band areas (**B**). MW: molecular weight standard. C: control extract. PLE: extract obtained by means of pressurized liquid extraction.

Both skin and tailfin extracts presented wider molecular weight ranges (≈6–140 kDa) than muscle, heads, and viscera extracts. Furthermore, for both samples, several protein bands in control extracts did not appear in PLE extracts. According to the gel image analysis, bands in 25–50 and 75–125 kDa ranges from control skin extracts were not present in the corresponding PLE extracts. Similarly, the 10–30 kDa protein fragments in tailfin control extracts were not found in those of PLE. The protein molecular weight distribution of discards

from Australian Atlantic salmon was evaluated previously [5]. The head and skin protein fragments were in the range of 25–250, whereas most of the viscera were below 10 kDa. Based on these results, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) revealed different protein profiles between the matrices studied. In addition, differences observed among control and PLE extracts for each side stream have shown that PLE-assisted extraction influenced the size of protein fragments obtained in the extracts. It should be noted that this electrophoretic technique provides additional information as to the total protein content. However, it does not allow the retention of peptides in the gel, which could be relevant to correlate the presence of peptides with the antioxidant capacity shown by the extracts.

2.4. *Identification of Peptides in Viscera Extracts*

As previously described, the salmon viscera extracts obtained through PLE-assisted extraction resulted the most interesting sample in terms of in vitro antioxidant capacity. Their TEAC and ORAC values not only stand out against the other salmon by-products studied here, but also in comparison with previously investigated PLE protein extracts from sea bass and sea bream viscera. For this reason, PLE protein extracts from salmon viscera were selected for the identification of antioxidant peptides. Control viscera extracts were also screened in order to compare peptides extracted through PLE and under stirring conditions. Only peptides with a confidence percentage ≥ 90% have been reported.

A total of 137 peptides were identified in the PLE viscera extracts (Table 1). In contrast, 67 peptides were identified in the viscera control extracts (Table 2).

Table 1. Peptides identified in salmon viscera extract obtained through pressurized liquid extraction.

Protein of origin of the identified peptide	Sequence	Obs MW	Obs m/z	Theor z
Collagen alpha-2(I) chain	GESGPTGNGGPVGA	1155.52	578.77	2
Collagen alpha-2(I) chain	GPAGPHGPPG	842.40	422.21	2
Collagen alpha-2(I) chain	SGETGSAGITGPAGPR	1413.68	707.85	2
Uncharacterized PE-PGRS family protein	GGNGGAGGAGGNGGAGGLGG	1370.62	686.32	2
Collagen alpha-3(V) chain	GIPGPLGPL	819.45	410.73	2
Collagen alpha-3(V) chain	GIPGPLGPLGP	973.52	487.77	2
Collagen alpha-3(V) chain	GPAGHPGPPG	842.40	422.21	2
Collagen alpha-1(I) chain	GETGPAGPAG	812.40	407.21	2
Collagen alpha-1(I) chain	GLPGSPGPAGEAGK	1193.60	597.81	2
Glycine-rich protein DOT1	GGGGGHGGGAGGGGGGPGG	1292.58	647.30	2
Collagen alpha-4(IV) chain	GPIGPLGPLGP	973.52	487.77	2
Probable heat shock protein ssa1	PGGAPGGMPGGAP	1021.47	511.74	2
WAG22 antigen	PAGTAAGGAGGAGGAPGL	1308.60	655.31	2
Collagen alpha-1(I) chain	GETGPAGPAG	812.40	407.21	2
Histone H2A	AQGGVLPNIQ	995.54	498.78	2
60 kDa heat shock protein, mitochondrial	VGGTSDVEVNEK	1232.58	617.30	2
Collagen alpha-1(XXII) chain	GYAKDGLPGIPGPQGET	1655.76	828.89	2
Filamin-A	VITPEEIVDPNVDEH	1704.81	569.28	3
Glycine-rich cell wall structural protein	GGGEGYGGGGANGGGY	1285.60	643.81	2
Fumarylacetoacetase	IGVAIGDQILDLSVIK	1652.97	827.49	2
Pulmonary surfactant-associated protein A	GPLGPPGGMPGH	1072.53	537.27	2
Collagen alpha-1(I) chain	GETGPAGPAG	812.40	407.21	2

Collagen alpha-4(IV) chain	GPPGLPGPPGPPGHKGF	1607.77	804.89	2
WAS/WASL-interacting protein family member	GGGGGGGGGGGGSGGNFGGGGPP	1586.64	794.33	2
Adenylate cyclase type 10	GRVNIQDLQKNKFLMRANT	2245.16	749.40	3
Forkhead box protein K1	QPPPGPPPPPP	1076.55	539.28	2
Exocyst complex component SEC5	ALMILIVVHSECFR	1629.91	815.96	2
tRNA dimethylallyltransferase	EAARDGWPAL	1084.53	543.27	2
Orotidine 5'-phosphate decarboxylase	RPAGAEAGDQK	1098.54	550.28	2
Homogentisate 1,2-dioxygenase	GPIGSNGLANPR	1152.52	577.27	2
Wolframin	NTAPLGPSCPQPPPAP	1508.68	755.35	2
Keratin, type II cytoskeletal I	TALGGAAGGMGGGGGMGGGM	1538.63	770.32	2
Integrin-linked-kinase-associated-serine/threonine phosphatase 2C	GLPPAGSGNSGSLATSGS	1515.64	758.83	2
Collagen alpha-4(IV) chain	ACAGMIGPPGPQGFP	1399.63	467.55	2
Collagen alpha-3(V) chain	GIPGPLGPLGP	973.52	487.77	2
Fatty acid-binding protein, liver	AIGLPDDLIQK	1181.67	591.84	2
Actin-related protein 3	VIDSGDGVTH	998.47	500.24	2
Collagen alpha-1(I) chain	GAPGPVGPAGKGETGPAGPAGPAG	1925.86	963.94	2
Chaperone protein DnaK	QAGEGGAGAGAGAAG	1100.52	551.27	2
Collagen alpha-2(V) chain	GNPGPLGPIGP	974.52	488.27	2
Collagen alpha-1(XVIII) chain	LPGPPGPPGPPGPRGYPG	1665.79	833.90	2
POTE ankyrin domain family member E	VMDSGDGVTH	1016.43	509.22	2
Uncharacterized protein SE_1560	GPLVLVDTDDL	1155.61	578.81	2
Serum albumin 1	AIQPDTEFTPPELDASS	1816.84	909.43	2
Phosphoenolpyruvate guanylyltransferase	SLAMLNDVLVAL	1257.73	629.87	2
Collagen alpha-1(I) chain	AGPPGADGQPGAK	1164.55	583.28	2

DNA (cytosine-5)-methyltransferase 3A	DPASPNVATTP	1068.50	535.26	2
Protein Shroom4	SQAPESHESRTGL	1397.61	699.81	2
Ataxin-2 homolog	PAGGGPQPAFTPP	1192.56	597.29	2
Magnesium-chelatase 38 kDa subunit	QSGENVVERDGL	1301.60	651.81	2
Uroporphyrinogen decarboxylase	DVAVQGNLDPL	1139.61	570.81	2
Collagen alpha-1(I) chain	AGAQGAPGPAGPA	1021.47	511.74	2
Collagen alpha-3(V) chain	GIPGPLGPLGP	973.53	487.77	2
Actin-related protein 3	DSGDGVTH	786.32	394.17	2
tRNA-N6-adenosine-threonylcarbamoyltransferase	LSLVVSGGHTELVL	1422.69	712.35	2
Calpain-12	AGTGAGGPQ	714.20	358.11	2
Collagen alpha-1(XVII) chain	QNLVGPPGPPGPPGVSGD	1623.77	812.89	2
Fumarylacetoacetase	IGVAIGDQILDLSVIK	1652.97	552.00	3
Probable aquaporin PIP2-6	DINAGGGACASVGLL	1316.67	659.34	2
60 kDa chaperonin	AAVEEGIVAGGGTAF	1347.58	674.80	2
Arginine kinase	KGDRFLEAAGVNKLWPE	1928.92	965.47	2
Collagen alpha-2(I) chain	GETGSAGITGPAGPR	1326.65	664.33	2
Cytoplasmic dynein 1 light intermediate chain 1	TGSPGGPGVSGGSPAGGAG	1425.64	713.83	2
Collagen alpha-2(I) chain	RGDGGPPGVTGFPGAA	1411.63	706.82	2
Collagen alpha-1(I) chain	AKGDTGAPGAPGSQGAP	1437.68	719.85	2
Zinc finger protein 831	ESEGEGGPGPGPGVAGAEP	1649.78	550.93	3
Collagen alpha-2(IV) chain	PGEKGDAGLPGLSGK	1363.64	682.83	2
Collagen alpha-2(I) chain	GPTGNGGPVGA	882.42	442.22	2
Translation initiation factor IF-2	GGGGGAPGRPGGGGGGGGAP	1405.65	703.83	2
Collagen alpha-2(I) chain	GPAGPHGPP	785.38	393.70	2

Serine/threonine-protein kinase ATG1	ESNMFVSEYL	1217.56	609.79	2
ATP-dependent RNA helicase DBP7	REGKWDIHATT	1312.67	657.34	2
Nucleoside diphosphate kinase B	ETNPADSKPGSI	1214.58	608.30	2
Glucosyl-3-phosphoglycerate synthase	VAGDLAGGRAPGALP	1320.64	661.33	2
Collagen alpha-6(IV) chain	VGPLGPSG	682.33	342.17	2
Collagen alpha-3(V) chain	GIPGPLGPLGP	973.53	487.77	2
5'-3' exoribonuclease 2	NNGGGGGGYGGQP	1090.51	546.26	2
PE-PGRS family protein PE_PGRS30	NGGAAGLIGNGGAGGAGGAGGAG	1639.72	820.87	2
Protein FAM81B	DTNVNKSASPTATAEEQPVEP	2184.09	1093.05	2
E3 ubiquitin-protein ligase Topors	DQGLFMGPSTSGAAANR	1679.70	560.91	3
(R)-2-hydroxyglutaryl-CoA-dehydratase activating ATPase	GIADKQMSELSCHA	1488.70	745.36	2
Uncharacterized TPR repeat-containing protein At1g05150	DALGLELNADE	1158.57	580.29	2
Collagen alpha-1(I) chain	DGNPGLPGPPGPPGPPG	1492.69	747.35	2
Golgin subfamily A member 6A	GNHEGHG	706.28	354.15	2
Collagen alpha-2(IV) chain	EVLGAQPGTRGDAGLPGQPG	1875.93	626.32	3
MTOR-associated protein MEAK7	DVDGLFDTLSGSSSSAAAKNGK	2126.05	1064.03	2
Transforming protein Maf	GSAAAVVSAVIAAA	1156.53	579.27	2
Glycine dehydrogenase (decarboxylating)	PGAMGADIAIG	971.40	486.71	2
L-lactate dehydrogenase A-like 6B	SVADLTESILK	1174.65	392.56	3
CTP synthase	PDGKLVEICEVTGHPF	1739.83	870.92	2
Collagen alpha-3(V) chain	GIPGPLGPL	819.45	410.73	2
T-related protein	VSGGGGGGGAGGGAGSGSPQ	1429.68	715.85	2
Glyceraldehyde-3-phosphate dehydrogenase 1	TVDGPSGK	759.37	380.69	2

UDP-3-O-acylglucosamine N-acyltransferase	ADGFGFAPDFGPQGGEW	1753.78	877.90	2
Protein prickle	GGGAGGSSGGPGGADAAAAPAAGQ	1767.76	884.89	2
Histone H2A	AQGGVLPNIQ	995.54	498.78	2
Putative cuticle collagen 155	GPSGPNGNPGAPGAPGQ	1430.71	716.36	2
BTB/POZ domain and ankyrin repeat-containing protein NH5.1	GGAGGGGGAP	656.34	329.18	2
PE-PGRS family protein PE_PGRS5	GAGGKGGNGGTGGAGGPGG	1341.64	671.83	2
Collagen alpha-5(IV) chain	PGIPGIGLPGPPGPKGFPGIP	1947.00	974.51	2
Glutamate dehydrogenase 1, mitochondrial	IGPGIDVPAPDMSTGE	1554.73	778.37	2
Collagen alpha-2(IV) chain	SGPSGIPGLPGPKGEPGY	1665.76	833.89	2
Collagen alpha-1(I) chain	GLPGSPGPAGEAGK	1193.60	597.81	2
TRPM8 channel-associated factor homolog	SEAVQTNLVPFFEAWGWPI	2190.10	1096.06	2
Collagen alpha-4(IV) chain	GPPGIPGPNGEDGLPGLP	1639.76	820.89	2
Elastin	VPGAVPGGVP	848.44	425.23	2
Multidrug resistance protein PE_PGR46	IMVVVQPFVLVAI	1426.82	714.42	2
Uncharacterized PE-PGRS family protein PE_PGRS46	GDGAPGGDGGAGPLLIGNG	1550.68	776.35	2
POTE ankyrin domain family member E	SGDGVTH	671.29	336.65	2
Actin-related protein 3	SEVVDEVIQN	1130.54	566.28	2
Actin-related protein 3	SGDGVTH	671.29	336.65	2
Protein Wiz	GPERLPGPAPRENIEGGAE	1944.94	973.48	2
DNA-directed RNA polymerase subunit beta	GKPIPESGLPE	1122.53	562.27	2
Histone H2A	AQGGVLPNIQ	995.54	498.78	2
Ribulose bisphosphate carboxylase/oxygenase activase 2, chloroplastic	TLMNIADNPTNVQLP	1639.72	820.87	2

FT-interacting protein 1	PEVFVKAQVGNQILK	1668.86	835.43	2
Collagen alpha-2(I) chain	GAVGPVGPVG	808.44	405.23	2
Collagen alpha-2(I) chain	GPIGPPGNPGA	932.47	467.24	2
Polyribonucleotide nucleotidyltransferase	TEAVVAEGLEAAKP	1383.75	692.88	2
Putative cuticle collagen 145	EGPAGPAGPAGPDGQPGA	1501.64	751.83	2
Contactin-3	VSGGGGSRSELVITWDPVP	1911.97	956.99	2
Collagen alpha-1(III) chain	EPGQAGPAGPPGPPG	1285.60	1286.61	1
Collagen alpha-2(I) chain	SIGEPGPIGIAG	1066.51	534.26	2
Collagen alpha-2(I) chain isoform X3	GDPGPGGPQGEPGAVGPAGITGDKGPSGES	2601.20	868.08	3
Uncharacterized protein	DIKPVTEIQQNGNDFVITSK	2245.16	749.40	3
Calmodulin	IDQLTEEQIAEF	1434.65	718.33	2
Mitochondrial fission regulator	HLSLPRFFPSRTGE	1643.18	548.73	3
Collagen, type V, alpha 3a	LIDVLRVLELSEDMEGVSV	2114.92	1058.47	2
Si:dkey-237h12.3	ELDASNMGGWSLDK	1521.81	761.91	2
Uncharacterized protein Salmo truta	AGAEGFDDIK	1021.47	511.74	2
Fatty acid-binding protein, liver	AIGLPDDLIQK	1181.67	591.84	2
Uncharacterized protein Sinocyclocheilus anshuiensis	DVFRDGFTMDT	1302.61	652.31	2
Collagen alpha-4(IV) chain	GSSPIGPPGSPGSPGASGQ	1592.74	797.38	2
Mucin-5AC-like	GGPTSGSEGGDNESIK	1490.65	746.33	2
D-dopachrome decarboxylase	MIVVVKPGLPMLM	1426.82	714.42	2
Uncharacterized protein OS=Echeneis naucrates	PKPLPFFGTMLSYR	1653.00	827.51	2
Fumarylacetoacetase	IGVAIGDQILDLSVIK	1652.97	827.49	2

Table 2. Peptides identified in salmon viscera extract obtained by conventional stirring.

Protein of origin of the identified peptide.	Sequence	Obs MW	Obs m/z	Theor z
Adenosylhomocysteinase	GVSEETTTGVH	1115.51	558.76	2
Hemoglobin subunit alpha	AIHFPADFTPEVH	1479.71	494.24	3
Forkhead box protein K1	PQPPPGPPPPP	1076.57	539.29	2
40S ribosomal protein	ADGYEPPIQET	1218.54	610.28	2
WW domain-binding protein 11	PGPPPGPPPP	908.48	455.24	2
Filamin-A	VITPEEIVDPNVDEH	1704.81	569.28	3
Collagen alpha-1(X) chain	ISVPGKPGPQ	978.47	490.24	2
Fatty acid-binding protein 10-A, liver basic	AQENYEEFLR	1297.59	649.80	2
Methionine import ATP-binding protein MetN	IDEIGGQHVGSLVLGVP	1688.81	845.41	2
Probable tRNA pseudouridine synthase	ENNVDFVNRKIKEGEAMVSGPI	2445.24	816.09	3
Mediator of RNA polymerase II transcription subunit 30	LAASGMAPGPFAGPQ	1370.71	686.36	2
1-(5-phosphoribosyl)-5-[(5-phosphoribosylamino)-methylideneamino] imidazole-4-carboxamide isomerase	HWVDQGGKRLHL	1444.89	723.45	2
Quinolinate synthase A	EGADEVHVDPGI	1236.58	619.29	2
40S ribosomal protein S17	DQEIIEVDPDT	1272.58	637.30	2
Uncharacterized PE-PGRS family protein PE_PGRS54	NGGNGGDGGNGGDGGNGAP	1627.66	814.84	2
Prostaglandin reductase 1	GPPPPGPPPEVVI	1251.65	626.83	2
NAD(P)H-hydrate epimerase	LVGAGNNGGDALLAAAELAR	1851.87	926.94	2
Cysteine--tRNA ligase	VLRFFMATTQYR	1531.90	766.96	2
Actin, cytoplasmic 1	DSGDGVTH	786.32	787.33	1
Putative adenosylhomocysteinase 3	LDRMKNSCIVCNIGH	1701.92	851.97	2
Spore membrane assembly protein 2	SSSSILVVIATL	1188.79	595.40	2

Adenosylhomocysteinase	IPAINVNDSVT	1141.60	571.81	2
Hemoglobin subunit alpha	IHFPADFTPEVH	1408.68	470.57	3
Uncharacterized protein y4iR	VFASYPQPLG	1077.53	539.77	2
2-C-methyl-D-erythritol 4-phosphate-cytidylyltransferase	LQSVIAVVPAAGV	1222.84	612.43	2
Zinc finger C2HC domain-containing protein 1A	NQVIKDGGPLPPPPPP	1621.80	811.91	2
Trichodiene synthase	VSEGITLNQALE	1272.58	637.30	2
60 kDa heat shock protein, mitochondrial	GTSDVEVNEK	1076.50	539.25	2
pH-response regulator protein palF/RIM8	PIRITHLTVAL	1232.80	617.41	2
Leucine-rich repeat-containing protein 56	LEQLEVLDLEGNS	1457.65	729.83	2
Tungsten-containing formylmethanofuran-dehydrogenase 2 subunit C	DVDVRVGGEMKAG	1331.66	666.84	2
Cyclic pyranopterin monophosphate synthase	NTNGEANMVDVSMKQ	1636.80	819.41	2
Acetylcholinesterase	FRHPRPAEKWTGV	1579.88	790.95	2
Uncharacterized PE-PGRS family protein PE	NGGNGGIGGP	798.43	400.22	2
Sulfocyanin	SPSASSSTGTSTGP	1222.59	612.30	2
Actin, cytoplasmic	VMDSGDGVTH	1016.42	509.22	2
40S ribosomal protein S3a	GEGGGSSAAKPSG	1060.47	531.24	2
DNA repair protein crb2	DSLYDRLLARKGPLFGK	1948.23	975.12	2
Argininosuccinate synthase	IEGGRLEDPSFVPP	1511.82	756.92	2
Collagen alpha-2(I) chain	GAVGPVGPVG	808.44	405.23	2
Thiazole synthase	GVLLNTAVSGAKDP	1340.73	671.37	2
Histone-lysine N-methyltransferase 2D	PLSPPPEDSPLSPPP	1525.79	509.61	3
Probable transcriptional regulatory protein Ecaj_0351	NFDSLFNIAI	1152.59	577.30	2
50S ribosomal protein L29	HAKKAELFELRVK	1567.85	784.93	2

Forkhead box protein K1	QPPPGPPPPPP	1076.57	539.29	2
Probable GPI-anchored adhesin-like protein PGA32	ATAAGTEVQGFTPI	1361.60	681.81	2
Replicase polyprotein 1ab	MAKMGKYGLGFK	1329.90	665.96	2
Stonin-2	VVDGGSQDHS	999.35	500.68	2
SLAIN motif-containing protein	AGGGGPEPGGAGTPPGAAAAP	1615.84	808.93	2
Structural maintenance of chromosomes protein 4	EIQNSILNVGGPQ	1367.67	684.84	2
Golgin-84	TPEIH	595.30	298.66	2
Keratin, type II cytoskeletal 5	LGGGAGFGGGYGGP	1122.54	562.28	2
Translation initiation factor IF-2	VEEGLTSDEPDLE	1431.60	716.81	2
Genome polyprotein	IDLSANAAGSDPP	1226.61	614.31	2
Collagen alpha-1(X) chain	ISVPGKPGPQ	978.47	490.24	2
Transcription-associated protein 1	VASVQPYAMPP	1158.53	580.27	2
MAM and LDL-receptor class A domain-containing-protein 2	LDDSPCPPE	971.37	972.38	1
Coiled-coil domain-containing protein CG32809	SSSKKKRKGRE	1289.85	645.93	2
Large tegument protein deneddylase	SVPAPPTLPP	974.52	488.27	2
Adenylyl cyclase-associated protein	GPPPPGPPPPP	1004.53	503.27	2
Neuroblast differentiation-associated protein AHNAK	VDIEGPDVDIEGSGG	1457.65	729.83	2
Mediator of RNA polymerase II transcription subunit 28	QPPGPPPPPPP	1076.56	539.29	2
Protein S100	DLDANSDGSVDFQ	1381.56	691.79	2
LisH domain-containing protein	VISYALDLIEVKHDSARVH	2164.32	1083.16	2
Adenylyl cyclase-associated protein	DGDYTEIPVPEQ	1361.59	681.80	2
Guanylate cyclase domain-containing protein	LISPGDAL	784.35	393.18	2
Insulin receptor substrate 2	VCGGSGPG	632.26	317.14	2

Despite using the same viscera sample, only five peptides matched in both extracts (color marked in both tables). These data show that the extraction conditions used for PLE-assisted extraction influence the peptides obtained from salmon viscera.

A common method currently used to speculate about peptide function is through an amino acid homology alignment against a database of known functional peptide sequences. The antioxidant activity of the identified peptides was thus predicted using the BIOPEP-UWM database, which is a bioinformatics tool for searching among bioactive peptides, mainly derived from foods [28]. None of the peptides identified in salmon viscera extracts were found among the antioxidant peptides inputted in the BIOPEP-UMP database. Therefore, a new search based on the profiles of the potential biological activity of peptides was performed. BIOPEP-UWM analysis results exhibited several antioxidant small peptides encrypted in amino acid sequences of PLE (Table 3) and control (Table 4) viscera extracts, with some of them known to be derived from marine species. Throughout the entire structure of peptides, 19 different sequences of peptides with antioxidant activity were found in the PLE extract, whereas there were 12 in the control extract. Most of these potential antioxidant peptides were di- and tripeptides. The sequence GPP was found in 15 peptides of the PLE extract, followed by GAA, which was found in five peptides. These sequences could be responsible for antioxidant activity, since antioxidant peptides from marine resources have been described to contain hydrophobic acids such as glycine (G), proline (P), and alanine (A) [8,9,29]. Furthermore, salmon antioxidant peptides from the pectoral fin (FLNEFLHV) and trimmings (GGPAGPAV, GPVA, PP, GP) have been reported [10,30].

Table 3. Comparison of peptides identified in salmon viscera extracts obtained through pressurized liquid extraction with potential antioxidant sequences contained in the BIOPEP-UWM database.

Sequence_modification	Sequence in BIOPEP-UWM database	Identity of sequences with antioxidant potential
GPAGPHGPPG	PHG	ID 8026 synthetic peptide
	GPP	ID 8987
GPAGHPGPPG	GPP	ID 8987
GYAKDGLPGIPGPQGET	KD	ID 8134 peptide from dried bonito
GGGEGYGGGGANGGGY	GGE	ID 8114 peptide from sardinelle byproducts
GPLGPPGGMPGH	GPP	ID 8987
GPPGLPGPPGPPGHKGF_ Carbamyl(K)@15	GPP	ID 8987
GGGGGGGGGGGGSGGNFGGGGPP	GPP	ID 8987
QPPPGPPPPPP	GPP	ID 8987
TALGGAAGGMGGGGGMGGGM_ Oxidation(M)@20	GAA	ID 8983
ACAGMIGPPGPQGFP_ Deamidated(Q)@12	GPP	ID 8987
	ACA	ID 10038
QAGEGGAGAGAGAAG	GAA	ID 8983
LPGPPGPPGPPGPRGYPG	GPP	ID 8987
AIQPDTEFTPPELDASS	EL	ID 7888
	PEL	ID 8139 synthetic peptide
	GPP	ID 8987
LSLVVSGGHTELVL	EL	ID 7888

QNLVGPPGPPGPPGVSGD_ Gln->pyro-Glu@N-term	GPP	ID 8987
DINAGGGACASVGLL	ACA	ID 10038
KGDRFLEAAGVNKLWPE	LW	ID 8462 peptide from marine bivalve
RGDGGPPGVTGFPGAA	GAA	ID 8983
	GPP	ID 8987
GPAGPHGPP	PHG	ID 8026
	GPP	ID 8987
ETNPADSKPGSI	KP	ID 8218
NGGAAGLIGNGGAGGAGGAGGAG	GAA	ID 8983
DQGLFMGPSTSGAAANR_ Deamidated(N)@16	GAA	ID 8983
GIADKQMSELSCHA	EL	ID 7888
DALGLELNADE	EL	ID 7888
DGNPGLPGPPGPPGPPG_ Pro->pyro-Glu(P)@16	GPP	ID 8987
SVADLTESILK	LK	ID 8217
ADGFGFAPDFGPQGGEW	GGE	ID 8114 peptide from sardinelle by-products
	ADGF	ID 9328
PGIPGIGLPGPPGPKGFPGIP_ Delta:H(2)C(2)(K)@15	GPP	ID 8987
SEAVQTNLVPFFEAWGWPI	WG	ID 9082
	EAVQ	ID 9881
GPPGIPGPNGEDGLPGLP	GPP	ID 8987
GKPIPESGLPE	KP	ID 8218
PEVFVKAQVGNQILK	LK	ID 8217
GPIGPPGNPGA	GPP	ID 8987

TEAVVAEGLEAAKP	KP	ID 8218
VSGGGGSRSELVITWDPVP	EL	ID 7888
	TW	ID 8459 peptide from marine bivalve
EPGQAGPAGPPGPPG_ Deamidated(Q)@4	GPP	ID 8987
DIKPVTEIQQNGNDFVITSK	KP	ID 8218
HLSLPRFFPSRTGE	HL	ID 3317
LIDVLRVLELSEDMEGVSV	EL	ID 7888
ELDASNMGGWSLDK	EL	ID 7888
GGPTSGSEGGDNESIK	GPP	ID 8987
MIVVVKPGLPMLM	KP	ID 8218
	VKP	ID 8434 peptide from jellyfish
PKPLPFFGTMLSYR	LPM	ID 9360
IGVAIGDQILDLSVIK	KP	ID 8218

Several antioxidant peptide sequences from the viscera of sardinella (LHT, LARL, GGE), black pomfret (AMT6GLEA), and mackerel (ACFL) have also been identified [9].

In addition to specific amino acids, peptides derived from fish sources, especially in the range of 0.5–1.5 kDa, have been assumed to be a key factor in terms of antioxidant activity [26]. The molecular weight of peptides in control viscera extracts ranged from 0.63 to 2.44 kDa (Table 4), whereas for viscera PLE extracts, the molecular weight of peptides was 0.67–2.60 kDa (Table 2). However, there was a greater amount of small peptides in the PLE extract. As can be seen in **Figure 4**, a higher intensity of analytes with shorter retention times was observed for the viscera PLE extract, which in the case of peptides usually corresponds to more polar and/or smaller compounds.

