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*Departament de Medicina Preventiva i Salut Pública, Ciències de
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**SOLVENTES NATURALES PROFUNDAMENTE EUTÉCTICOS
PARA UNA EXTRACCIÓN SOSTENIBLE DE COMPUESTOS
BIOACTIVOS DE SUBPRODUCTOS DE *CITRUS SINENSIS*:
CARACTERIZACIÓN FÍSICOQUÍMICA, ESTABILIDAD,
ACTIVIDAD BIOLÓGICA Y APLICACIÓN EN PRODUCTOS
ALIMENTARIOS**

**NATURAL DEEP EUTECTIC SOLVENTS FOR A SUSTAINABLE
EXTRACTION OF BIOACTIVE COMPOUNDS FROM *CITRUS
SINENSIS* BY-PRODUCTS: PHYSICOCHEMICAL
CHARACTERIZATION, STABILITY, BIOLOGICAL ACTIVITY
AND FOOD PRODUCTS APPLICATIONS**

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Dirigida per

Dra. Ana Frígola Cánoves

Dra. M^a José Esteve Mas

Dr. Jesús Blesa Jarque

Presentada per:

Clara Gómez Urios

Ana M^a Frígola Cánoves, M^a José Esteve Mas, Catedráticas de Universidad, y Jesús Blesa Jarque, Profesor Permanente Laboral, del área de Nutrición y Bromatología 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 Clara Gómez Urios ha realizado bajo su dirección el trabajo que lleva por título: "Solventes naturales profundamente eutécticos para una extracción sostenible de compuestos bioactivos de subproductos de *Citrus sinensis*: caracterización fisicoquímica, estabilidad, actividad biológica y aplicación en productos alimentarios" para optar al Título de Doctora Internacional por la Universitat de València, en Ciencias de la Alimentación

Y para que así conste, expiden y firman el presente certificado en Burjassot (València), 9 de enero de 2025.

Dra. Ana Frigola Canoves Dra M^a José Esteve Mas Dr. Jesús Blesa Jarque

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de les masoveres
els sabers populars
que sempre perseveren
vinc de la mort
l'exili i la pena
els camps d'extermini
les fosses de Paterna
les mestres d'escola
que mai claudicaren
el fusell de La Pastora
a les nostres muntanyes
vinc del puny alçat
d'Alejandra Soler
l'etern somriure
de Carme Miquel
les cançons de lluita
de Vicent Torrent
el batec immortal
de milers de Guillems

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ABREVIATURAS

ABBREVIATIONS

AA	Ácido ascórbico	Ascorbic acid
ABTS	2,2'-Azino-bis(3- etilbenzotiazolina-6-sulfónica)	2,2'-Azino-bis(3- ethylbenzothiazoline-6-sulfonic acid)
AC	Capacidad Antioxidante	Antioxidant capacity
AESAN	Agencia Española de Seguridad Alimentaria y Nutrición	Spanish Agency for Food Safety and Nutrition
AGREE	Calculadora de sostenibilidad	Analytical Greenness Calculator
ANOVA	Análisis de Varianza	Analysis of Variance
Bet	Betaína	Betaine
C/C0	Concentración final / Concentración inicial	Final concentration / Initial concentration
CA	Ácido Cítrico	Citric acid
CE	Catequina equivalente	Catechin equivalent
CFU	Unidades Formadoras de Colonias	Colony Forming Units
ChCl	Cloruro de Colina	Choline chloride
DELTA-E	Incremento de color	Color increment
DES	Solventes profundamente eutécticos	Deep eutectic solvents
DPPH	2,2-difenil-1-picrilhidrazilo	2,2-Diphenyl-1-picrylhydrazyl
dw	Peso seco	Dry weight
EFSA	Autoridad Europea de Seguridad Alimentaria	European Food Safety Authority
EPA	Agencia de Protección Ambiental	Environmental Protection Agency
EtOH	Etanol	Ethanol
FAO	Organización de las Naciones Unidas para la Alimentación y la Agricultura	Food and Agriculture Organization of the United Nations
FDA	Administración de Alimentos y Medicamentos	Food and Drug Administration
FRAP	Poder Reductor del Ion Férrico	Ferric Reducing Antioxidant Power
Fruc	Fructosa	Fructose

FTIR	Espectroscopía Infrarroja por Transformada de Fourier	Fourier-Transform Infrared Spectroscopy
GAE	Equivalente de Ácido Gálico	Gallic Acid Equivalent
GC	Cromatografía de Gases	Gas Chromatography
GI	Gastrointestinal	Gastrointestinal
Glu	Glucosa	Glucose
Gly	Glicerol	Glycerol
GRAS	Generalmente Reconocido como Seguro	Generally Recognized As Safe
HAT	Transferencia de Átomo de Hidrógeno	Hydrogen Atom Transfer
HBA	Aceptor de Enlace de Hidrógeno	Hydrogen Bond Acceptor
HBD	Donante de Enlace de Hidrógeno	Hydrogen Bond Donor
HP	Altas Presiones	High Pressures
HPLC-UV	Cromatografía Líquida de Alta Resolución con Detección Ultravioleta	High-Performance Liquid Chromatography with Ultraviolet Detection
HS	Extracción por Calentamiento y Agitación	Heating-Stirring Extraction
IDA	Adquisición Dependiente de Información	Information-Dependent Acquisition
ILs	Líquidos Iónicos	Ionic Liquids
K	Pendiente de la curva	Slope of the curve
LA	Ácido Láctico	Lactic Acid
LAB	Bacterias Lácticas	Lactic Acid Bacteria
LC/MS	Cromatografía Líquida con Espectrometría de Masas	Liquid Chromatography-Mass Spectrometry
LOD	Límite de Detección	Limit of Detection
LOQ	Límite de Cuantificación	Limit of Quantification
LP	Prolina	Proline
MA	Ácido Málico	Malic Acid
MAE	Extracción Asistida por Microondas	Microwave-Assisted Extraction

MetOH	Metanol	Methanol
MRS	Medio de cultivo Man Rogosa and Sharpe	Man Rogosa and Sharpe Medium
MS	Espectrómetro de Masas	Mass Spectrometer
NADES	Solventes Naturales Profundamente Eutécticos	Natural Deep Eutectic Solvents
ND	No Detectado	Not Detected
ODS	Objetivos de Desarrollo Sostenible	Sustainable Development Goals
OJ	Jugo de Naranja	Orange Juice
ORAC	Capacidad de Absorción de Radicales de Oxígeno	Oxygen Radical Absorbance Capacity
PAMPA	Ensayo de Permeabilidad de Membrana Artificial Paralela	Parallel Artificial Membrane Permeability Assay
PCA	Análisis de Componentes Principales	Principal Component Analysis
PCAg	Recuento en Placa con Agar	Plate Counting Agar
Pe	Coeficiente de Permeabilidad	Permeability Coefficient
PEF	Campos Eléctricos Pulsados	Pulsed Electric Fields
%NADES	Porcentaje de NADES en agua (v/v)	NADES percentage in water (v/v)
RSD	Desviación Estándar Relativa	Relative Standard Deviation
RSM	Metodología de Superficie de Respuesta	Response Surface Methodology
SCF	Fluidos Supercríticos	Supercritical Fluids
SD	Desviación Estándar	Standard Deviation
SE	Error Estándar	Standard Error
SET	Transferencia de Electrón Único	Single Electron Transfer
SPME	Microextracción en Fase Sólida	Solid Phase Microextraction
TA	Ácido Tartárico	Tartaric Acid
TE	Equivalente de Trolox	Trolox Equivalent
TEAC	Capacidad Antioxidante Equivalente a Trolox	Trolox Equivalent Antioxidant Capacity
TFC	Contenido Total de Flavonoides	Total Flavonoid Content

TMAB	Bacterias Aerobias Mesófilas Totales	Total Mesophilic Aerobic Bacteria
TPC	Contenido Total de Polifenoles	Total Polyphenol Content
UAE	Extracción Asistida por Ultrasonidos	Ultrasound-Assisted Extraction
UE	Unión Europea	European Union
UV/VIS	Ultravioleta/Visible	Ultraviolet/Visible
XYL	Xilosa	Xylose
YM	Levaduras y Mohos	Yeast and Molds
YPD	Extracto de Levadura Peptona Dextrosa	Yeast Extract Peptone Dextrose



RESUMEN

ABSTRACT

Resum

La taronja dolça és una de les fruites més cultivades a escala mundial per la seua versatilitat. Es pot consumir directament en fresc, en elaboracions culinàries o en sucs. L'elevat consum de taronges genera una quantitat significativa de residus, especialment en la indústria citrícola, on aproximadament el 50% del pes de la fruita es descarta a escala mundial.

En la cerca de processos sostenibles i d'economia circular, resulta necessari reaprofitar aquests residus (que inclouen la pell, la polpa i les llavors) a causa del seu alt contingut en compostos de gran valor biològic, com els compostos fenòlics, l'àcid ascòrbic (AA) i els carotenoides, destacant especialment per les seues propietats antioxidants.

Per a dur a terme un procés de reaprofitament plenament sostenible, l'extracció d'aquests compostos bioactius ha de basar-se en la química verda i ser sostenible. En els darrers anys, una nova classe de dissolvents ha sorgit com una alternativa als dissolvents tradicionals, oferint propietats d'extracció diverses i adequades, característiques fisicoquímiques úniques i gran versatilitat. Aquests dissolvents naturals profundament eutèctics (NADES) són mescles de dos o més compostos naturals en una proporció molar específica que es fonen en el seu punt eutèctic. Una de les seues desavantatges és la seua alta viscositat, però es pot controlar mitjançant la dilució amb aigua en certs percentatges. A més, els seus components estan reconeguts per la FDA com a segurs per al consum, la qual cosa fa possible el seu ús com a additius o ingredients dins de la matriu alimentària sense necessitat de purificar l'extracte.

L'objectiu d'aquesta tesi doctoral comprén l'ús dels NADES per a l'extracció de compostos fenòlics i àcid ascòrbic dels subproductes de la pell de la taronja

(*Citrus sinensis*), comparant també mètodes d'extracció convencionals i no convencionals, com l'extracció assistida per ultrasons i l'extracció amb agitació magnètica en calent. També es caracteritzen els extractes obtinguts amb NADES i s'estudia el paper protector d'aquests dissolvents sobre la degradació dels compostos bioactius extrets. Finalment, es formula una beguda a base de suc de taronja amb l'addició de l'extracte de subproductes de taronja obtingut amb NADES, analitzant la vida útil mitjançant proves microbiològiques i del perfil volàtil en condicions de refrigeració (4 °C) i abús tèrmic (8 °C).

Capítol 1: Es realitza una caracterització dels dissolvents mitjançant infraroig, pH, densitat, polaritat i capacitat antioxidant. Es desenvolupa un estudi *in silico* sobre la solubilitat i l'estabilitat de l'AA en diversos NADES, així com l'avaluació de la permeabilitat de membrana gastrointestinal i cutània dels NADES amb AA. Els resultats mostren que els NADES tenen un efecte protector sobre l'AA al llarg del temps, augmentant-ne la vida útil i prevenint-ne la degradació. Aquests resultats són prometedors per a la formulació de productes alimentaris, cosmètics i farmacèutics.

Capítol 2: Se centra en l'optimització dels millors paràmetres d'extracció per al contingut fenòlic total (TPC) i el contingut de flavonoides totals (TFC) en diversos NADES, així com en l'estabilitat del TPC en les millors condicions optimitzades. S'utilitza un model *in silico* de *screening* mitjançant el programari COSMOtherm. Els resultats indiquen que els millors NADES són l'àcid làctic:glucosa (LA:Glu) i la prolina:àcid màlic (LP:MA). L'optimització d'aquests dos NADES es duu a terme mitjançant metodologia de superfície de resposta, tenint en compte variables com el temps (minuts), la proporció (p/v) i el percentatge d'aigua en el NADES (v/v). Els resultats mostren que els paràmetres d'optimització tenen una influència significativa en l'extracció de TPC i TFC, i demostren que els NADES

augmenten l'estabilitat del TPC, revelant-ne l'eficiència per a l'extracció de compostos bioactius de la pell de la taronja.

Capítol 3: Estudia les tècniques d'extracció més sostenibles mitjançant l'eina AGREE. Es realitza un *screening* inicial dels dos millors NADES per a l'extracció de l'hesperidina, classificant-los segons els seus components (basats en sucres o poliols i basats en àcids) i analitzant la capacitat antioxidant i l'extracció d'AA. També s'avalua l'estabilitat dels compostos fenòlics i de l'AA mitjançant HPLC-UV/VIS i la capacitat antioxidant. Els resultats mostren que el clorur de colina:àcid màlic (ChChl:MA) i el clorur de colina:glicerol (ChChl:Gly) són els NADES més adequats per a l'extracció de compostos bioactius. Els compostos fenòlics i l'AA són més estables en ChChl:MA, la qual cosa ressalta la seua capacitat per millorar l'estabilitat dels compostos bioactius i potenciar la capacitat antioxidant, fent l'extracte adequat per a aplicacions en formulacions alimentàries.

Capítol 4: Compara dos mètodes d'extracció, l'extracció assistida per ultrasons (UAE) i l'extracció amb agitació en calent (HS), per a extreure compostos fenòlics i capacitat antioxidant en quatre NADES diferents. Els resultats mostren que l'UAE ofereix millors rendiments d'extracció de compostos fenòlics i capacitat antioxidant, particularment amb ChChl:Fruc i ChChl:LA. Tanmateix, l'HS demostra una major estabilitat per als compostos bioactius extrets en la majoria de NADES.

Capítol 5: Consta de dues parts. La primera es dedica a caracteritzar quatre extractes de NADES basats en clorur de colina per a l'ús com a additius o ingredients en productes alimentaris. S'avaluen el perfil fenòlic, el perfil volàtil, la capacitat antioxidant i les propietats antimicrobianes. Els resultats mostren que els NADES basats en clorur de colina tenen potencial com a agents

antimicrobians i antioxidants. La segona part se centra en formular un suc de taronja amb extractes de NADES. Els estudis indiquen que el suc formulat amb NADES té una vida útil superior i millor seguretat microbiològica respecte al control.

En conclusió, aquesta tesi demostra el potencial dels NADES com a agents protectors i vehicles per a compostos bioactius. Els NADES no sols allarguen la vida útil de l'àcid ascòrbic, sinó que també optimitzen l'extracció i l'estabilitat dels polifenols i antioxidants, depenent de la composició i condicions d'emmagatzematge. Finalment, la incorporació d'aquests extractes en suc de taronja millora significativament la seua vida útil i qualitat sensorial, destacant la seua aplicabilitat comercial.