According to these results, both the specific amino acid sequences encrypted in the identified peptides and a molecular weight below 1.5 kDa could be related to the antioxidant capacity exhibited by the PLE extract from salmon viscera.

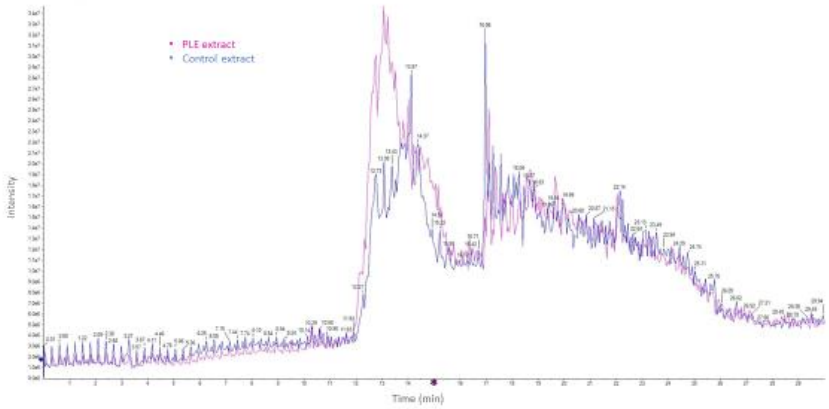

Figure 4. Cromatogram of total ions counts of salmon viscera protein extracts obtained by conventional stirring and Pressurized Liquid Extraction (PLE).

Table 4. Comparison of peptides identified in salmon viscera extract obtained through conventional stirring with potential antioxidant sequences contained in the BIOPEP-UWM database.

Sequence_modification	Sequence in BIOPEP-UWM database	Identity of sequences with antioxidant potential
AIHFPADFTPEVH	ADF	ID 7868 peptide from Okara protein
PQPPPGPPPPP	GPP	ID 8987
PGPPPGPPPP	GPP	ID 8987
ISVPGKPGPQ	KP	ID 8218
HWVDQGGKRLHL	LH	ID 3305
	HL	ID 3317
	LHL	ID 7995 synthetic peptide
GPPPPGPPPEVVI	GPP	ID 8987
LVGAGNNGGDALLAAAELAR	EL	ID 7888
IHFPADFTPEVH	ADF	ID 7868 peptide from Okara protein
NQVIKDGGPLPPPPPP	KD	ID 8134 peptide from dried bonito
PIRITHLTVAL	HL	ID 3317
	IR	ID 8215
DVDVRVGGEMKAG	GGE	ID 8114 peptide from sardinelle by-products
GEGGGSSAAKPSG	KP	ID 8217
GVLLNTAVSGAKDP	KD	ID 8134 peptide from dried bonito
HAKKAELFELRVK	EL	ID 7888
QPPPGPPPPPP	GPP	ID 8987
AGGGGPEPGGAGTPPGAAAAP	GAA	ID 8983

2.5. *Determination of Heavy Metals and Mycotoxins in Salmon Side Streams*

The concentrations of As, Hg, Cd, and Pb in salmon muscle, head, viscera, skin, and tailfin are shown in **Table 5**. Mean concentration ranges, expressed as μg/g of wet weight (*ww*), were 0.4186-0.6922, 0.0095-0.0408, 0.0004-0.0104, and 0.0071-0.0859 for As, Hg, Cd, and Pb, respectively. For all salmon side streams, the most abundant element was As, whereas the lowest concentration was observed for Cd. There is a lack of information in the literature on heavy metals content in salmon discards. For instance, one study reported liver Hg accumulation in four wild species of Pacific salmon [31]. The results (0.120-0.192 μg/g, *ww*) were higher than those found in the present study for viscera samples, which include more organs than liver. The content of As, Hg, Cd, and Pb in several fish side streams of sea bass, sea bream, and meager has also been described [18,19,23,25]. The arsenic levels in viscera (1.867-2.587 μg/g, *ww*) of these fish species were higher than those of salmon viscera.

Table 5. Concentration of heavy metals in salmon side streams.

Salmon Side Streams	Heavy metals (μg/g of wet weight)			
	As	**Hg**	**Cd**	**Pb**
Muscle	0.5413 ± 0.0068	0.0238 ± 0.0005	0.0004 ± 0.0001	0.0269 ± 0.0002
Head	0.6922 ± 0.0072	0.0157 ± 0.0005	0.0011 ± 0.0001	0.0190 ± 0.0001
Viscera	0.4617 ± 0.0055	0.0095 ± 0.0002	0.0044 ± 0.0002	0.0071 ± 0.0001
Skin	0.4504 ± 0.0032	0.0077 ± 0.0003	0.0019 ± 0.0001	0.0247 ± 0.0001
Tailfin	0.4186 ± 0.0054	0.0408 ± 0.0015	0.0104 ± 0.0003	0.0859 ± 0.0016
[Legislation*]	< 13.5	< 0.50	< 0.05	< 0.30

*values referred to fish muscle tissue [23,25,35].

The data available on toxic elements in fish usually refer to edible muscle due to the potential health risk for consumers. In this sense, levels of Cd and Pb in 21 samples of smoked salmon from a Polish market were determined [32]. The results were on the order of 0.0040–0.0196 μg/g (*ww*) for Cd and 0.0109–0.1559

μg/g (*ww*) for Pb, both of which are considered safe for consumers. In addition, As, Hg, Cd, and Pb contents in fresh salmon muscle were evaluated [33,34]. It should be noted that the limits for heavy metals in fish side streams are not currently regulated. Therefore, the safety assessment could be based on the limit values established for edible muscles of fish (μg/g): 13.5 for As, 0.5 for Hg, 0.05 for Cd, and 0.30 for Pb [23,25,35]. According to this, the toxic elements analyzed in all salmon side streams in this study are below the limits set by authorities and could be considered safe for consumers in terms of As, Hg, Cd, and Pb content.

Nostbakken et al. [33] showed a trend towards a decrease in As and Hg content in farmed Atlantic salmon, which was related to the decline in the use of fish meal and fish oil in commercial fish feed. However, the replacement of marine ingredients by others of plant origin can lead to the presence of contaminants such as mycotoxins in both aquafeeds and fish tissues. In this way, Bernhoft et al. [36] conducted a toxicokinetic study of deoxynivalenol (DON) and ochratoxin A (OTA) mycotoxins in farmed salmon fed with contaminated feeds for 8 weeks. The authors observed an even distribution in the liver, kidney, brain, skin, and muscle for DON, as well as a distribution mainly in the liver and kidney for OTA. According to this, the possible occurrence of mycotoxins in the muscle, head, viscera, skin, and tailfin of farmed salmon was investigated in the present study. Through a simultaneous multi-mycotoxin evaluation using a non-targeted screening approach, no mycotoxins or related metabolites were identified in salmon side streams. These results are in agreement with those found by Nácher-Mestre et al. [37,38] on the carry-over of common and emerging mycotoxins from feeds to edible parts of farmed Atlantic salmon fed with high plant based diets. In addition, there was no presence detected of

several mycotoxins, such as aflatoxins, fumonisins, enniatins, or ochratoxin A, in smoked salmon and raw salmon sushi commercial products [39].

3. Materials and Methods

3.1. Reagents

AAPH (2,2´-azobis (2-amidinopropane)) (Acros Organics), sodium phosphate dibasic, sodium chloride, potassium dihydrogen phosphate, potassium sulphate, TRIS (ultrapure), glycine (proteomics grade), ortho-boric acid, and methanol (HPLC grade) were obtained from VWR International Eurolab S.L. (Barcelona, Spain). Trizma® base, ABTS (2,2´-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)), DTT (DL-Dithiothreitol), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), fluorescein sodium salt, formic acid (reagent grade ≥ 95%), and diatomaceous earth (Hyflo® Super Cel®) were provided by Sigma-Aldrich (Steinheim, Germany). Sodium hydroxide, glacial acetic acid, and sulfuric acid were supplied by Fisher Scientific (Madrid, Spain). SDS (sodium dodecyl sulfate) and nitric acid (65% *p/p*) were purchased from Panreac (Barcelona, Spain). Bromophenol blue indicator (ACS reagent), acetonitrile (HPLC grade), trifluoroacetic acid, acetone, and glycerol were provided by Merck (Darmstadt, Germany). Absolute ethanol was obtained from J.T. Baker (Deventer, The Netherlands), Octadecyl C18 sorbent was obtained from Phenomenex (Madrid, Spain), and anhydrous magnesium sulfate (99.5% min powder) was obtained from Alfa Aesar (Karlsruhe, Germany). Deionized water with a resistivity of >18 MW/cm was obtained through a Milli-Q SP® Reagent Water System (Millipore Corporation Bedford, MA, USA).

3.2. *Raw Material and Sample Preparation*

Whole salmon fish (Salmo salar) from Norwegian aquaculture were purchased in a local market in Valencia (Spain) during different weeks of June 2019. They were immediately transported to the laboratories of the University of Valencia under refrigerated conditions. Individual salmon were dissected as a simulation of fish processing for human consumption. Then, muscle leftovers, complete heads, viscera, flesh-free skin, and tailfins were placed separately inside aluminum containers and frozen at -80 °C for 48 h. Next, they were freeze-dried (LABCONCO, 2.5. FREE ZONE, USA) for 72 h, and keep in a desiccator until reaching a constant weight. Then, water content was determined gravimetrically. The moisture percentages were 67.61% ± 1.04%, 61.66% ± 2.52%, 52.31% ± 1.98%, 45.04% ± 1.60%, and 45.63% ± 0.71% for muscle remains, heads, viscera, skin, and tailfins, respectively. Similar values for salmon head, viscera, and skin were reported by Aspevik et al. [6] and He et al. [5]. Each type of sample was ground in an analytical mill (A11 basic IKA® WERKE, Staufen, Germany) and stored at -25 °C until the extraction process and the determination of possible food contaminants.

3.3. *Pressurized Liquid Extraction (PLE) Process*

Antioxidant protein extracts from salmon side stream materials were obtained using an accelerated solvent extractor ASE 200 Dionex (Sunnyvale, CA, USA) equipped with a solvent controller. Dried samples were mixed with diatomaceous earth before being introduced into 22-mL stainless steel cells with a glass fiber filter placed in the end part. The standard operation parameters were as follows: preheating period (1 min), heating period (5 min), and flush volume (60%), and nitrogen purge (145 psi for 1 min). The extractions were performed

under a pressure of 1500 psi with distilled water as a solvent. The pH, temperature, and time conditions for PLE-assisted extraction were selected based on the optimization of the extraction conditions to obtain antioxidant protein extracts from sea bass side streams [18]: pH 7, 20 °C, 5 min for muscle; pH 4, 60 °C, 15 min for heads; pH 7, 50 °C, 15 min for viscera; pH 7, 55 °C, 5 min for skin; and pH 7, 60 °C, 15 min for tailfins. For all samples, control extracts were also carried out in parallel by stirring for 30 min with distilled water at room temperature. Both types of extractions were performed at least in duplicate. The extracts obtained were homogenized individually, divided into several replicates and stored at -25 °C for subsequent analyses. Protein recovery, protein molecular weight distribution, and total antioxidant capacity were evaluated and compared (PLE vs control extracts).

3.4. *Evaluation of Total Antioxidant Capacity*

3.4.1. Trolox Equivalent Antioxidant Capacity Assay (TEAC)

The TEAC assay measures the inhibition of the radical cation ABTS+ by antioxidant compounds, which is compared to the activity of a reference antioxidant standard (Trolox). The spectrophotometric method proposed by de la Fuente et al. [18] was used. ABTS reagent (7 mM) and $K_2S_2O_8$ (140 mM) were mixed and maintained at room temperature in darkness for 16 h to generate the ABTS+ stock solution. Then, it was diluted in ethanol until an absorbance of 0.700 ± 0.020 at 734 nm and 30 °C to obtain the ABTS+ working solution. Proper dilution of each fish extract to achieve a percentage of absorbance inhibition of approximately 50% was required. A range of Trolox standard solutions (0–300 μM) were prepared. The absorbance of 2 mL of ABTS+ working solution was considered the initial point of reaction (A_0). Then, 100 μL

of diluted extracts or Trolox standards were added immediately. After 3 min of reaction, the absorbance was measured and considered the final point (A_f). All measures were conducted in a thermostatized UV–vis spectrophotometer. The percentages of absorbance inhibition were calculated using the following equation: 1 - (A_f/A_0) x 100 and were compared to the Trolox standard curve. The results were expressed as μM Trolox Equivalents.

3.4.2. Oxygen Radical Absorbance Capacity Assay (ORAC)

The ORAC assay measures the scavenging of the peroxyl radical AAPH by antioxidant compounds. The fluorometric method described by de la Fuente et al. [18] was applied. Sodium fluorescein (0.015 mg/mL), AAPH radical solution (120 mg/mL), and Trolox standard solution (100 μM) were prepared with phosphate buffer (75 mM, pH 7). Adequate diluted extracts were required. The operating conditions for the final reaction consisted of 50 μL of diluted extract, Trolox standard or phosphate buffer (blank), 50 μL of fluorescein, and 25 μL of AAPH incubated at 37 °C in a Multilabel Plate Counter VICTOR3 1420 (PerkinElmer, Turku, Finland). Fluorescence filters for an excitation wavelength (485 nm) and an emission wavelength (535 nm) were selected. The fluorescence was recorded every 5 min over 60 min, where the fluorescence in the assay was less than 5% of the initial value. Differences of areas under the fluorescence decay curve (AUC) between the blank and the sample over time were compared and the results were expressed as μM Trolox Equivalents.

3.5. *Determination of Protein Recovery*

The total nitrogen content in salmon side stream materials and extracts obtained by conventional stirring and PLE-assisted extraction was determined

using the Kjeldahl method [40]. The total protein content was calculated based on the total nitrogen values and the protein–nitrogen conversion factor (6.25) for fish and fish side streams. Then, the following formula was applied for protein recovery: (protein in extract/protein in side stream) x 100.

3.6. *Molecular Weight Distribution of Protein Fragments*

SDS-PAGE was used to investigate the protein molecular weight distribution of both control (stirring) and optimal (PLE) extracts from salmon side stream materials. Acetone was added to the extracts at a 4:1 ratio (*v*/*v*) and they were mixed by means of a vortex. For protein precipitation, the mixture was centrifuged at 11,000 rpm, 4 °C, and 10 min. The supernatant was then removed and the pellet was dissolved and distilled. Afterwards, equal volumes of SDS-PAGE sample buffer solution (62.5 mM Tris-HCl (pH 6.8), 2% SDS, 20% glycerol, 0.01% bromophenol blue, and 50 mM dithiothreitol) and protein solution were mixed and heated in a thermoblock (95 °C, 5 min). Next, 10 µL were loaded onto 8–16% Mini-PROTEAN® TGX™Precast gels (Bio-Rad). The electrophoresis was performed using a Mini-PROTEAN® Tetra Cell (Bio Rad) under a constant voltage of 80 V for 120 min. The running buffer consisted of Trizma® base (25 mM), glycine (192 mM), and SDS (0.1%). The gels obtained were stained in Coomassie brilliant blue R-250 (0.125%) and destained through a solution of water:methanol:acetic acid (70:20:10) until the background was as clear as possible. In order to estimate the molecular weight of protein bands obtained in the electrophoretic gels, a standard molecular weight of protein bands (5–250 kDA, Precision Plus Protein™, Bio-Rad) was used. The images of the gels were also evaluated using ImageJ® software, a public domain digital image processing program developed at the National Institutes of Health (NIH).

For a better visualization of protein bands, background subtraction and 8-bit format were selected.

3.7. *Identification of Peptides in Viscera Extracts*

3.7.1. Sample Preparation

The salmon viscera extracts obtained through shaking and PLE were frozen and lyophilized. Freeze-dried samples (100 mg) were resuspended in MilliQ water (200 μL). Then, 200 μL of acetonitrile (ACN) were added and the mixture was kept overnight at 4 °C for protein precipitation. Next, samples were centrifuged at 5000 rpm for 5 min and the supernatants, which contained soluble peptides, were dried in a speed vacuum (Eppendorf, Hamburg, Germany). The resulting pellets were dissolved in 27 μL of aqueous solution, containing 2% ACN and 0.1% trifluoroacetic acid (TFA), and sonicated for 5 min. Afterwards, 0.5 μL of sample solution was diluted with 6 μL water with ACN (0.2%) and TFA (0.1%).

3.7.2. Mass Spectrometry Analysis

Peptides were analyzed in a nanoESI qTOF mass spectrometer (6600plus TripleTOF, ABSCIEX, Framingham, MA, USA). A total of 5 μL of sample was loaded onto a trap column (ChromXP C18, 3 μm 120, 350 μm, 0.5 mm; Eksigent) and desalted with 0.1% TFA at a flow rate of 5 μL/min for 5 min. The peptides were then loaded onto an analytical column (3μ C18-CL 120, 0.075 x 150 mm; Eksigent) equilibrated in 5% ACN and 0.1% TFA. Elution was carried out with a linear gradient from 7% to 40% B in A for 45 min. (A: 0.1% formic acid (FA); B: ACN, 0.1% FA) at a flow rate of 300 nL/min.

Sample was ionized by applying 3.0 kV to the spray emitter at 175 °C. Analysis was performed in a data-dependent mode. Survey MS1 scans were acquired from 350–1400 m/z for 250 ms. The quadrupole resolution was set to 'LOW' for MS2 experiments, which were acquired 100–1500 m/z for 25 ms in 'high sensitivity' mode. The following switch criteria were used: charge: 2+ to 4+; minimum intensity; 250 counts per second (cps). Up to 100 ions were selected for fragmentation after each survey scan. Dynamic exclusion was set to 15 s. The system sensitivity was controlled by analyzing 0.5 μg of K562 trypsin digestion (Sciex). In these conditions, 2230 proteins were identified (FDR <1%) in a 45 min gradient.

3.7.3. Data Analysis

After LC-MS/MS, the identification of peptides was carried out with the software ProteinPilot v5.0 search engine (AB SCIEX). ProteinPilot default parameters were used to generate the peak list directly from 6600 plus TripleTOF wiff files. The Paragon algorithm [41] in ProteinPilot v 5.0 was used to search against the Swiss Prot (Inr 200602) and Uniprot Chordata (Inr 2007721) protein sequence databases with the following parameters: none digestion, none cys-alkylation, taxonomy non restricted, and the search effort set to thorough.

The BIOPEP-UWM database was used in the search for similar previously identified sequences showing antioxidant activity (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep accessed on 28 April 2021). The search option "profiles of potential biological activity" was then employed, in which antioxidant activity was selected.

3.8. *Analysis of Heavy Metals in Salmon Side Stream Materials*

The presence of As, Hg, Cd, and Pb in side stream materials of farmed salmon was studied. Muscle, heads, viscera, skin, and tailfins were mineralized in a microwave oven (MARS, CEM, Vertex, Spain). Approximately 0.30 g of sample was placed in a Teflon reactor vessel. Next, 1 mL of H_2O_2 (30% v/v) and 4 mL of HNO_3 (14M) were added and the digestion was conducted under a microwave irradiation power of 800 W at 180 °C for 15 min. The digested samples were left to cool at room temperature. After eliminating the nitrogenous vapour, they were filtered and brought up to volume with distilled water.

The identification and quantification of toxic metals was carried out using an inductively coupled plasma spectrometer mass detector (ICP-MS, Agilent model 7900). The analytical conditions were as follows: carrier gas (1.07 L/min), Ar gas flow (15.0 L/min), reaction gas (He), RF power (1550 W), nebulizer pump speed (0.10 rps), and RF matching (1.80 V). To correct matrix-induced signal fluctuations and instrumental drift, internal standard solutions of ^{72}Ge, ^{103}Rh, and ^{19}3Ir (ISC Science) at 20 μg/g were used. For the quantification of As, Cd, and Pb, standard calibration curves from 0 to 1000 μg/L were used. As for the quantification of Hg, a standard calibration curve from 0 to 100 μg/L was utilized. Limits of detection (LODs) were calculated according to the following equation: LOD = 3sB/a where "3sB" is 3 times the standard deviation at zero concentration and "a" is the slope of the calibration curve. LOD values obtained for As, Hg, Cd, and Pb were 0.012, 0.0015, 0.004, and 0.0015 μg/L, respectively. The concentrations of heavy metals in the digested blank (distilled water) were subtracted from the values of samples. The results were expressed as μg of element/g of side stream material in wet weight. To confirm the accuracy of the

method, the fish protein powder DORM-3 was used as the Certified Reference Material for Trace Metals. It was prepared and analysed simultaneously to the salmon samples. The recovery percentages were 98%, 86%, 76%, and 77% for As, Hg, Cd, and Pb, respectively.

3.9. *Analysis of Mycotoxins in Salmon Side Stream Materials*

High-performance liquid chromatography coupled with electrospray ionizationquadrupole-time of flight-mass spectrometry (LC-ESI-qTOF-MS) was employed to investigate the occurrence of mycotoxins in salmon side stream materials. An Agilent 1200-LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a Gemini® column NX-C18 (3 M, 150 x 2 mm ID) (Phenomenex), as well as a vacuum degasser, binary pump, and autosampler, were used to achieve the chromatographic separations The mobile phases consisted of acidified (0.1% of formic acid) water (A) and acetonitrile (B). A gradient program of 50% B (0–6 min); 100% B (7–12 min); and 50% B (13–20 min) was applied. Samples (5 μg L) were injected at a flow rate of 0.2 mL/min. Mass spectrometry (MS) analysis was carried out using a 6540 Agilent Ultra-High-Definition-Accurate-Mass-q-TOF-MS coupled to the HPLC, equipped with an Agilent Dual Jet Stream electrospray ionization (Dual AJS ESI) interface in positive and negative ionization modes. The operational conditions were as follows: nebulizer pressure (50 psi); capillary voltage (3500 V); fragmenter voltage (160 V); scan range (m/z 50–1500); drying gas temperature (370 °C); and nitrogen drying gas flow (12.0 L/min). Automatic MS/MS experiments were performed under the following collision energy values: m/z 100, 30 eV; m/z 500, 35 eV; m/z 1000, 40 eV; and m/z 1500, 45 eV. For data acquisition and integration, Mass Hunter Workstation software was used.

The QuEChERS procedure to extract mycotoxins from fish discards, previously reported by de la Fuente et al. [18], was applied. Approximately 3 g of salmon samples were mixed with 30 mL of acidified water (2% formic acid) in an orbital shaker (IKA KS 260) for 30 min. Then, 10 mL of acetonitrile were added and the mixture was stirring again for 30 min. Next, 8 g of $MgSO_4$ and 2 g of NaCl were added to the mixture, vortexed for 30 s and centrifuged at 4000 rpm for 10 min. Afterward, 0.1 g of Octadecyl C18 sorbent and 0.3 g of $MgSO_4$ were mixed with 2 mL of supernatant. Additional shaking and centrifugation under the same conditions as reported previously were performed. The supernatant was then filtered (13 mm/0.22 μm nylon filter) and 20 μL were injected into the LC-ESI-qTOF-MS system.

3.10. *Statistical Analysis*

Experimental data were subjected to one-way analysis of variance (ANOVA) to determine the significant differences among samples. Tukey's honestly significant difference (HSD) multiple range test, at a significance level of $p < 0.05$ was applied. Statistical analyses were performed with Statgraphics Centurion XVI.I software (Statpoint Technologies, Inc., The Plains, VA, USA).

4. Conclusions

The Pressurized Liquid Extraction (PLE) technique allowed us to obtain, for the first time, protein extracts with in vitro antioxidant capacity from Atlantic salmon processing side streams. PLE-assisted extraction influenced the size of the protein fragments obtained in the extracts, since extracts from muscle leftovers, heads, viscera, skin, and tailfins showed different SDS-PAGE profiles. Both the highest protein recovery percentage (92%) and the highest antioxidant

capacity were observed in the viscera PLE extract. As 40% of the peptides identified in the PLE extract contained small peptide sequences with known antioxidant activity, salmon viscera could be considered an interesting source of antioxidant peptides. Further research on the relationship between antioxidant activity and specific peptides from salmon viscera PLE extract is required. The levels of toxic metals (As, Hg, Cd, and Pb) and the absence of mycotoxins in salmon processing side streams contribute not only to increasing the limited data in the literature about these contaminants in farmed fish, but also provide information about their safety as candidates for use in the food industry.

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3.4. Nutritional and bioactive oils from Salmon (*Salmo salar*) side streams obtained by Soxhlet and microwave-assisted extraction

Food Chemistry (under review)

Nutritional and bioactive oils from salmon (*Salmo salar*) side streams obtained by Soxhlet and optimized microwave-assisted extraction

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Abstract

The efficiency of the microwave-assisted extraction (MAE) technique on recovering nutritional and bioactive oils from salmon (*Salmo salar*) side streams was evaluated and compared to Soxhlet extraction. The response surface methodology (RSM) coupled with a central composite rotatable design was used to optimize time, microwave power, and solid/liquid ratio of the MAE process in terms of oil yield. The optimal MAE conditions were 14.6 min, 291.9 W, 80.1 g/L for backbones, 10.8 min, 50.0 W, 80.0 g/L for heads, and 14.3 min, 960.6 W, 99.5 g/L for viscera, which resulted in a recovery of 69% of the total lipid content for backbones and heads and 92% for viscera. The oils obtained under optimal MAE conditions showed a healthy lipid profile as well as cytotoxic, antioxidant, anti-inflammatory, or antimicrobial properties. These results highlight that oils from underutilized salmon by-products could be exploited by different industrial sectors under the circular economy approach.

Keywords: salmon side streams, fish oil, fatty acids, microwave-assisted extraction (MAE), bioactive properties, cellular assays

1. Introduction

The concept of circular economy applied to the food industry has been promoted by the European Union in recent years. The transformation of food bio-waste into value-added products is now a challenge for both the food industry and the researchers. This approach is particularly interesting for the seafood processing industry since it can generate up to 80% by-products from the whole organism (Nawaz et al., 2020). In this context, several studies have highlighted the nutritional value and functional properties of fish processing side streams, showing their relevance from a food perspective (Khawli et al., 2019; Nawaz et al., 2020). A case study of salmon aquaculture concluded that there are economic, environmental, and food security benefits through an appropriated management of salmon by-products, which were also considered food grade raw material for human consumption (Stevens, Newton, Tlusty, & Little, 2018).

Atlantic salmon (*Salmo salar*) is currently the most important farmed fish in Europe, reaching 1.3 million tons and 5 billion EUR in 2017 (European Commision 2020). About 50% of a whole salmon is transformed into marketable edible products while the remaining 50% (mainly backbones, heads, and viscera) is available for the recovery of quality nutrients and bioactive compounds (He, Franco, & Zhang, 2011; Inguglia et al., 2020). In this sense, salmon side streams are considered to be rich in fat (15-30%) and a good source of polyunsaturated fatty acids (PUFAs), especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (He et al., 2011).

EPA and DHA are recognized to be the most important fatty acids of the omega-3 PUFAs family due to their proven and potential biological activities. These fish fatty acids are accepted to prevent risk factors associated with the

development of various disorders such as cardiovascular and neurological diseases, inflammation, hypertension, obesity, osteoarthritis, and different types of cancer (Kapoor et al., 2021; Oppedisano et al., 2020). A great number of studies have also shown cytotoxic or anti-proliferative effects of omega-3 PUFAs from fish on different human cancer cell lines without affecting normal cells (Jameel, Agarwal, Arshad, & Serajuddin, 2019). In addition, the antibacterial activity related to specific compounds such as EPA, DHA, and linolenic acid have been described (Inguglia et al., 2020).

Dietary foods containing PUFAs have been recommended to include in a routine diet in order to maintain a good health (Kapoor et al., 2021). Fish oil has been widely applied for the fortification of different food products, an innovative strategy accepted by consumers interested in healthy foodstuffs (Jamshidi, Cao, Xiao, & Simal-Gandara, 2020). The growing demand for fish oil has led to the use of fish processing by-products (mainly cod liver) for oil production by the industry (Liu, Ramakrishnan, & Dave, 2020). At industrial scale, wet pressing is the conventional technique globally applied to obtain fish oil. However, this process involves two important disadvantages, an inefficient oil extraction and the degradation of thermolabile compounds. Due to moderate extraction conditions, enzymatic extraction became an initial alternative for the extraction of oil from fish side streams. Nevertheless, the oil yield was not as efficient as expected (Bruno, Ekorong, Karkal, Cathrine, & Kudre, 2019). Recently, non-conventional extraction techniques such as supercritical fluid extraction, ultrasound-assisted extraction, and microwave-assisted extraction (MAE) have been proposed as potential sustainable methods for oil extraction

from fish by-products (Alfio, Manzo, & Micillo, 2021; Bruno et al., 2019; Khawli et al., 2019; Marsol-Vall, Aitta, Guo, & Yang, 2020).

MAE is based on the use of microwave energy to rapidly heat solid samples in contact with solvents to improve the extraction of compounds (Llompart, Garcia-Jares, Celeiro, & Dagnac, 2018). The increase in temperature causes an increase in pressure in the cells of the sample until a massive disruption of cell membranes, facilitating the transfer of compounds to the solvent (Alfio et al., 2021; Marsol-Vall et al., 2020; Ozogul et al., 2018). As a modern technique, MAE is also appreciated for the possibility of automating the extraction process as well as for using short extraction times and few amount of solvent. A greater extraction yields of bioactive compounds from plant matrices by MAE have been reported (Bagade & Patil, 2019). However, studies related to MAE technique for the extraction of fish oils are scare. For instance, MAE was used to recover oil from edible muscles of different fish species and authors observed different effects of MAE on oil yield and quality (Afolabi, Mudalip, & Alara, 2018; Costa & Bragagnolo, 2017; Ozogul et al., 2018). As far as we know, only one study about MAE and fish by-products oil has been published. In that work, MAE was employed effectively as a pre-treatment for the enzymatic extraction of head oil from the freshwater fish *Labeo rohita* (Bruno et al., 2019). Therefore, there is currently a research gap in this field and further research is required.

The main goal of the present study was to explore, for the first time, the application of MAE technique to recover fish oil enriched in omega-3 PUFAs from salmon side streams. Backbones, heads, and viscera were used to optimize the MAE of oils applying the response surface methodology (RSM). Oil yield as well as nutritional (fatty acid profile) and bioactive (cytotoxic, antioxidant, anti-

inflammatory, and antimicrobial) properties of salmon oils obtained under MAE optimal conditions were evaluated and compared with those of the traditional Soxhlet extraction.

2. Material and methods

2.1. Standards and reagents

The fatty acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U), and the standards dexamethasone, quercetin, and ellipticine were provided by Sigma-Aldrich (St. Louis, MO, USA). Commercial antibacterials methicillin, streptomycin, and ampicillin were purchased from Fisher Scientific (Janseen Pharmaceutical, Belgium) while the commercial antifungal ketoconazole was from Frilabo (Porto, Portugal). Dulbecco's modified Eagle's medium (DMEM), Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS), L-glutamine, penicillin (100 U/mL)/streptomycin (100 mg/mL) solution, Hank's balanced salt solution (HBSS), trypsin–EDTA (ethylenediaminetetraacetic acid) were obtained from Hyclone (Logan, Utah, USA). Muller-Hinton Broth (MHB) and Malt Extract Broth (MEB) were from Biolab® (Budapest, Hungary) while Blood Agar (Sheep blood 7%) was from LiofilChemsrl (Roseto d. Abruzzi (TE), Italy. Trichloroacetic acid (TCA), tris(hydroxymethyl)aminomethane (Tris), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), 2',7'-dihydrodichlorofluorescein diacetate (DCFH-DA), 2,2'-azobis(2-amidinopropane) dihydrochloride (APPH), and sodium nitrate were also from Sigma-Aldrich. *p*-Iodonitrotetrazolium chloride (INT) and sodium sulfate were purchased from Panreac Applichem (Barcelona, Spain). Sulfuric acid (98%), n-hexane (95%), methanol, toluene, and diethyl ether were provided by Fisher Scientific (Leicestershire, UK). Water was treated through a

Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Fish material and sample preparation

Salmon backbones, heads, and viscera were provided by the Department of Nutrition and Feed Technology of the Nofima Food, Fisheries and Aquaculture Research Institute (Bergen, Norway). The salmon side stream materials were collected fresh from the filleting factory at Sotra Seafood (Oygaraden, Norway), stored frozen (-80 °C for 48 h) and freeze-dried (-56 °C for 72 h). Lyophilized samples were then vacuum packed and transported under refrigeration conditions to the Mountain Research Center (CIMO, Bragança, Portugal). Each type of salmon side stream was minced in order to obtain a smaller particle size for a better sample homogenization. Fish samples were stored at -25 °C until use.

2.3. Experimental design for MAE optimization

A central composite rotatable design (CCRD) combining five-levels of the independent variables X_1 (time, t, 1–20 min), X_2 (microwave power, P, 50–1000 W), and X_3 (solid/liquid ratio, R, 80–120 g/L) was implemented to optimize the extraction of fatty acids from salmon by-products using RSM. These variables and the respective range of values were selected based on preliminary experiments and literature data (Afolabi et al., 2018; Costa & Bragagnolo, 2017; Ozogul et al., 2018). Design-Expert software, Version 11 (Stat-Ease, Inc., Minneapolis, MN, USA) was used to generate the experimental points of the CCRD design, which included eight factorial points, six axial or star points, and

six replicated center points. The 20 runs were randomized to minimize the effects of unexpected variability.

2.4. Extraction methods

2.4.1. Soxhlet extraction (SE)

A conventional SE was performed using 5 g of fish sample and ~250 mL of n-hexane in a laboratory Soxhlet extractor (Behr Labor Technik™, Düsseldorf, Germany) at 80 °C for 6 h. Following the extraction process, the solvent was completely removed using a rotary vacuum evaporator (Hei-VAP Silver 4, Schwabach, Germany) with a water bath at 40 °C. All extractions were carried out at least in duplicate.

2.4.2. Microwave-assisted extraction (MAE)

The MAE process was conducted in a Nμ Tech microwave extractor (NuWav-Uno, Sonilex, West Bengal, India) equipped with a circulating cool-water reflux system, time controller, and manual electromagnetic stirrer. The Intelli-System's internal also controls the microwave power conditions (max. 1000 W) thus eliminating any temperature overshoot. The quantities of fish sample (backbones, heads or viscera) and solvent (n-hexane) required to obtain the designed solid-liquid ratio with a constant volume of 50 mL were introduced into a flask, which was placed in the microwave chamber. For each extraction (performed according to the CCRD), time and power were set by the digital panel. After extraction, samples were filtered and the solvent was removed as described for SE.

2.5. Oil yield determination

For both extraction methods, the resulting amount of oil from salmon side streams was calculated gravimetrically as follows: extraction yield of oil (%) = (weight of extracted oil / weight of fish material) × 100. Since lyophilized fish samples were used, percent recovery values refer to dry weight (dw).

2.6. Characterization of the fatty acid profile

The fatty acids of salmon oils extracted by SE and MAE were subjected to methyl esterification according to Reis et al. (2012) with some modifications. Thus, 5 mL of a catalytic solution of methanol:sulfuric acid:toluene (2:1:1, *v*/*v*/*v*) were mixed with 500 μL of oil sample and kept overnight in a bath at 50 °C and 60 rpm. Then, 3 mL of water and 3 mL of diethyl ether were added and vortexed for 30 s. After phase differentiation, the obtained FAMEs were recovered from the upper layer, mixed with sodium sulfate and filtered (0.22 μm nylon filters). All samples were diluted 1/10 in ethyl ether before being stored at -20 °C until analysis.

Fatty acid profile analysis was performed using a gas-liquid chromatography with flame ionization detection (GC-FID). A DANI model GC 1000 instrument (Milan, Italy) equipped with a split/splitless injector, a FID detector, and a Macherey-Nagel capillary column (30 m × 0.32 mm ID × 0.25 μm d_f) was employed for chromatographic determinations. Hydrogen at a flow rate of 4 mL/min was used as gas carrier. The injector and detector temperatures were 250 °C and 260 °C, respectively. The oven temperature program was as follows: 50 °C for 2 min, increased to 125 °C at 30 °C/min, increased to 160 °C at 20 °C/min, increased to 180 °C at 3 °C/min, increased to 200 °C at 20 °C/min and increased to 220 °C for 15 min. One microliter of sample was injected and the

fatty acids identification was carried out by comparing the relative retention times of FAMEs peaks from salmon oils with a reference standard FAMEs mixture. For data recorded and processed, CSW 1.7 software (DataApex 1.7) was used. The results were expressed in relative percentage of each fatty acid.

2.7. Response variables, extraction process modelling and statistical analysis

The fatty acid profile of the lipid extracts obtained with the 20 runs of the experimental design was analyzed by GC-FID for each sample (results in **Tables A.1–A.3** provided in supplementary material). Since fatty acids were not significantly affected by the applied MAE conditions, these were not used as response variable. Therefore, the oil yield (g/100 g dw) was the dependent variable selected to optimize the extraction process.

The response surface models were fitted by means of least squares calculation using the second-order polynomial equation (1):

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{i=1}^{n} b_{ii} {X_i}^2 + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j \qquad \text{Eq. (1)}$$

where Y corresponds to the dependent variable to be modelled (oil yield), X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of the linear effect, b_{ii} is the coefficient of the quadratic effect, b_{ij} is the coefficient of the interaction effect, and n is the number of variables ($n = 3$).

Fitting procedures, coefficient estimates, and statistical analysis were performed using Design-Expert software. Analysis of variance (ANOVA) was used to assess the significance of the models and of all the terms that make up the models, as well as the lack-of-fit. Only the statistically significant terms ($p < 0.05$) were used in the models' construction (except those required to ensure

hierarchy). Coefficient of determination (R^2), adjusted coefficient of determination (R^2_{adj}), and adequate precision were used to estimate the adequacy of the polynomial equation to the response. Since the lack-of-fit measures the quality of the model's fit to the experimental data, it must be non-significant ($p > 0.05$).

2.8. Biological activities of salmon oils obtained under optimized conditions

2.8.1. Cell lines and culture conditions for cellular assays

Different cell lines were used to evaluate different *in vitro* bioactivities of salmon oils extracted by SE and optimized MAE: Human gastric (AGS), colorectal (CaCo-2), breast (MCF-7), and lung (NCI-H460) cancer cells; non-tumor porcine liver primary culture (PLP2) cells, as well as murine macrophage (RAW 264.7) cells. AGS, Caco-2 and RAW cells were purchased from the European Collection of Authenticated Cell Cultures (ECACC) while MCF-7 and NCI-H460 cells were provided by Leibniz-Institute DSMZ. In compliance with the authors (Mandim et al. 2022), the primary culture PLP2 was established in the laboratory using porcine liver tissue in order to obtain tissue explants for the proliferation of non-tumor liver cells.

Tumor and non-tumor cell lines were grown and maintained in RPMI 1640 while RAW cells did so in DMEM. Both culture mediums were supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL). All cell types were incubated in culture flasks (75 cm^2) at 37 °C, a CO_2 flow of 5% and under a humid atmosphere. The medium was changed every 2-3 days until the cell monolayer reached 70-80% confluence. Afterward, tumor and non-tumor cells were harvested by trypsinization whilst

murine macrophages were scraped. Then, cells were seeded in 96-well plates at a density (cells/cm^2) depended on the cell type.

Tumor cells were employed for cytotoxic evaluation. PLP2 cells were used to verify that the effect of salmon oils only affected to tumor cells. RAW cells were used for antioxidant and anti-inflammatory tests. For all cellular assays, the incubation conditions were the same as for cell culture maintenance (37 °C/ 5% CO_2/ humid atmosphere). All assays were performed with at least two technical replicates in two independent days.

2.8.2. Fish oil sample preparation for cellular assays

Salmon backbones, heads, and viscera oils extracted by SE and optimized MAE were used for cell bioactivity studies. Each oil sample was previously dissolved in DMSO:H_2O (1:1, *v*/*v*) at a concentration of 8 mg/mL (stock solution). Serial dilutions were then prepared in a microplate at a concentration range of 0.125–8 mg/mL, before performing the specific cell assay. The final concentrations of salmon oils tested were 6.25–400 μg/mL for cytotoxic and anti-inflammatory cell assays, while 500–2000 μg/mL were used for the cellular antioxidant activity (CAA) assay.

2.8.3. Cytotoxic activity

Tumor and non-tumor cells mentioned above were used to evaluate the cytotoxic effect of salmon oils extracted by SE and MAE. All cell lines were seeded at a density of 10,000 cells/well. According to the procedure previously described by Mandim et al. (2022), 190 μL of cell suspension were added to 10 μL of different concentrations of oil solutions and kept for 40 min at room temperature. After verifying the correct cellular distribution and adherence in

the wells, the plates were incubated at 37 °C for 72 h. Then, sulforhodamine B (SRB) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) for the cytotoxicity screening of compounds to adherent cells was applied (Vichai & Kirtikara, 2006). For this, 100 μL of cold 10% (*w*/*v*) TCA were added to the wells and the plates were incubated at 4 °C for 1 h. Next, TCA was removed and adhered cells were washed three times with water and dried. Cells were then stained with 100 μL of 0.057% (w/v) SRB solution for 30 min at room temperature. Excess dye was removed by washing three times with 1% (*v*/*v*) acetic acid. Once the plates were dried, 200 μL of 10 mM Tris base were used to dissolve the cells and the absorbance at 510 nm of protein-bound dye was measured in a microplate reader (Biotek ELX800, Bio-Tek Instruments, Inc., Winooski, VT, USA). For each cell line, plated cells without salmon oil were used as a negative control and their absorbance values were considered time zero for the calculations. In addition, the antitumor drug ellipticine (10 mM) was used as a positive control. The results were expressed as GI_{50} values (oil concentration capable of inhibiting 50% of cancer cell growth).

2.8.4. Antioxidant activity

The evaluation of the antioxidant capacity of salmon oils was carried out using the cellular antioxidant activity (CAA) assay described by (Wolfe & Rui, 2007), with some modifications. This method is based on the determination of intracellular reactive oxygen and nitrogen species (ROS/RNS) by measuring the ability of compounds to prevent the oxidation of intracellular dihydrodichlorofluorescein ($DCFH_2$), which is easily oxidizable to fluorescent dichlorofluorescein (DCF) by peroxyl radicals ($ROO^{\bullet}$). The RAW 264.7 cells were seeded at a density of $2x10^4$ cells/well and incubated for 48 h. After this

period, the culture medium was discarded and the cells were washed twice with 100 μL of HBSS (100 mM, pH 7.4). Then, 200 μL of oil at different concentrations and 100 μL of DCFH-DA (50 μM) were added to each well and co-incubated at 37 °C for 1 h. After incubation, the mixtures were removed and cells were washed twice with 100 μL of HBSS. Next, 100 μL of AAPH (600 μM), an azo compound that generates ROO• by thermal decomposition, were added to each well. The reaction was performed at 37 °C in a plate reader (Biotek FLX800, Bio-Tek Instruments, Inc., Winooski, VT, USA) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The fluorescence was recorded every 5 min over 1 h and the differences of areas under the curve (AUC) were considered for calculations. Therefore, CAA values were calculated according to Equation (2), where ∫ AUCs is the integrated area under the sample fluorescence *versus* time curve and ∫ AUCc is the integrated area from the control curve.

$$\text{CAA unit} = 100 - (\int \text{AUCs} / \int \text{AUCc}) \times 100 \qquad \text{Eq.(2)}$$

The results were expressed as a percentage of inhibition of the oxidation reaction. Quercetin was used as a positive control.

2.8.5. Anti-inflammatory activity

The inhibition of nitric oxide (NO) produced by LPS-stimulated RAW 264.7 cells was determined according to Sobral et al. (2016), with some modifications. RAW cells were seeded at $1.5x10^5$ cells/well and growth overnight under incubation conditions in order to attach to the plate. Then, cells were exposed to the different concentrations of each fish oil (15 μL) and they were incubated for 1 h. The anti-inflammatory corticosteroid dexamethasone (50 mM) was applied as a positive control while salmon oils in the absence of LPS were

considered as a negative control. Macrophage stimulation was carried out by adding 30 μL of LPS (1 mg/mL in DMEM) followed by 24 h incubation. After this period, 100 μL of the cell culture supernatants and 100 μL of the standard calibration curve (sodium nitrate, 1.6 - 100 mM, $y = 0.0065X + 0.1282$; $R^2 = 0.9991$) were placed into a new 96-well plate. Next, the quantification of NO was performed using a Griess Reagent System kit (Promega, Madison, WI, USA). After reaction, the absorbance of the NO produced was measured at 540 nm in the microplate reader referred above and values of the samples were compared to those of the standard. The concentrations of salmon oils needed to inhibit 50% of the NO production were determined and, therefore, the results were expressed as IC_{50} (mg/mL).

2.8.6. Antibacterial activity

Eight bacteria related to food contamination were selected to evaluate the antibacterial activity of salmon oils: *Bacillus cereus* (ATCC 11778), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 49741), *Listeria monocytogenes* (ATCC 19111), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), *Staphylococcus aureus* (ATCC 11632), and *Yersinia enterocolitica* (ATCC 8610). All microorganisms were obtained from Frilabo (Porto, Portugal). In order to maintain the exponential growth phase, Gram positive bacteria were incubated in fresh Blood Agar (7% sheep blood) and Gram negative bacteria in Muller Hilton Agar, all of them at 37 ºC for 24 h before the analysis. Then, bacterial suspensions were prepared at 1.5×10^6 CFU/mL.

The broth microdilution method (96-well plates) and the rapid INT colorimetric assay previously described by Pires et al. (2018) were applied to determine the antibacterial potential of the samples. Salmon oils were firstly

dissolved 50% in MHB (0.5% Tween 80). Through serial dilutions, they were tested from 50 to 0.39%. Briefly, 90 μL of each oil concentration were mixed with 10 μL of bacterial suspension and the microplates were incubated under shaking at 37 °C for 18-24 h. Afterward, 40 μL of INT dye (0.2 mg/mL) was added to each well and plates were incubated again at 37 °C for 30 min. Then, bacterial growth was monitored by changing the color of viable cells from yellow to pink. The lowest concentration of oil, which showed no color change, was considered as minimum inhibitory concentration (MIC). Ampicillin (20 μg/mL) and streptomycin (1 mg/mL) were used as positive controls for all bacteria, except for *S. aureus* where methicillin (1 mg/mL) was employed. Two negative controls (MHB and fish oil sample) were also prepared for each inoculum.

The determination of the minimum bactericidal concentration (MBC) was performed by transferring 10 μL of liquid from each well that showed no color change into solid medium (Blood Agar, 7% sheep blood) and incubated at 37 °C for 24 h. MBC was defined as the lowest concentration required to kill bacteria.

2.8.7. Antifungal activity

Two foodborne fungi were used to assess the antifungal capacity of salmon oils: *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404). Both fungal strains were also provided by Frilabo (Porto, Portugal). The micromycetes were cultured in malt agar plates at 25 ºC for 72h. Next, the spores were recovered from the agar surface with sterile 0.85% saline containing 0.1% Tween 80 (*v*/*v*) and fungal spore suspensions were adjusted at 1.0×10^5 UFC/mL.

The antifungal activity was carried out in 96 well-plates by a serial dilution technique according to Heleno et al. (2013), with some modifications. Salmon oils were prepared in MEB with 0.5% Tween 80 at concentrations ranging from

50 to 0.39% (*v*/*v*). Briefly, 90 μL of each oil concentration were mixed with 10 μL of fungal suspension and the microplates were incubated at 25 °C for 72 h. The growth of fungi was then observed under binocular microscope and the lowest oil concentrations without visible growth were defined as MICs. The minimum fungicidal concentration (MFC) was performed using serial subculture technique from the wells where fish oils showed antifungal capacity. Two microliters were transferred to microplates containing 100 μL of MEB per well and incubated at 25 °C for 72 h followed by additional incubation (25 °C/72 h). The lowest concentration with no visible fungal growth was defined as MFC indicating 99.5% killing of the original inoculum. Ketoconazole (1 mg/mL) was used as positive control for both MIC and MFC.

2.9. Statistical analysis

Experimental data were subjected to one-way analysis of variance (ANOVA) to determine the significance differences among samples. Tukey Honestly Significant Difference (HSD) multiple range test ($p < 0.05$) was applied. The Statgraphics Centurion XVI® software (Statpoint Technologies, Inc., The Plains, VA, USA) was used for statistical analysis.

3. Results and discussion

3.1. MAE process optimization

The influence of the independent variables time, microwave power, and solid/liquid ratio on the extraction of oil from salmon side streams (backbones, heads, and viscera) by MAE was studied using RSM. The results of the 20 experimental runs carried out under the CCRD design matrix for the oil content obtained from each salmon by-product are shown in **Table 1**.

Table 1. Experimental responses obtained under the extraction conditions defined by the CCRD design for the extraction oil yield from salmon side streams and statistical information of the models fitting procedure.

Runs	RSM experimental domain			Experimental response: oil yield (%)		
	Time (min)	Power (W)	Ratio (g/L)	Backbones	Heads	Viscera
1	5	243	88	34.67	38.28	49.28
2	16	243	88	43.24	37.92	48.45
3	5	807	88	42.83	32.68	56.74
4	16	807	88	43.15	40.54	66.29
5	5	243	112	39.84	38.10	57.05
6	16	243	112	36.23	37.21	61.24
7	5	807	112	38.23	30.99	58.20
8	16	807	112	35.96	34.62	65.11
9	1	525	100	35.37	30.12	52.87
10	20	525	100	38.89	32.03	65.71
11	10.5	50	100	39.78	50.63	62.89
12	10.5	1000	100	38.99	25.63	71.10
13	10.5	525	80	44.79	41.51	43.06
14	10.5	525	120	35.58	34.49	60.72
15	10.5	525	100	35.58	38.28	61.86
16	10.5	525	100	33.91	38.85	64.94
17	10.5	525	100	40.24	39.82	62.96
18	10.5	525	100	39.90	38.91	66.06
19	10.5	525	100	42.86	41.21	63.95
20	10.5	525	100	38.38	39.09	61.83
	Statistical data					
	Model F-value			25.40	126.70	83.41
	Lack-of-fit			0.2443	0.5678	0.5857
	R^2			0.9486	0.9713	0.9570
	R^2_{adj}			0.9113	0.9636	0.9455
	Adequate precision			19.65	34.19	31.04
	Coefficient of variance (%)			3.49	1.12	2.71

The oil yield range from approximately 34–45%, 26–51%, and 43–71% for backbones, heads, and viscera, respectively. In general, the combination of extraction parameters to obtain the best oil recovery was different for each sample, which revealed the influence of the matrix intrinsic nature in the MAE process, as well as the preference for conducting an optimization study (Llompart et al., 2018).

The response values in **Table 1** were fitted to the second-order polynomial Equation (1) using Design-Expert software. The significant parameters, assessed at a 95% confidence level, were used in the development of the theoretical models, as well as the non-significant ones needed for the hierarchy. Thus, a regression equation capable of explaining the effects of the independent variables of the MAE process on the oil yield response obtained from each salmon side stream was constructed. These polynomial models, expressed in coded values, are presented in Equations (3)–(5):

For salmon viscera:

$$Y = 63.6 + 3.0t + 3.2P + 3.4R - 1.7t^2 + 1.0P^2 - 4.8R^2 + 1.6tP - 2.5PR \quad \text{Eq. (3)}$$

For salmon backbones:

$$Y = 39.8 + 0.7t - 2.1SR - 0.8t^2 - 1.6tR \quad \text{Eq. (4)}$$

For salmon heads:

$$Y = 39.0 + 0.3t - 3.5P - 1.0R - 3.2t^2 \quad \text{Eq. (5)}$$

The coefficients of each term in the polynomial models illustrate the effect of independent variables (*t*, *P*, and *R*) on the oil yield response and indicate the expected change in response per unit change in factor value when the other factors are held constant. The higher the parametric value (regardless of its sign),

the more significant the independent variable will be. Thus, the extraction trends are translated by the model equations complexity and the replacement of *t*, *P*, and *R* values in these models result in a theoretical value for the oil yield.

As observed in **Table 1**, all models presented a non-significant lack-of-fit ($p > 0.24$) and an adequate precision greater than 19, which indicates that the model equations adequately describe the effects of the independent variables on the oil yield. The variables involved in the extraction also explained the variability of the response, since R^2 and R^2_{Adj} values higher than 0.94 and 0.91, respectively, were obtained. Thus, the three models were statistically valid and suitable to navigate the design space in the optimization stage.

Information regarding the general effects of the independent variables on oil extraction from salmon side streams can be inferred from the complexity of Equations (3)–(5). For backbones, the variables solid/liquid ratio and time significantly affected the oil yield through liner and/or quadratic terms (**Fig. 1**), and also interacted significantly, while no significant effects were induced by microwave power. For heads, solid/liquid ratio was the least influential parameter on oil extraction. Increasing microwave power negatively affected oil recovery trough a linear effect, while moderate extraction times did so positively due to the notable quadratic term. Equation (5) describing the MAE of oil from viscera was the most complex, containing linear and quadratic terms for the three factors involved in the extraction, mainly solid/liquid ratio. The interaction of microwave power with solid/liquid ratio and time also made up this model. For this matrix, higher microwave powers favored the oil recovery rate, while low values were preferable for the other two side streams, which also demanded a lower solid/liquid ratio.

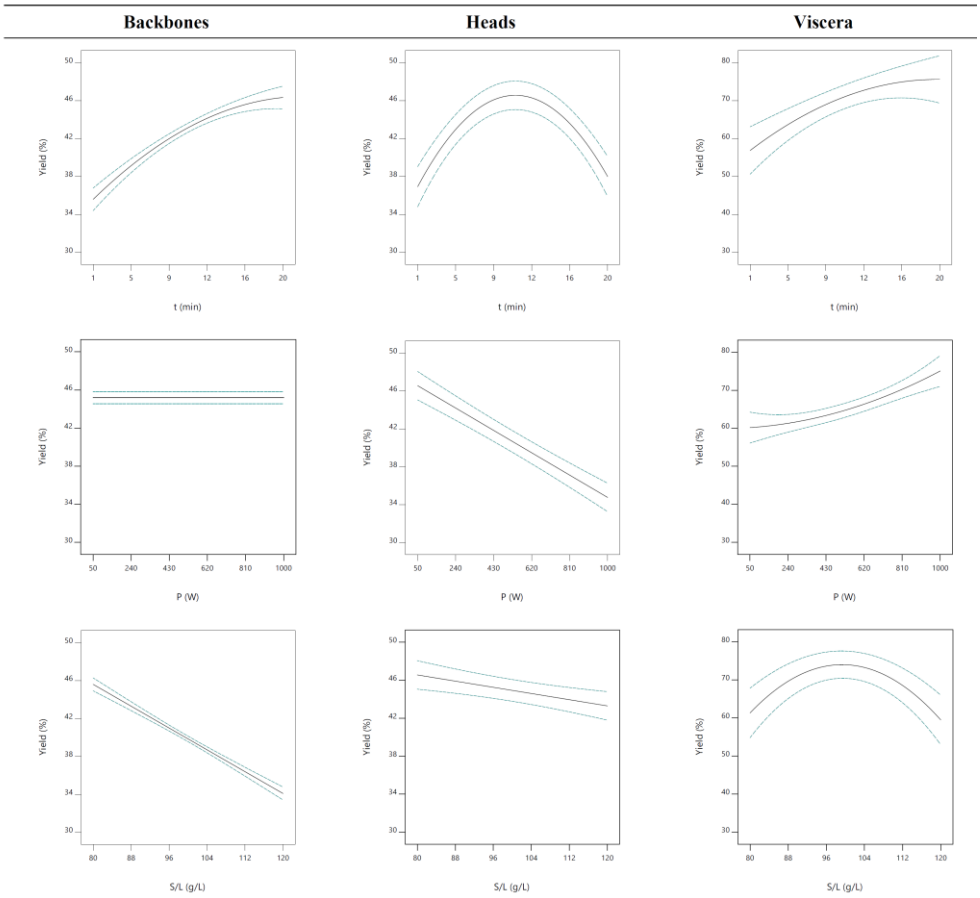

Figure 1. 2D response graphs for the effects of the independent variables on microwave-assisted extraction of oil from salmon side streams.

All these extraction trends (illustrated in **Fig. 1**) confirm the strong influence of the matrix type in the MAE process, as well as the importance of carrying out an optimization of this process using RSM.