Resumen

La naranja dulce es una de las frutas más cultivadas a nivel mundial, debido a su versatilidad. Se puede consumir directamente en fresco, en elaboraciones culinarias y en zumos. Debido a su alto consumo, se genera una gran cantidad de residuos, sobre todo por parte de la industria citrícola, donde a nivel mundial un 50% del peso de la fruta se descarta.

En la búsqueda de procesos sostenibles y de economía circular, resulta necesario el reaprovechamiento de estos residuos (que engloban la piel, la pulpa y las semillas) debido a su alto contenido en compuestos de gran valor biológico como los compuestos fenólicos, el ácido ascórbico (AA) y los carotenoides, los cuales destacan sobre todo por sus propiedades antioxidantes.

Para poder llevar a cabo un proceso de reaprovechamiento totalmente sostenible, la forma en que se extraen estos compuestos bioactivos debe basarse en la química verde y ser, a su vez, sostenible. En los últimos años, una

nueva clase de solventes se ha postulado como alternativa a los solventes tradicionales, debido a sus variadas y adecuadas propiedades extractantes, características físico-químicas únicas y gran versatilidad, ya que con la selección de sus componentes pueden modificarse sus propiedades para un uso en concreto. Así, los solventes naturales profundamente eutécticos (NADES), son mezcla de dos o más compuestos naturales en un ratio molar concreto, que se funden en su punto eutéctico. Una de sus desventajas es su alta viscosidad, pero esta puede ser controlada por dilución con agua en ciertos porcentajes. Además, sus componentes están reconocidos por la FDA como seguros para su consumo, lo que hace que sea posible su uso como aditivos o ingredientes dentro de la matriz alimentaria sin necesidad de purificar el extracto.

El objetivo de la presente tesis doctoral engloba, el uso de NADES para la extracción de compuestos fenólicos y ácido ascórbico de los subproductos de la piel de la naranja (*Citrus sinensis*), comparando también métodos de extracción no convencionales y convencionales, como la extracción asistida por ultrasonidos y la extracción con agitación magnética en caliente. La caracterización de los extractos obtenidos de la piel de naranja con el uso de NADES, así como el papel protector de estos solventes sobre la degradación de los compuestos bioactivos extraídos. Y, finalmente, la formulación de una bebida a base de zumo de naranja, con la adición del extracto de subproductos de la naranja obtenidos con NADES, estudiando la vida útil mediante pruebas microbiológicas y el perfil volátil a temperatura de refrigeración (4 °C) y abuso térmico (8 °C).

El primer capítulo, engloba una caracterización de los solventes, mediante su espectro de infrarrojo y los valores de pH, densidad, polaridad y capacidad antioxidante. Un estudio *in-silico* de la solubilidad y de la estabilidad del AA en diferentes NADES, y el estudio de la permeabilidad de membrana

(gastrointestinal y cutánea) del AA solubilizado en los NADES. Los resultados muestran que los NADES tienen un papel protector del AA a lo largo del tiempo, aumentando su vida útil y previniendo la degradación. Estos resultados resultan prometedores para la formulación de productos alimentarios, cosméticos y farmacéuticos.

El segundo capítulo se centra en la optimización de los mejores parámetros de extracción para fenoles solubles totales (TPC) y flavonoides totales (TFC) en diferentes NADES y la estabilidad de TPC con los mejores parámetros de optimización. Para ello se realiza un modelo *in-silico* de screening utilizando el software COSMOtherm. Los resultados sugieren que los mejores NADES eran ácido láctico:glucosa (LA:Glu) y prolina:ácido málico (LP:MA). La optimización de estos dos se llevó a cabo mediante metodología de respuesta de superficie, teniendo en cuenta las variables tiempo (min), ratio (p/v) y porcentaje de agua en el NADES (v/v). Los resultados muestran que los parámetros de optimización tienen una influencia significativa en la extracción de TPC y TFC. También se demuestra que los NADES son capaces de aumentar la estabilidad de los TPC, revelando que los NADES son eficientes para la extracción de compuestos bioactivos de la piel de la naranja.

El capítulo tres, tiene como objetivo estudiar las técnicas de *screening* más sostenibles utilizando la herramienta AGREE. Un *screening* inicial de los dos mejores NADES para la extracción de la hesperidina, separándolos según sus componentes (basados en cloruro de colina con azúcares o polialcoholes y basados en cloruro de colina con ácidos), así como el estudio de la capacidad antioxidante y la extracción de AA. Además, se estudia la estabilidad en base a los compuestos fenólicos y al AA analizados por HPLC-UV/VIS, y a la capacidad antioxidante. Los resultados muestran que el cloruro de colina:ácido málico (ChChl:MA) y el cloruro de colina:glicerol (ChChl:Gly) son los NADES más

adecuados para la extracción de compuestos bioactivos. Los compuestos fenólicos y el AA son más estables en ChChl:MA, por lo que la capacidad de este NADES para mejorar la estabilidad de los compuestos bioactivos y potenciar la capacidad antioxidante hace que el extracto sea adecuado para su aplicación en las formulaciones alimentarias.

El capítulo cuatro, tiene como objetivo comparar dos métodos de extracción, extracción asistida por ultrasonidos (UAE) y extracción por placa magnética calefactada (HS), para la extracción de compuestos fenólicos y la capacidad antioxidante en cuatro NADES diferentes. Los resultados de capacidad antioxidante muestran que la UAE tiene mejores resultados que la HS con ChChl:Fruc y ChChl:LA. Además, las muestras extraídas por UAE tienen un rendimiento de extracción superior para los compuestos fenólicos, particularmente para el ácido cafeico y la hesperidina, donde ChChl:Fruc y ChChl:LA son los mejores NADES. En cuanto a la estabilidad, el extracto obtenido por HS muestra una capacidad antioxidante más estable que al utilizar UAE, excepto para ChChl:Fruc. La extracción por UAE demuestra una estabilidad mayor para los compuestos fenólicos en ChChl:LP:MA y ChChl:Gly, mientras que HSE muestra mayor eficacia para ChChl:LA. Queda demostrado que la selección de NADES y los métodos de extracción deben adaptarse para lograr un equilibrio óptimo entre rendimiento, capacidad antioxidante y estabilidad de los compuestos bioactivos.

El capítulo cinco engloba dos partes, la primer se centra la caracterización de cuatro extractos de NADES basados en cloruro de colina para su futura aplicación como aditivo o ingrediente en la formulación de nuevos productos alimentarios. Para ello se analiza el perfil fenólico por UPLC-TOF-MS/MS, el estudio de los compuestos volátiles por GC-MS/MS, un análisis del perfil infrarojo de los extractos, la capacidad antioxidante por cuatro métodos

diferentes (DPPH, FRAP, TEAC y ORAC), la capacidad de los extractos para modular la actividad de las enzimas digestivas y las propiedades antimicrobianas de los extractos. El análisis de volátiles muestra que el perfil está compuesto principalmente por alcoholes, aldehídos y terpenos, y se observa que todos los extractos mejoran la actividad de la pepsina sin afectar la de la quimotripsina. La actividad antimicrobiana de los extractos es limitada para cepas seleccionadas de patógenos comunes transmitidos por los alimentos, a saber, *E. coli*, *S. aureus* y *S. epidermidis*, aunque ninguno de los extractos tuvo un efecto inhibitor sobre el crecimiento de *L. monocytogenes*. El extracto con cloruro de colina:glicerol:ácido cítrico (ChChl:Gly:CA) presenta una mayor actividad antimicrobiana hacia las especies sensibles. Estos resultados refrendan que los NADES a base de cloruro de colina pueden representar un método sostenible para reutilizar los subproductos de naranja, así como un gran avance para su uso como ingredientes en la formulación de nuevos alimentos debido a su acción antimicrobiana y antioxidante.

La segunda parte del capítulo cinco, tiene como objetivo la formulación de un zumo a base de naranja con la adición de extracto de NADES (ChChl:Fru, ChChl:Gly, ChChl:Xyl, ChChl:Gly:CA). Para ello se realiza un estudio sensorial previo con una lengua y una nariz electrónica para conocer la cantidad de extracto admisible en el nuevo zumo sin cambiar sus propiedades organolépticas. Se realiza posteriormente un estudio de la vida útil microbiológica y del perfil volátil a temperatura de refrigeración (4 °C) y abuso térmico (8 °C), así como del color y del pH. Además, se realiza una prueba *challenge test* contra microorganismos contaminantes de los zumos de naranja. Los resultados de este estudio demuestran que la vida útil del control es de nueve días a ambas temperaturas de almacenamiento, en contraste, el nuevo zumo formulado permanece inalterado hasta 13 días. Al comparar con el zumo

control los resultados del *challenge test* indican que el zumo adicionado exhibe una seguridad microbiológica mayor en comparación con el control. Esto demuestra que es factible prolongar la vida útil, mejorar la funcionalidad y la seguridad del zumo de naranja sin comprometer sus propiedades organolépticas.

En conclusión, esta tesis evidencia el potencial de los NADES como agentes protectores y vehículos para compuestos bioactivos. Los NADES no solo alargan la vida útil del AA, sino que también optimizan la extracción y la estabilidad de los polifenoles y los compuestos antioxidantes, aunque varían en función de la composición y de las condiciones de almacenamiento. Finalmente, la incorporación de estos extractos en zumos de naranja mejora significativamente la vida útil y la calidad sensorial del producto, lo que demuestra su aplicabilidad comercial.

Abstract

Sweet orange is one of the most widely cultivated fruits globally due to its versatility. It can be consumed fresh, used in culinary preparations, or processed into juices. The high consumption of oranges generates significant amounts of waste, especially in the citrus industry, where approximately 50% of the fruit's weight is discarded worldwide.

In the pursuit of sustainable and circular economy processes, the reuse of these by-products (including peel, pulp, and seeds) is necessary due to their high content of biologically valuable compounds such as phenolic compounds, ascorbic acid (AA), and carotenoids, which are particularly notable for their antioxidant properties.

To implement a fully sustainable reuse process, the extraction of these bioactive compounds must adhere to green chemistry principles and be environmentally sustainable. In recent years, a new class of solvents has emerged as an alternative to traditional solvents, offering diverse and suitable extracting properties, unique physicochemical characteristics, and high versatility. These natural deep eutectic solvents (NADES) are mixtures of two or more natural compounds at specific molar ratios that melt at their eutectic point. Although their high viscosity is a drawback, it can be controlled by diluting them with water at certain percentages. Furthermore, their components are recognized as safe for consumption by the FDA, enabling their use as additives or ingredients in food matrices without the need to purify the extract.

The objective of this doctoral thesis encompasses the use of NADES for the extraction of phenolic compounds and ascorbic acid from orange peel (*Citrus sinensis*) by-products, comparing conventional and unconventional extraction methods, such as ultrasound-assisted extraction and hot magnetic stirring extraction. It also involves characterizing the extracts obtained using NADES, as well as evaluating the protective role of these solvents against the degradation of extracted bioactive compounds. Finally, it investigates the formulation of an orange juice beverage incorporating the orange by-product extract obtained with NADES, assessing its shelf life through microbiological tests and volatile profile analysis under refrigerated (4 °C) and thermal abuse (8 °C) conditions.

Chapter 1 provides a characterization of the solvents using infrared spectroscopy, pH, density, polarity, and antioxidant capacity. An in-silico study of solubility and AA stability in various NADES, as well as an assessment of gastrointestinal and cutaneous membrane permeability, was conducted. Results demonstrate that NADES have a protective effect on AA over time, extending

its shelf life and preventing degradation. These findings are promising for applications in food, cosmetics, and pharmaceuticals.

Chapter 2 focuses on optimizing the best extraction parameters for total phenolic content (TPC) and total flavonoid content (TFC) in different NADES and evaluating TPC stability under optimized conditions. An in-silico screening model using COSMOtherm software suggested that the best NADES were lactic acid:glucose (LA:Glu) and proline:malic acid (LP:MA). Optimization was carried out using response surface methodology, considering variables such as time (min), ratio (p/v), and water percentage in NADES (v/v). Results indicate significant influences of optimization parameters on TPC and TFC extraction and reveal that NADES enhance TPC stability, highlighting their efficiency in extracting bioactive compounds from orange peel.

Chapter 3 investigates sustainable extraction techniques using the AGREE tool. A preliminary screening of the two best NADES for hesperidin extraction, categorized by their components (sugar- or polyol-based and acid-based), was performed alongside an analysis of antioxidant capacity and AA extraction. The stability of phenolic compounds and AA was studied using HPLC-UV/VIS and antioxidant capacity analysis. Results show that choline chloride:malic acid (ChCl:MA) and choline chloride:glycerol (ChCl:Gly) are the most suitable NADES for bioactive compound extraction. Phenolic compounds and AA are more stable in ChCl:MA, underscoring its potential for improving the stability of bioactive compounds and antioxidant capacity, making it suitable for food formulations.

Chapter 4 compares two extraction methods: ultrasound-assisted extraction (UAE) and hot stirring extraction (HS), for extracting phenolic compounds and

antioxidant capacity in four different NADES. Antioxidant capacity results reveal that UAE outperforms HS in ChChl:Fruc and ChChl:LA. UAE also yields higher extraction efficiency for phenolic compounds, particularly caffeic acid and hesperidin, with ChChl:Fruc and ChChl:LA emerging as the best NADES. Stability analysis shows that HS provides more stable antioxidant capacity than UAE, except for ChChl:Fruc. The study demonstrates that the selection of NADES and extraction methods must be tailored to balance yield, antioxidant capacity, and stability of bioactive compounds.

Chapter 5 comprises two parts. The first part characterizes four NADES-based extracts (using choline chloride) for their future application as additives or ingredients in new food formulations. Phenolic profiles were analyzed using UPLC-TOF-MS/MS, volatile compounds with GC-MS/MS, and infrared profiles of the extracts. Antioxidant capacity was assessed through four different methods (DPPH, FRAP, TEAC, and ORAC), along with evaluations of the extracts' ability to modulate digestive enzyme activity and their antimicrobial properties. Results indicate that the extracts enhance pepsin activity without affecting chymotrypsin and exhibit limited antimicrobial activity against selected common foodborne pathogens. ChChl:Gly:citric acid extract shows higher antimicrobial activity against sensitive species, demonstrating that choline chloride-based NADES can sustainably reuse orange by-products and advance their use as food ingredients due to their antimicrobial and antioxidant actions.