The 3D response surface graphs constructed to visually interpret the effect of the time-power-solid/liquid ratio combination on the analyzed response (oil yield) are illustrated in **Fig. 2**. In each graph, the excluded variable was fixed at its optimal value. As already discussed, the oil extraction process from each salmon side stream showed specific extraction trends. Thus, in order to find a set of conditions that maximized the extraction, the three independent variables were set within the experimental range, while the response was set at the maximum. The model-predicted MAE conditions that maximized the oil yield to optimal values were as follows: 291.9 W for 14.6 min at 80.1 g/L for backbones; 50.0 W for 10.8 min at 80.0 g/L for heads; and 960.6 W for 14.3 min at 99.5 g/L for viscera.

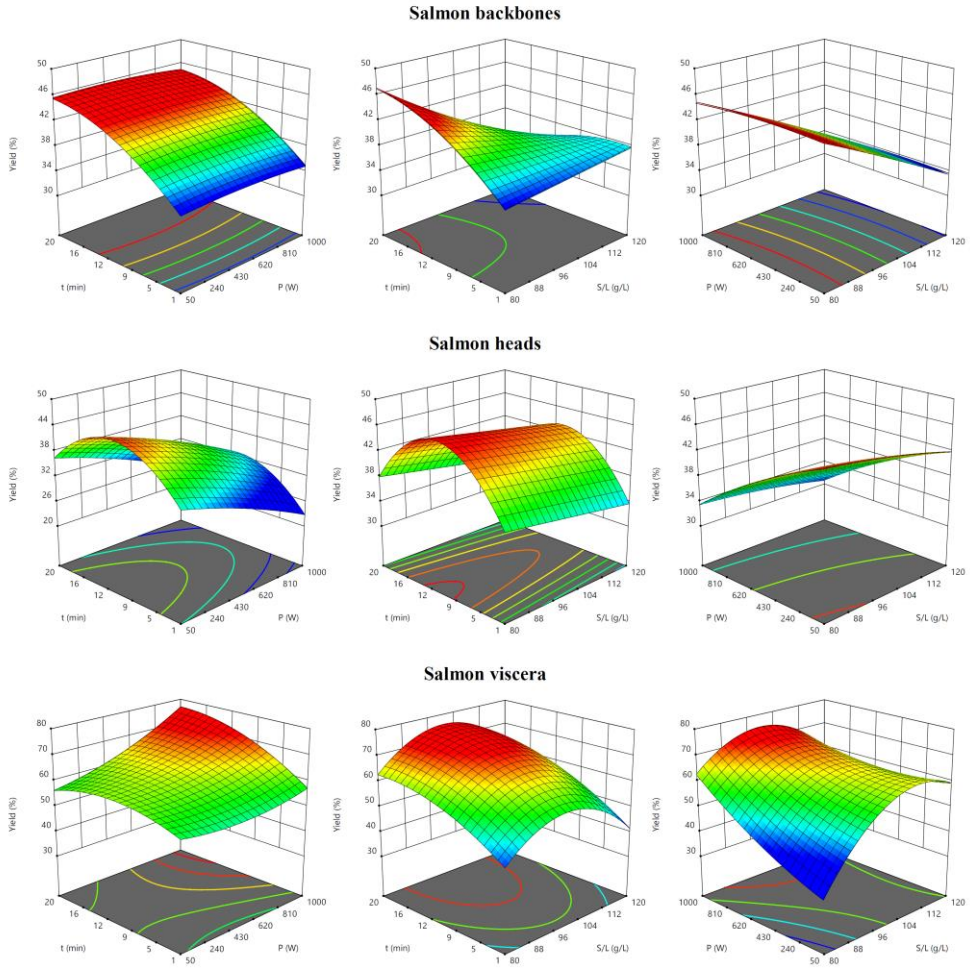

Figure 2. Response surface graphs for the combined effects of the independent variables on oil yield (g/100 g dw) obtained by microwave-assisted extraction from salmon side streams.

3.2. Fish oil extraction yield

The oil yield results of salmon side streams after applying SE and the optimized MAE extraction conditions are shown in **Table 2**. The Soxhlet method resulted in extraction yields of 56.81±1.01%, 55.76±1.83%, and 76.85±2.49% for backbones, heads, and viscera, respectively. Regarding MAE technique, oil yields were 39.36±0.33% for backbones, 38.42±1.37% for heads, and 71.00±2.23% for viscera. For both extraction methods, the highest amount of oil was found in viscera, while there were no differences of oil content among backbones and heads. Aspevik et al. (2021) observed similar results of lipid content in raw salmon by-products using the Bligh and Dyer method. More quantity of oil was also determined in salmon viscera than in a mixture of salmon heads and frames by Liu et al. (2020). In addition, SE with n-hexane as a solvent was applied to dried salmon viscera (excluding liver), obtaining an oil percentage of 65% (Rincón-Cervera, Villarreal-Rubio, Valenzuela, & Valenzuela, 2017).

In addition, Costa and Bragagnolo (2017) optimized and validated a MAE method for fish lipids using tilapia fillet. Then, optimal extraction conditions were applied to fish species with different lipid content and compared to conventional Folch method. The results did not show differences in the amount of oil obtained by MAE and Folch extraction. It is noteworthy that no information was found in the literature on the use of MAE to recover oil from fish processing by-products.

Table 2. Yield and fatty acid profile of oil extracted from salmon side streams using Soxhlet extraction (SE) and optimized microwave-assisted extraction (MAE).

	Backbones		**Heads**		**Viscera**	
	SE	**MAE**	**SE**	**MAE**	**SE**	**MAE**
Oil (g/100 g dw)	56.81±1.01 b	39.36±0.33 a	55.76±1.83 b	38.42±1.37 a	76.85±2.49 d	71.00±2.23 c
			Fatty acid profile (%)			
C12:0	0.42±0.03 ab	0.58±0.02 c	0.36±0.04 a	0.46±0.01 b	nd	nd
C14:0	2.47±0.05 ab	2.77±0.05 b	2.54±0.26 ab	2.72±0.10 b	2.13±0.09 a	2.09±0.03 a
C15:0	0.18±0.02 a	0.18±0.01 a	0.18±0.02 a	0.18±0.01 a	0.17±0.01 a	0.16±0.01 a
C16:0	10.09±0.06 ab	10.12±0.06 ab	10.32±0.48 ab	10.47±0.14 b	9.97±0.12 ab	9.58±0.07 a
C16:1	2.51±0.02 a	2.69±0.01 b	2.59±0.01 ab	2.58±0.06 ab	2.05±0.01 a	2.08±0.06 a
C17:0	nd	nd	nd	nd	0.14±0.01 a	0.14±0.01 a
C17:1	nd	nd	nd	nd	0.11±0.01 a	0.10±0.01 a
C18:0	2.83±0.06 a	2.81±0.03 a	2.86±0.03 a	2.92±0.08 a	3.15±0.06 b	3.00±0.04 ab
C18:1n9c	38.69±0.05 c	38.17±0.19 bc	38.07±0.11 bc	37.78±0.26 ab	37.07±0.37 a	37.65±0.13 ab
C18:2n6t	nd	nd	nd	nd	0.21±0.02 b	0.13±0.04 a
C18:2n6c	14.57±0.03 a	14.43±0.06 ab	14.05±0.16 b	14.14±0.20 ab	18.30±0.03 c	18.30±0.08 c
C18:3n6	nd	nd	nd	nd	0.12±0.03 a	0.14±0.01 b
C18:3n3	7.19±0.11 a	7.23±0.04 a	6.99±0.04 a	6.96±0.01 a	8.96±0.04 b	9.05±0.12 b
C20:0	nd	nd	nd	nd	0.18±0.01 b	0.14±0.01 a
C20:1	2.79±0.01 c	2.79±0.04 cd	2.65±0.01 d	2.71±0.03 d	2.35±0.06 b	2.20±0.03 a
C20:2	1.01±0.01 a	1.11±0.04 ab	1.09±0.03 ab	1.08±0.08 ab	1.07±0.01 ab	1.21±0.06 b

Table 2. *Cont.*

C20:3n6	0.23±0.01 a	0.22±0.04 a	0.27±0.02 a	0.25±0.01 a	0.23±0.01 a	0.24±0.01 a
C20:4n6	0.28±0.01 b	0.28±0.01 b	0.31±0.01 c	0.32±0.01 c	0.23±0.01 a	0.23±0.01 a
C20:3n3	0.68±0.01 a	0.64±0.02 a	0.64±0.01 a	0.64±0.01 a	0.82±0.02 b	0.80±0.02 b
C22:0	0.09±0.01 a	0.10±0.01 a	nd	nd	0.21±0.02 b	0.20±0.01 b
C20:5n3	5.92±0.08 b	5.86±0.04 b	6.14±0.31 b	6.16±0.04 b	3.79±0.01 a	3.72±0.06 a
C22:2	0.09±0.01 b	0.09±0.01 b	0.07±0.01 a	0.09±0.01 b	0.09±0.01 b	0.10±0.01 b
C24:1	1.74±0.07 d	1.57±0.03 cd	1.61±0.08 cd	1.52±0.07 bc	1.30±0.04 ab	1.29±0.02 a
C22:6n3	8.21±0.24 b	8.35±0.04 b	9.25±0.28 c	9.04±0.07 c	7.31±0.048 a	7.45±0.02 a
Total fatty acid class						
SFA	16.09±0.05 a	16.56±0.16 a	16.16±0.82 a	16.75±0.31 a	15.94±0.28 a	15.30±0.08 a
MUFA	45.73±0.05 d	45.22±0.20 cd	44.91±0.03 bc	44.59±0.09 b	42.88±0.28 a	43.31±0.05 a
PUFA	37.08±0.01 a	37.02±0.02 a	38.83±0.84 b	38.66±0.22 b	41.06±0.03 c	41.26±0.13 c
n6/n3	0.69±0.02 b	0.68±0.01 b	0.64±0.01 a	0.65±0.01 a	0.92±0.01 c	0.91±0.01 c

C12:0 lauric acid; C14:0 mystiric acid; C15:0 pentadecylic acid; C16:0 palmitic acid, C16:1 palmitoleic acid; C17:0 heptadecanoic acid; C17:1 heptadecenoic acid; C18:0 stearic acid; C18:1n9c oleic acid; C18:2n6t linolelaidic acid C18:2n6c linoleic acid; C18:3n6 γ-linoleic acid; C18:3n3 α-linolenic acid; C20:0 arachidic acid; C20:1 eicosenoic acid; C20:2 eicosadienoic acid; C20:3n6 eicosadienoic acid; C20:4n6 arachidonic acid; C20:3n3 eicosatrienoico; C22:0 docosanoic acid; C20:5n3 eicosapentaenoic acid (EPA); C22:2 docosadienoic acid; C24:1 nervonic acid; C22:6n3 docosahexaenoico acid (DHA); nd: not detected; SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids; n6/n3: omega-6/omega-3 ratio. In each row, different letters mean statistical differences among samples.

Considering SE as a reference method for the extraction of total lipid content, the optimization of MAE allowed to recover 69% of total fat content in salmon backbones and heads as well as 92% in salmon viscera, which was also carried out in less than 15 min for all samples. The reduction of extraction time is one of the main advantages linked to the MAE technique (Llompart et al., 2018). In this work, 360 min were required for SE while 10-14 min (depending on the sample) were used for MAE. Microwaves are supposed to cause rupture of the fish tissues, allowing the oil to be released and transferred to the solvent more quickly and efficiently than traditional methods (Costa & Bragagnolo, 2017). The variety of tissues that constitute fish by-products compared to fish muscle tissue could influence the oil extraction capacity in short periods of time as used here in MAE. In the case of viscera sample, it took approximately 25 times more extraction time to recover 7% more oil. Therefore, MAE could be considered as an interesting non-conventional technique to recover oil from salmon side streams, especially for salmon viscera.

3.3. Fatty acid profiles

Fatty acid composition of salmon side streams oils obtained by SE and optimal MAE are listed in **Table 2**. For all samples, the main fatty acids identified were oleic acid (C18:1n9c, ≈ 38%), linoleic acid (C18:2n6c, 14-18%), and palmitic acid (C16:0, ≈ 10%). Equal profiles (oleic > linoleic > palmitic) in salmon oils obtained by enzymatic hydrolysis of heads and mixture of viscera and backbones were previously reported (Liu et al., 2020). Salmon head oil extracted after heat treatment (90 °C for 1 h) and protein coagulation also showed the same lipid profile (Inguglia et al., 2020). Despite the use of different extraction techniques, the composition of the most representative fatty acids

seems to remain stable in heads, viscera and backbones of Atlantic salmon. According to Rincón-Cervera et al. (2017), application of temperatures above 100 °C (not used in these studies) could affect the fatty acids that are more susceptible to thermal degradation and, therefore, modify the lipid profile. In addition, microwave energy did not alter the fatty acid composition of fish muscle (Costa & Bragagnolo, 2017).

Contents of saturated fatty acids (SFA, 15-17%), monounsaturated fatty acids (MUFA, 43-45%), and polyunsaturated fatty acids (PUFA, 37-41%) were similar for both extraction methods and salmon oil samples. Different percentages of the fatty acids classes of Atlantic salmon by-products have been reported (Gbogouri, Linder, Fanni, & Parmentier, 2006; Inguglia et al., 2020; Liu et al., 2020), which may due to the various extraction methods and solvents used.

The fatty acids docosahexaenoic (DHA) and eicosapentaenoic (EPA), characteristic of fatty fish, were found in the ranges of 4-6% and 7-9%, respectively. Different values of DHA and EPA in salmon side stream materials have been previously reported (Gbogouri et al., 2006; Inguglia et al., 2020; Liu et al., 2020). It should be noted that salmon viscera oil had a higher amount of linoleic and linolenic acids as well as a lower content of DHA and EPA compared to salmon head and backbone oils, which showed very few differences in their complete lipid profile. According to the European Regulation on nutritional and health claims made on foods, "high omega-3 fatty acids" content is attributed to samples with at least 80 mg of DHA+EPA/100 g of product and per 100 Kcal (Regulation (EU) No 1924/2006) . Therefore, lipid fraction from salmon backbones, heads, and viscera here investigated complied with this

health claim, making them interesting candidates for developing products with a healthier lipid profile through fortification.

Based on the Food and Agriculture Organization (FAO) recommendations, the values of n6/n3 ratio for human diet should be below 4 (Food and Agriculture Organization of the United Nations, 2010). All samples studied ranged from 0.64-0.92, confirming the nutritional quality of salmon by-products oils. As can be seen from **Table 2**, no differences in fatty acid composition were observed among Soxhlet and microwave extractions. Therefore, the conditions of power, solid/liquid ratio, and extraction time used in MAE did not influence the lipid profile of oils extracted from salmon by-products. In this sense, there were also no differences in the fatty acid profile of lipids extracted by solvent extraction and controlled proteolysis of salmon heads (Gbogouri et al., 2006).

3.4. Bioactivities of salmon oils obtained by SE and optimal MAE conditions

Data regarding cytotoxic, antioxidant, and anti-inflammatory activities of salmon oils extracted by SE and optimal MAE are presented in **Table 3**. All tested oils showed cytotoxic effects against all studied tumor cell lines. In general, the oils of each salmon by-product (extracted by Soxhlet and MAE) exhibited similar cytotoxicity for each cell line. Therefore, the extraction method does not seem to be decisive in the cytotoxic activity of the oils. The human adenocarcinoma cells (Caco-2) were the least susceptible to any of the tested oils. However, some studies revealed that fish-oil derived DHA reduced the viability of Caco-2 cells by increasing apoptosis and caspase-3 activity (Ahangar, Sam, Nejati, & Habibian, 2016; Jameel et al., 2019).

Table 3. Cytotoxic, antioxidant, and anti-inflammatory activities of oil extracted from salmon side streams using Soxhlet extraction (SE) and optimized microwave-assisted extraction (MAE).

	Backbones oil		**Heads oil**		**Viscera oil**	
	SE	**MAE**	**SE**	**MAE**	**SE**	**MAE**
	Cytotoxic activity (GI_{50} μg/mL)					
AGS	219.28±19.10 ab	229.64±14.08 b	248.73±11.55 bc	285.83±6.53 c	234.05±14.68 bc	173.37±12.47 a
Caco-2	297.44±28.94 a	303.20±27.23 a	306.44±28.45 a	277.33±24.75 a	363.54±14.18 a	355.53±23.24 a
MCF-7	205.00±5.66 b	207.97±18.61 b	221.85±3.36 b	131.62±7.95 a	231.58±20.98 b	207.34±8.18 b
NCI-H460	142.43±11.66 b	76.22±5.16 a	235.62±18.12 c	144.49±9.31 b	233.37±21.30 c	217.34±15.33 c
PLP2	201.11±13.33 a	230.08±23.62	253.33±14.40 a	241.23±2.41 a	249.48±11.02 a	215.95±9.72 a
	Cellular antioxidant activity (% inhibition of oxidation at 2 mg/mL)					
RAW 264.7	nd	nd	nd	36.07±3.24 a	78.63±7.33 b	76.17±6.62 b
	Anti-inflammatory activity (IC_{50} μg/mL)					
RAW 264.7	34.49±2.05 a	62.76±2.57 cd	50.85±0.81 bc	64.79±4.57 d	42.41±3.79 ab	32.03±3.31 a

GI_{50} values for Ellipticine: 1.23±0.03 μg/mL (AGS), 1.21±0.02 μg/mL (Caco-2), 1.02±0.02 μg/mL (MCF-7), 1.02±0.01 μg/mL (NCI-H460), 1.4±0.1 μg/mL (PLP2). IC_{50} values for Dexamethasone: 6.3±0.4 μg/mL (RAW 264.7). Quercetin: 95.3±4.6% oxidation inhibition at 0,3 μg/mL; nd: not detected. Different letters in each row correspond to significant differences ($p < 0.05$) among oil samples.

The lung cancer cells (NCI-H460) demonstrated the highest susceptibility to the oil samples, especially for salmon backbone oil extracted by MAE (GI_{50} = 76 μg/mL). According to Yin et al. (2017), DHA not only induced the apoptosis of non-small cell lung cancer cells *in vitro*, but also suppressed the migration and invasion of the cells.

Salmon viscera oil obtained by MAE was the most effective oil (GI_{50} = 173 μg/mL) against the proliferation of stomach cancer cells (AGS). In this sense, different types of fish PUFAs decreased the growth of both tumor and non-tumor gastric cell lines (Dai, Shen, Pan, Shen, & Das, 2013). Among all fish oils, salmon head oil obtained by MAE exhibited the highest cytotoxicity (GI_{50} = 131 μg/mL) against the breast cancer cells (MCF-7). Fish DHA and related molecules have also showed antiproliferative effect against triple-negative, luminal, and MCF-7 breast cancer cell lines (Guo, Zhu, Wu, He, & Chen, 2017; Jameel et al., 2019). In addition, a meta-analysis of published research articles during 18 years concluded that fish oil consumption had a protective effect in breast cancer patients (Lachance, Radhakrishnan, Madiwale, Guerrier, & Vanamala, 2020).

Regarding the antioxidant activity, only the highest concentration (2 mg/mL) of some salmon oils were able to inhibit the oxidation reaction. The antioxidant compounds present in salmon viscera oils inhibited more than 75% of the oxidation generated in the *in vitro* macrophage cells. The slight differences in the lipid profile of salmon viscera compared to that of salmon heads and backbones (Table 2) could influence the different antioxidant activity observed. Chemical antioxidant assays have also showed the antioxidant capacity of oil from salmon

belly part, trimmed muscle, backbones, and skin (Haq, Ahmed, Cho, & Chun, 2017) as well as from hake heads (Karoud et al., 2020).

As for the anti-inflammatory potential, all salmon oils demonstrated relevant NO inhibition in LPS-stimulated macrophages. The fish oil concentration needed to inhibit 50% the NO production ranged from 32 to 64 μg/mL. Ahmad et al. (2019) correlated low levels of MUFAs and high levels of EPA and DHA with inhibition of NO and TNFα in LPS-stimulated macrophages, indicating that the overall lipid composition of both edible flesh and by-products from marine sources could influence the anti-inflammatory activity. Therefore, the amount of EPA and DHA found in salmon backbones, heads, and viscera here studied (Table 2) could be partly responsible for the observed anti-inflammatory effect.

Results of antibacterial and antifungal activities of salmon oils extracted by SE and optimal MAE are shown in **Table 4**. The backbone and head oils showed the same inhibition efficiency of bacterial growth for all strains tested without differences among extraction methods. Thus, the highest oil concentration tested (50%) displayed antibacterial activity against both Gram-positive and Gram-negative bacteria. The growth of *E. cloacae*, *E. coli*, *Y. enterocolitica*, *B. cereus*, and *S. aureus* was inhibited with lower concentrations of viscera oil (3.125-25%) than backbone and head oils (50%). There were no differences in the antibacterial activity of viscera oil depending on the extraction technique, except for *Y. enterocolitica*. The different antibacterial capacity of salmon by-products oils could be probably related to their fatty acid composition since the complete lipid profile of viscera oil differs from backbone and head oils, which were similar to each other (**Table 2**).

Table 4. Antibacterial and antifungal activities of oil extracted from salmon side streams using Soxhlet extraction (SE) and optimized microwave-assisted extraction (MAE).

	Backbones oil				**Heads oil**				**Viscera oil**			
	SE		**MAE**		**SE**		**MAE**		**SE**		**MAE**	
Antibacterial activity	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria												
E. cloacae	50	nd	50	nd	50	nd	50	nd	25	nd	25	nd
E. coli	50	nd	50	nd	50	nd	50	nd	25	nd	25	nd
P. aeruginosa	50	nd	50	nd	50	nd	50	nd	50	nd	50	nd
S. enterica	50	nd	50	nd	50	nd	50	nd	50	nd	50	nd
Y. enterocolitica	50	nd	50	nd	50	nd	50	nd	3.125	nd	25	nd
Gram-positive bacteria												
B. cereus	50	nd	50	nd	50	nd	50	nd	12.5	nd	12.5	nd
L. monocytogenes	50	nd	50	nd	50	nd	50	nd	50	nd	50	nd
S. aureus	50	nd	50	nd	50	nd	50	nd	25	nd	25	nd
Antifungal activity	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. brasiliensis	25	nd	50	nd	25	nd	50	nd	50	nd	nd	nd
B. fumigatus	50	10	nd	nd	50	10	nd	nd	nd	nd	nd	nd

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration; nd: not detected; MIC and MBC values for positive controls: streptomycin (0.007-0.01 mg/mL), methicillin (0.007 mg/mL), ampicillin (0.15-0.63 mg/mL), and ketoconazole (0.06-0.5 mg/mL); MFC values for positive controls: Ketoconazole (0.125-1.0 mg/mL

Oil extracted from salmon soft tissue and salmon heads also inhibited the growth of *P. aeruginosa* and *S. aureus* (Inguglia et al., 2020). In addition, pressing and maceration methods used for the extraction of fish oil provided different activity against two bacteria strains (Simplice et al., 2018). Regarding antifungal activity and in contrast to bacterial activity, backbone and head oils appeared to have a greater effect against tested fungi compared to viscera oil. In addition, the oil extracted by Soxhlet seemed to be more effective than the oil obtained by MAE. However, to the best of our knowledge there are no reports regarding the antifungal activity of oil from fish processing by-products. Therefore, these results could be interesting for further research in this field.

4. Conclusions

The MAE technique could be considered as an interesting tool for oil extraction from salmon side streams since it allowed to recover 69% of total lipid content from backbones and heads, as well as 92% from viscera in less than 15 min. Salmon by-product oils have a healthy lipid profile due to their percentages of saturated and unsaturated fatty acids as well as their EPA and DHA content, making them potential candidates as ingredients in fortified food products. According to the displayed bioactivities, in particular for antimicrobial and anti-inflammatory properties, the application of salmon by-product oils could be further exploited beyond the food industry. Overall, this study shows the possibility of valorization of salmon side streams from a circular economy point of view.

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Conflicts of interest: The authors declare no conflict of interest.

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3.5. Sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) head oils recovered by microwave-assisted extraction: nutritional and biological properties

Food Chemistry (under review)

Sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) head oils recovered by microwave-assisted extraction: nutritional and biological properties

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Abstract

Microwave-assisted extraction (MAE) technique was used to obtain oil from European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) heads. The MAE extraction conditions (10.8 min, 50.0 W, 80.0 g/L), previously optimized for salmon heads, allowed the recovery of >50% of the total lipid content for both fish species. Based on their fatty acid composition, the oils presented a healthy lipid profile and were found to be a good source of docosahexaenoic acid (DHA, 11-14%). The fish head oils exhibited the higest cytotoxic effect (GI_{50} = 38-46 μg/mL) against breast cancer cells (MCF-7). Anti-inflammatory potential (IC_{50} = 14-21 μg/mL), cellular antioxidant capacity (29-35% inhibition oxidation) as well as relevant antibacterial and antifungal activity were also observed. These results show that sea bass and gilthead sea bream heads could be exploited for the production of oil with nutritional and bioactive properties from the circular economy point of view.

Keywords: sea bass, sea bream, fish heads, fish oil, fatty acids, microwave-assisted extraction (MAE), biological properties

1. Introduction

The use of fish by-products throughout the world for different purposes, including human consumption is not new. In 1903, million tons of cod heads were used for the elaboration of feed and fertilizer in Norway while fish skin has traditionally been used for clothing, bags, and carrier packs in different Nordic countries. Cleaned fish stomach and fried fish milt in Asia as well as fish liver in Eastern Europe have been usually consumed (Rustad, Storrø, & Slizyte, 2011). Changes in the lifestyle of the population have led to an increased demand for fish fillets rather than whole fish. As a consequence, it is gradually more common to find this type of convenience fish products in the markets of developed countries.

European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) are amongst the main finfish species farmed in the European Union (EU) (The EU Fish Market, 2020). Their consumption is very popular in Mediterranean countries since ancient times, where they are currently being traded as whole fish gutted head-off. After industrial processing, the heads of sea bass and sea bream represent about 19% and 17% of the total fish weight, respectively (Valcarcel, Sanz, & Vázquez, 2020). The increasing transformation of both fish species means a greater amount of heads in the near future, which is an opportunity for their valorization by the fish processing industry.

The nutritional characterization of several by-products from sea bass and sea bream have been recently reported (Munekata et al., 2020; Pateiro et al., 2020; Valcarcel et al., 2020). The heads were found to contain 29-34% and 39-46% fat (dry weight) for sea bass and sea bream, respectively. The extracted oils presented similar fatty acids composition for both fish species, showing a healthy

lipid profile. However, there are no studies in the literature regarding the potential biological activities of oils from sea bass and sea bream side streams.

Since the valorization is an environmentally friendly concept, sustainable technology must be used for the production of value-added products from underutilized natural resources (Chemat et al., 2020). In this sense, several non-conventional techniques have been investigated to recover valuable compounds from seafood processing by-products (Bruno, Ekorong, Karkal, Cathrine, & Kudre, 2019). Among them, the microwave-assisted extraction (MAE) is considered a fast and efficient technique due to the microwave energy effect on the sample (Llompart, Garcia-Jares, Celeiro, & Dagnac, 2018). Theoretically, the microwaves increase the temperature of the sample cells, causing the disruption of cell membranes and the consequent transfer of compounds into the solvent (Alfio, Manzo, & Micillo, 2021; Llompart et al., 2018). For instance, MAE was successfully applied for the extraction of chitosan from shrimp by-products, increasing the extraction yield and reducing the extraction time compared to conventional methods (Bruno et al., 2019). In a previous study, MAE was applied to obtain oil with nutritional and bioactive properties from salmon backbones, heads, and viscera using the response surface methodology (under review).

The objective of this study was to recover oil from sea bass and gilthead sea bream heads using the MAE technique. The fatty acid composition of the obtained oils was evaluated as an indicator of their nutritional quality. Several bioactive properties such as antimicrobial, antioxidant, anti-inflammatory, and cytotoxic, were also studied in order to provided valuable information for future valorization of these fish processing side streams.