The second part of Chapter 5 focuses on formulating an orange juice beverage with NADES extracts (ChChl:Fruc, ChChl:Gly, ChChl:Xyl, ChChl:Gly:CA). A preliminary sensory study with electronic tongue and nose systems determined the acceptable extract concentration for preserving the juice's organoleptic properties. Microbiological shelf-life, volatile profile, color, and pH were

evaluated under refrigeration (4 °C) and thermal abuse (8 °C). A challenge test against contaminating microorganisms was also performed. Results reveal a shelf life of nine days for the control juice, whereas the formulated juice remains unaltered for 13 days. The formulated juice exhibits enhanced microbiological safety compared to the control. These findings confirm that extending shelf life, improving functionality, and ensuring juice safety without compromising sensory properties is feasible.

In conclusion this PhD thesis demonstrates the potential of NADES as protective agents and carriers for bioactive compounds. NADES not only extend the shelf life of ascorbic acid but also optimize the extraction and stability of polyphenols and antioxidants, depending on their composition and storage conditions. Incorporating these extracts into orange juice significantly improves its shelf life and sensory quality, showcasing their commercial applicability



INTRODUCCIÓN

INTRODUCTION

1. Industria citrícola y economía circular

En la producción mundial de cítricos como naranjas, limones, mandarinas y pomelos destaca la importancia de la industria citrícola en la economía agroalimentaria global. Según una estimación reciente de la Organización de las Naciones Unidas para la Alimentación y la Agricultura, la producción mundial de cítricos ha alcanzado los 144 millones de toneladas y está aumentando a un ritmo anual constante (Benny et al., 2023). La Unión Europea (UE) es uno de los mayores productores de frutas cítricas del mundo, elevándose a 11 millones de toneladas entre los años 2016-2020 (FAO, 2020). Así mismo España se sitúa como el primer país de la UE, con una producción media de 6,8 millones de toneladas y una producción en la campaña de 2023-2024 de 5,8 millones de toneladas (un 14,4% menos que los anteriores 5 años). Le siguen Italia y Grecia. El clima mediterráneo, donde predominan veranos cálidos e inviernos suaves y la composición de la tierra de cultivo, hace de esta zona europea una localización idónea para la producción de los frutos cítricos. De esta producción de cítricos, la naranja dulce (*Citrus Sinensis*) es la más cultivada, especialmente la variedad Navel, con una producción de 1,9 millones de toneladas (Ministerio de Agricultura, Pesca y Alimentación, 2021).

España además de ser uno de los principales productores a nivel mundial de naranjas, también es el principal exportador mundial de cítricos frescos. Por otra parte, alrededor del 49% de los productos comerciales obtenidos de la naranja están relacionados con la producción de zumo (Ferreira et al., 2023).

La industria citrícola es importante desde un punto de vista económico, pero produce una gran cantidad de subproductos y desechos durante el proceso industrial, ya que solo una parte de la fruta se destina al consumo directo

durante el procesamiento de cítricos, mientras que una gran cantidad se convierte en subproductos y desperdicios (de Castro et al., 2020). Estos incluyen pieles, semillas, pulpa y otros restos que tradicionalmente se han desechado o utilizado para la producción de alimentos para animales o el compostaje (Maqbook et al., 2023). La gestión eficaz de estos desechos y subproductos es importante para el medio ambiente y el desarrollo de un proceso productivo finalmente sostenible, con la implementación de modelos de economía circular en la industria y el aprovechamiento de los residuos, convirtiendo de esta manera los procesos en eficaces.

En los últimos años a través de la implementación de la Agenda 2020-2030 y de los Objetivos de Desarrollo Sostenible (ODS), la industria alimentaria y farmacéutica muestran gran interés en disminuir el desperdicio y utilizar procesos más sostenibles, así como en la revalorización de los residuos aprovechando sus compuestos bioactivos (United Nations, 2024).

Los subproductos de la industria citrícola, como la piel (albedo y flavedo), la pulpa y las semillas, contienen compuestos de alto valor añadido, como aceites esenciales, compuestos fenólicos, pectina, vitaminas, pigmentos (clorofilas y carotenoides), tocoferoles y compuestos volátiles y aromáticos (Anticono et al., 2020; Maqbool et al., 2023; Matharu et al., 2016; Murador et al., 2021). Se ha demostrado que los extractos de piel de naranja disminuyen el riesgo de desarrollar cáncer en modelos de ratón (Tajaldini et al., 2020). Estos beneficios biológicos se pueden atribuir a la amplia gama de biomoléculas presentes en la piel de naranja como azúcares fermentables, polímeros de carbohidratos, flavonoides, polifenoles, vitaminas, carotenoides y aceites esenciales (Anticono et al., 2020). Especialmente, los polifenoles han ganado mucho interés debido a sus efectos antioxidantes e inflamatorios (Ozturk et al., 2018; Panić et al.,

2020a). Estos compuestos se pueden utilizar en una variedad de industrias, incluida la producción de alimentos y suplementos nutricionales funcionales, así como su uso en la industria cosmética y farmacéutica. La extracción y reaprovechamiento de estos subproductos puede generar más ingresos para la industria, además de mejorar la sostenibilidad de todo el proceso de producción (Fraga et al., 2019; Smeu et al., 2022).

En lugar de seguir el modelo lineal tradicional de "producir, usar y desechar" el proceso de aprovechamiento de residuos agroalimentarios da pie a la economía circular, donde se propone un modelo económico regenerativo en el que los productos y materiales se reutilizan y revalorizan a lo largo de su ciclo de vida (Lüdeke-Freund et al., 2019). Este enfoque en la industria citrícola fomenta el uso completo de las frutas y está ganando terreno debido a la creciente presión por crear soluciones de gestión de residuos sostenibles y reducir el impacto ambiental de las actividades industriales. De esta manera, una de las principales estrategias para alcanzar los ODS en la industria citrícola es convertir los desechos en recursos. El uso de tecnologías verdes, como la generación de biogás a partir de residuos cítricos, pueden aprovechar en gran medida los residuos generados mientras se produce energía renovable o productos de gran valor añadido (Mahato et al., 2021). De esta manera, se cierra el ciclo de vida, lo que promueve la eficiencia de los recursos y reduce la huella ambiental. La transición hacia una industria más sostenible y circular requiere este aprovechamiento integral de los residuos (Pedro et al., 2024).

2. Contaminantes microbiológicos de las naranjas y sus derivados.

El deterioro microbiológico de los alimentos está causado por el crecimiento de microorganismos que producen enzimas que degradan el alimento y se generan compuestos que alteran sus características organolépticas (Krisch et al., 2016;

Odeyemi et al., 2020). El deterioro físicoquímico de los alimentos se produce cuando diferentes componentes del alimento reaccionan entre sí o con algún componente añadido que altera sus características organolépticas, así como cuando los alimentos húmedos se deshidratan en exceso o los alimentos secos absorben demasiada humedad (Oduoye et al., 2024).

La contaminación microbiana de las naranjas puede suponer un riesgo significativo para la seguridad y la calidad de los alimentos, y afecta a la vida útil de los productos cítricos. Las bacterias, las levaduras y los mohos son los principales agentes microbianos que pueden contaminar las naranjas, especialmente durante la manipulación posterior a la cosecha, el almacenamiento y la distribución (Izah & Odubo, 2023; Mitsagga et al., 2021).

El zumo recién exprimido de frutas es muy susceptible de perder su calidad por el deterioro de las características organolépticas y físicoquímicas, lo que provoca el rechazo del producto por parte de los consumidores. El proceso de exprimido implica una gran manipulación humana que puede contaminar el producto final (Kaddumukasa et al., 2017). Además de la manipulación, el crecimiento bacteriano en los zumos de frutas depende del pH, la temperatura de almacenamiento, los tipos de material de envasado, la humedad, la actividad del agua, la concentración de conservantes, la aplicación de tratamientos UV durante la producción, el contenido de azúcar, la limpieza de la maquinaria, las materias primas y la calidad de las frutas, entre otros factores (Alegbeleye et al., 2022).

En cuanto a las bacterias más comunes presentes en las naranjas y sus derivados durante la manipulación se encuentran *Salmonella* spp, y *Escherichia coli*. Las bacterias *Pseudomonas* spp son organismos de deterioro que prosperan en

condiciones húmedas y pueden provocar sabores y olores desagradables en los productos a base de naranja (Kaddumukasa et al., 2017).

Las condiciones ácidas del zumo de naranja favorecen la presencia de bacterias ácido lácticas, en particular del género *Lactiplantibacillus*, estas producen metabolitos como ácido láctico, ácido fórmico, ácido acético y etanol, entre otros, que cambian totalmente las características organolépticas de los zumos de naranja (Sourri et al., 2022). No obstante, no todas las bacterias ácido lácticas son perjudiciales, algunas bacterias son capaces de alargar la vida útil de los mismos (Quan et al., 2022), por ejemplo, *Limosilactobacillus fermentum* puede ayudar a promover la producción de compuestos volátiles en los zumos de frutas mejorando así sus características organolépticas (Cui et al., 2019). Por otra parte, *Lactiplantibacillus plantarum* suele estar presente en los zumos de frutas y verduras fermentados y entre otras funciones favorecen la absorción de nutrientes por su acción probiótica (Marrero et al., 2019).

Los mohos y las levaduras son los microorganismos predominantes en los zumos de frutas cítricas, debido a su alta tolerancia a condiciones ácidas, en combinación con la presencia de los azúcares naturales de la fruta y las condiciones de almacenamiento y refrigeración de los productos, todo ello favorece la contaminación por estos microorganismos (Sourri et al., 2022). El género más común de levaduras en zumos de frutas cítricas es *Saccharomyces*, que producen metabolitos secundarios y compuestos volátiles como etanol y dióxido de carbono, lo que provoca un aumento de la turbidez y la producción de gas en los zumos (Feliciano et al., 2019). El moho más común en las frutas cítricas es *Penicillium*, afectando a las características organolépticas de los productos cítricos.

3. Tratamientos térmicos y no térmicos para aumentar la seguridad y vida útil de los zumos de cítricos

Los tratamientos para disminuir la carga microbiana de las naranjas y sus derivados son una necesidad para poder comercializar de manera segura los productos. Existen tratamientos que son ampliamente utilizados, térmicos y no térmicos. En la Tabla 1, se muestran las ventajas y desventajas de los mismos.

La pasteurización y la esterilización son métodos convencionales utilizados para reducir los microorganismos y destruir las enzimas que causan cambios indeseables en los zumos (Bevilacqua et al., 2018; Umair et al., 2022). Sin embargo, las altas temperaturas pueden degradar los compuestos sensibles al calor, como las vitaminas y los compuestos fenólicos, lo que provoca cambios negativos en las propiedades físicas, nutricionales y sensoriales (Aghajanzadeh et al., 2023; Morales-de la Peña et al., 2019). Para satisfacer las demandas de los consumidores de alimentos mínimamente procesados, la industria alimentaria está explorando nuevos procesos sostenibles que preserven la calidad nutricional y sensorial, al tiempo que garanticen la estabilidad microbiológica, vida útil y mejoran su funcionalidad (Fonteles et al., 2024; Jiménez-Sánchez et al., 2017). Entre las tecnologías no térmicas aplicada a zumos de frutas, en los últimos años se han propuesto con resultados prometedores la homogeneización a alta presión, las altas presiones hidrostáticas, los campos eléctricos pulsados y los ultrasonidos (Gottardi et al., 2021; Satari & Karimi, 2018; Siroli et al., 2023a). Sin embargo, estas tecnologías todavía tienen varios inconvenientes, incluidos los altos costos del equipo y el consumo de energía, la efectividad limitada contra las esporas bacterianas y los posibles cambios en la calidad de los productos (Allai et al., 2023). El uso de antimicrobianos naturales, como aceites esenciales y extractos, puede representar una

alternativa ecológica y sostenible para la estabilización y mejora de bebidas innovadoras. Debido a sus propiedades antimicrobianas y antioxidantes, se pueden utilizar en la formulación de bebidas innovadoras como agentes aromatizantes (Bukvicki et al., 2023; Patrignani et al., 2020; Wu et al., 2023). En el contexto de la economía circular y la sostenibilidad de los procesos productivos, es de gran interés el uso de extractos de residuos y subproductos de la industria cítrica (Panić, Andlar, et al., 2021a; Romano et al., 2022) como una alternativa a los aditivos utilizados en la producción de alimentos debido a su composición.

Tabla 1. Ventajas y desventajas de los tratamientos térmicos y no térmicos para la conservación de los zumos de frutas cítricas

Tratamiento	Ventajas	Desventajas	Referencia
Pasteurización	- Mantiene la mayoría de los nutrientes y características organolépticas del producto. - Método ampliamente aceptado y seguro.	- No elimina todos los microorganismos, solo reduce los patógenos. - Puede alterar el sabor y la textura de ciertos alimentos.	Wibowo et al., 2015
Esterilización	- Elimina casi todos los microorganismos, incluidos esporas. - Prolonga la vida útil de los productos.	- Puede afectar en gran medida las propiedades sensoriales y nutricionales del producto. - Requiere altas temperaturas.	Aghajanzadeh et al., 2016)

Continúa

Continuación

Microondas	- Conserva nutrientes sensibles al calor por el corto tiempo de tratamiento. - Método eficiente y económico.	- No garantiza la eliminación completa de microorganismos. - Puede causar texturas indeseadas en algunos alimentos	Pratap Singh et al., 2018
Homogenización Altas presiones	- Inactiva microorganismos sin necesidad de altas temperaturas. - Mantiene propiedades sensoriales y nutricionales casi intactas.	- Alto coste de los equipos. - No es efectiva contra esporas sin combinación de calor.	Gottardi et al., 2021
Altas presiones hidrostáticas	- Tratamiento no térmico adecuado para alimentos líquidos y sólidos. - Excelente para productos frescos y de valor agregado, manteniendo el sabor y las características nutricionales	- Alto coste operativo y de maquinaria. - Limitaciones en productos con envases rígidos o de estructura específica.	Pokhrel et al., 2022
Campos eléctricos pulsados	- Conserva propiedades organolépticas y nutricionales. - Rápido y eficiente en el tratamiento de líquidos y purés. - No generan residuos químicos indeseados.	- Eficacia limitada frente a esporas bacterianas. - Costes iniciales elevados. - Requiere equipamiento especializado.	Ranjha et al., 2021
Antimicrobianos naturales	- Alta aceptación entre consumidores que buscan productos "naturales". - Pueden tener beneficios adicionales, como antioxidantes.	- Eficacia limitada contra ciertos patógenos. - Variabilidad en la actividad según la fuente y las condiciones del alimento.	Patrignani et al., 2020b

4. Importancia y caracterización de compuestos bioactivos en subproductos de la naranja

Los desechos de la industria citrícola son una fuente abundante de compuestos bioactivos que tienen propiedades biológicas importantes, como antioxidantes, antiinflamatorias y antimicrobianas, entre otras (Cirkovic Velickovic & Stanic-Vucinic, 2018). Los compuestos fenólicos y el ácido ascórbico son dos de los principales compuestos que han demostrado ser capaces de neutralizar radicales libres y proteger a las células del daño oxidativo, lo que los convierte en ingredientes susceptibles de sustituir a aditivos utilizados habitualmente en la industria alimentaria (Maya-Cano et al., 2021; Skrzydlewska et al., 2022).