2. Material and methods

2.1. Standards and regeants

The commercial antibacterial ampicillin, methicillin, and streptomycin were acquired from Fisher Scientific (Janseen Pharmaceutical, Belgium) while the commercial antifungal ketoconazole was provided by Frilabo (Porto, Portugal). The standards ellipticine, dexamethasone, and quercetin, as well as the fatty acid methyl ester (FAME) reference standard mixture 37 (standard 47885-U) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Analytical grade reagents were used for all experiments. Sulfuric acid (98%), n-hexane (95%), diethyl ether, methanol, and toluene were purchased from Fisher Scientific (Leicestershire, UK). Roswell Park Memorial Institute medium (RPMI 1640), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine, penicillin (100 U/mL)/streptomycin (100 mg/mL) solution, Hank's balanced salt solution (HBSS), and trypsin–EDTA (ethylenediaminetetraacetic acid) were provided by Hyclone (Logan, Utah, USA). Malt Extract Broth (MEB) and Muller-Hinton Broth (MHB) were from Biolab® (Budapest, Hungary) while Blood Agar (Sheep blood 7%) was from LiofilChemsrl (Roseto d. Abruzzi (TE), Italy). 2',7'-dihydrodichlorofluorescein diacetate (DCFH-DA), 2,2'-azobis(2-amidinopropane) dihydrochloride (APPH), tris(hydroxymethyl)aminomethane (Tris), trichloroacetic acid (TCA), dimethyl sulfoxide (DMSO), lipopolysaccharide (LPS), and sodium nitrate were also from Sigma-Aldrich. *p*-Iodonitrotetrazolium chloride (INT) and sodium sulfate were provided by Panreac Applichem (Barcelona, Spain). Water was treated through a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Fish material and sample preparation

Farmed European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) were obtained in a local market of Valencia (Spain) during different days of February and April 2019. They were immediately transported from the market to the laboratories of the University of Valencia under refrigerated conditions. Individual fish were dissected as a simulation of fish processing for human consumption. The different resulting by-products, including heads, were separated, frozen, lyophilized, grounded and stored at -25ºC until experimental use (de la Fuente, Pallarés, Barba, & Berrada, 2021; de la Fuente, Pallarés, Berrada, & Barba, 2021). Freeze-dried heads were transported from the University of Valencia to the Mountain Research Center (CIMO, Bragança, Portugal) under refrigeration conditions for oil extraction and characterization.

2.3. Microwave-assisted extraction (MAE)

Oil extraction was performed in a microwave extractor (Nμ Tech, NuWav-Uno, Sonilex, West Bengal, India) equipped with a circulating cool-water reflux system, manual electromagnetic stirrer, and time controller. Microwave power conditions (max. 1000 W) and temperature overshoot were also controlled by the internal Intelli-System. The extraction conditions were established according to the values of microwave power (50.0 W), extraction time (10.8 min) and solid to liquid ratio (80 g/L) previously optimized for obtaining oil from salmon heads (under review). Total volume of solvent was fixed in 50 mL. After extraction process, samples were filtered and hexane solvent was completely removed using a rotary vacuum evaporator (Hei-VAP Silver 4, Schwabach, Germany) at 40 °C. Obtained oils were stored at -25 ºC until subsequent characterization assays. At least five extractions were carried out for each fish head sample.

At the same time, the conventional Soxlhet extraction (SE) was used as a reference method for total lipid extraction. Head sample (5 g) and n-hexane (250 mL) were introduced in a laboratory Soxhlet extractor (Behr Labor Technik™, Düsseldorf, Germany), where the oil extraction was conducted at 80 °C for 6 h. After finishing the extraction time, solvent removal and oil storage were made as in the MAE process. All extractions were carried out at least in duplicate.

2.4. Oil yield determination

The amount of oil from sea bass and sea bream heads was calculated gravimetrically by applying the following formula: extraction yield of oil (%) = (weight of extracted oil / weight of fish material) × 100. The percentage recovery values were expressed in dry weight (dw) due to the use of freeze-dried fish head samples for both extraction processes (SE and MAE).

2.5. Lipid profile of head oils

Fatty acid methyl esters (FAMEs) were prepared from sea bass and sea bream head oils (500 μL) by transesterification with a methanol:sulfuric acid:toluene (2:1:1, *v/v/v*) catalytic solution (5 mL) overnight at 50 ºC y 600 rpm. Then, 3 mL of water and 3 mL of diethyl ether were added and vortexed for 30 s in order to achieve phase differentiation. FAMEs were recovered from the upper layer and mixed with sodium sulfate. After filtering (0.22 μm nylon filters), samples were diluted 1/10 in diethyl ether and stored at -20 °C until fatty acid analysis.

Fatty acid composition analysis was carried out using a gas chromatograph (GC) constituted by a DANI model GC 1000 instrument (Milan, Italy), a flame ionization detector (FID), a split/splitless injector, and a Macherey-Nagel capillary column (30 m × 0.32 mm ID × 0.25 μm d_f). Fatty acids were then identified by comparing the relative retention times of FAMEs peaks from fish

head oils with a reference standard FAMEs mixture. Details of chromatography separation and determinations were described by Reis, Barros, Martins, & Ferreira, (2012).

2.6. Evaluation of biological properties of head oils

2.6.1. Antimicrobial activity

Inhibitory activity of the obtained fish head oils was tested against eight bacteria (*Enterobacter cloacae* (ATCC 49741), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 9027), *Salmonella enterica* subsp (ATCC 13076), *Yersinia enterocolitica* (ATCC 8610), (*Bacillus cereus* (ATCC 11778), *Listeria monocytogenes* (ATCC 19111), and *Staphylococcus aureus* (ATCC 11632)); and two fungal strains (*Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404)). All commercial microorganisms related to food contamination were purchased from Frilabo (Porto, Portugal).

Gram positive bacteria were incubated in fresh Blood Agar (7% sheep blood) and Gram negative bacteria in Muller Hilton Agar at 37 ºC for 24 h in order to maintain the exponential growth phase. Bacterial suspensions were prepared at 1.5×10^6 CFU/mL. The micromycetes were grown in Malt Agar plates at 25 ºC for 72h. After this culture period, the spores were recovered from the agar surface with sterile 0.85% saline containing 0.1% Tween 80 (*v*/*v*). Fungal spore suspensions were adjusted at 1.0×10^5 UFC/mL. Before antimicrobial analysis, fish head oil samples were dissolved 50% in MHB (0.5% Tween 80) and then final concentrations to be tested were prepared by serial dilutions (50% - 0.39%).

To assess the antibacterial potential, the minimum inhibitory and minimum bactericidal concentrations (MIC and MBC, respectively) were evaluated using the broth microdilution method and the rapid INT colorimetric assay described by Pires et al. (2018). Ampicillin (20 μg/mL) and streptomycin (1 mg/mL) were

used as positive controls for all bacteria, except for *S. aureus*, where methicillin (1 mg/mL) was employed. Two negative controls (MHB and oil sample) were also prepared for each bacterial inoculum.

To determine the antifungal potential, the minimum inhibitory and minimum fungicidal concentrations (MIC and MFC, respectively) were evaluated according to the procedure reported by Heleno et al. (2013). Ketoconazole (1 mg/mL) was used as positive control while MHB and oil samples were utilized as negative control for each fungal inoculum.

2.6.2. Antioxidant activity

The cellular antioxidant activity (CAA) assay described by (Wolfe & Rui, 2007) and adapted by de la Fuente et al. (2021) (under review) for salmon by-products oils was applied to evaluate the antioxidant capacity of sea bass and sea bream head oils. Murine macrophage cell line (RAW 264.7) was acquired from the European Collection of Authenticated Cell Cultures (ECACC). Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL) at 37 °C, 5% of CO_2 flow and humid atmosphere until 70-80% confluence of cell monolayer. Next, cells were scraped, seeded in 96-well plates at a density of $2x10^4$ cells/well, and incubated for 48 h. Afterward, the culture medium was removed and the cells were washed twice with 100 μL of HBSS (100 mM, pH 7.4). Then, 200 μL of fish head oil at different concentrations (500 – 2000 μg/mL in DMSO:H_2O_2 1:1, *v/v*) and 100 μL of DCFH-DA (50 μM) were added to each well and co-incubated at 37 °C for 1 h. After incubation, the mixture was discarded and cells were washed twice with 100 μL of HBSS before adding 100 μL of AAPH (600 μM). The reaction was carried out in a plate reader Biotek FLX800 (Bio-Tek Instruments, Inc., Winooski, VT, USA) with fluorescence filters for an excitation

wavelength of 485 nm and an emission wavelength of 535 nm at 37 °C. The fluorescence values were recorded every 5 min over 1 h and the differences of areas under the curve (AUC) were considered for calculations. Therefore, CAA values were calculated according to the following equation: CAA unit = 100 - (∫ AUCs / ∫ AUCc) × 100, where ∫ AUCs is the integrated area under the sample fluorescence *versus* time curve and ∫ AUCc is the integrated area from the control curve. The results were expressed as a percentage of inhibition of the oxidation reaction. Quercetin standard was used as a positive control.

2.6.3. Anti-inflammatory activity

The procedure reported by Sobral et al., (2016) to measure the inhibition of nitric oxide (NO) produced by LPS-stimulated macrophages was reproduced to evaluate the anti-inflammatory potential of sea bass and sea bream head oils. The quantification of NO was performed using a Griess Reagent System kit (Promega, Madison, WI, USA). For this method, RAW 264.7 cells were grown and maintained under the same culture conditions as for the aforementioned CAA assay. Then, they were seeded at 1.5x105 cells/well. Fish head oils were firstly dissolved in DMSO:H2O (1:1, v/v) at a concentration of 8 mg/mL. Through serial dilutions, the final concentrations of fish oils tested were 6.25 - 400 μg/mL. Dexamethasone standard at 50 μM was used as positive control and head oil samples without LPS were employed as negative controls. Results were expressed as the oil concentration that caused 50% of NO production inhibition (IC50, μg/mL).

2.6.4. Cytotoxic activity

Four human tumour cell lines (gastric adenocarcinoma, AGS; colon adenocarcinoma, CaCo-2; breast adenocarcinoma, MCF-7; and non-small cell

lung cancer, NCI-H460) were used to evaluate the cytotoxic potential of sea bass and sea bream head oils. AGS and Caco-2 cells were provided by ECACC while MCF-7 and NCI-H460 cells were purchased from Leibniz-Institute DSMZ. All human tumour cell lines were grown and maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), penicillin (100 U/mL) and streptomycin (100 mg/mL). They were incubated at 37 ºC with 5% CO_2 and humid atmosphere until 70-80% confluence of cell monolayer. All cancer cells were trypsinized and seeded at a density of 10,000 cells/well. In addition, one non-tumour cell line obtained from the primary culture of porcine liver (PLP2) was established in the laboratory and maintained according to the authors (Mandim et al., 2022). PLP2 cells were employed to verify that oil samples only affected to cancer cells. On the other hand, the fish head oils were prepared at a final concentrations ranged from 6.25 to 400 μg/mL in DMSO:H_2O (1:1, *v*/*v*).

The sulforhodamine B (SRB) colorimetric assay (Sigma-Aldrich, St. Louis, MO, USA) described by Vichai & Kirtikara, (2006) and adapted by de la Fuente et al. (2021) (under review) for salmon by-products oils was applied. Briefly, 100 μL of cold 10% (*w*/*v*) TCA were added to the wells and the microplates were incubated at 4 °C for 1 h. After removing the TCA, adhered cells were washed three times with water and were dried. Cell staining was then carried out by adding 100 μL of 0.057% (w/v) SRB solution at room temperature for 30 min. Excess dye was eliminated by washing three times with 1% (*v*/*v*) acetic acid and plates were left to dry. Next, 200 μL of 10 mM Tris base were used to dissolve the cells and the absorbance of protein-bound dye was measured in a microplate reader (Biotek ELX800, Bio-Tek Instruments, Inc., Winooski, VT, USA) at 510 nm. The antitumor drug ellipticine at 10 mM was used as a positive control.

Plated cells without fish head oil were used as a negative control and their absorbance values were considered time zero for the calculations. Results were expressed as extract concentration responsible for 50% of cell growth inhibition (GI50, μg/ mL).

2.7. Statistical analysis

Experimental data were subjected to one-way analysis of variance (ANOVA) to determine the significance differences among samples. Tukey Honestly Significant Difference (HSD) multiple range test ($p < 0.05$) was applied. The Statgraphics Centurion XVI® software (Statpoint Technologies, Inc., The Plains, VA, USA) was used for statistical analysis.

3. Results

3.1. Fish head oil extraction yield

The oil yield results of sea bass and sea bream head oils obtained by SE and MAE are shown in **Table 1**. The MAE technique resulted in extraction yields of 21.50±1.85% and 20.75±1.17% for sea bass and sea bream heads, respectively. Regarding SE, oil yields were 39.14±0.65% for sea bass heads and 41.58±0.67% for gilthead sea bream heads. Since Soxhlet method is supposed to extract total fat content from food samples, the extraction conditions of MAE allowed to recover 55% of total lipid content in sea bass heads and 52% in sea bream heads in less than 11 min. This means that MAE took about 33 times less extraction time (360 min *vs* 10.8 min) and 5 times less solvent amount (250 mL *vs* 50 mL) than SE, which is in accordance with the advantages related to the non-conventional MAE technique (Llompart et al., 2018).

Table 1. Yield and fatty acid profile of oil extracted from sea bass and sea bream heads using Soxhlet extraction (SE) and microwave-assisted extraction (MAE).

	Sea bass head oil		**Sea bream head oil**	
	SE	**MAE**	**SE**	**MAE**
	Oil yield (%)			
g oil/ 100g dw	39.14±0.65 b	21.50±1.85 a	41.58±0.67 b	20.75±1.17 a
	Fatty acid profile (%)			
C12:0	0.03±0.01 a	0.04±0.01 a	0.04±0.01 a	0.04±0.01 a
C14:0	2.22±0.01 a	2.26±0.01 a	2.67±0.01 b	2.77±0.05 b
C15:0	0.24±0.01 a	0.22±0.01 a	0.31±0.01 b	0.32±0.01 b
C16:0	14.72±0.07 a	15.01±0.06 a	14.37±0.11 a	14.52±0.16 a
C16:1	3.75±0.04 a	3.80±0.01 a	5.03±0.06 b	5.14±0.04 b
C17:0	0.18±0.01 a	0.19±0.01 b	0.23±0.01 c	0.25±0.01 d
C17:1	0.17±0.01 a	0.16±0.01 a	0.26±0.02 b	0.29±0.01 b
C18:0	2.73±0.08 a	2.75±0.14 a	2.69±0.02 a	2.77±0.01 a
C18:1n9c	33.73±0.18 a	34.41±0.41 a	35.30±0.13 b	36.64±0.41 b
C18:2n6t	0.44±0.13 a	0.33±0.01 a	nd	nd
C18:2n6c	16.29±0.32 b	16.52±0.13 b	12.90±0.07 a	13.06±0.19 a
C18:3n6	0.32±0.05 b	0.26±0.01 b	0.20±0.01 a	0.19±0.01 a
C18:3n3	3.73±0.02 b	3.60±0.11 b	3.03±0.01 a	3.00±0.01 a
C20:0	0.08±0.01 a	0.07±0.01 a	0.13±0.01 b	0.12±0.01 b
C20:1	1.91±0.05 b	1.88±0.02 b	1.50±0.01 a	1.56±0.02 a
C20:2	0.95±0.02 b	0.95±0.02 b	0.39±0.01 a	0.41±0.01 a
C20:3n6	0.21±0.01 a	0.20±0.01 a	0.19±0.01 a	0.19±0.01 a
C20:4n6	0.47±0.02 ab	0.44±0.01 a	0.52±0.01 b	0.48±0.02 ab
C20:3n3	0.26±0.01 c	0.26±0.01 c	0.19±0.01 a	0.21±0.01 b
C22:0	0.05±0.01 a	0.08±0.01 a	0.12±0.01 b	0.14±0.01 b
C20:5n3	4.60±0.13 a	4.37±0.20 a	4.33±0.14 a	4.41±0.16 a
C22:2	0.04±0.01 a	0.05±0.01 a	0.05±0.01 a	0.05±0.01 a
C24:1	1.33±0.03 a	1.10±0.01 a	1.94±0.04 a	1.64±0.12 a
C22:6n3	11.54±0.18 ab	11.02±0.10 a	13.61±0.17 c	12.79±0.21 b

Table 1. *Cont.*

Total fatty acid class				
SFA	20.25±0.02 a	20.68±0.12 ab	20.57±0.08 ab	20.94±0.25 b
MUFA	40.89±0.23 a	41.34±0.38 a	44.03±0.16 b	45.27±0.59 b
PUFA	37.87±0.22 b	36.98±0.29 b	35.35±0.25 a	34.73±0.57 a
n6/n3	0.88±0.01 b	0.92±0.02 c	0.65±0.01 a	0.68±0.01 a

C12:0 lauric acid; C14:0 mystiric acid; C15:0 pentadecylic acid; C16:0 palmitic acid, C16:1 palmitoleic acid; C17:0 heptadecanoic acid; C17:1 heptadecenoic acid; C18:0 stearic acid; C18:1n9c oleic acid; C18:2n6t linolelaidic acid C18:2n6c linoleic acid; C18:3n6 γ-linoleic acid; C18:3n3 α-linolenic acid; C20:0 arachidic acid; C20:1 eicosenoic acid; C20:2 eicosadienoic acid; C20:3n6 eicosadienoic acid; C20:4n6 arachidonic acid; C20:3n3 eicosatrienoico; C22:0 docosanoic acid; C20:5n3 eicosapentaenoic acid (EPA); C22:2 docosadienoic acid; C24:1 nervonic acid; C22:6n3 docosahexaenoico acid (DHA); nd not detected; SFA saturated fatty acids; MUFA monounsaturated fatty acids; PUFA polyunsaturated fatty acids; n6/n3 ratio omega-6/omega-3. In each row, different letters mean statistical differences among samples.

On the other hand, a greater amount of fish oil was extracted by traditional Soxhlet method than MAE process for both fish head samples. Recently, MAE has been used to optimize the oil recovery from salmon side streams, including heads (under review). In spite of using the same extraction conditions, higher oil yield (38%) was obtained for salmon heads compared to sea bass and sea bream heads (21%). According to the data reported on chemical composition of fish processing by-products, salmon heads contain higher total lipid fraction than sea bass and sea bream heads (He, Franco, & Zhang, 2011; Valcarcel et al., 2020). Therefore, this difference could be due to the specific fish species employed for oil extraction, which is agreement with the conclusions reached by Ozogul et al., (2018). In addition, some studies have been carried out to compare the oil yield of fish fillets using traditional extraction methods and MAE technique (Costa & Bragagnolo, 2017; Ozogul et al., 2018; Ramalhosa et al., 2012). Different results were obtained in terms of oil yield after applying the different extraction

processes. As a consequence, further research is required, especially for fish processing side streams.

3.2. Lipid profile of obtained fish head oils

Fatty acid composition of sea bass and sea bream head oils recovery by SE and MAE are listed in **Table 1**. The results showed that the extraction method did not affect the fatty acid composition of head oils. In addition, few variations with respect to the lipid profile were observed among fish species. The predominant fatty acid identified in both oil samples was oleic acid (C18:1n9c), with a slight difference ($p<0.05$) between sea bass (33-34%) and sea bream (35-36%). Linoleic acid (C18:2n6c, 13-16%), palmitic acid (C16:0, 14-15%), docosahexaenoic acid (DHA, 11-14%), eicosapentaenoic acid (EPA, ≈4%), and linolenic acid (C18:3n3, 3-4%) were then the most representative fatty acids for both fish head oils. Total saturated fatty acids (SFA, 20-21%) were equivalent between oil samples. However, some differences ($p<0.05$) in the percentages of monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) were found. Thus, oil from sea bass heads showed ≈41% MUFA and ≈37% PUFA while oil from sea bream heads presented ≈44% MUFA ≈37% PUFA.

Similar percentages of oleic, linoleic, and palmitic acids were found in head oils of European sea bass (Munekata et al., 2020) and gilthead sea bream (Pateiro et al., 2020). Few differences were also observed between MUFA and PUFA content, which seemed to be linked to the greater amount of EPA and, mainly, DHA, in heads samples analyzed in the present study. Interestingly, the levels of DHA were higher than those found in heads from the same fish species (Munekata et al., 2020; Pateiro et al., 2020), as well as equivalent or higher than those reported for salmon heads (under review; He et al., 2011; Inguglia, Chiaramonte, Di Stefano, et al., 2020).

"High omega-3 fatty acids" is a food claim established by European authorities when a food product contain at least 80 mg of DHA+EPA per 100 g and per 100 Kcal (Regulation (EU) No 1924/2006). Since sea bass and gilthead sea bream head oils here investigated presented an average of 15.7 and 17.6 g of DHA+EPA/100 g (fresh weight), respectively, both fish oils comply with this health claim. According to the nutritional guidance of the Food and Agriculture Organization of the United Nations (FAO), values of omega-6/omega-3 lower than 4 are related to a healthy diet (Food and Agriculture Organization of the United Nations., 2010). The omega-6/omega-3 ratios of fish head oil samples were 0.88-0.92 (sea bass) and 0.64-0.68 (sea bream).

Therefore, the studied head oils could be part of people's diet. Based on the nutritional properties showed by sea bass and sea bream head oils, these underutilized fish processing by-products are interesting candidates for the production of oil intended for feed and food fortification in order to achieve a better lipid profile of the final product. In this sense, meat, milk, bakery products, and livestock feed are currently fortified using fish oil (Jamshidi, Cao, Xiao, & Simal-Gandara, 2020).

3.3. Biological activities of sea bass and sea bream head oils

3.3.1. Antimicrobial activity

Results of antibacterial and antifungal activities of sea bass and sea bream head oils extracted by SE and MAE are presented in **Table 2**. All fish oils inhibited the growth of all bacteria and fungi tested. It should be highlighted that the head oils obtained by MAE exhibited higher antibacterial effect than the oils extracted by Soxhlet, except for *P. aeruginosa*, against which there were no differences between extraction methods. In addition, sea bream head oil was more effective against *E. cloacae*, *S. enterica*, and *S. aurus* than sea bass head oil. In

the same way, salmon head oil inhibited the growth of *P. aeruginosa* and *S. aureus* (Inguglia, Chiaramonte, Stefano, et al., 2020). Recently, the antibacterial potential of cephalopod liver viscera oil against several clinical bacteria has also been reported (Moovendhan, Kavisri, Vairamani, & Shanmugam, 2021). Regarding antifungal activity, similar results were observed for both fish species against *A. brasiliensis* and *A. fumigatus*. No fish head oil provided a bactericidal or fungicidal effect on any microorganism tested.

Table 2. Antibacterial and antifungal activities of oil extracted from sea bass and sea bream heads using Soxhlet extraction (SE) and microwave-assisted extraction (MAE).

	Sea bass head oil				**Sea bream head oil**			
	SE		**MAE**		**SE**		**MAE**	
Antibacterial activity	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria								
E. cloacae	50	nd	12.5	nd	50	nd	3.125	nd
E. coli	25	nd	25	nd	25	nd	12.5	nd
P. aeruginosa	50	nd	50	nd	50	nd	50	nd
S. enterica	25	nd	25	nd	25	nd	6.25	nd
Y. enterocolitica	12.5	nd	3.125	nd	12.5	nd	3.125	nd
Gram-positive bacteria								
B. cereus	25	nd	12.5	nd	25	nd	12.5	nd
L. monocytogenes	50	nd	50	nd	25	nd	6.25	nd
S. aureus	12.5	nd	6.25	nd	25	nd	3.125	nd
Antifungal activity	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
A. brasiliensis	50	nd	25	nd	25	nd	25	nd
A. fumigatus	50	nd	25	nd	50	nd	25	nd

MIC: minimum inhibitory concentration; MBC: minimum bactericidal concentration; MFC: minimum fungicidal concentration; nd: not detected; MIC and MBC values for positive controls: streptomycin (0.007-0.01 mg/mL), methicillin (0.007 mg/mL), ampicillin (0.15-0.63 mg/mL), and ketoconazole (0.06-0.5 mg/mL); MFC values for positive controls: Ketoconazole (0.125-1.0 mg/mL).

Because there are no differences in the fatty acid composition of the head oils with respect to the extraction techniques (Table 1), other constituents present in these oils could influence the antimicrobial activity. Although the literature information on the content of fat-soluble vitamins in fish by-products is scarce, fish liver oils from tuna, shark, and cod are considered important sources of vitamins A and E (Moovendhan et al., 2021). Since vitamins are known to be heat sensitive, the temperatures reached in the Soxhlet extraction process (≈80 ºC) are likely to degrade the vitamins present in fish head oils. In contrast, the MAE technique, applied here at a maximum temperature of 50 ºC, could have prevented the degradation of vitamins in the obtained oils. Therefore, sea bass and sea bream head oils extracted by MAE could exert antibacterial activity due to the content of vitamins or other thermolabile compounds. Further research is required in this regard.

3.3.2. Cellular antioxidant activity

Results of antioxidant activity of sea bass and sea bream head oils extracted by SE and MAE are shown in **Table 3**. The highest oil concentration tested (2 mg/mL) inhibited the oxidation reaction generated in the RAW macrophages by 29 to 56 %, except for the sea bream head oil extracted by Soxlhet, which did not show antioxidant capacity. In a previous study, head oil from Atlantic salmon (*Salmo salar*) obtained by MAE also inhibited the oxidation reaction by 36% while that extracted by Soxhlet did not exhibit antioxidant potential (under review). The traditional Folch method was used to recover oil from hake (*Merluccius merluccius*) heads and the results revealed high antioxidant activity based on DPPH and β-carotene bleaching assays (Karoud et al., 2020).

Table 3. Cytotoxic, antioxidant, and anti-inflammatory activities of oil extracted from sea bass and sea bream heads using Soxhlet extraction (SE) and microwave-assisted extraction (MAE).

	Sea bass head oil		Sea bream head oil	
	SE	**MAE**	**SE**	**MAE**
Cytotoxic activity (GI_{50} μg/mL)				
AGS	240.39±10.89 a	250.21±10.77 a	249.12±16.31 a	273.94±8.95 a
Caco-2	>400 b	224.00±12.63 a	361.76±33.97 a	325.73±21.49 a
MCF-7	93.07±0.99 b	38.17±2.67 a	39.43±0.92 a	46.23±4.65 a
NCI-H460	144.18±14.19 a	235.70±23.28 b	233.63±3.53 b	178.67±2.55 ab
PLP2	227.65±6.24 b	236.73±17.54 b	237.68±12.96 b	156.50±10.72 a
Cellular antioxidant activity (% inhibition of oxidation at 2 mg/mL)				
RAW 264.7	56.00±5.74 b	35.00±4.45 a	nd	29.00±3.20 a
Anti-inflammatory activity (IC_{50} μg/mL)				
RAW 264.7	14.94±0.14 a	20.84±1.30 b	14.84±0.70 a	13.89±1.28 a

GI_{50} values for Ellipticine: 1.23±0.03 μg/mL (AGS), 1.21±0.02 μg/mL (Caco-2), 1.02±0.02 μg/mL (MCF-7), 1.02±0.01 μg/mL (NCI-H460), 1.4±0.1 μg/mL (PLP2). IC_{50} values for Dexamethasone: 6.3±0.4 μg/mL (RAW 264.7). Quercetin: 95.3±4.6% oxidation inhibition at 0,3 μg/mL; wa: without activity. Different letters in each row correspond to significant differences ($p < 0.05$) among oil samples.

3.3.3. Anti-inflammatory activiy

Results of anti-inflammatory activity of sea bass and sea bream head oils extracted by SE and MAE are shown in **Table 3**. Regardless of the extraction method used, all fish head oils tested exhibited important NO inhibition in LPS-stimulated RAW macrophage cells. The concentration of fish head oils required to inhibited 50% NO production was from 14 to 21 μg/mL. Thus, the anti-inflammatory potential of sea bass and sea bream head oils are higher than that of salmon head (51-65 μg/mL), backbones (34-63 μg/mL), and viscera (32-42 μg/mL) oils also obtained by Soxhlet method and MAE technique (under review). In addition, the administration of oil from heak head reduced significantly the edema in a carrageenan-induced mice paw edema model

(Karoud et al., 2020). The authors suggested that the anti-inflammatory effect of hake head oil could be due to the presence of EPA and DHA, since these fatty acids act as competitor substrates for the inhibition oxidation of arachidonic acid. In the same way, high levels of EPA and DHA as well as low levels of MUFA from marine sources and related by-products were associated with the inhibition of NO and TNFα in LPS-stimulated macrophage cells (Ahmad et al., 2019). Therefore, the anti-inflammatory activity showed by sea bass and sea bream head oils could be due to their relevant content of EPA and, specially, DHA (Table 1).

3.3.4. Cytotoxic activity

Data regarding cytotoxicity of sea bass and sea bream head oils obtained by SE and MAE are presented in **Table 3**. The inhibition of cancer cell growth by the fish head oils tested did not seem to be related to the oil extraction techniques used. The breast cancer cells (MFC-7) exhibited the highest susceptibility to the oil samples (GI_{50} = 38-93 μg/mL) while the colon cancer cells (Caco-2) showed the lowest, at the tested concentrations. Similar growth inhibition effects of stomach (AGS) and lung cancer cell (NCI-H460) lines were observed for all fish head oils. Salmon head oil obtained by MAE technique also inhibited proliferation of MFC-7 cells (GI_{50} = 131 μg/mL) (under review). According to the literature, the cytotoxic or antiproliferative *in vitro* studies have been performed using isolated fish oil fatty acids instead of oil from fish or fish processing by-products. As a result of the data reviewed by Jameel, Agarwal, Arshad, & Serajuddin, (2019), different omega-3 fatty acids from fish oil could be considered anti-cancer agents since they influence in multiple mechanisms involved in cancer development. As for the omega-3 fatty acid DHA, found in high levels in the fish head oil samples studied, some works have revealed its

antiproliferative effect against colon and lung cancer cells (Ahangar, Sam, Nejati, & Habibian, 2016; Yin, Sui, Meng, Ma, & Jiang, 2017).

4. Conclusions

The MAE technique allowed the recovery of 55% and 52% of total lipid content from heads of European sea bass and gilthead sea bream, respectively, which was carried out in less than 11 minutes. The obtained oils showed a healthy lipid profile and high levels of DHA. There was a remarkable bacterial inhibition, which could be due to non-degraded thermolabile compounds during oil extraction by MAE. The head oils also exhibited anti-inflammatory potential, cellular antioxidant capacity, as well as cytotoxicity against breast cancer cells. Overall, this work shows the first step towards the possible valorization of sea bass and sea bream heads through the sustainable extraction of healthy oil for the fish processing and food industries under a circular economy approach.

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3.6. Marine resources and cancer therapy: from current evidence to challenges for functional foods development

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Marine resources and cancer therapy: from current evidence to challenges for functional foods development

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Abstract

Cancer is currently the second cause of death worldwide. Dietary habits are one the main factors involved in certain cancer onset. While some anticancer drugs are not completely effective in killing the tumor, they lead to adverse effects in patients. For instance, a growing interest has been shown regarding new natural molecules with anticancer properties, including those of marine origin. The state-of-the-art suggest that marine bioactive compounds exhibit in vitro and in vivo anticancer activities. Their potential use as ingredients for functional foods in cancer therapy as well as the different limitations in achieving a scientifically proven functional food products for cancer patients are here reviewed.

Keywords: Marine resources, functional foods, cancer therapy, bioactive compounds, marine by-products

Introduction

According to the World Health Organization (WHO), cancer is defined as a wide group of diseases that can start in almost any organ or tissue due to an abnormal and uncontrollable cell growth, which causes cell invasion and/or migration to other body parts [1]. Despite advances in medical research in recent decades, cancer is currently the second leading cause of death worldwide, with 9.6 million deaths in 2018 and about 29.5 million new cases expected by 2040 [2]. Most of types of cancer are mainly treated with surgery, chemotherapy, radiotherapy, immunotherapy and their combinations [3,4]. Unfortunately, some of these treatments are not completely effective removing tumors while they are responsible of several adverse effects on healthy cells (multiple drug resistance and toxicity) and on patient well-being (immune system depression and physical discomfort) [5,6]. In order to solve these problems, alternative and/or complementary therapies are required. In this sense, a growing research on new natural substances with anticancer properties has been observed over the last years. For instance, vegetables, marine resources, and microorganisms have become the main candidates for this purpose [5].

Marine ecosystem presents a great biodiversity of plants, animals, and microorganisms with a wide variety of bioactive compounds produced as response to adverse environmental conditions. Therefore, a large amount of molecules from marine origin are considered as possible therapeutic agents in oncology [7–10]. In addition to marine life, bioactive compounds can be found in the biomass discarded in seafood processing industry. For instance, several studies have reported anticancer activity of protein hydrolysates and peptides from tunicate, sepia, shellfish, and oyster side streams in different cancer cell

lines [11]. It should be noted that some of these studies are not only based on *in vitro* and *in vivo* models but also in human clinical trials [8,12].

Cancer is known to be a multifactorial disease, having dietary factors a significant role in the development of some specific types of cancers [1]. Thus, it is reasonable to assume that food plays an important role in the onset of cancer. On the one hand, dietary interventions to alleviate the side effects of cancer treatments have been proposed [13]. On the other hand, the population's concerns about the relationship between diet and cancer have led to the search for new food products with healthy attributes by both the industry and the consumers. In this context, functional foods are a promising tool against the establishment and progress of chronic degenerative diseases, including cancer. Although right now there is no consensus definition or internationally unified regulation on the concept of functional food, the incorporation of bioactive compounds into a specific food is the main approach for functional food development by the food industry [14].

The objective of this review is to describe the scientific evidence on anticancer properties of marine bioactive compounds as potential ingredients for functional foods. The limitations for the development of authorised marine-based functional foods focused on cancer therapy are also briefly analysed.

Current and potential anticancer agents of marine origin

Since the first marine clinical drug was discovered in 1945, the use of marine compounds in cancer therapy treatment has been frequently described [15]. To date, several bioactive compounds obtained from different species of sponges, tunicates, mollusks, and cyanobacteria have been approved as anticancer marine molecules (i.e. Cytostar-U®, Depocyt®, Halaven®, Fludara®, Arranon®,

Yondelis®, Aplidin®, Adcetris®, and Polivy®) [10]. For instance, they are used to treat soft tissue sarcoma, different types of leukemia and lymphoma, as well as ovarian and breast cancer. In addition, several molecules of marine origin are being evaluated in different phases of human clinical trials [8,11].

The current understanding of the complexity of cancer biology has boosted the knowledge of bioactive compounds as modulators of different cellular mechanisms such as proliferation, differentiation, apoptosis, angiogenesis and metastasis through multiple signalling pathways and biological reactions [4,5]. In this sense, several nitrogenous and non-nitrogenous organic compounds from Mediterranean ascidians exerted cytotoxic activity against different cancer cell lines [16]. Deshmukh et al. also reported 199 different metabolites related to diverse marine fungi with *in vitro* anticancer activity, in some cases, greater than drugs used as positive controls. [17] Furthermore, bioactive peptides obtained from *Perinereis aibuhitensis* and rainbow trout (*Oncorhynchus mykiss*) skin after applying enzymatic hydrolysis exhibited antiproliferative effects toward human lung and colon cancer cells, respectively [18,19]. Other small molecules interesting for their direct effect on the development and progression of cancer are protein kinase inhibitors. Marine organisms such as bacteria, fungi, soft corals, sponges, algae, and animals present protein kinase inhibitors. Some of them are being evaluated in human clinical trials (i.e. lestaurtinib, enzastaurin, kahalide F, elisidepsin, staurosporine, UCN-01, and CEP-2563) or have recently been approved (i.e. plitidepsin and midostaurin) for cancer treatment [11]. Regarding algae, both alcoholic and aqueous extracts, as well as isolated compounds (i.e. carotenoids, aldehydes, polysaccharides, fatty acids, and phytosterols) showed antitumor effect in a wide variety of cancer cell lines. Among the microalgae used, *Chlorella sorokiniana* and *Chaetoceros calcitrans* were the

most relevant sources of anticancer compounds compared to marine anticancer drugs available for clinical use. [20]. Phycocyanin, a phycobiliprotein derived from *Spirulina*, has shown considerable antineoplastic effects in different types of cancer cell lines. For the first time, Hao et al. [21] described the theoretical basis for the ability of phycocyanin to down-regulate the TIRAP/NF-κB signalling in human non-small cell lung cancer cells. A large amount of studies has revealed the anticancer properties of fucoidan (brown seaweeds polysaccharide) against numerous human cancer cell lines via multiple signal pathways [4]. In addition, novel porphyran extracted from the edible red algae *Pyropia yezoensis* as well as its derivatives obtained by gamma-irradiation displayed a significant reduction of cell viability in human hepatic, cervical, and breast cancer cell lines [22]. Docosahexanoic (DHA) and eicosapentanoic (EPA) acids from algal oils inhibited relevant angiogenic factors related to the growth of pre-existing adenocarcinoma, colorectal, and breast cancer cells [23]. Carotenoids and tannins derived from algae have also shown anti-leukemic activity in different leukemia models [24]. Moreover, the *in vitro* antitumor activities of the compounds from marine flora (fungi, macro- and micro-algae, higher plants), invertebrate fauna (sponges, tunicates, mollusks, bryozoans), bacteria, and symbiotic microbes have been recently reviewed [10,25].

Complementary actions on cancer therapy by marine compounds

Apart from acting on tumoral cell death, certain marine compounds have demonstrated to be effective as chemotherapeutic adjuvants by alleviating the negative side effects of drugs. For instance, the combination of fucoidan with different anticancer drugs such as cyclophosphamide, FOL-FOX, tamoxifen, cisplatin, and paclitaxel reduced the toxicity of the drugs, thus allowing the

inhibition of various tumors and/or cancers in mice [4]. In the same way, the carotenoid fucoxanthin exhibited a synergistic effect with the drug Doxonibicin on anticancer activity, by protecting myocardial cells against drug-induced cardiotoxicity [26]. Fucoxanthin and pholorotannins in combination with Itamib have been also suggested to reduce drug resistance for the treatment of leukemia [24]. Another strategy to improve the effectiveness of drugs in cancer treatments, is their combination with chemosensitizers, molecules capable of reversing or decreasing drug resistance of cancer cells. Polyoxygenated steroids, sipholane triterpenoids, derivatives of Agosterol A, and the peptide Kendarimide A are examples of chemosensitizers isolated from marine organisms [3].

In addition to pharmacological approaches, nutritional interventions have been shown effective to alter cancer metabolism. For example, according to Lévesque et al. [13], these dietary interventions limited the progression of established cancers and enhanced the efficacy of anticancer treatments in animal studies. In this sense, fucoidan has been recently investigated as a dietary supplement in complementary cancer therapy [4]. For instance, the oral administration of fucoidan slowed down the appearance of fibrosis in lung cancer mice-treated with thoracic radiation. On the other hand, the colorectal tumor was mitigated in mice fed wakame enriched with fucoxanthin in a dose-dependent manner, since the algae with the highest content of fucoxanthin markedly suppressed the tumor microenvironment formation as well as different cancer-associated cells [27]. Moreover, the intake of substances regulating the patient's immune system is also another promising strategy in cancer therapy [28,29]. This is the case of the oral administration of eckol, a phloroglucinol from brown algae, that notably inhibited the tumor growth in an *in vivo* model of

sarcoma by stimulating the mouse's innate and adaptive immune responses [29]. Randomized controlled trials based on high energy oral nutritional supplements enriched with protein and unsaturated fatty acids to evaluate both the weight loss and the metabolic alterations of cancer patients have also been performed [30]. Positive effects on body weight during chemo- and radio- therapy were observed. Similarly, DHA and EPA were useful in anorexia/cachexia conditions for pancreatic cancer patients [31].

Challenges for marine functional foods development

As shown, there is an important number of scientific studies that have showed anticancer properties of diverse marine compounds using *in vitro* and *in vivo* models. There are also currently several regulated cancer drugs based on marine molecules (see previous section). Therefore, the development of marine-based functional foods for cancer therapy is considered a health and economic opportunity.

As for any functional food, the first obstacle is the meaning of functional food itself. Although it is a term that was first coined in the 80s, today there is no an internationally unified regulation on the concept of functional food [14,32,33]. This situation not only places states with more stringent health laws at a clear commercial disadvantage, but also harms consumers who could be misinformed or misled, highlighting the need for regulated labels for functional food products [33].

Despite the lack of a definitive definition, the key factors to consider a food as functional are both the proven scientific evidence on such functionality and the appearance as a usual foodstuff. It should be pointed out that the health effects exhibited by functional foods are based on the fraction of bioactive

compounds (natural o added) that reach the systemic circulation after the gastrointestinal digestion process and are then distributed to organs and tissues to finally display their bioactivity [34]. Since the health effects of these biologically active substances could be affected during the gastrointestinal digestion, bioaccessibility and bioavailability are essential assays to know possible modifications. Pre-clinical studies with isolated compounds are required in order to obtain valuable information about safety, mechanisms of action or potential physiological effects. However, they do not provide overwhelming proofs on the functionality of a complete food, since a bioactive agent can exhibit different effects as an isolated compound than as an ingredient in a food matrix [35,36]. Therefore, studies on the beneficial activity of bioactive compounds are usually carried out before and after their incorporation into a food product [37].

In addition to previous *in vitro* models, research evidence must be supported by human clinical trials[36]. Specific plasma biomarkers of the functional food tested is one of the most important challenges in human studies [14,36]. Few clinical trials have been performed to evaluate the effect of marine functional foods on cancer patients. Colorectal cancer patients undergoing chemotherapy treatment showed significantly lower levels of inflammatory cytokines after consuming 2 g of fish oil for 9 weeks compared to control patients [38]. However, in other study under the same fish oil consumption guidelines, there were no differences between colon cancer patients and control group. Similarly, women with breast cancer who ingested fish oil for 30 days did not obtain healthy benefits in comparison with the control group [38]. Through a meta-analysis of published research articles during 18 years, the authors concluded that fish oil consumption had a protective effect in breast cancer patients [39].

On the other hand, the functionality of bioactive compounds can also be affected by the industrial processing of the final product. Non-thermal innovative processing technologies have been proposed in order to preserve the properties of bioactive compounds from foods and related by-products [14]. The application of marine lipids, carotenoids, proteins and peptides in the food industry is often limited due to specific properties of each substance such as low solubility in water, poor oral bioavailability, incompatibility of the food matrix and negative impact on sensory attributes [40,41]. Bioavailability of iodine, pholorotannins, and vitamin B12 of algae origin have been evaluated [42]. Finding effective strategies to solve these inconveniences is a relevant challenge for the food industry. In this way, different delivery systems for marine bioactive ingredients into food matrices to enhance their organoleptic properties, absorption, release, and safety have been recently reviewed [40].

In addition to this, there are also limiting factors to obtain the marine bioactive compounds of interest. Since stressful conditions seems to be crucial for the production of bioactive metabolites, reproducing specific environmental conditions such as nutrients availability, light intensity, temperature, or seasonal variation at the laboratory level is required [20,43]. Furthermore, sustainable and efficient extracting technologies of target compounds must be applied. In this line, several green extraction techniques to obtain bioactive compounds from marine sources and related by-products have been reviewed [44,45].

Due to the complexity of the complete process to develop a functional food supported by scientific evidence (especially for diseased population as cancer patients), collaboration of food science, food health, and food technology professionals has been proposed [14]. Regardless of the health reason, obtaining functional foods based on the incorporation of bioactive compounds into a food

matrix is a long-term, laborious and expensive process, similar to drug development (pre-clinical testing, human clinical trials, and regulatory approval). Finding out which natural foods provide benefits against diseases such as cancer would considerably reduce the time for the designation of a food as functional. In this sense, clinical trials have identified several natural food products (cranberries, pomegranate, whole grains, low fat dairy products, almonds, chia seeds, cardamom, algae oil, etc.) to regulate inflammation [38]. Authors recommend a frequent consumption of these functional foods as part of the habitual diet, for individuals with chronic diseases, including cancer.

Conclusions

There is an extensive scientific evidence in the available literature showing that a great variety of marine compounds such as carotenoids, polysaccharides, aldehydes, phytosterols, fatty acids, tanins, peptides, steroids, triterpenoids, protein kinase inhibitors, etc. that present anticancer properties via multiple signal pathways supported by both *in vitro* and *in vivo* models. Moreover, marine molecules can be also used to develop functional foods. It may be concluded that the combination of nutritional intervention together the use of anticancer drugs is a promising strategy in cancer therapy due to the positive side-effect of marine substances compared to the conventional cancer treatments. In addition, there is a need of additional clinical trials in cancer patients to design new functional food based on marine substances. It is also necessary to ascertain which natural functional foods display benefits in cancer therapy for faster and cheaper access to successful functional foods for cancer patients.

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4. GENERAL DISCUSSION

4. General Discussion

Durante el desarrollo de los objetivos planteados en la presente Tesis Doctoral, se ha utilizado la extracción mediante líquidos presurizados (PLE) para obtener extractos acuosos a partir de restos de músculo, cabezas, vísceras, piel y colas de lubina, dorada y salmón. Las condiciones de extracción fueron previamente optimizadas utilizando la metodología de superficie de respuesta (RSM, *response surface methodology*) y los subproductos de lubina. Las condiciones óptimas obtenidas se aplicaron tanto a los subproductos de lubina como a los de dorada y salmón. Paralelamente, se prepararon extractos acuosos mediante agitación y posterior filtrado. Todos los extractos fueron caracterizados en base a su contenido en proteínas, distribución del peso molecular proteico y capacidad antioxidante total. Los resultados de los extractos obtenidos bajo las condiciones óptimas de extracción de la técnica PLE (extractos óptimos) se compararon con los extractos resultantes de la técnica convencional (extractos control). Adicionalmente, los extractos de víscera de salmón fueron seleccionados para llevar a cabo un análisis peptídico mediante espectrometría de masas (nanoESI qTOF), seguido de un estudio bioinformático con la finalidad de identificar péptidos con actividad antioxidante establecida.

También se ha empleado la extracción asistida por microondas (MAE) para obtener aceite de cabezas, esqueletos y vísceras de salmón, así como de cabezas de lubina y dorada. Las condiciones de extracción se optimizaron a través de la RSM utilizando los subproductos de salmón. Las condiciones óptimas obtenidas se aplicaron entonces a los subproductos correspondientes de salmón, lubina y dorada. Al mismo tiempo, se realizaron extracciones de la fracción grasa mediante el método tradicional Soxhlet. La caracterización de todos los aceites se realizó en función de la composición de los ácidos grasos y de las propiedades

citotóxicas, antioxidantes, antiinflamatorias y antimicrobianas. Los resultados de los aceites extraídos bajo las condiciones óptimas de la técnica MAE (aceites óptimos) se compararon con los extraídos por el método Soxhlet (aceites control).

Por otra parte, se ha evaluado la presencia de posibles contaminantes relacionados con el entorno del cultivo de pescado en diferentes subproductos (restos de músculo, cabezas, vísceras, piel y colas) de lubina, dorada y salmón. La espectrometría de plasma acoplada inductivamente a detector de masa (ICP-MS) se utilizó para identificar y cuantificar los niveles de arsénico, mercurio, cadmio y plomo. La cromatografía líquida acoplada a espectrometría de masa de alta resolución TOF (LC-ESI-qTOF-MS) se empleó para investigar el contenido de micotoxinas de origen alimentario y sus metabolitos.

4.1. Metodología de superficie de respuesta

La RSM es un conjunto de técnicas matemáticas y estadísticas empleadas para mejorar y optimizar procesos. Una de sus principales aplicaciones es modelar y analizar situaciones en las que una o varias respuestas de interés están influenciadas por más de un factor cuantitativo, consiguiendo optimizar las respuestas mediante la determinación de los valores óptimos de los factores implicados (Dean, Voss, & Draguljić, 2017). Estas características han convertido a la RSM en una herramienta útil para la optimización de tecnologías novedosas enfocadas hacia la extracción de compuestos nutricionales y bioactivos a partir de matrices alimentarias y subproductos relacionados.

Debido a que cada subproducto de pescado tiene una estructura particular en cuanto a la composición de sus tejidos, cabía esperar un comportamiento diferente entre el solvente y la matriz frente a las mismas condiciones de

extracción para cada técnica utilizada (PLE y MAE). Por este motivo, se investigaron las condiciones óptimas de extracción para los subproductos de forma individual a través de la RSM. Los resultados confirmaron que se requerían diferentes combinaciones de los parámetros de extracción para conseguir la respuesta óptima de cada uno de los subproductos estudiados. En el anexo 1 se muestra el material suplementario correspondiente a la evaluación de la RSM aplicada a la PLE para los subproductos de lubina. En el anexo 2 se encuentra el material suplementario con los resultados de la composición de ácidos grasos de los subproductos de salmón empleados para la optimización de la MAE.

4.2. Extracción proteica mediante líquidos presurizados

4.2.1. Recuperación proteica

A partir de los datos del contenido proteico total de los subproductos de pescado liofilizados y de sus correspondientes extractos, se calculó el porcentaje de recuperación de proteínas para cada subproducto y especie. Los resultados muestran que se recuperan más proteínas en los extractos óptimos que en los controles, lo que pone de manifiesto que las condiciones de pH, temperatura y tiempo de extracción óptimos, junto con la presión aplicada por la técnica PLE influyen de forma positiva en la extracción de proteínas de los subproductos de pescado.

Para todas las especies de pescado estudiadas, el mejor porcentaje de recuperación proteica se observó en las vísceras, independientemente del método de extracción empleado. Mediante PLE se alcanzaron valores de 61, 78 y 92 % de recuperación de proteínas a partir de vísceras de lubina, dorada y salmón, respectivamente. El incremento más significativo de la extracción de

proteínas tras aplicar la técnica PLE se produjo en las colas de dorada (3% *vs* 26%) y de salmón (6% *vs* 28%). Esta eficiencia también se mostró en las colas de lubina (14% *vs* 30%), en la piel (11% *vs* 24%) y cabezas (19% *vs* 33%) de dorada, así como en la piel (18% *vs* 29%) y vísceras de salmón (57% *vs* 92%). Por contra, para los recortes de músculo de pescado de las tres especies junto con las cabezas y vísceras de lubina, el aumento de la recuperación proteica fue menos relevante entre los extractos óptimos y los controles. A pesar de que la PLE se ha utilizado para recuperar compuestos nutricionales y bioactivos a partir de diferentes fuentes naturales, principalmente de origen vegetal, solo dos estudios se han llevado a cabo para la extracción de proteínas. En uno de ellos se observaron porcentajes de recuperación proteica de aproximadamente un 5% en algas (Harrysson et al., 2018), mientras que en el otro trabajo se recuperaron la mitad de las proteínas de las semillas de pimiento rojo empleando la RSM (Firatligil-Durmus & Evranuz, 2010).

4.2.2. Capacidad antioxidante total

Se ha evaluado la capacidad antioxidante total de los extractos óptimos y los extractos control mediante dos ensayos espectrofotométricos basados en diferentes mecanismos de acción de los compuestos antioxidantes: el método TEAC (Trolox equivalent antioxidant capacity o capacidad antioxidante equivalente al Trolox) y el método ORAC (oxygen radical antioxidant capacity o capacidad de absorción de radicales de oxígeno). El ensayo TEAC mide la capacidad de las sustancias antioxidantes de reducir radicales catiónicos, mientras que el ensayo ORAC mide la capacidad de secuestrar radicales de tipo peróxido. Los resultados de ambos métodos muestran que los extractos óptimos exhiben más actividad frente a radicales libres que los extractos control en todos

los subproductos y especies de pescado (excepto para el músculo y la piel de salmón, donde los valores TEAC mostraron un ligero descenso no significativo estadísticamente). Por lo tanto, en general, las condiciones de extracción de la técnica PLE también influyen positivamente en la capacidad antioxidante total de los extractos obtenidos a partir de los subproductos de pescado.

Para cada subproducto de la misma especie de pescado, los valores TEAC y ORAC fueron distintos, lo que podría deberse a diferencias propias de cada matriz. Una vez aplicada la técnica PLE, tanto para la lubina como para la dorada, la capacidad antioxidante más alta (TEAC y ORAC) se encontró en los extractos de los recortes de músculo, mientras que la actividad más baja se observó en los extractos de las colas. En el caso del salmón, el extracto óptimo de las vísceras fue el que exhibió los mejores valores TEAC y ORAC (≈ 3700 y 7800 equivalentes de Trolox μM, respectivamente), mientras que los extractos óptimos de la piel mostraron los valores más bajos por ambos métodos antioxidantes.

Se considera que tanto el tamaño de los fragmentos proteicos como la presencia de aminoácidos hidrofóbicos están relacionados con las propiedades antioxidantes de los subproductos obtenidos de diferentes fuentes marinas (Sila & Bougatef, 2016; Ucak et al., 2021; Zamora-Sillero, Gharsallaoui, & Prentice, 2018). Puesto que se han identificado distintos aminoácidos hidrofóbicos en cabezas, agallas, intestinos, hígado y piel de lubina (Munekata et al., 2020) y dorada (Pateiro et al., 2020), así como en cabezas, vísceras y piel de salmón (He, Franco, & Zhang, 2011), estos compuestos podrían encontrarse en los extractos de los subproductos estudiados, dando lugar a los valores TEAC y ORAC mostrados.

4.2.3. Distribución del peso molecular de la fracción proteica

Se ha utilizado la electroforesis en gel de poliacrilamida (PAGE) en condiciones desnaturalizantes con el detergente dodecil sulfato sódico (SDS) para separar las proteínas de los extractos óptimos y de los extractos control de las tres especies de pescado estudiadas. Las imágenes de los geles se analizaron con el software ImageJ®, un programa de procesado de imágenes de dominio público. La estimación del peso molecular de las bandas de proteínas obtenidas en los geles electroforéticos se realizó por comparación frente a un estándar de pesos moleculares (5-250 kDa). Los resultados de la electroforesis SDS-PAGE muestran que las condiciones de extracción de la técnica PLE aplicadas a los subproductos de pescado afectan al tamaño de las proteínas obtenidas en los extractos, formándose más fragmentos proteicos de menor peso molecular.

Por una parte, el perfil electroforético de los geles fue diferente para cada subproducto de una misma especie, lo que parece corresponder con los distintos tejidos y, por tanto, distintos componentes proteicos que forman las diversas partes del cuerpo del pescado. Comparando entre tipo de subproducto, un amplio rango de tamaño de bandas resultó ser común entre muestras, independientemente de la especie. Por ejemplo, el peso molecular de los extractos de vísceras de lubina, dorada y salmón fue de 7-60, 8-61 y 7-73 kDa, respectivamente. Se han realizado estudios sobre proteínas musculares de carpa y siluro (Tadpitchayangkoon, Park, & Yongsawatdigul, 2010), proteínas de cabezas de salmón y pez loro (He et al., 2011; Prihanto, Nurdiani, & Bagus, 2019), y proteínas de vísceras de bacalao y salmón (Aspmo, Horn, & Eijsink, 2005; He et al., 2011). Los perfiles electroforéticos coincidieron en ciertos rangos de peso molecular con los de los extractos del subproducto correspondientes de lubina, dorada y/o salmón.

Por otra parte, el estudio digital de las imágenes de los geles permitió confirmar diferencias respecto de los métodos de extracción empleados. Se pudo observar que los extractos obtenidos mediante PLE contenían mayor cantidad de fragmentos proteicos de bajo peso molecular que los extractos obtenidos por agitación convencional, circunstancia que se produjo en todos los extractos de los subproductos y especies investigadas.

4.2.4. Identificación de secuencias peptídicas antioxidantes

Debido a que los extractos óptimos de las vísceras de salmón exhibieron una actividad antioxidante muy elevada, especialmente frente a radicales peróxido, se procedió a su análisis peptídico y posterior estudio bioinformático para la identificación de péptidos con actividad antioxidante establecida. Tras el análisis de espectrometría de masas y la utilización de dos bases de datos de secuencias proteicas, se identificaron 137 péptidos en los extractos obtenidos mediante PLE frente a los 67 péptidos encontrados en los extractos obtenidos mediante agitación convencional. A pesar de tratarse de la misma muestra de partida, solo cinco péptidos coincidieron en ambos extractos de vísceras de salmón. Estos resultados muestran que las condiciones de extracción de la técnica PLE influyen en la cantidad y tipo de péptidos presentes en los extractos.