4.1. Compuestos fenólicos en subproductos de las naranjas

Los compuestos fenólicos son una amplia gama de metabolitos secundarios y se encuentran en altas concentraciones en los subproductos cítricos, especialmente en la piel, y son esenciales para proteger la planta contra el estrés ambiental. No obstante, también juegan un papel crucial en el organismo humano ya que tienen propiedades antioxidantes y antiinflamatorias (Shehata et al., 2021). La capacidad de los fenoles para neutralizar los radicales libres ayuda a prevenir el daño celular causado por el estrés oxidativo (Zeb, 2020). Además, se ha demostrado que los compuestos fenólicos de los residuos cítricos pueden prevenir el cáncer, las enfermedades cardiovasculares y los trastornos neurodegenerativos (Labbé et al., 2015).

La forma más común de clasificar los compuestos fenólicos es teniendo en cuenta su estructura química (ácidos fenólicos, flavonoides, isoflavonoides, estilbenos, polímeros fenólicos, lignanos y cumarinas), siendo, los flavonoides, los ácidos fenólicos y los lignanos los principales compuestos fenólicos

presentes en la naranja, concentrándose en la piel y las membranas internas del fruto. Su estructura molecular confiere a estos compuestos diferentes características químicas, así como su actividad biológica (Singh et al., 2020). En la Figura 1 se muestra la clasificación detallada de los compuestos fenólicos presentes en la naranja.

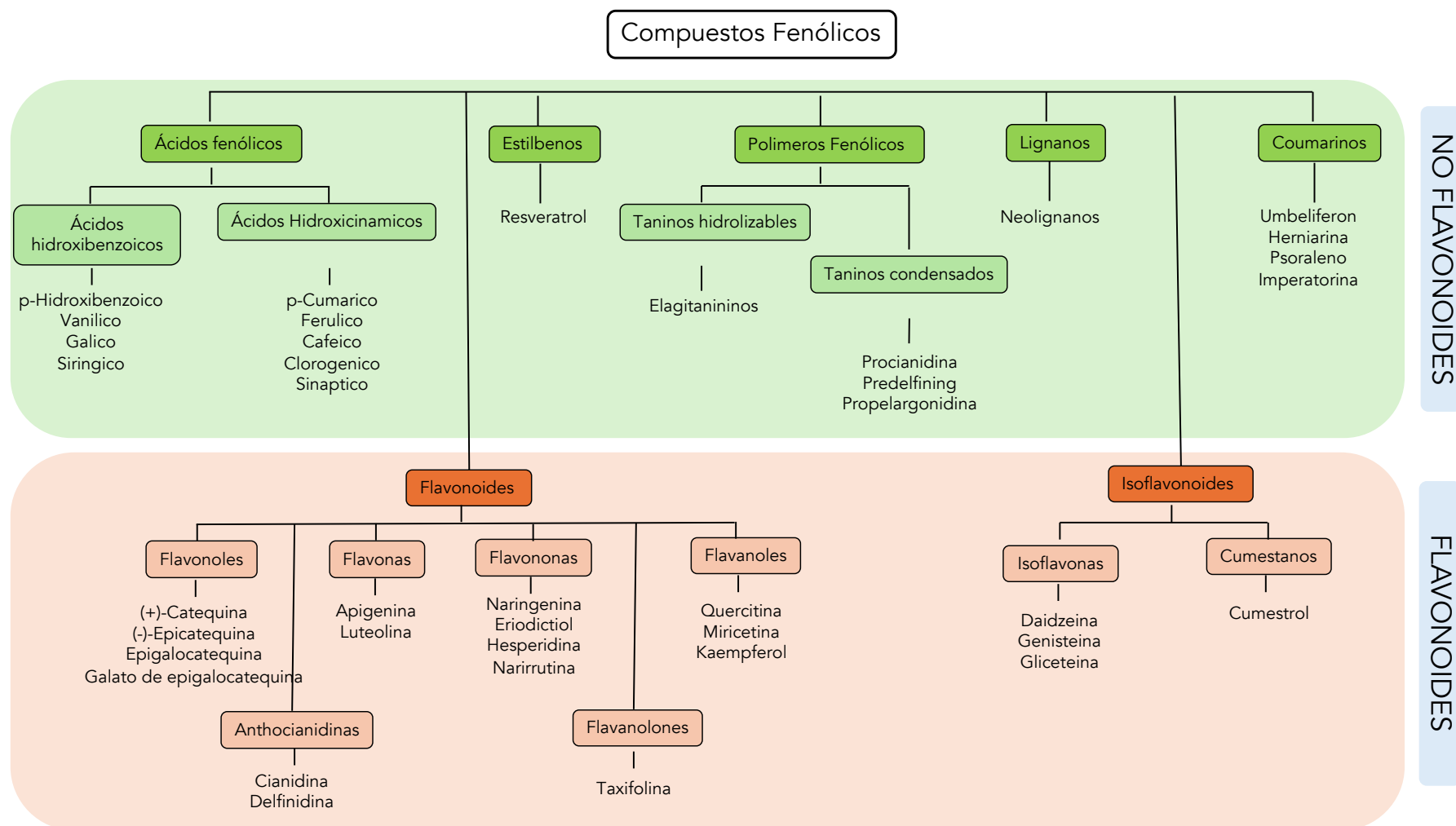


Figura 1. Compuestos fenólicos y su clasificación

4.1.1. Flavonoides

La figura 2 muestra las estructuras químicas de los flavonoides mayoritarios presentes en la piel de las naranjas.

Los compuestos fenólicos más comunes en la naranja son las flavononas, flavonas y flavonoles.

Flavononas

Son el tipo predominante de flavonoides en los cítricos, especialmente en las naranjas. La naringina y la hesperidina son las más comunes y mayoritarias. La hesperidina, que se encuentra principalmente en las membranas y la piel de la fruta, tiene propiedades antioxidantes, antiinflamatorias y cardioprotectoras (Akiyama et al., 2010) y constituye el compuesto fenólico mayoritario presente en la piel de naranja. La naringina, que se encuentra en concentraciones más bajas, destaca por sus efectos antioxidantes su capacidad para reducir los niveles de colesterol y su acción hepatoprotectora (Tajaldini et al., 2020). Ambos compuestos están especialmente presentes en su forma glicosilada, lo que significa que están unidas a un glúcido, dicha unión afecta a su solubilidad y consiguiente absorción.

No obstante, debido a los procesos de hidrólisis por las enzimas gastrointestinales y las condiciones de pH básico del intestino delgado humano, ambas pasan a su forma aglicona (Rha et al., 2019). En el caso de la hesperidina por la acción de la microbiota colónica pasa a su forma hesperetina. La naringina debido a la acción de la alfa-L-ramnosidasa, y de la beta-glucosidasa, pasa a su forma aglicona, la naringenina. Ambas formas tienen una solubilidad mayor y de esta manera aumenta su absorción (Arafah et al., 2020). La piel de naranja,

también contiene de forma natural ambas formas agliconas pero en menor cantidad (Jokić et al., 2020a).

Flavonas

Las flavonas, como la apigenina y la luteolina, se encuentran en la piel y otras partes del fruto de naranja, pero en menor concentración que las flavononas. Las flavonas pueden actuar como agentes antioxidantes al donar protones y estabilizar los radicales libres, evitando el daño celular. Las propiedades bioactivas de las flavonas se deben a su esqueleto químico C6-C3-C6, el doble enlace del anillo C y la presencia de grupos funcionales como los hidroxilos (Ali et al., 2017).

Flavonoides

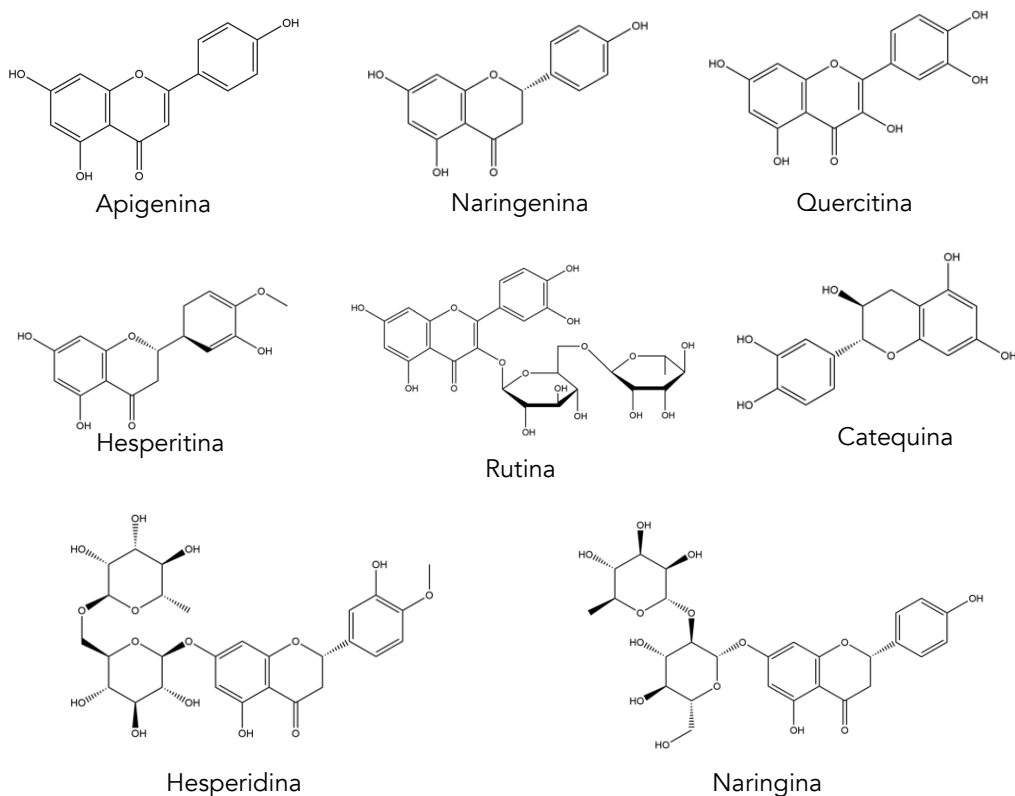


Figura 2. Estructuras químicas de los principales flavonoides presentes en la piel de la naranja

Se ha estudiado la actividad antiinflamatoria y antitumoral de estas moléculas ya que actúan en procesos que detienen el crecimiento de células cancerosas y protegen las células contra el daño oxidativo a nivel celular. Una dieta rica en apigenina puede también tener un papel protector contra el riesgo de cáncer de mama y de próstata (Labbé et al., 2015). Estas flavonas pueden también ser beneficiosos para prevenir enfermedades cardiovasculares (Zhou et al., 2017).

Flavonoles

Los flavonoles, como la quercetina, son conocidos por sus potentes efectos antioxidantes y su capacidad para proteger las células contra el daño por radicales libres, y se encuentran en cantidades menores que los otros flavonoides en la naranja. Las investigaciones sobre la quercetina se han centrado principalmente en su capacidad para prevenir enfermedades neurodegenerativas y su efecto antiinflamatorio, ya que pueden atravesar la barrera hematoencefálica (Wang et al., 2016). La estructura química de los flavonoles se basa en dos anillos aromáticos conectados por un anillo que contiene un anillo de pireno (véase Figura 2). La quercetina suele encontrarse en forma glucosilada, en la que uno o más grupos hidroxilo se sustituyen por diferentes tipos de grupos de glucídicos. Esta estructura les confiere una baja solubilidad en agua, una inestabilidad química y una vida media corta, reduciendo su eficacia en alimentos y medicamentos (Zhao et al., 2013); no obstante, ha demostrado ser un potente antioxidante en estudios *in vitro* siendo uno de más potentes contra las especies reactivas del oxígeno (Bentz, 2017).

4.1.2. Ácidos fenólicos

Estos compuestos son comunes en las plantas y frutas incluyendo a los cítricos y tienen propiedades antioxidantes, antiinflamatorias y antimicrobianas.

La figura 3, muestra las estructuras químicas de los ácidos fenólicos mayoritarios presentes en la piel de naranja. Los ácidos hidroxicinámicos y los ácidos hidroxibenzoicos son los más importantes

Ácidos hidroxicinámicos

Los ácidos hidroxicinámicos son compuestos fenólicos que derivan del ácido cinámico, tienen la fórmula general C₆-C₃, y están compuestos por un anillo bencénico (C₆) unido a una cadena de tres carbonos (C₃) con un grupo carboxilo (-COOH) en el extremo. Los ácidos hidroxicinámicos tienen características bioactivas debido a la presencia de uno o más grupos hidroxilo (-OH) en el anillo bencénico.

La piel de naranja contiene altas concentraciones de ácido ferúlico, lo que lo convierte en un ingrediente importante en los productos cosméticos debido a su capacidad para neutralizar radicales libres y proteger la piel de la radiación ultravioleta. Se ha demostrado que el ácido cafeico tiene propiedades antioxidantes y antimicrobianas, así como también se ha estudiado por su potencial para prevenir enfermedades metabólicas como la diabetes y el síndrome metabólico al afectar la regulación de la glucosa (Nia & Hadjmohammadi, 2021).

Ácidos hidroxibenzoicos

La presencia de uno o más grupos hidroxilo (-OH) unidos al anillo bencénico (C₆) del ácido benzoico define esta clase de compuestos fenólicos. El ácido 4-

hidroxibenzoico y el ácido vanílico se utilizan en la industria alimentaria como conservantes. Por otra parte han demostrado ser capaces de inhibir ciertas respuestas inflamatorias en estudios preclínicos (da Silva et al., 2023).

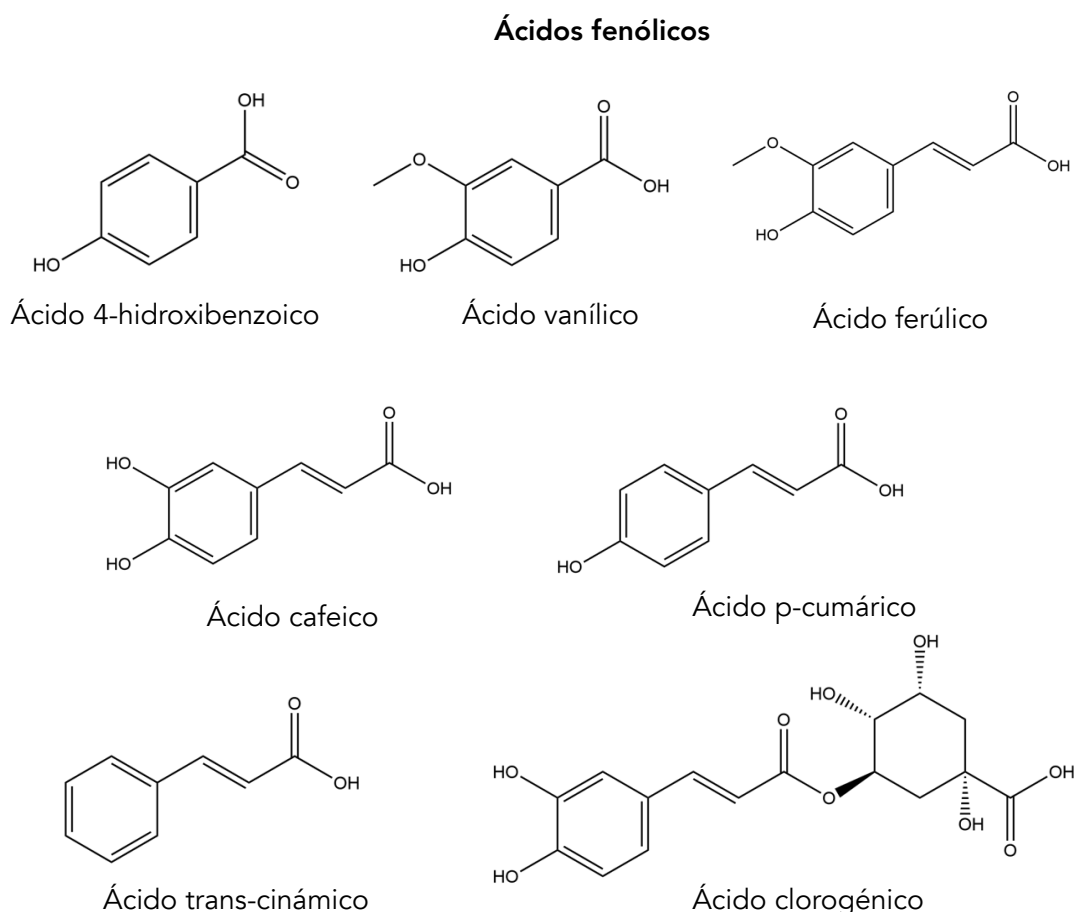


Figura 3. Estructuras químicas de los principales ácidos fenólicos presentes en la piel de la naranja

Los compuestos fenólicos presentes en los subproductos de naranja actúan como antioxidantes neutralizando los radicales libres, moléculas extremadamente reactivas que pueden dañar las células al oxidar lípidos, proteínas y ADN. Los compuestos fenólicos actúan al proporcionar electrones a los radicales libres, lo que los estabiliza y evita el daño oxidativo. Además,

pueden estimular la actividad de enzimas antioxidantes endógenas como la catalasa y la superóxido dismutasa, estas enzimas son esenciales para mantener la defensa antioxidante del organismo humano (Vuolo et al., 2019; Zeb, 2020).

4.2. Ácido ascórbico en subproductos de las naranjas.

El ácido ascórbico (AA), es una molécula que contiene seis átomos de carbono con un grupo enediol (dos grupos hidroxilo adyacentes a un doble enlace), responsable de su capacidad antioxidante (Njus et al., 2020). El ácido ascórbico es una forma reducida de fácil oxidación, convirtiéndose en ácido dehidroascórbico de forma reversible dando lugar a compuestos menos reactivos que el ácido ascórbico (Chen et al., 2021; Smirnoff, 2018) (Figura 4).

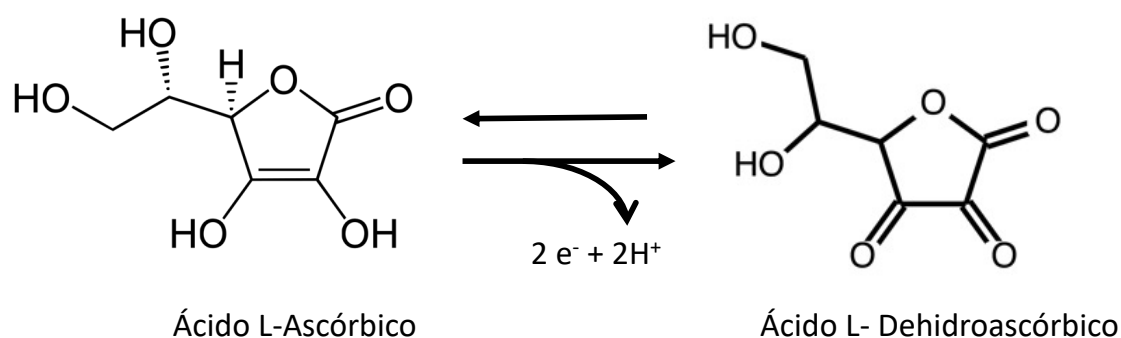


Figure 4. Oxidación del ácido ascórbico

El AA es un poderoso antioxidante que ayuda a prevenir la oxidación de los alimentos, evitando así su alteración en cuanto a sabor, color y textura, reduciendo la necesidad de utilizar conservantes químicos adicionales. (Tucaliuc et al., 2022).

Su solubilidad en agua es propiedad muy importante en la industria alimentaria, ya que facilita su dispersión en productos líquidos, sin embargo, la luz, el calor

y el oxígeno pueden degradar el ácido ascórbico. Si no se maneja adecuadamente durante el procesamiento, se oxida rápidamente, lo que puede reducir su eficacia como antioxidante (Njus et al., 2020; Yin et al., 2022a). Por lo tanto, se deben utilizar condiciones de almacenamiento controladas, como temperaturas bajas y envases opacos.

4.2.1. Beneficios del ácido ascórbico sobre la salud

El AA juega un papel fundamental en la biosíntesis de colágeno, proteína clave en la piel, los cartílagos y los huesos. Su ingesta ayuda a la cicatrización de heridas, fortalece el sistema inmunológico al promover la función y producción de leucocitos (Jamshidovich, 2023), y mejora la absorción del hierro no hemo en el intestino. Al reducir el daño oxidativo y la inflamación, el AA ayuda a prevenir enfermedades crónicas como enfermedades cardiovasculares (Berretta et al., 2020; Khan et al., 2020; Maurya et al., 2023), ciertos tipos de cáncer y patologías neurodegenerativas como el Alzheimer (Moretti et al., 2017). Por otra parte, también participa en el mantenimiento del equilibrio redox en el sistema antioxidante endógeno (Skrzydłowska et al., 2022b). Además, la Agencia Española de Seguridad Alimentaria y Nutrición (AESAN), reporta otros beneficios del AA, como su contribución al funcionamiento del sistema inmunitario durante y después del ejercicio físico intenso y en la recuperación de este, así como en la disminución del cansancio y la fatiga y a regenerar la forma reducida de la vitamina E (AESAN, 2024.)

4.2.2. Aplicaciones del ácido ascórbico en subproductos de naranja

Los subproductos de la industria cítrica, como piel, semillas y pulpas de las naranjas, contienen AA, lo que permite su aprovechamiento. El AA desempeña un papel importante en la industria alimentaria al proporcionar beneficios tanto

para la calidad del producto y su conservación como para la salud del consumidor. Se utiliza como conservante natural, protege los alimentos de la oxidación y deterioro por el contacto con el oxígeno. Su utilización predomina sobre todo en la fabricación de zumos y mermeladas. Además, también se utiliza como aditivo fortificando productos alimentarios aumentando sus propiedades antioxidantes (Yin et al., 2022b).

El ácido ascórbico, al ser termolábil se emplea como un parámetro de la calidad de los alimentos, lo que permite identificar la alteración de los alimentos procesados (Moradinezhad et al., 2020).

Además, también es ampliamente utilizado en la formulación de productos cosméticos. Su capacidad antioxidante favorece la síntesis de colágeno mejorando la estabilidad de la piel y previniendo el envejecimiento prematuro (Napolitano & Chool Boo, 2022). En la industria farmacéutica se utiliza en la formulación de suplementos nutricionales, que ayudan a reforzar el sistema inmunológico y prevenir el daño celular (Rondanelli et al., 2021).

5. Extracción de compuestos bioactivos: solventes y métodos.

5.1. Química Sostenible y Verde

Uno de los retos más importantes a los que se enfrenta la industria es la química verde, que se esfuerza por reducir los impactos nocivos sobre la salud humana y minimizar el daño medioambiental mediante el uso de recursos renovables y el desarrollo de nuevas tecnologías (Dai et al., 2013a). En los últimos años, se ha vuelto cada vez más importante reemplazar los solventes peligrosos y petroquímicos en la extracción de compuestos bioactivos de matrices de vegetales con alternativas innovadoras (Ling et al., 2020), como la implementación de nuevas técnicas que utilizan solventes de acuerdo con el

principio de la reducción del consumo de energía, más seguros y la reducción de los productos derivados (Chemat et al., 2019).

Por esta razón, el concepto de "Química Sostenible" surgió en los años noventa, como una idea para diseñar productos y procesos químicos que impliquen reducir o eliminar sustancias nocivas para el ser humano o el medio ambiente (Anastas & Eghbali, 2009). De hecho, surgió de la idea de que el desarrollo económico podía conducir a un deterioro de la calidad de vida, en lugar de una mejora. Los doce principios de la Química Verde fueron propuestos por la Agencia de Protección Ambiental (EPA, EE.UU.), utilizando los principios establecidos por Anastas y Warner (1988). Dichos principios se muestran detallados en la Tabla 2. El objetivo principal es el uso de tecnologías que generen productos no tóxicos, y el uso de solventes verdes y naturales (Ardila-Fierro & Hernández, 2021).

La química verde es actualmente un aspecto esencial de la industria para generar nuevos productos alimentarios y extraer compuestos bioactivos para alimentos funcionales, productos farmacéuticos y cosméticos. El aprovechamiento sostenible de los residuos y subproductos agroalimentarios para la producción de productos de valor añadido también ofrece una oportunidad para cumplir con la Agenda 2030 y los Objetivos de Desarrollo Sostenible (United Nations, 2024). En respuesta al imperativo de procesos ambientalmente responsables, se están explorando nuevas tendencias dentro de la industria alimentaria, incluida la utilización de técnicas de extracción no térmicas y solventes naturales no convencionales (Rente et al., 2022).

Tabla 2. Doce principios de la química verde

1°	Prevención
2°	Economía del átomo
3°	Síntesis de químicos menos peligrosos
4°	Diseño de químicos más seguros
5°	Solventes auxiliares más seguros
6°	Diseñar para la eficiencia energética
7°	Utilización de materias primas renovables
8°	Reducir los productos derivados
9°	Catálisis
10°	Diseñar productos degradables
11°	Realizar estudios <i>real-time</i> para la prevención de la contaminación
12°	Química intrínsecamente más segura para la prevención de accidentes

5.2. Procesos de extracción alternativos y sostenibles

Las condiciones de extracción con disolventes orgánicos, las altas temperaturas y los largos tiempos de extracción siempre se han utilizado en la industria alimentaria (Reddy et al., 2020). La extracción de compuestos bioactivos se ha realizado convencionalmente mediante el método de extracción por calentamiento-agitación (HS) utilizado (Popovic et al., 2022a; Santana et al., 2019a) lo que permite la difusión de los compuestos diana a los disolventes y no implica una alta inversión, ni un alto consumo energético a la industria alimentaria. Recientemente, ha surgido la necesidad de implementar métodos de extracción verdes, y se ha incrementado el uso de técnicas de extracción como fluidos supercríticos, pulsos eléctricos, extracción asistida por ultrasonidos y extracción asistida por microondas, para minimizar los efectos negativos de

métodos convencionales, lo que ayuda y contribuye a la sostenibilidad y seguridad alimentaria (Chemat et al., 2019).

5.2.1. Extracción asistida por ultrasonidos

La extracción asistida por ultrasonidos (UAE, por sus siglas en inglés *ultrasound-assisted extraction*) es un procedimiento versátil que se puede utilizar tanto en la conservación de alimentos como en la extracción de compuestos bioactivos. En la conservación de alimentos, los UAE se consideran una tecnología limpia, con menor tiempo y temperatura de procesamiento, reduciendo el coste de procesado y las modificaciones organolépticas (Guntero et al., 2015).

Es una técnica de extracción ecológica y eficaz que utiliza ondas ultrasónicas para mejorar la extracción de compuestos de diversas matrices (Lukić et al., 2019), especialmente en las industrias alimentaria, farmacéutica y cosmética. Este proceso se basa en la cavitación acústica: la formación, el crecimiento y el colapso de burbujas dentro del disolvente provocados por dichas ondas (véase Figura 5). Además, produce efectos acoplados a la cavitación, como inestabilidades de interfaz, compresiones y expansiones sucesivas que generan fuerzas de ruptura en la célula, y disrupción de la membrana celular por la implosión de burbujas y el choque de ondas de UAE (Anticono et al., 2021; Yusoff et al., 2022).

La extracción puede realizarse de distinta manera según el equipo. Destaca la extracción en baño de ultrasonidos, donde las muestras se colocan en un recipiente sumergido en un baño, y por sonda de ultrasonidos donde se introduce la sonda directamente en la muestra, lo que permite un mayor control de la extracción. Ambos métodos destacan por ser rápidos, de bajo coste y ser adaptables a todo tipo de solventes (Bosiljkov et al., 2017a; Oney-Montalvo et

al., 2022). Entre sus ventajas, los UAE mejoran las propiedades fisicoquímicas de los productos alimentarios al mismo tiempo que le confieren seguridad y mantienen sus características organolépticas (Lukić et al. 2019).