En cuanto a las propiedades antioxidantes de los péptidos identificados, se realizó una predicción de su actividad a través de la base de datos BIOPEP-UWN, herramienta bioinformática para la búsqueda de péptidos bioactivos derivados principalmente de alimentos (Minkiewicz, Iwaniak, & Darewicz, 2019). No se encontró homología entre las secuencias de aminoácidos de los péptidos contenidos en los extractos de vísceras de salmón y las secuencias de aminoácidos de los péptidos antioxidantes introducidos en la base de datos. Se

realizó entonces una nueva búsqueda sobre la actividad antioxidante potencial, basada en la presencia de secuencias peptídicas antioxidantes de pequeño tamaño encriptadas en cadenas polipeptídicas. Los resultados del análisis bioinformático mostraron la presencia de varias secuencias de aminoácidos distintas con potencial antioxidante dentro de las cadenas peptídicas de los extractos de vísceras de salmón, algunas de las cuales han sido previamente identificadas en péptidos procedentes de bonito, sardina, moluscos, medusas o subproductos de sardina. En concreto, se identificaron 19 secuencias de 2 a 4 aminoácidos en los extractos óptimos y 12 secuencias de dipéptidos y tripéptidos en los controles. Las secuencias GPP y GAA fueron las más repetidas en los péptidos de los extractos obtenidos por la técnica PLE. Debido a que el contenido de aminoácidos hidrofóbicos como glicina (G), prolina (P) y alanina (A) se ha relacionado con la actividad antioxidante de péptidos de origen marino (Cheung, Ng, & Wong, 2015; Sila & Bougatef, 2016; Zamora-Sillero et al., 2018), estas secuencias podrían ser las responsables de la actividad antioxidante *in vitro* mostrada por los extractos óptimos de las vísceras de salmón. En este contexto, se han identificado varias secuencias de péptidos antioxidantes en vísceras de sardina (LHT, LARL, GGE) y caballa (ACFL) (Sila & Bougatef, 2016), así como péptidos antioxidantes en aletas (FLNEFLHV) y recortes de músculo (GGPAGPAV, GPVA, PP, GP) de salmón (Ahn, Kim, & Je, 2014; Neves, Harnedy, O'Keeffe, & FitzGerald, 2017).

Péptidos de pescado de peso molecular entre 0.5 y 1.5 kDa han sido relacionados con propiedades antioxidantes (Ucak et al., 2021). Los péptidos identificados en ambos extractos de vísceras de salmón se corresponden con pesos moleculares desde 0.63 hasta 2.60 kDa. Sin embargo, la alta intensidad de analitos con tiempos de retención cortos observada en el cromatograma

peptídico, indica que hubo más cantidad de péptidos de pequeño tamaño en los extractos de víscera obtenidos mediante PLE.

De acuerdo con los resultados, tanto la presencia de determinadas secuencias de aminoácidos encriptadas en los péptidos identificados, como un peso molecular en el rango 0.5-1.5 kDa, serían factores relevantes en relación a la actividad antioxidante mostrada por los extractos óptimos de vísceras de salmón.

4.3. Extracción asistida por microondas (MAE)

4.3.1. Recuperación de aceite

Considerando la extracción Soxhlet como método de referencia para obtener la cantidad total de grasa de los alimentos, la optimización de la técnica MAE permitió recuperar el 92% del contenido total de lípidos de las vísceras de salmón y el 69% de las espinas y cabezas, así como el 52 y 55% de las cabezas de dorada y lubina, respectivamente. A pesar de que el rendimiento en aceite de los subproductos de pescado fue mayor con el método tradicional, cabe destacar que la técnica MAE redujo de 25 a 33 veces el tiempo de extracción (360 min *vs* <15 min), dependiendo del tipo de subproducto. En este sentido, una de las principales ventajas asociadas a la MAE es la reducción del tiempo de extracción de los compuestos de interés (Llompart, Garcia-Jares, Celeiro, & Dagnac, 2018). De acuerdo con Costa & Bragagnolo (2017), las microondas romperían los tejidos del pescado, permitiendo la liberación y transferencia del aceite hacia el solvente de forma más rápida y eficiente que los métodos convencionales. Sin embargo, en bibliografía existen datos contradictorios al respecto. La técnica MAE se ha utilizado para obtener aceite de la parte comestible de diferentes especies de pescado y el rendimiento se ha comparado con distintos métodos tradicionales de extracción grasa (Costa & Bragagnolo, 2017; Ozogul et al., 2018;

Ramalhosa et al., 2012). Mientras que algunos estudios no mostraron diferencias en la cantidad de aceite obtenido mediante MAE y las técnicas convencionales, otros trabajos revelaron que diferentes métodos de extracción proporcionan distintos rendimientos de aceite, por lo que la eficiencia de la extracción dependería tanto del método aplicado como del tipo de pescado utilizado. En lo que respecta a subproductos de pescado, las microondas se han empleado de manera efectiva como tratamiento previo a la extracción enzimática de aceite a partir de cabezas de carpa (Bruno, Ekorong, Karkal, Cathrine, & Kudre, 2019).

4.3.2. Propiedades nutricionales

La composición de los ácidos grasos de los aceites extraídos de los subproductos de pescado se llevó a cabo mediante cromatografía de gases con detector de llama (GC-FID). No se observaron diferencias entre los aceites obtenidos mediante las técnicas MAE y Soxlhet para cada uno de los subproductos de pescado estudiados (Anexo 2). Los resultados mostraron un perfil lipídico saludable en todos los aceites analizados debido a la presencia de una proporción mayor de ácidos grasos poliinsaturados (AGPI) frente a ácidos grasos saturados (AGS) (Chen & Liu, 2020). En concreto, los AGPI de los aceites de cabezas, esqueletos y vísceras de salmón representaron más del 82% del total de ácidos grasos. Estos porcentajes coinciden con los obtenidos en aceite de cabezas y tejidos blandos de salmón tras aplicar una extracción de una hora a 90 °C (Inguglia et al., 2020). De forma similar, los AGPI de los aceites de cabezas de lubina y dorada constituyeron el 78 y 80% del total de ácidos grasos, respectivamente. Resultados equivalentes han sido previamente publicados en estudios sobre la composición nutricional de cabezas de lubina (Munekata et al., 2020) y dorada (Pateiro et al., 2020).

Los principales ácidos grasos identificados en todos los aceites fueron el ácido oleico (C18:1n9c), el ácido linoleico (C18:2n6c) y el ácido palmítico (C16:0), con algunas diferencias entre subproductos y especies de pescado. Por ejemplo, el contenido de ácido oleico fue equivalente en los tres subproductos de salmón (38%) y mayor que en el aceite de cabezas de lubina y dorada (≤36%). El mismo comportamiento se observó en el ácido palmítico, con porcentajes del 15% para los subproductos de salmón y del 10% para los de lubina y dorada. En cuanto al ácido linoleico, la cantidad más elevada se encontró en el aceite de vísceras de salmón (18%), seguida del aceite de cabezas de lubina (16%), de los aceites de cabezas y esqueletos de salmón (14%), y finalmente, del aceite de cabezas de dorada (13%). Otro importante AGPI identificado en todos los aceites de pescado en proporciones variables fue el ácido linolénico (C18:3n3). Mientras que supuso un 10% del total de ácidos grasos para el aceite de vísceras de salmón, solo representó el 7% para los aceites de cabezas y esqueletos de salmón y aproximadamente el 3% para los de cabezas de lubina y dorada. En general, los ácidos grasos predominantes en los aceites de los subproductos de salmón, lubina y dorada extraídos por Soxhlet y MAE concuerdan con aquellos obtenidos por otros métodos de extracción (Inguglia et al., 2020; Liu, Ramakrishnan, & Dave, 2020; Munekata et al., 2020; Pateiro et al., 2020).

Los dos ácidos grasos de la familia omega-3 más estudiados por su relevancia en cuanto a propiedades saludables son el DHA y el EPA. Ambos compuestos fueron identificados en todos los aceites en un rango de 7-13% y 4-6%, respectivamente. Comparando los subproductos de salmón, el contenido de DHA y EPA en el aceite de vísceras fue menor que en el aceite de cabezas y esqueletos. Estos resultados coinciden con los publicados por Liu et al. (2020) tras analizar el perfil lipídico del aceite extraído de estos subproductos de salmón

mediante hidrólisis enzimática. En lo que se refiere a las cabezas de lubina y dorada, los valores de DHA y EPA del presente estudio son superiores a los encontrados en publicaciones recientes sobre la caracterización nutricional de las cabezas de ambas especies (Munekata et al., 2020; Pateiro et al., 2020). A pesar de que el salmón es considerado como un alimento saludable por su contenido en ácidos grasos omega-3 (Haq, Ahmed, Cho, & Chun, 2017), los aceites procedentes de los subproductos de pescado blanco (cabezas de lubina y dorada) presentaron niveles más elevados de DHA en comparación con los aceites extraídos de los subproductos de pescado azul (cabezas, esqueletos y vísceras de salmón). Esta circunstancia pone de manifiesto la importancia de investigar las características nutricionales de la amplia variedad de subproductos generados en la transformación industrial de diferentes especies de pescado.

Según el Reglamento Europeo (UE) No 1924/2006 sobre declaraciones nutricionales y propiedades saludables en los alimentos, un contenido de ácidos grasos omega-3 elevado se atribuye a alimentos o productos alimentarios con al menos 80 mg de DHA+EPA por 100 g y 100 Kcal. De acuerdo con las recomendaciones de la FAO, una relación omega-6/omega-3 con un valor inferior a 4 se corresponde con una dieta saludable (FAO, 2010). Todos los aceites extraídos de los subproductos de salmón, lubina y dorada cumplen con la declaración de contenido elevado en ácidos grasos omega-3 y con valores omega-6/omega-3 saludables, lo que los convierte en candidatos relevantes para desarrollar productos con un perfil lipídico beneficioso para la salud. En este contexto, la fortificación de distintos productos alimentarios con aceite de pescado es una estrategia ampliamente utilizada y aceptada por los consumidores (Jamshidi, Cao, Xiao, & Simal-Gandara, 2020).

4.3.3. Propiedades bioactivas

4.3.3.1. Efecto citotóxico

Para evaluar la capacidad citotóxica de los aceites extraídos de los subproductos de pescado se han llevado a cabo estudios *in vitro* utilizando células de cáncer de estómago (AGS), de colon (Caco-2), de pecho (MCF-7) y de pulmón (NCI-H460). Con la finalidad de verificar que el efecto citotóxico de los aceites solo afectaba a las células tumorales, también se utilizaron células no tumorales obtenidas a partir de cultivo primario de tejido de hígado porcino (PLP2). En general, todos los aceites mostraron actividad frente a las líneas celulares estudiadas. Sin embargo, también se observó cierta capacidad citotóxica frente a las células PLP2. La variabilidad de los resultados observados en la actividad de los aceites respecto a los métodos de extracción (MAE y Soxhlet), a los diferentes subproductos (cabezas, esqueletos y vísceras) y a las distintas especies de pescado (salmón, lubina y dorada), no ha permitido establecer en todos los casos una relación directa entre el tipo de aceite y el efecto citotóxico.

Los resultados sí muestran claramente que la línea celular de cáncer de pecho es la más susceptible al efecto de los aceites de cabezas de lubina y dorada (GI_{50}= 38-93 μg/mL). Las células de cáncer de pulmón también mostraron cierta susceptibilidad a los aceites de pescado, especialmente a los procedentes de esqueletos de salmón (GI_{50}= 76-142 μg/mL). Sin embargo, todos los aceites fueron menos eficaces frente a la proliferación celular de las líneas cancerígenas gástricas y colónicas. A pesar de no disponer de datos en bibliografía sobre la utilización de aceite de pescado o de subproductos en ensayos de citotoxicidad celular, algunos estudios *in vitro* han relacionado diferentes AGPI omega-3 de pescado con la reducción de la viabilidad celular en diferentes líneas tumorales,

incluyendo células de cáncer de pecho, de estómago y de colon (Ahangar, Sam, Nejati, & Habibian, 2016; Dai, Shen, Pan, Shen, & Das, 2013; Guo, Zhu, Wu, He, & Chen, 2017; Jameel, Agarwal, Arshad, & Serajuddin, 2019). Por otra parte, varios estudios han mostrado efectos antiproliferativos de hidrolizados proteicos y péptidos obtenidos de subproductos de pescado en diferentes líneas celulares tumorales (Ishak & Sarbon, 2018). También existe evidencia científica sobre una amplia variedad de compuestos marinos de origen vegetal y animal con propiedades anticancerígenas. Caronetonides, polisacáridos, ácidos grasos, aldehídos, fitoesteroles, péptidos, taninos, esteroides, triterpenoides, etc. han mostrado actividad anticancerígena en modelos celulares *in vitro* e *in vivo* (Alves et al., 2018; Khalifa et al., 2019; Li et al., 2019; Wali et al., 2019; Wang, Sorolla, Gopal Krishnan, & Sorolla, 2020).

4.3.3.2. Capacidad antioxidante celular

Debido a la existencia de limitaciones tanto en los experimentos *in vivo* como en los métodos químicos *in vitro*, los ensayos basados en células se consideran actualmente como la mejor alternativa para el estudio de las propiedades antioxidantes de alimentos a nivel biológico (Martinelli et al., 2021). En este sentido, se han llevado a cabo ensayos celulares con macrófagos de ratón (RAW 264.7) para estimar la actividad antioxidante de los aceites óptimos y los aceites control. Tras determinar el porcentaje de inhibición de la oxidación generada en las células de macrófagos, los mejores resultados se han obtenido utilizando los aceites de vísceras de salmón, con porcentajes de inhibición por encima del 75%. Por el contrario, los aceites de los esqueletos de salmón no fueron capaces de inhibir la reacción de oxidación, mostrando no poseer capacidad antioxidante frente a especies reactivas de oxígeno (ROS) y nitrógeno (RNS). En cuanto a los

aceites de cabezas de pescado, los procedentes de lubina consiguieron inhibir la oxidación entre un 35% (óptimo) y un 56 % (control). Los aceites óptimos de cabezas de dorada y salmón también mostraron porcentajes de inhibición de la oxidación del 29 y 36 % respectivamente, mientras que no se observó efecto antioxidante en los controles. La variabilidad de los resultados no ha permitido establecer una relación entre la composición de ácidos grasos o las técnicas de extracción lipídica empleadas y las propiedades antioxidantes de las muestras analizadas. A través de ensayos químicos *in vitro*, aceites de subproductos de salmón (Haq et al., 2017) y de merluza (Karoud et al., 2020) han mostrado tener capacidad antioxidante.

4.3.3.3. Efecto antiinflamatorio

Se ha evaluado el potencial antiinflamatorio de los aceites óptimos y control midiendo su capacidad de inhibición del NO producido por la estimulación de los macrófagos RAW 264.7. Todos los aceites de los subproductos de pescado redujeron los niveles de NO celular con concentraciones inferiores a 65 μg/mL. Los aceites de las cabezas de lubina y dorada mostraron mayor efecto antiinflamatorio que los aceites de los subproductos de salmón. En este sentido, la administración de aceite de cabezas de merluza extraído por el método tradicional Folch, redujo el edema en un modelo de inflamación *in vivo* (Karoud et al., 2020). El hecho de que niveles altos de DHA y EPA en las partes comestibles y en los subproductos de fuentes marinas hayan sido relacionados con actividad antiinflamatoria *in vitro* (Ahmad et al., 2019), podría indicar que los resultados obtenidos en este ensayo se deben al mayor porcentaje de DHA que presentan los aceites de cabezas de lubina y dorada en comparación con los aceites de subproductos de salmón.

4.3.3.4. Capacidad antimicrobiana

Se ha investigado tanto la capacidad antibacteriana como antifúngica de todos los aceites extraídos a partir de subproductos de pescado. Los resultados mostraron que la actividad antibacteriana de los aceites de salmón fue similar, independientemente del método de extracción. El aceite de las vísceras inhibió el crecimiento de *Enterobacter cloacae*, *Escherichia coli*, *Yersinia enterocolitica*, *Bacillus cereus* y *Staphylococcus aureus* a concentraciones más bajas (3.125-25%) que los aceites de esqueletos y cabezas (50%). Al contrario, el aceite de las vísceras mostró poca o nula capacidad antifúngica, mientras que los aceites de esqueletos y cabezas, además de exhibir actividad frente al crecimiento de los hongos, también mostraron efecto fungicida a una concentración de 10 mg/mL (en el caso de extracciones llevadas a cabo con el método Soxhlet). De acuerdo con Inguglia et al. (2020), aceites de salmón procedente de cabezas y tejidos blandos inhibieron el crecimiento de *Pseudomonas aeruginosa* y *S. aureus*.

Los aceites recuperados de las cabezas de lubina y dorada por la técnica MAE fueron más eficaces frente al crecimiento de bacterias y hongos que los extraídos por Soxhlet. Los aceites óptimos de ambas especies mostraron el mismo efecto de inhibición del crecimiento de *P. aeruginosa*, *Y. enterocolitica*, *B. cereus* y *Aspergillus fumugatus*. Sin embargo, para el resto de las bacterias Gram-postivo (*Listeria monocytogenes* y *S. aureus*) y Gram-negativo (*E. clocae*, *E. coli* y *Salmonella enterica*) estudiadas, el aceite de cabezas de dorada fue más eficaz que el aceite de cabezas de lubina. En cuanto a los aceites de cabezas de lubina y dorada obtenidos por el método Soxhlet, su comportamiento frente a los microorganismos analizados fue similar.

Las diferencias en la capacidad antimicrobiana exhibida por los distintos aceites de subproductos de pescado podrían estar relacionadas con su composición en ácidos grasos. Por ejemplo, los perfiles lipídicos completos de los aceites de esqueletos y cabezas de salmón son muy similares entre sí y difieren en el contenido de los ácidos grasos linoleico y linolénico, así como en ácidos grasos minoritarios respecto al aceite de las vísceras. Teniendo en cuenta que la lubina y la dorada mediterráneas son especies estrechamente relacionadas, los perfiles lipídicos completos de los aceites de las cabezas de estas dos especies también son similares entre sí y muestran algunas diferencias con los aceites de los subproductos de salmón, especialmente en la proporción de DHA. Puesto que el aceite de cabeza de dorada es el que contiene mayor cantidad de DHA (13%), este ácido graso podría ser el responsable de la actividad antibacteriana mostrada por dicho aceite. En este sentido, la capacidad del aceite extraído de la parte muscular de dos especies de pescado de agua dulce para inhibir el crecimiento de *S. aureus*, fue relacionada con su composición en AGPI omega-3 (Simplice et al., 2018). En una revisión sobre la efectividad de los AGPI omega-3 frente a microorganismos patógenos, el ácido linolénico y sus ácidos grasos derivados (DHA y EPA) han sido considerados como potenciales agentes antimicrobianos para mejorar la salud de las personas y de los animales, a falta de más estudios clínicos al respecto (Chanda et al., 2018).

4.4. Presencia de contaminantes

4.4.1. Metales pesados

Se ha evaluado la presencia de arsénico, mercurio, cadmio y plomo en subproductos de lubina, dorada y salmón. Teniendo en cuenta las tres especie de pescado, los rangos de concentración obtenidos y expresados en μg/g de peso fresco, fueron de 0.541-0.938 (músculo), 0.346-0.859 (cabezas), 0.462-2.586 (vísceras), 0.387-0.969 (piel) y 0.388-.04406 (colas) para el arsénico; 0.024-0.106 (músculo), 0.016-0.059 (cabezas), 0.009-0.047 (vísceras), 0.007-0.026 (piel) y 0.041-0.042 (colas) para el mercurio; 0.0004-0.0010 (músculo), 0.001-0.003 (cabezas), 0.004-0.068 (vísceras), 0.004-0.010 (piel) y 0.001-0.010 (colas) para el cadmio; y 0.005-0.027 (músculo), 0.019-0.063 (cabezas), 0.007-0.046 (vísceras), 0.025-0.061 (piel) y 0.033-086 (colas) para el plomo. Aunque actualmente el contenido de contaminantes en subproductos de pescado no está regulado, los resultados de metales pesados se compararon con los niveles máximos permitidos por la agencia europea de seguridad alimentaria (EFSA, *European Food Safety Authority*) para productos pesqueros comercializados (Reglamento CE No 1881/2006). Las concentraciones de los cuatro elementos tóxicos analizados se encontraron por debajo de los límites legales establecidos en todos los subproductos de pescado, excepto en el caso de las vísceras de dorada, donde los niveles de cadmio (0.068 μg/g) superaban el valor máximo permitido para filetes de pescado (0.05 μg/g).

Cabe señalar que existe poca información en bibliografía sobre el contenido de elementos tóxicos en subproductos de pescado. En algunos estudios se han determinado las concentraciones de arsénico, mercurio, cadmio y/o plomo, no solo en subproductos de lubina, dorada y corvina de cultivo, sino también en el

pienso y ambiente acuícolas (Kalantzi et al., 2016; Kandyliari et al., 2020). Los autores concluyeron que factores como la especie y la localización parecían influir en la acumulación de metales pesados en los distintos tejidos del pescado. Además, debido a la presencia de dichos elementos en niveles traza, los subproductos fueron considerados seguros tanto para consumo humano como para su posible incorporación en la industria alimentaria. En lo que respecta a subproductos de salmón, no se han encontrado datos de elementos metálicos tóxicos en salmón procedente de acuicultura. Sin embargo, el contenido de mercurio en vísceras de varias especies de salmón de distintas zonas del mar Pacífico ha sido descrito (Khristoforova, Tsygankov, Lukyanova, & Boyarova, 2018). Las concentraciones de mercurio en las vísceras de salmón, lubina y dorada de cultivo del presente estudio fueron inferiores a las de salmón silvestre.

4.4.2. Micotoxinas

Se ha investigado la presencia de micotoxinas en subproductos de lubina, dorada y salmón a través de un cribado no dirigido frente a una biblioteca formada por 223 micotoxinas de origen alimentario y metabolitos relacionados. No se observó contaminación por micotoxinas en ninguno de los subproductos de pescado analizados, excepto en las vísceras de lubina, donde la micotoxina deoxivalenol (DON) se identificó en niveles traza (<0.5 ppb). La concentración máxima de DON permitida por la Comisión Europea en los ingredientes de piensos es de 12 mg/kg para subproductos de maíz, 8 mg/kg para otros cereales y hasta 5 mg/kg para piensos completos (Oliveira & Vasconcelos, 2020). Por lo tanto, los niveles de DON en las vísceras de dorada se encontraron muy por debajo de los permitidos por las autoridades para ingredientes y piensos destinados a alimentación animal.

DON es producida principalmente por hongos de la familia *Fusarium*, que se encuentran normalmente en los granos de los cereales utilizados para elaborar piensos para peces. Por este motivo, DON es conocida en Europa por su alta prevalencia e incidencia tanto en los ingredientes como en los productos finales para alimentación acuícola (Pietsch, 2020). Por ejemplo, se ha informado de una concentración alta de DON en piensos para dorada de cultivo como consecuencia de la utilización de trigo en su composición (Nácher-Mestre et al., 2015). A pesar de que se considera que hay una baja retención de DON en los tejidos del pescado debido a su rápida metabolización y excreción (Pietsch, 2020), se ha observado una distribución uniforme en músculo, hígado, riñones, piel y cerebro de salmón del Atlántico alimentado durante 2 meses con pienso contaminado con DON (Bernhoft et al., 2017).

La ingesta de piensos contaminados puede afectar a la salud de los peces de cultivo, así como a la del consumidor final de la cadena alimentaria. Los piensos destinados a la alimentación de animales criados mediante acuicultura están constituidos por una mezcla de ingredientes de origen marino y vegetal, lo que les hace susceptibles a ser contaminados por sustancias tóxicas diversas como los metales pesados (Adamse, Van der Fels-Klerx, & de Jong, 2017; Kalantzi et al., 2016; Kandyliari et al., 2020) y las micotoxinas (Nácher-Mestre et al., 2015; Tolosa, Font, Mañes, & Ferrer, 2014). Mantener unos niveles de sustancias tóxicas por debajo de los límites máximos permitidos por las normativas de seguridad alimentaria, es necesario tanto en las partes del pescado destinadas directamente a consumo humano como en los subproductos generados en la industria de transformación de productos pesqueros que puedan ser incorporados hacia nuevos procesos de producción de la industria alimentaria para su valorización.

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5. CONCLUSIONS

5. CONCLUSIONES

1. La optimización de las condiciones de extracción de la técnica PLE ha puesto de manifiesto que se trata de una herramienta rápida y sostenible para obtener extractos acuosos proteicos con capacidad antioxidante a partir de subproductos de pescado, especialmente en el caso de las vísceras de salmón.

2. El estudio de la distribución del peso molecular de la fracción proteica de los extractos reveló un contenido mayor de fragmentos proteicos de bajo peso molecular en los extractos óptimos, sugiriendo que la técnica PLE influye en la cantidad y el tamaño de las proteínas extraídas.

3. Las secuencias peptídicas antioxidantes GPP y CAA identificadas en diferentes péptidos de los extractos de vísceras de salmón obtenidos mediante PLE, podrían estar relacionadas con los altos valores de actividad antioxidante exhibida.

4. Las condiciones de extracción optimizadas para la técnica MAE evidencian que es un sistema rápido para la recuperación de aceite de subproductos de pescado.

5. La composición de ácidos grasos demostró que todos los aceites de subproductos de pescado presentan un perfil lipídico saludable, apoyando la posibilidad de su incorporación en productos alimentarios fortificados.

6. La susceptibilidad de las células de cáncer de pecho y de pulmón a ciertos aceites estudiados supone un punto de partida para futuras investigaciones sobre los compuestos y mecanismos de acción implicados.

7. La elevada actividad antioxidante a nivel celular mostrada por el aceite de vísceras de salmón no ha podido relacionarse de forma clara con su composición en ácidos grasos, por lo que se necesita más investigación al respecto.

8. Todos los aceites extraídos de los subproductos de pescado exhibieron un importante efecto antiinflamatorio, probablemente debido a su contenido DHA y EPA.

9. La marcada actividad antibacteriana y antifúngica mostrada por los aceites de cabezas de lubina y dorada extraídos mediante MAE podría estar relacionada con la presencia de sustancias termolábiles.

10. En la gran mayoría de subproductos analizados no hubo presencia de micotoxinas y los niveles de arsénico, mercurio, cadmio y plomo se encontraron muy por debajo de los niveles máximos establecidos para productos pesqueros. Sin embargo, los niveles traza de DON y el contenido de cadmio en algunas vísceras reflejan la necesidad de confirmar la calidad de los subproductos de pescado antes de su valorización.

11. La extracción de nutrientes y/o compuestos con propiedades bioactivas a partir de subproductos de pescado mediante tecnologías de extracción no convencionales de forma sostenible supone una estrategia de valorización desde el punto de vista de la economía circular para el sector acuícola

5. CONCLUSIONS

1. The optimization of the extraction conditions of the PLE technique has shown that it is a fast and sustainable tool to obtain protein extracts with antioxidant capacity from fish side streams, especially in the case of salmon viscera.

2. The study of the molecular weight distribution of the extracts' protein fractions revealed a higher content of low molecular weight protein fragments in the optimal extracts, which suggest that the PLE technique influences the quantity and size of the extracted proteins.

3. The antioxidant peptide sequences GPP and CAA, identified in different peptides from extracts of salmon viscera obtained by PLE, could be related to the high values of antioxidant activity exhibited.

4. The extraction conditions optimized for the MAE technique show that it is a rapid system for the recovery of oil from fish side streams.

5. The fatty acid composition demonstrated that all fish side stream oils have a healthy lipid profile, which support the possibility of their incorporation into fortified food products.

6. The susceptibility of breast and lung cancer cells to the oils studied is a starting point for future research on both the compounds and the mechanisms of action involved.

7. The high cellular antioxidant activity shown by salmon viscera oil has not been clearly related to its fatty acid composition, more research is therefore required in this regard.

8. All oils extracted from fish side streams exhibited a significant anti-inflammatory effect, probably due to their DHA and EPA content.

9. The marked antibacterial and antifungal activity of sea bass and sea bream head oils, extracted by PLE, could be related to the presence of thermolabile substances.

10. In the vast majority of side streams analyzed there were no mycotoxins and the levels of arsenic, mercury, cadmium and lead were far below the maximum levels established for fishery products. However, the trace levels of DON and cadmium content in some viscera samples reflect the need to confirm the quality of fish by-products prior to their valorization.