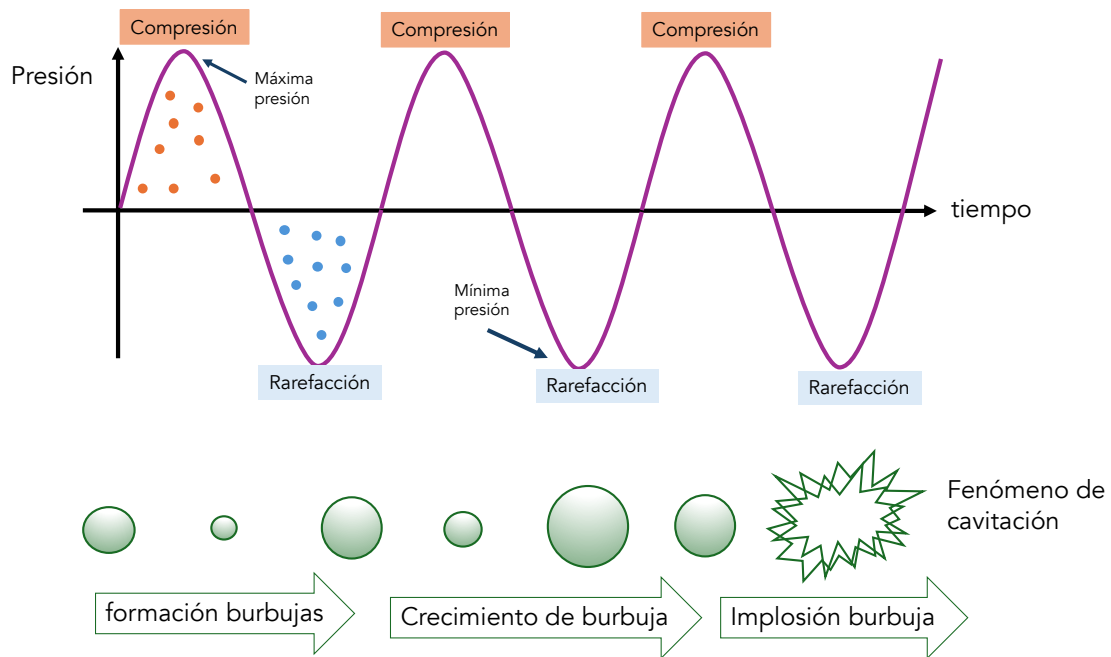


Figura 5. Mecanismo de la extracción por sonda de ultrasonidos

5.2.2. Extracción con altas presiones

Las altas presión (HP, por sus siglas en inglés *high-pressure*), es una tecnología no térmica. Sus características tales como, ecológica, limpia y económica, y ser capaz de mantener el sabor y el color originales de los alimentos procesados (Gottardi et al., 2021; Huang et al., 2017; Siroli et al., 2023b), la hacen una técnica ampliamente utilizada.

La extracción con HP emplea presiones que suelen oscilar entre 100 y 800 MPa, dependiendo de los compuestos de interés y la matriz. La alta presión altera la

estructura de las células y mejora la transferencia de masa a través de la disrupción celular, una mayor penetración de los solventes en la matriz vegetal y una difusión mejorada. Además, la técnica de extracción con HP tiene un efecto antimicrobiano, aumentado la seguridad de los extractos y así su vida útil (Guo et al., 2012; Siroli et al., 2023b). Las HP tiene diversas ventajas, como un menor gasto de energía y tiempo de extracción, así como una mayor extracción de compuestos bioactivos y un adecuado escalado industrial (Limsangouan et al., 2020).

5.2.3. Extracción asistida por microondas

La extracción asistida por microondas (MAE) es una técnica de extracción no convencional que utiliza la energía microondas para calentar y alterar la matriz de la muestra, de 0 mHz a 300 Ghz (Destandau & Michel, 2022), permitiendo la liberación de los compuestos de interés al solvente. Es un método apto para ser utilizado en la industria alimentaria y farmacéutica por su eficacia, selectividad y sostenibilidad, reduciendo así el consumo de energía, tiempo y uso de disolventes (Alvi et al., 2022).

La MAE debe su funcionamiento al principio del calentamiento dieléctrico. Cuando una muestra que contiene agua u otras moléculas polares se expone a la radiación de microondas, las moléculas oscilan rápidamente debido al campo electromagnético alterno. Esta oscilación genera calor y presión dentro de la matriz, lo que altera las paredes celulares y así favorece la liberación de compuestos bioactivos al solvente, además mejora también la transferencia de masa ya que es capaz de aumentar la penetración del solvente y ayudar a la disolución de los compuestos bioactivos (Guzik et al., 2022; Nonglait & Gokhale, 2024). Es un proceso sencillo, que permite controlar las condiciones de

extracción como la temperatura, la presión y el tiempo (González-de-Peredo et al., 2022).

5.2.4. Pulsos eléctricos

En los últimos años también ha ido ganando interés el método de extracción utilizando pulsos eléctricos (PEF, por sus siglas en inglés, *pulsed electric fields*).

La extracción por PEF se basa en la electroporación, un proceso en el que los campos eléctricos de alto voltaje crean poros en las membranas celulares de las matrices alimentarias, que permiten que los compuestos bioactivos difundan en el solvente, mientras mejora la transferencia de masa garantizando una extracción selectiva (Arshad et al., 2020). El proceso consiste en introducir la muestra en una solución conductora y, a continuación, aplicar una serie de pulsos eléctricos (que duran de microsegundos a milisegundos) de moderada a alta intensidad que oscila entre 100 y 300 V/cm en modo discontinuo y entre 20 y 80 kV/cm en modo continuo (Xi et al., 2021).

Esta técnica, al igual que las demás que siguen los principios de la química verde, trata de minimizar los efectos nocivos de los procesos de extracción convencionales. Se ha utilizado para separar compuestos bioactivos, así como para estabilizar y deshidratar los compuestos de interés sin afectar a sus propiedades nutritivas (Ranjha et al., 2021).

5.3. Solventes alternativos y ecológicos

La liberación de compuestos orgánicos volátiles a la atmósfera es un importante problema ambiental y de salud para los organismos vivos. Esto ha aumentado el interés por crear una industria más concienciada con el medio ambiente, centrándose en encontrar nuevos medios de reacción que permitan diseñar

procesos más sostenibles y dar respuesta a las demandas de la sociedad. En este sentido, existe la posibilidad de utilizar fluidos supercríticos (SCF), líquidos iónicos (ILs), solventes profundamente eutécticos (NADES) como sustitutos de los solventes orgánicos convencionales, favoreciendo así un proceso más verde y sostenible (Mehariya et al., 2021).

5.3.1. Fluidos supercríticos

La extracción con fluidos supercríticos, es un método ampliamente utilizado en diversas industrias gracias a su eficacia y su sostenibilidad. Esta técnica utiliza fluidos que se elevan a una temperatura y presión por encima de su punto crítico, donde existen en una fase única con una densidad similar a la de los líquidos y una difusividad similar a la de los gases (Preetam et al., 2023).

Cada fluido tiene un punto crítico único y un conjunto de características de solubilidad que lo hacen adecuado para extraer distintos tipos de compuestos. Tienen propiedades intermedias entre las de los gases y las de los líquidos, como su alta densidad, esto les permite disolver compuestos eficazmente, además tienen baja viscosidad favoreciendo la extracción de los compuestos de interés, pudiendo penetrar fácilmente en la matriz de la muestra y disolver los compuestos objetivo, que se separan cuando el fluido vuelve a su estado gaseoso (Jokić et al., 2020b). Entre sus propiedades, cabe destacar su eficacia de extracción, temperaturas de extracciones bajas lo que protege a los compuestos bioactivos susceptibles de degradación, y tiene una fácil separación y reciclaje. Además, es un fluido no inflamable, no tóxico y fácilmente disponible convirtiéndolo en un método sostenible y verde de extracción.

El más utilizado para la extracción es el dióxido de carbono (CO₂), principalmente por su baja toxicidad, no inflamabilidad y condiciones críticas

suaves (alrededor de 7,38 MPa, 31,3 °C) (Guo et al., 2022). Sin embargo, también pueden utilizarse otros fluidos, como el agua, que se utiliza a presiones y temperaturas muy altas. El metanol, etanol y propano, aunque resultan menos comunes, también pueden utilizarse para aplicaciones específicas que requieran diferentes propiedades de solubilidad (Montalbán et al., 2022). No obstante resulta un método caro con una maquinaria especializada y una inversión inicial grande (Y. H. Fan et al., 2022).

5.3.2. Líquidos iónicos

Los líquidos iónicos (ILs, por sus siglas en inglés *ionic liquids*) son sales compuestas por cationes orgánicos y aniones orgánicos o inorgánicos, son líquidos a temperatura ambiente y su punto de fusión es inferior a 100 °C, dependiendo de los aniones y cationes seleccionados (Hermanutz et al., 2020). La mayoría de los cationes ILs estudiados son imidazol, piridina y tetraalquilamonio, (Seddon et al., 2000).

Las propiedades fisicoquímicas de los ILs resultan importantes para entender su comportamiento ya que presentan un punto de fusión más bajo que sus componentes por separado (Bogdanov et al., 2012; Passos et al., 2014). En cuanto a la degradación de los ILs, hay pocos estudios, pero se ha descrito que también depende de la cadena alquilada de las mismas, por lo que, al aumentar su longitud, la velocidad de degradación disminuye, haciéndose más persistente en el medio (Stepnowski & Zaleska, 2005). La desventaja de los ILs es el uso de disolventes orgánicos para su síntesis (Benvenutti et al., 2019).

5.3.3. Solventes profundamente eutécticos

Los solventes profundamente eutécticos (DES, por sus siglas en inglés Deep Eutectic Solvents) fueron citados por primera vez por Abbott et al, en 2002, cuyo

interés ha crecido en la última década; teniendo, al igual que los ILs, grandes aplicaciones en diferentes campos. Desde que fueron descritos, se convierten en una alternativa interesante a los disolventes orgánicos. Los DES son mezclas eutécticas de dos o más componentes sólidos o líquidos con un punto de fusión inferior a 100 °C. Uno de los componentes actúa como aceptor de enlaces de hidrógeno (HBA, por sus siglas en inglés *hydrogen bond acceptor*), y el otro como donante de enlaces de hidrógeno (HBD por sus siglas en inglés *hydrogen bond donor*) (Germani et al., 2017). Las interacciones entre ellos están condicionadas por enlaces de hidrógeno intermoleculares que provocan una disminución de la temperatura de su punto de fusión (Xu et al., 2017). De esta manera, dos sustancias que son sólidas forman una fase líquida a su temperatura eutéctica, que se puede utilizar como disolvente para diversas aplicaciones. Uno de los ejemplos más representativos es la mezcla de cloruro de colina y urea (ChCl:U) (1:2, mol:mol) que tiene un punto de fusión de 12 °C, mientras que ambos compuestos por separado tienen puntos de fusión de 302 y 133 °C, respectivamente (Durand et al., 2012).

Dependiendo de la naturaleza de las especies utilizadas en la preparación de estos solventes, se pueden diferenciar cuatro clases de DES con diferentes propiedades intrínsecas según el tipo de enlace de hidrógeno presente en su red (véase Tabla 3). La clase III es la más investigada, debido al fácil proceso de preparación, la alta biodegradabilidad, la baja reactividad en agua y el bajo costo de los materiales. Las clases I y IV no han sido muy estudiadas hasta ahora, probablemente debido a la naturaleza altamente higroscópica de las sales metálicas utilizadas en su preparación, que a su vez es la principal diferencia con la clase II (Smith et al., 2014). Los DES se consideran una subclase de ILs por sus características similares, como la facilidad de producción, la baja toxicidad y la alta biodegradabilidad (Shishov et al., 2017).

Tabla 3. Clasificación de los solventes profundamente eutécticos

Tipos	Composición
Tipo I	Sal orgánica + cloruro metálico
Tipo II	Sal orgánica + cloruro metálico hidrato
Tipo III	Sal orgánica + donador de puentes de hidrógeno
Tipo IV	donador de puentes de hidrógeno + cloruro metálico hidrato

Adaptado de Długosz (2023).

Recientemente, ha aparecido otra clase de DES, los solventes naturales profundamente eutécticos (NADES, por sus siglas en inglés, *Natural Deep Eutectic Solvents*) que utilizan metabolitos primarios como aminoácidos, azúcares, colina y sales orgánicas que actúan como HBD. Los NADES siguen los principios de la química verde, siendo solventes verdes y respetuosos con el medio ambiente (Panić et al., 2021). Tienen muchas ventajas, como bajo costo, alta biodegradabilidad, preparación simple, baja toxicidad, no son inflamables, son miscibles con agua, biocompatibles y tienen un alto poder de extracción de diferentes sustancias (polares y apolares) (Panić et al., 2019b).

Estructuralmente, los NADES son mezclas de dos o tres componentes naturales, una sal de amonio cuaternario como el cloruro de colina y la betaína (HBA), y un HBD (azúcares, aminas, aminoácidos, polialcohol, etc.), (Figura 6), interactúan entre sí a través de enlaces de hidrógeno intermoleculares, en una relación molar determinada (mol:mol, HBA:HBD), una vez que se cambia la relación sus propiedades también cambian, de esta manera, los NADES son los solventes más versátiles en la actualidad (Dai et al., 2015a).

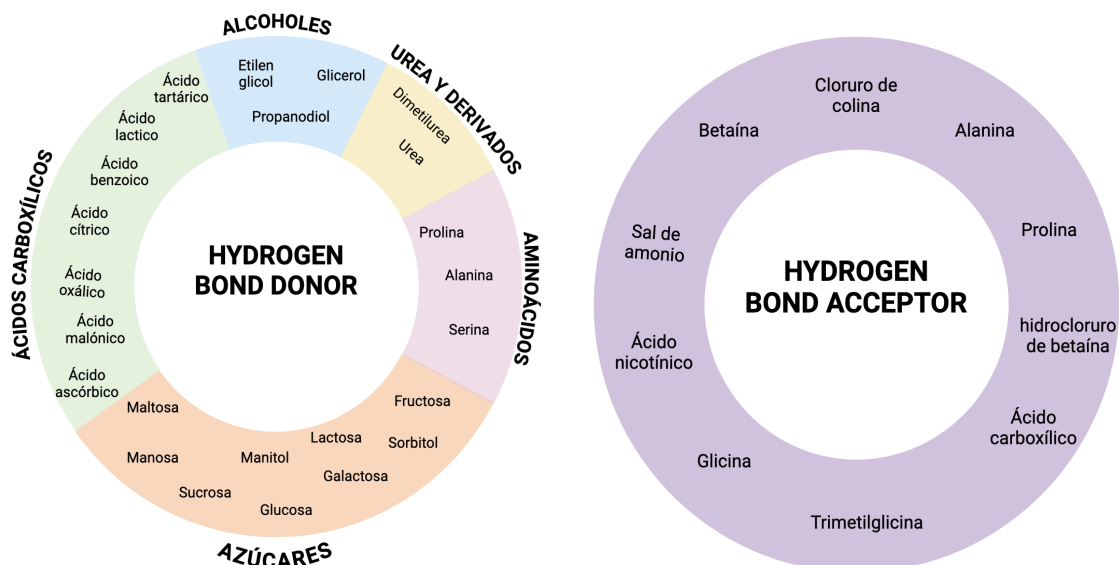


Figura 6. Principales componentes de solventes naturales profundamente eutécticos

La formación de los NADES es un proceso sencillo que requiere poco gasto de energía (Figura 7). No obstante, esta síntesis puede ser llevada a cabo mediante diferentes técnicas, descritas en la Tabla 4, entre las que se encuentran la mezcla en baño de agua o utilizando el vacío. En general la síntesis de los NADES puede tardar entre 30 minutos y 6 horas, dependiendo del HBD utilizado (Hayyan et al., 2014).

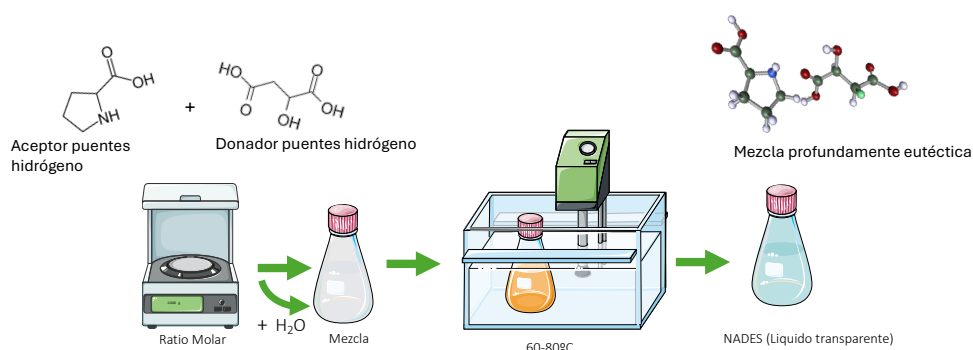


Figure 7. Proceso de síntesis de los solventes naturales profundamente eutécticos

Los DES y NADES tienen muchas ventajas en comparación con los ILs, como una preparación más sencilla y de menor coste que los líquidos iónicos, a pesar de sus beneficios, el mayor inconveniente es su viscosidad, la cual puede ser disminuida agregando agua (Dai et al., 2016; Martins Ferreira et al., 2017). Recientemente, el agua se ha considerado como un buen HBD para formar los NADES debido a su capacidad para formar enlaces de hidrógeno (Triolo et al., 2021).

En cuanto a la biodegradabilidad de los NADES, se considera que gran parte de ellos se degradan rápidamente debido al origen natural de sus componentes. Por ejemplo, todos los componentes iniciales de los NADES son fácilmente metabolizados por cualquier organismo de la naturaleza. Por lo tanto, se puede decir que los NADES tienen baja toxicidad y que la mayoría se pueden clasificar como disolventes biodegradables (Benlebna et al., 2018; Dai et al., 2013b)

Tabla 4. Diferentes técnicas para la síntesis de NADES

Método	Proceso	Características	Componentes	Referencia
Agitación magnética con calor	Mezcla de componentes bajo condiciones de agitación	Fácil de operar	Los NADES que no sean sensibles a la temperatura	Florindo et al., 2014
Liofilización	Mezcla de componentes con la adición de una pequeña cantidad de agua destilada, con la posterior liofilización	Requiere instrumentación especializada	NADES con componentes sensibles a la temperatura	Gutiérrez et al., 2009
Molienda	Mezclar y moler los componentes a temperatura ambiente	Puede absorber humedad fácilmente	Componentes que no sean sensibles de absorber agua	Florindo et al., 2014
Evaporación rotatoria	Disolver los componentes en agua y evaporar	Proceso muy largo	Componentes hidrosolubles	Dai et al., 2013b
Asistida por microondas	Mezcla de componentes en un reactor microondas	Simple y rápido	Todos los componentes	Gomez et al., 2018
Asistida por ultrasonido	Mezcla de componentes en un baño de ultrasonidos	Simple y rápido	Todos los componentes	Santana et al., 2019b
Extrusión de doble tornillo	Mezcla de componentes dentro del extrusor	Rápido y se puede utilizar para producir a gran escala	Los requeridos por la industria	Farooq et al., 2020

Adaptado de Zhou et al., 2022

Los NADES presentan capacidad antioxidante y se ha demostrado que juegan un papel protector en la degradación de ciertos compuestos bioactivos, como los polifenoles y el ácido ascórbico, haciendo de ellos unos solventes clave para su futuro uso en la industria alimentaria, cosmética y farmacéutica (Fu et al., 2021; Ling et al., 2021). Se ha visto que los NADES son capaces de extraer eficazmente compuestos bioactivos, como los polifenoles, de una gran variedad de subproductos alimentarios, incluso en ocasiones más que los solventes convencionales, como el etanol o el metanol. Los NADES pueden ser formulados para maximizar la extracción del compuesto bioactivo de interés (Dai et al., 2015a; Jeong et al., 2015). Sus propiedades fisicoquímicas, como densidad, polaridad, conductividad y baja o nula toxicidad (Kovács et al., 2020), así como su biodegradabilidad y biocompatibilidad (Popović et al., 2022) los convierten en disolventes adecuados para los procesos de extracción en matrices alimentarias. Por otra parte, se ha demostrado que disminuyen la degradación de compuestos naturales de las altas temperaturas y durante el almacenamiento (Choi et al., 2011). Debido a que se pueden añadir directamente a la formulación de alimentos, con o sin eliminación previa de NADES (Misan et al., 2019), son una gran alternativa a los solventes orgánicos (Panić et al., 2021).

Los componentes de los NADES están aceptados para su uso en la industria alimentaria, por la Food and Drug Administration de Estados Unidos que ha considerado los componentes NADES como GRAS (Generally Recognized as Safe) para consumo humano, también la AESAN (Agencia Española de Seguridad Alimentaria y Nutrición, 2024) los ha considerado seguros para el ser humano. Además, la Agencia Europea de Seguridad Alimentaria (EFSA), reconoce que todos los componentes de NADES son aptos para el consumo

humano y su uso en la formulación de nuevos alimentos, excepto el cloruro de colina, que se acepta como ingrediente para la alimentación animal. Sin embargo, no existe una declaración negativa contra este componente (EFSA, 2011),

Para hacer el proceso más sostenible, es fundamental el reciclado de los solventes después de la extracción y recuperación de los compuestos bioactivos. En este caso, los NADES pueden ser recuperados y reutilizados como solventes, aumentando así su ciclo de vida y disminuyendo el gasto de solventes. Diferentes estudios muestran que los NADES pueden ser regenerados y reciclados utilizando técnicas como la liofilización, la SPE o la precipitación por modificación del pH (Fan et al., 2020; Jeong et al., 2015; Liu et al., 2019). Además, los NADES reciclados son capaces de mantener alta eficiencia de extracción después incluso de múltiples ciclos, de hecho, Jeong et al., (2015) han observado rendimientos de un 92%, 85%, y 83% en el primer, segundo y tercer reciclado, respectivamente.

Los NADES pueden contribuir a que los compuestos bioactivos formen parte de nuevos alimentos, aumentando su solubilidad, estabilidad y biodisponibilidad, permitiendo una mayor incorporación de estos ingredientes a los productos alimentarios sin alterar su estructura ni sabor (Mercadal et al., 2024).

A pesar de sus numerosas ventajas, los NADES también plantean un reto para la industria alimentaria debido a su viscosidad, las nuevas formulaciones de productos y el coste del desarrollo de nuevos productos (Siripattanakulkajorn et al., 2025). Recientemente, se han mostrado los beneficios de enriquecer productos alimentarios con extractos de NADES. Siripattanakulkajorn et al (2025) han formulado una mayonesa con diferentes tipos de NADES basados en betaína como HBA, y han demostrado que alarga la vida útil del producto, y

disminuyendo la oxidación lipídica, sin modificar las características organolépticas necesarias para la aceptación de los consumidores.

La formulación de bebidas basadas en extractos de NADES ricos en compuestos bioactivos, ha ido en aumento en los últimos años. Salazar-Bermeo et al., (2023) ha formulado un *smoothie*, siguiendo la formulación de las bebidas comercializadas, a la que le añadieron extractos de subproductos del caqui extraídos con NADES, mejorando así sus propiedades funcionales. Además, Panić et al., (2020), enriquecieron varias bebidas, una de leche con chocolate y otra a base de yogur líquido de fresa, con extractos de NADES ricos en compuestos fenólicos, donde concluyeron que añadir entre 1 % – 10 % de estos extractos es aceptado por los consumidores, aumentando la capacidad antioxidante de dichos productos alimentarios.

Los NADES no solo se han utilizado como ingrediente en la formulación de nuevos alimentos, sino también se han realizado investigaciones en el uso de estos solventes para la elaboración de films de uso alimentario, también llamados eutectogels (Rozaria Nefeli Pontillo et al., 2021).

5.3.4. Otros solventes naturales

El bioetanol, derivado de biomasa como la caña de azúcar o el maíz, es un disolvente verde utilizado habitualmente en extracciones (Sunwoo et al., 2024) eficaz para extraer compuestos fenólicos y otros compuestos bioactivos polares. Su biodegradabilidad y baja toxicidad lo hacen apto para aplicaciones alimentarias, farmacéuticas y cosméticas (Toledo Hijo et al., 2022).

El agua a alta temperatura es un buen solvente para la extracción de compuestos polares como los compuestos fenólicos y las vitaminas hidrosolubles. Es

respetuoso con el medio ambiente, económico y no requiere grandes equipos (Di Domenico Ziero et al., 2020; Vidana Gamage & Choo, 2023).

Los aceites naturales como el de oliva, coco, sésamo, palma o soja se utilizan como solventes para la extracción de compuestos liposolubles, como aceites esenciales, carotenoides y vitaminas lipofílicas. Son especialmente valiosos en las industrias alimentaria y cosmética por su naturaleza no tóxica y comestible. Entre sus características destaca que son biodegradables y no tóxicos, baja volatilidad, son estables térmicamente, y están compuestos por triglicéridos y ácidos grasos. (Chutia & Mahanta, 2021; Kultys & Kurek, 2022; Lara-Abia et al., 2022).

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OBJETIVOS

OBJECTIVES

El objetivo general de la presente tesis doctoral es la extracción sostenible de compuestos bioactivos de subproductos de naranja con solventes hidrofílicos naturales profundamente eutécticos y la aplicación en productos alimentarios de los extractos obtenidos.

Para alcanzar este objetivo general se plantean los siguientes objetivos específicos:

1. Estudiar el papel protector de los NADES frente a la degradación de los compuestos bioactivos y evaluar su eficacia como vehículo para la liberación de compuestos de interés a través de membranas biológicas.
2. Optimizar la extracción de compuestos fenólicos de la piel de naranja utilizando diferentes NADES y realizar el control de la estabilidad de los polifenoles durante el periodo de almacenamiento.
3. Aplicar los principios de la química verde a una selección de NADES, basados en cloruro de colina, para la extracción y determinación de compuestos fenólicos y ácido ascórbico, así como el estudio de la estabilidad de los extractos de piel de naranja.
4. Comparar el contenido en compuestos fenólicos, estabilidad de polifenoles y actividad antioxidante mediante dos métodos de extracción (ultrasonidos y agitación en placa) utilizando la piel de naranja y NADES basados en cloruro de colina.
5. Analizar la actividad biológica y el efecto antimicrobiano de los extractos de piel de naranja extraídos con NADES basados en cloruro de colina.
6. Formular una bebida a base de zumo de naranja con la adición de extractos de piel de naranja obtenidos con NADES basados en cloruro de colina y estudiar su vida útil.

The general objective of this doctoral thesis is to sustainably extract bioactive compounds from orange by-products with natural hydrophilic deep eutectic solvents and the application of the resulting extracts in food products.

To achieve this general objective, the following specific objectives are proposed:

1. To study the protective role of NADES against bioactive compounds' degradation and evaluate their capacity as carriers for delivering compounds of interest across biological membranes.
2. Optimize the extraction of phenolic compounds from orange peel using different NADES and monitor the stability of polyphenols during the storage period.
3. Apply the green chemistry principles to a selection of eight NADES, based on choline chloride, for the extraction and determination of phenolic compounds and ascorbic acid, as well as studying the stability of orange peel extracts.
4. Compare the phenolic compound content, polyphenol stability, and antioxidant activity using two extraction methods (ultrasound-assisted and hot plate agitation) with orange peel and NADES based on choline chloride.
5. Analyze the biological activity and antimicrobial effects of orange peel extracts obtained with choline chloride-based NADES.
6. Formulate an orange juice-based beverage with the addition of orange peel extracts obtained using choline chloride-based NADES and study its shelf life.



METHODOLOGY

METODOLOGÍA

Chemicals and reagents

The chemicals used in the study were obtained from the suppliers described below:

General reactives	Purchased from
(±)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) 2,2-diphenyl-1-picrylhydrazyl (DPPH) 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) and sodium fluorescein 9-(Diethylamino)-5H-benzo[alpha]phenoxazin-5-one (Nile red) - Choline chloride (≥98%) - D-(-)-fructose (≥99%) - D-(+)-xylose (≥99.0%), DL-lactic acid (LA) (90%) - Folin-Ciocalteu reagent, metaphosphoric acid (HPO₃) - α-D-glucose (96%)	Sigma-Aldrich (Steinheim, Germany)
Glycerol (≥ 99.5%)	Glentham Life Science (Corsham, UK)
- DL-malic acid (≥98%) - Formic acid (98.0%)	Thermo Fisher (Kendel, Germany)
L-(+)-tartaric acid (>99.0%)	TCI (Zwijndrecht, Belgium)
- Anhydrous sodium carbonate (Na₂CO₃) - Citric acid (≥99.9%) - Sodium nitrite (nano₂)	VWR Chemicals (Leuven, Belgium)
L-proline	Guinama (Valencia, Spain)
Betaine	Fluorochem (Hadfield, UK)
Aluminum chloride (AlCl₃·6H₂O)	Acofarma (Terrassa, Barcelona).
Acetonitrile (>99.9% LC-MS grade)	Fisher Scientific (Loughborough, England).
- Potassium persulfate - Ferric chloride - Sodium carbonate - Sodium acetate - Disodium hydrogen phosphate	Panreac Química (Castellar del Vallès, Spain).