11. The extraction of nutrients and/or compounds with bioactive properties from fish side streams using non-conventional extraction technologies in a sustainable way, represents a valorization strategy from the circular economy point of view for the aquaculture sector.

ANEXOS

Anexo 1. Foods (2021)

An integrated approach for the valorization of sea bass (*Dicentrarchus labrax*) side streams: Evaluation of contaminants and development of antioxidant protein extracts by Pressurized Liquid Extraction

Table S1. Experimental and predicted data for the response variables obtained from the central composite design for sea bass muscle.

RSM				MUSCLE					
Run	pH	Tª (°C)	Time (min)	Protein (mg)		TEAC (µM Trolox Eq)		ORAC (µM Trolox Eq)	
				experimental	predicted	experimental	predicted	experimental	predicted
1	7	20	10	456	461	2079	1777	4572	3843
2	7	40	5	504	576	1407	1531	2779	3027
3	7	40	10	549	494	1356	1498	3058	3006
4	7	40	10	540	494	1222	1498	2837	3006
5	7	40	15	510	488	2111	1778	3060	2753
6	7	60	10	386	431	1166	1259	1665	2335
7	4	20	5	454	408	1166	1145	2428	2540
8	4	20	15	226	268	1273	1412	1465	1881
9	4	40	10	285	311	981	943	1933	1579
10	4	60	5	242	251	1140	1066	918	845
11	4	60	15	312	280	859	853	1016	917
12	10	20	5	301	320	955	1013	2241	2355
13	10	20	15	135	113	1593	1719	1647	1735
14	10	40	10	207	231	1003	832	941	1236
15	10	60	5	301	246	621	534	705	304
16	10	60	15	178	210	688	762	512	415

Regression equations provided by Response Surface Methodology and Statgraphics Centurion XVI.I for sea bass muscle.

Equation S1: Protein = -351,446 + 329,988*pH + 2,05575*Tª (ºC) - 49,0105*Time (min) - 24,7399*pH^2 + 0,351208*pH*Tª (ºC) - 1,09267*pH*Time (min) - 0,119173*Tª (ºC)^2 + 0,426025*Tª (ºC)*Time (min) + 1,53923*Time (min)^2

Equation S2: TEAC = -1154,72 + 924,751*pH + 6,70513*Tª (ºC) - 103,388*Time (min) - 67,8735*pH^2 - 1,66194*pH*Tª (ºC) + 7,33508*pH*Time (min) + 0,049372*Tª (ºC)^2 - 1,19826*Tª (ºC)*Time (min) + 6,23355*Time (min)^2

Equation S3: ORAC = -3289,88 + 2482,73*pH - 62,1289*Tª (ºC) - 12,0103*Time (min) - 177,646*pH^2 - 1,48406*pH*Tª (ºC) + 0,65075*pH*Time (min) + 0,207019*Tª (ºC)^2 + 1,82696*Tª (ºC)*Time (min) - 4,6517*Time (min)^2

Table S2. Experimental and predicted data for the response variables obtained from the central composite design for sea bass head.

RSM				HEAD					
Run	pH	Tª (°C)	Time (min)	Protein (mg)		TEAC (µM Trolox Eq)		ORAC (µM Trolox Eq)	
				experimental	predicted	experimental	predicted	experimental	predicted
1	7	20	10	151	136	514	407	911	1063
2	7	40	5	156	157	539	635	983	1194
3	7	40	10	161	175	622	618	1342	1307
4	7	40	10	189	175	650	618	1379	1307
5	7	40	15	201	201	861	783	1949	1791
6	7	60	10	245	260	365	490	1210	1112
7	4	20	5	149	160	503	515	1781	1489
8	4	20	15	162	165	594	742	1129	1409
9	4	40	10	192	179	986	782	1576	1474
10	4	60	5	213	209	531	578	952	1203
11	4	60	15	289	292	986	984	1794	1657
12	10	20	5	132	129	531	529	571	695
13	10	20	15	130	134	469	418	1700	1436
14	10	40	10	172	184	335	558	1005	1159
15	10	60	5	253	249	605	453	839	545
16	10	60	15	342	332	537	521	1540	1819

Regression equations provided by Response Surface Methodology and Statgraphics Centurion XVI.I for sea bass head.

Equation S4: Protein = 307,374 - 21,7314*pH - 5,53485*Tª (ºC) - 6,51543*Time (min) + 0,767816*pH^2 + 0,296229*pH*Tª (ºC) - 0,00725*pH*Time (min) + 0,0577384*Tª (ºC)^2 + 0,193313*Tª (ºC)*Time (min) + 0,162614*Time (min)^2

Equation S5: TEAC = 244,619 - 38,9942*pH + 35,4742*Tª (ºC) - 36,8202*Time (min) + 5,79935*pH^2 - 0,580958*pH*Tª (ºC) - 5,63667*pH*Time (min) - 0,422627*Tª (ºC)^2 + 0,447375*Tª (ºC)*Time (min) + 3,65617*Time (min)^2

Equation S6: ORAC = 2588,45 - 226,216*pH + 27,9453*Tª (ºC) - 237,431*Time (min) + 1,02586*pH^2 + 0,5635*pH*Tª (ºC) + 13,6735*pH*Time (min) - 0,550043*Tª (ºC)^2 + 1,33307*Tª (ºC)*Time (min) + 7,40531*Time (min)^2

Table S3. Experimental and predicted data for the response variables obtained from the central composite design for sea bass viscera.

RSM				VISCERA					
Run	pH	Tª (°C)	Time (min)	Protein (mg)		TEAC (µM Trolox Eq)		ORAC (µM Trolox Eq)	
				experimental	predicted	experimental	predicted	experimental	predicted
1	7	20	10	101	95	240	272	978	884
2	7	40	5	111	108	479	480	1212	1169
3	7	40	10	117	117	507	470	1440	1368
4	7	40	10	115	117	474	470	1241	1368
5	7	40	15	115	117	472	492	1290	1306
6	7	60	10	125	129	464	454	1333	1399
7	4	20	5	69	69	220	198	436	399
8	4	20	15	68	73	156	158	401	484
9	4	40	10	107	102	376	396	1209	1199
10	4	60	5	101	105	432	450	1195	1252
11	4	60	15	124	120	434	417	1320	1228
12	10	20	5	78	82	267	279	520	619
13	10	20	15	87	84	357	334	968	918
14	10	40	10	103	107	450	452	1260	1242
15	10	60	5	108	103	392	384	981	905
16	10	60	15	116	117	430	447	1050	1095

Regression equations provided by Response Surface Methodology and Statgraphics Centurion XVI.I for sea bass viscera.

Equation S7: Protein = -51,906 + 23,9487*pH + 2,02711*Tª (ºC) + 4,13325*Time (min) - 1,45167*pH^2 - 0,0608125*pH*Tª (ºC) - 0,03725*pH*Time (min) - 0,0128375*Tª (ºC)^2 + 0,0277625*Tª (ºC)*Time (min) - 0,2054*Time (min)^2

Equation S8: TEAC = -453,438 + 89,2597*pH + 30,0081*Tª (ºC) - 23,7856*Time (min) - 5,10586*pH^2 - 0,609729*pH*Tª (ºC) + 1,58758*pH*Time (min) - 0,266932*Tª (ºC)^2 + 0,0176625*Tª (ºC)*Time (min) + 0,65629*Time (min)^2

Equation S9: ORAC = -2093,27 + 296,113*pH + 77,5411*Tª (ºC) + 104,62*Time (min) - 16,4249*pH^2 - 2,36308*pH*Tª (ºC) + 3,563*pH*Time (min) - 0,567536*Tª (ºC)^2 - 0,272625*Tª (ºC)*Time (min) - 5,24757*Time (min)^2

Table S4. Experimental and predicted data for the response variables obtained from the central composite design for sea bass skin.

RSM				SKIN					
Run	pH	Tª (°C)	Time (min)	Protein (mg)		TEAC (μM Trolox Eq)		ORAC (μM Trolox Eq)	
				experimental	predicted	experimental	predicted	experimental	predicted
1	7	20	10	90	123	323	336	525	571
2	7	40	5	153	152	376	315	881	805
3	7	40	10	153	176	403	271	686	793
4	7	40	10	166	176	381	271	749	793
5	7	40	15	184	169	159	245	817	818
6	7	60	10	353	304	nd	13	1517	1396
7	4	20	5	74	59	298	331	264	337
8	4	20	15	79	81	372	298	362	309
9	4	40	10	155	140	185	267	685	561
10	4	60	5	214	232	129	92	1124	1132
11	4	60	15	267	277	nd	-4	1204	1299
12	10	20	5	92	87	413	411	717	640
13	10	20	15	92	77	336	367	487	498
14	10	40	10	149	149	346	290	691	739
15	10	60	5	252	255	nd	68	1228	1300
16	10	60	15	249	267	nd	-39	1407	1353

Regression equations provided by Response Surface Methodology and Statgraphics Centurion XVI.I for sea bass skin.

Equation S10: Protein = -135,323 + 56,624*pH - 3,47822*Tª (ºC) + 15,4937*Time (min) - 3,49216*pH^2 - 0,0224375*pH*Tª (ºC) - 0,529917*pH*Time (min) + 0,0951888*Tª (ºC)^2 + 0,0558375*Tª (ºC)*Time (min) - 0,613979*Time (min)^2

Equation S11: TEAC = 126,966 + 11,7984*pH + 15,945*Tª (ºC) - 6,55872*Time (min) + 0,797184*pH^2 - 0,435125*pH*Tª (ºC) - 0,183*pH*Time (min) - 0,242376*Tª (ºC)^2 - 0,1574*Tª (ºC)*Time (min) + 0,357386*Time (min)^2

Equation S12: ORAC = -294,209 + 294,055*pH - 18,3493*Tª (ºC) - 19,5601*Time (min) - 15,9097*pH^2 - 0,563375*pH*Tª (ºC) - 1,905*pH*Time (min) + 0,475495*Tª (ºC)^2 + 0,48815*Tª (ºC)*Time (min) + 0,731917*Time (min)^2

Table S5. Experimental and predicted data for the response variables obtained from the central composite design for sea bass tailfin.

RSM				TAILFIN					
Run	pH	Tª (°C)	Time (min)	Protein (mg)		TEAC (µM Trolox Eq)		ORAC (µM Trolox Eq)	
				experimental	predicted	experimental	predicted	experimental	predicted
1	7	20	10	98	103	323	322	605	655
2	7	40	5	130	116	399	414	660	736
3	7	40	10	130	132	422	416	918	832
4	7	40	10	134	132	345	416	723	832
5	7	40	15	145	159	592	545	1179	1091
6	7	60	10	231	226	512	481	1177	1116
7	4	20	5	121	130	357	353	597	623
8	4	20	15	105	99	400	424	829	801
9	4	40	10	146	133	454	401	806	753
10	4	60	5	181	185	392	407	816	776
11	4	60	15	264	269	601	619	1253	1348
12	10	20	5	102	97	315	305	662	569
13	10	20	15	103	99	363	355	663	707
14	10	40	10	116	127	357	378	749	791
15	10	60	5	168	174	445	429	914	945
16	10	60	15	298	289	608	620	1501	1477

Regression equations provided by Response Surface Methodology and Statgraphics Centurion XVI.I for sea bass tailfins.

Equation S13: Protein = 289,54 - 7,1171*pH - 6,84346*Tª (ºC) - 14,9605*Time (min) - 0,195421*pH^2 + 0,0872708*pH*Tª (ºC) + 0,536917*pH*Time (min) + 0,080778*Tª (ºC)^2 + 0,284237*Tª (ºC)*Time (min) + 0,204248*Time (min)^2

Equation 14: TEAC = 405,904 + 28,7305*pH + 1,25441*Tª (ºC) - 49,57*Time (min) - 2,91966*pH^2 + 0,292521*pH*Tª (ºC) - 0,345917*pH*Time (min) - 0,0352047*Tª (ºC)^2 + 0,351362*Tª (ºC)*Time (min) + 2,55232*Time (min)^2

Equation 15: ORAC = 786,501 + 70,0734*pH - 15,4249*Tª (ºC) - 64,1887*Time (min) - 6,73596*pH^2 + 0,930187*pH*Tª (ºC) - 0,67025*pH*Time (min) + 0,132328*Tª (ºC)^2 + 0,985637*Tª (ºC)*Time (min) + 3,24746*Time (min)^2

Anexo 2. Food Chemistry (under review)

Nutritional and bioactive oils from salmon (*Salmo salar*) side streams obtained by Soxhlet and optimized microwave-assisted extraction

Tables A.1 Fatty acid composition of salmon backbone oils obtained during the optimization process of microwave-assisted extraction.

	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12	B13	B14	B15	B16	B17	B18	B19	B20
Compoun	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%
C12:0	0,556	0,576	0,552	0,568	0,563	0,535	0,577	0,552	0,564	0,516	0,556	0,595	0,558	0,592	0,593	0,501	0,539	0,613	0,569	0,563
C14:0	2,838	2,936	2,766	2,833	2,809	2,753	2,863	2,896	2,822	2,63	2,825	2,883	2,818	3,001	2,864	2,569	2,778	2,883	2,863	2,833
C15:0	0,188	0,194	0,191	0,192	0,192	0,184	0,187	0,194	0,182	0,176	0,186	0,191	0,194	0,196	0,191	0,174	0,188	0,187	0,181	0,19
C16:0	10,355	10,539	10,178	10,306	10,211	10,2	10,347	10,625	10,124	9,82	10,524	10,394	10,171	10,655	10,539	9,772	10,447	10,432	10,294	10,398
C16:1	2,69	2,673	2,651	2,576	2,611	2,668	2,561	2,681	2,626	2,588	2,595	2,698	2,632	2,689	2,65	2,567	2,629	2,662	2,686	2,614
C18:0	2,828	2,895	2,856	2,868	2,851	2,81	2,795	2,92	2,814	2,763	2,882	2,787	2,816	2,928	2,942	2,771	2,9	2,822	2,792	2,871
C18:1n9c	37,379	37,378	37,654	37,434	37,795	38,141	37,866	37,69	38,003	38,05	37,947	37,819	37,907	36,737	36,899	37,748	37,94	37,796	37,633	37,955
C18:2n6c	14,469	14,299	14,395	14,327	14,304	14,653	14,337	14,065	14,392	14,575	14,18	14,404	14,336	14,373	14,483	14,681	14,413	14,455	14,296	14,14
C18:3n3	7,209	7,071	7,169	7,152	7,116	7,27	7,077	6,94	7,136	7,256	6,862	7,118	7,088	7,104	7,189	7,312	7,021	7,035	7,149	6,886
C20:1	2,789	2,758	2,809	2,808	2,804	2,747	2,921	2,927	2,97	2,802	2,682	2,73	2,962	2,913	2,704	2,812	2,703	2,897	2,947	2,949
C20:2	1,146	1,115	1,127	1,128	1,144	1,11	1,098	1,129	1,184	1,098	1,077	1,104	1,093	1,121	1,088	1,119	1,101	1,068	1,127	1,124
C20:3n6	0,267	0,264	0,259	0,237	0,263	0,263	0,259	0,243	0,253	0,242	0,208	0,213	0,227	0,227	0,239	0,264	0,249	0,239	0,245	0,253
C20:4n6	0,279	0,304	0,295	0,301	0,29	0,286	0,283	0,284	0,279	0,285	0,295	0,288	0,28	0,295	0,306	0,289	0,288	0,281	0,284	0,291
C20:3n3	0,682	0,677	0,676	0,684	0,678	0,668	0,664	0,666	0,662	0,683	0,681	0,667	0,672	0,682	0,646	0,686	0,663	0,659	0,668	0,672
C22:0	0,181	0,184	0,176	0,184	0,187	0,182	0,176	0,169	0,179	0,188	0,183	0,179	0,173	0,182	0,186	0,183	0,176	0,18	0,18	0,169
C20:5n3	5,85	5,818	5,879	5,865	5,83	5,816	5,806	5,86	5,803	5,939	5,894	5,787	5,831	5,956	6,008	5,931	5,761	5,72	5,827	5,843
C22:2	0,103	0,109	0,138	0,117	0,125	0,109	0,083	0,076	0,091	0,115	0,12	0,111	0,114	0,1	0,095	0,1	0,082	0,112	0,111	0,115
C24:1	1,712	1,694	1,718	1,732	1,708	0,955	1,689	1,643	1,669	1,736	1,71	1,669	1,691	1,72	1,726	1,758	1,681	1,651	1,695	1,673
C22:6n3	8,479	8,515	8,509	8,688	8,52	8,65	8,411	8,438	8,248	8,537	8,59	8,366	8,438	8,529	8,651	8,762	8,439	8,309	8,455	8,461
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
SFA	16,946	17,324	16,719	16,951	16,813	16,664	16,945	17,356	16,685	16,093	17,156	17,029	16,73	17,554	17,315	15,97	17,028	17,117	16,879	17,024
MUFA	44,57	44,503	44,832	44,55	44,918	44,511	45,037	44,941	45,268	45,176	44,934	44,916	45,192	44,059	43,979	44,885	44,953	45,006	44,961	45,191
PUFA	38,381	38,063	38,309	38,382	38,145	38,716	37,935	37,625	37,957	38,615	37,787	37,947	37,965	38,287	38,61	39,044	37,935	37,766	38,051	37,67
Σ UFA	82,951	82,566	83,141	82,932	83,063	83,227	82,972	82,566	83,225	83,791	82,721	82,863	83,157	82,346	82,589	83,929	82,888	82,772	83,012	82,861

Tables A.2 Fatty acid composition of salmon head oils obtained during the optimization process of microwave-assisted extraction.

	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12	H13	H14	H15	H16	H17	H18	H19	H20
Compoun	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%
C12:0	0,424	0,385	0,432	0,388	0,424	0,414	0,393	0,372	0,397	0,426	0,423	0,446	0,411	0,411	0,39	0,348	0,421	0,421	0,418	0,418
C14:0	2,804	2,717	2,762	2,667	2,804	2,739	2,738	2,59	2,692	2,827	2,719	2,849	2,704	2,704	2,606	2,484	2,786	2,786	2,724	2,724
C15:0	0,201	0,182	0,205	0,22	0,201	0,191	0,193	0,18	0,184	0,197	0,19	0,19	0,188	0,188	0,184	0,174	0,194	0,194	0,186	0,186
C16:0	10,902	11,066	10,666	10,854	10,902	10,654	10,935	10,331	10,765	11,046	10,608	10,907	10,649	10,649	10,375	10,062	10,84	10,84	10,705	10,705
C16:1	2,644	2,52	2,626	2,603	2,644	2,631	2,631	2,65	2,471	2,535	2,609	2,604	2,61	2,61	2,638	2,569	2,499	2,499	2,638	2,638
C18:0	2,965	3,177	3,067	3,248	2,965	2,953	3,041	2,923	2,954	2,976	3,028	3,185	2,978	2,978	2,952	2,82	3,011	3,011	3,062	3,062
C18:1n9c	36,657	37,514	37,7	37,691	36,657	37,336	37,669	37,619	37,399	37,389	37,017	36,971	37,184	37,184	38,018	41,605	37,445	37,445	37,303	37,303
C18:2n6c	13,893	13,602	13,618	13,322	13,893	13,823	13,602	13,872	14,063	13,836	13,698	13,423	13,699	13,699	13,747	12,71	13,816	13,816	13,69	13,69
C18:3n3	6,815	6,467	6,628	6,393	6,815	6,856	6,678	6,903	6,737	6,605	6,825	6,596	6,815	6,815	6,811	6,356	6,62	6,62	6,656	6,656
C20:1	2,9	2,796	2,994	3,031	2,9	2,863	2,882	2,932	2,762	2,713	2,942	2,97	2,943	2,943	2,74	2,554	2,771	2,771	2,75	2,75
C20:2	1,196	1,082	1,148	1,148	1,196	1,058	1,132	1,104	0,976	0,977	1,048	1,069	1,139	1,139	1,118	1,027	0,952	0,952	1,139	1,139
C20:3n6	0,247	0,221	0,263	0,278	0,247	0,23	0,234	0,24	0,22	0,225	0,239	0,347	0,254	0,254	0,241	0,231	0,223	0,223	0,265	0,265
C20:4n6	0,349	0,333	0,304	0,306	0,349	0,363	0,324	0,333	0,317	0,338	0,346	0,363	0,347	0,347	0,34	0,317	0,323	0,323	0,341	0,341
C20:3n3	0,665	0,636	0,673	0,69	0,665	0,641	0,638	0,643	0,655	0,641	0,655	0,636	0,666	0,666	0,657	0,599	0,64	0,64	0,663	0,663
C20:5n3	6,145	6,094	5,825	5,916	6,145	6,065	5,683	5,924	5,964	6,067	6,155	6,177	5,932	5,932	5,906	5,711	5,997	5,997	5,933	5,933
C22:2	0,107	0,096	0,139	0,182	0,107	0,113	0,123	0,096	0,096	0,091	0,09	0,093	0,111	0,111	0,13	0,081	0,089	0,089	0,156	0,156
C24:1	1,711	1,622	1,696	1,63	1,711	1,687	1,713	1,726	1,754	1,676	1,724	1,707	1,748	1,748	1,706	1,559	1,744	1,744	1,685	1,685
C22:6n3	9,375	9,491	9,254	9,434	9,375	9,381	9,392	9,562	9,596	9,435	9,685	9,465	9,621	9,621	9,443	8,793	9,625	9,625	9,686	9,686
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
SFA	17,296	17,527	17,132	17,377	17,296	16,951	17,3	16,396	16,992	17,472	16,968	17,577	16,93	16,93	16,507	15,888	17,252	17,252	17,095	17,095
MUFA	43,912	44,452	45,016	44,955	43,912	44,517	44,895	44,927	44,386	44,313	44,292	44,252	44,485	44,485	45,102	48,287	44,459	44,459	44,376	44,376
PUFA	38,792	38,022	37,852	37,669	38,792	38,53	37,806	38,677	38,624	38,215	38,741	38,169	38,584	38,584	38,393	35,825	38,285	38,285	38,529	38,529
Σ UFA	82,704	82,474	82,868	82,624	82,704	83,047	82,701	83,604	83,01	82,528	83,033	82,421	83,069	83,069	83,495	84,112	82,744	82,744	82,905	82,905

Tables A.3 Fatty acid composition of salmon viscera oils obtained during the optimization process of microwave-assisted extraction.

	V1	V2	V3	V4	V5	V6	V7	V8	V9	V10	V11	V12	V13	V14	V15	V16	V17	V18	V19	V20
Compoun	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%	mount [%
C14:0	1,928	2,129	2,166	2,006	2,149	2,186	2,06	2,104	2,138	2,088	1,815	2,033	2,09	2,051	2,006	2,105	2,158	2,166	2,095	2,092
C15:0	0,147	0,165	0,163	0,161	0,165	0,168	0,157	0,16	0,164	0,167	0,135	0,16	0,162	0,147	0,144	0,159	0,163	0,163	0,159	0,16
C16:0	9,181	9,817	9,829	9,719	9,707	9,872	9,581	9,88	9,957	9,759	8,622	9,315	9,786	9,73	9,244	9,552	9,622	9,757	9,496	9,53
C16:1	1,981	2,087	2,012	2,023	2,059	2,08	2,078	2,019	1,992	2,042	1,953	2,133	2,053	1,985	2,164	2,208	2,113	2,07	2,087	2,091
C17:0	0,132	0,141	0,141	0,138	0,157	0,137	0,14	0,138	0,14	0,136	0,116	0,129	0,14	0,141	0,129	0,131	0,15	0,134	0,134	0,137
C17:1	0,105	0,09	0,088	0,109	0,105	0,112	0,109	0,09	0,11	0,082	0,071	0,086	0,103	0,085	0,096	0,114	0,11	0,089	0,103	0,116
C18:0	2,987	3,043	3,074	3,064	3,05	3,043	2,954	3,034	3,184	3,087	2,711	2,955	3,098	3,093	2,949	2,914	2,927	3,048	3,001	2,972
C18:1n9c	37,111	37,718	37,366	37,441	37,144	37,497	38,004	37,068	36,846	38,001	41,219	37,838	37,605	37,462	37,897	37,707	37,701	36,538	37,647	37,856
C18:2n6t	0,18	0,181	0,202	0,164	0,178	0,2	0,144	0,208	0,192	0,172	0,023	0,034	0,196	0,204	0,019	0,042	0,134	0,175	0,131	0,132
C18:2n6c	18,875	18,299	18,246	18,357	18,414	18,308	18,218	18,204	17,929	18,036	17,609	18,409	18,225	18,233	18,357	18,458	18,267	18,548	18,206	18,278
C18:3n6	0,128	0,164	0,16	0,149	0,164	0,124	0,129	0,123	0,124	0,122	0,141	0,12	0,15	0,163	0,126	0,125	0,125	0,123	0,148	0,151
C18:3n3	9,273	8,964	8,935	9,043	9,061	8,948	8,99	8,906	8,764	8,844	8,675	9,197	8,941	8,943	9,283	9,226	9,032	9,157	9,02	9,059
C20:0	0,146	0,139	0,141	0,144	0,141	0,136	0,141	0,133	0,131	0,125	0,15	0,144	0,129	0,131	0,141	0,156	0,151	0,136	0,148	0,15
C20:1	2,319	2,187	2,207	2,236	2,206	2,163	2,204	2,156	2,135	2,226	1,979	2,15	2,214	2,195	2,163	2,134	2,288	2,275	2,25	2,213
C20:2	1,21	1,076	1,122	1,184	1,162	1,326	1,208	1,119	1,131	1,16	1,163	1,271	1,103	1,11	1,29	1,242	1,434	1,195	1,174	1,179
C20:3n6	0,229	0,24	0,228	0,236	0,265	0,267	0,253	0,237	0,255	0,24	0,26	0,257	0,236	0,24	0,261	0,252	0,222	0,228	0,238	0,248
C20:4n6	0,211	0,212	0,234	0,227	0,236	0,217	0,216	0,268	0,283	0,247	0,214	0,217	0,227	0,251	0,235	0,232	0,211	0,229	0,227	0,219
C20:3n3	0,823	0,793	0,825	0,795	0,819	0,801	0,782	0,792	0,797	0,801	0,761	0,802	0,798	0,809	0,801	0,817	0,809	0,816	0,809	0,803
C22:0	0,21	0,222	0,204	0,198	0,204	0,196	0,194	0,195	0,195	0,199	0,191	0,181	0,199	0,195	0,187	0,182	0,183	0,206	0,203	0,197
C20:5n3	3,842	3,701	3,808	3,779	3,775	3,609	3,734	3,868	3,91	3,778	3,669	3,705	3,652	3,835	3,602	3,668	3,622	3,878	3,641	3,627
C22:2	0,125	0,123	0,091	0,097	0,103	0,091	0,095	0,098	0,095	0,098	0,103	0,103	0,098	0,098	0,096	0,101	0,089	0,099	0,094	0,091
C24:0	0,026	0,027	0,027	0,028	0,029	0,029	0,027	0,026	0,027	0,029	0,026	0,037	0,028	0,027	0,027	0,027	0,027	0,029	0,028	0,026
C24:1	1,33	1,276	1,311	1,292	1,306	1,279	1,271	1,326	1,35	1,3	1,216	1,285	1,294	1,311	1,281	1,225	1,237	1,34	1,316	1,253
C22:6n3	7,502	7,206	7,419	7,41	7,401	7,21	7,313	7,85	8,151	7,26	7,18	7,441	7,473	7,56	7,5	7,222	7,226	7,6	7,643	7,42
	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
SFA	14,757	15,683	15,745	15,458	15,602	15,767	15,254	15,67	15,936	15,59	13,766	14,954	15,632	15,515	14,827	15,226	15,381	15,639	15,264	15,264
MUFA	42,846	43,358	42,984	43,101	42,82	43,131	43,666	42,659	42,433	43,651	46,438	43,492	43,269	43,038	43,601	43,388	43,449	42,312	43,403	43,529
PUFA	42,273	40,836	41,179	41,344	41,475	41,01	40,987	41,575	41,536	40,66	39,695	41,453	41,001	41,348	41,474	41,284	41,082	41,949	41,237	41,116
Σ UFA	85,119	84,194	84,163	84,445	84,295	84,141	84,653	84,234	83,969	84,311	86,133	84,945	84,27	84,386	85,075	84,672	84,531	84,261	84,64	84,645