• Potassium dihydrogen phosphate	
• Analysis grade formic acid	
• Plate Count Agar (pcag)	Oxoid Ltd. (Basingstoke, United Kingdom)
• Man Rogosa and Sharpe (MRS)	
• Yeast Extract Peptone Dextrose (YPD)	
• Ethanol absolute	J.T. Baker Chemical Co. (Deventer, The Netherlands)
• HPLC grade methanol ($\geq 99.8\%$)	
• Analysis grade hydrochloric acid	
• HPLC grade acetonitrile ($\geq 99.9\%$)	
Analytical standards	
• 4-hydroxybenzoic acid (99%)	Sigma-Aldrich (Steinheim, Germany)
• Catechin ($\geq 99.0\%$)	
• Vanillic acid ($\geq 97.0\%$)	
• Chlorogenic acid ($\geq 95.0\%$)	
• Narirutin ($\geq 98\%$)	
• Caffeic acid ($\geq 99.0\%$)	
• Naringin ($\geq 95.0\%$)	
• Hesperidin ($\geq 97.0\%$)	
• p-coumaric acid ($\geq 98.0\%$)	
• Ferulic acid ($\geq 99.0\%$), naringenin ($\geq 95\%$)	
• Hesperetin ($\geq 98.0\%$)	
• Trans-cinnamic acid ($\geq 98.0\%$)	
• L-ascorbic acid ($\geq 99.7\%$)	
• Apigenin (96.86%)	HWI Analytik GmbH (Ruelzheim, Germany)
• Quercetin dihydrate (97.02%)	
• Rutin trihydrate (97.67%)	
Enzymes	
• Alpha-amylasa Assay Kit	Megazyme, Wicklow, Ireland
• Pepsine	Sigma-Aldrich (Steinheim, Germany)
• Trypsine	
• Chymotrypsin	

Deionized and distilled water were purified through a Milli-Q water system (Millipore, Molsheim, France).

Instrumentation

- Conductimetro TDS Meter HI5321 (Woonsocket, RI, USA), with an accuracy of $\pm 1\%$ of reading ($\pm 0.01 \mu\text{S}/\text{cm}$)
- e-nose (PEN3; Win Muster Air sense Analytics Inc., Schwerin, Germany)
- Eppendorf Centrifuge 5810 R
- Eppendorf Centrifuge 5810 R
- Espectrofotometro (Perkin Elmer ® Massachusetts, USA)
- GC 6890N, Network GC System with mass spectrometry (MS) 5970 MSD (Agilent Hewlett–Packard, Geneva, Switzerland)
- HPLC system (Agilent Technologies 1120 Compact LC) with an ultraviolet light detector
- LC/MS/MS TripleTOFTM 5600 (SCIEX) system. PeakViewTM 2.2 program is used to qualitatively analyze the data, and the MultiquantTM-3.0.3 program is used to quantify the phenolic chemicals found
- Milli-Q water system (Millipore, Molshein, France)
- Q500 ultrasonic processor (Qsonica, USA)
- SA 402B flavor detection system (Intelligent Sensor Technology Co., Ltd., Atsugi, Japan)
- Sension+ MM340 pH-meter (Hach, Germany)
- tristimulus spectrophotometer (mod. A60-1010-615 ColorFlex, HunterLab, Reston, USA)
- Vertex 70 spectrometer (Bruker Optics, Milan, Italy) with a spectral range of $4000\text{--}600 \text{ cm}^{-1}$, a resolution of 4 cm^{-1} , and 24 scans. The acquisition system was controlled by OPUS software (Bruker Optics, Milan, Italy).
- Wallac 1420 VICTOR2 multilabel counter equipment (PerkinElmer, MA, USA)

Methodology

Various scientific methodologies have been used to carry out this doctoral thesis, framed within analytical chemistry and food microbiology:

1. Raw material

The primary raw material employed in this study is orange peel (*Citrus sinensis*) of the Navel cultivar, provided by the Agricultural Cooperative Sant Bernat de Carlet (Valencia). The oranges, classified as a caliber between 4 and 5, were harvested in the initial harvest (mid-November) and were not waxed before processing. To ensure the absence of any soil or insect residue, the oranges were meticulously cleaned with distilled water. Subsequently, the oranges were stored at 8°C until used.

The peel was then separated from the fruit using a conventional kitchen knife, which was accurately cleaned and disinfected. Afterward, the skin is either crushed or cut into squares measuring 5 millimeters on a side, depending on the extraction method (maximum or minimum extraction).

2. Natural Deep Eutectic Solvents Preparation

The different NADES used throughout this doctoral thesis are shown in Table 5. The components of the NADES used for the realization of this doctoral thesis are accepted by the FDA as components generally recognized as safe (GRAS) for human consumption (FDA, 2024). AESAN has deemed all components safe for additives or food ingredients (AESAN, 2021). They are prepared following the method proposed by Dai et al (2015) with some modifications (Figure 7). The components of the NADES were weighed according to the specific molar ratio, then 25% (w/w) of distilled water was added. The mixture was placed in a water

bath at 60-80 °C for 6 hours until a transparent and homogeneous mixture was obtained. Each of the NADES systems has an approximate preparation time, ranging from 30 min to 6 h as well as a final appearance, which can be from a transparent colorless liquid to a liquid with a transparent orange color. The speed of preparation is related to the components, so those liquid components had a significantly shorter preparation time than those that were solid. NADES are kept in transparent test bottles for up to 2 months until use.

Table 5. Characteristics of the NADES used throughout this doctoral thesis

Acronym	Hydrogen Bond Acceptor	Hydrogen Bond Donor	Molar Ratio	Preparation time (hours)	Appearance
ChChI:Glu	Choline chloride	Glucose	2:1	2.5	Clear colorless liquid
ChChI:Fruc		Fructose	1.9:1	2.3	Clear colorless liquid
ChChI:Xyl		Xylose	2:1	2.3	Clear colorless liquid
ChChI:Gly		Glycerol	1:2	1.0	Clear colorless liquid
ChChI:Gly:CA		Glycerol:Citric Acid	1:1:1	4.0	Clear colorless liquid
ChChI:MA		Malic Acid	1:1	3.5	Transparent orange liquid
ChChI:TA		Tartaric acid	2:1	4.0	Clear colorless liquid
ChChI:LA		Lactic acid	1:3	0.5	Clear colorless liquid
ChChI:CA		Citric acid	2:1	5.1	Clear colorless liquid
ChChI:LP:MA		L-Proline:Malic Acid	1:1:1	3.0	Transparent orange liquid
Bet:MA	Betaine	Malic Acid	1:1	5.3	Transparent orange liquid
Bet:CA		Citric acid	1:1	5.0	Clear colorless liquid
LP:MA	L-Proline	Malic Acid	1:1	4.1	Transparent orange liquid
LA:Glu	Lactic acid	Glucose	5:1	1.5	Clear colorless liquid
MA:Glu	Malic Acid	Glucose	1:1	5.5	Transparent orange liquid

3. Extraction methods

Different extraction methods, conventional and unconventional, were used for the extraction of bioactive compounds from orange peel.

3.1. Heating-stirring extraction

For magnetic stirring extraction (Figure 8), orange peel samples were placed in beakers in a given solid/liquid ratio with the different NADES. The extraction process was carried out with agitation and constant temperature (40 ± 5 °C). Subsequently, the samples were centrifuged for 10 minutes at 15 °C, with 2500 rpm (Eppendorf Centrifuge 5810 R), and the supernatant was collected and used for the subsequent determinations of bioactive compounds.

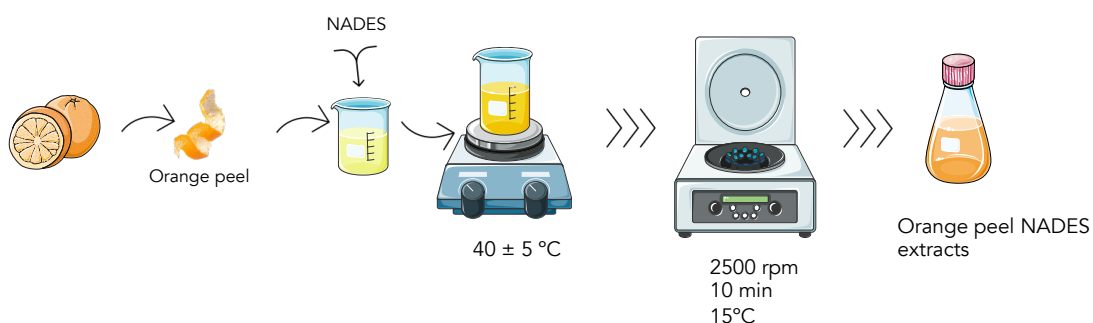


Figure 8. Procedure for the extraction of bioactive compounds by magnetic stirring

3.2. Ultrasound-assisted extraction

Ultrasound-assisted extraction (UAE) is performed using a Q500 ultrasonic processor (Qsonica, USA), with a frequency duty cycle of 40% amplitude and a maximum temperature of 40 ± 5 °C according to the method described by (Montero-Calderon et al., 2019) (Figure 9). To do this, the orange peel was

introduced into an Erlenmeyer together with the different NADES in a solid/liquid ratio of 1g/15mL (w/v). Samples were ultrasonicated for 20 minutes (in 1-minute cycles at 40% amplitude, with 2 minutes of cooling between cycles)

The samples were then centrifuged for 10 minutes at 15 °C, at 2500 rpm (Eppendorf Centrifuge 5810 R), and the supernatant was collected and used for the determination of subsequent bioactive compounds.

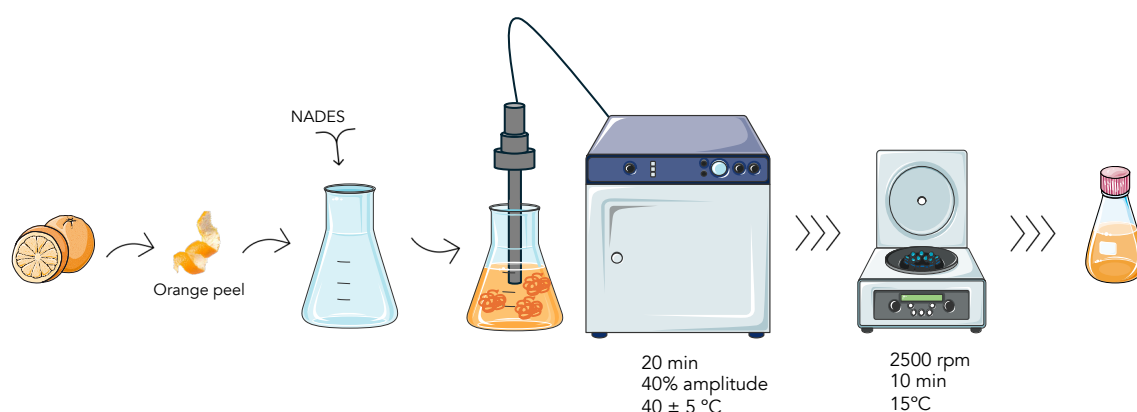


Figure 9. Ultrasound-assisted extraction procedure

4. Physicochemical characterization

4.1. Polarity

The polarity of NADES is measured using the Red Nile technique and reagent (1g/L ethanol) according to the method described by Craveiro et al (2016). Briefly, 50 μ L of the Red Nile reagent was added to a cuvette where 1 mL of NADES had previously been added. Absorbance was measured at each wavelength in the range of 400 to 700 nm (Perkin Elmer ® Massachusetts, USA).

The wavelength with the maximum absorption was interpolated into the following equation:

Equation 1:

$$E_{NR} = \frac{hcN_A}{\lambda_{max}} \quad (1)$$

Where, h represents Planck's constant (6.63×10^{-34} J·s), c the speed of light in a vacuum (2.997×10^8 m/s), and N_A Avogadro's constant (6.022×10^{23} mol⁻¹). The final polarity results were expressed in kcal/mol

4.2. Density

The density of NADES is determined by measuring the mass of 1 mL of NADES on an analytical balance (Kern, ABT 120-5DM. ± 1 mg, Darmstadt, Germany) on triplicate at 20° C. The results are expressed in g/cm³ with an accuracy of ± 0.005 g/cm³.

4.3. pH

The pH of NADES was measured with the Sension+ MM340 pH-meter (Hach, Germany). The analysis was performed for each water quantity of fourteen NADES. All measurements were made at room temperature (25 °C).

4.4. Conductivity

Electrical conductivity is measured with a TDS Meter HI5321 (Woonsocket, RI, USA), with an accuracy of $\pm 1\%$ of reading ($\pm 0.01 \mu\text{S/cm}$). The measurement is made at room temperature (25 °C) and is expressed in mScm⁻¹.

4.5. Color

The color was determined using a tristimulus spectrophotometer (A60-1010-615 ColorFlex, HunterLab, Reston, USA) Equipped with a sample holder (12 mm diameter). Color is measured using the CIE L*a*b* color space and D65 illuminant.

The color was expressed as L^* , a^* , and b^* as reported by Glicerina et al (2021). Color stability is expressed as the total color difference (ΔE^*), between day 0, and days 6 and 13. The calculation is done as follows:

$$\text{Equation 2: } \Delta E = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2} \quad (2)$$

Where L^* represents the luminosity of the color, a^* is the position between red/green, and b^* represents the position between yellow and blue.

4.6. FTIR Infrared Analysis

Fourier-transform near-infrared spectroscopy (FTIR)) were collected using a single-reflection ATR module on a Vertex 70 spectrometer (Bruker Optics, Milan, Italy) with a spectral range of $4000\text{--}600\text{ cm}^{-1}$, a resolution of 4 cm^{-1} , and 24 scans, at 25°C (Quijano-Ortega et al., 2020). A background was collected before the analysis of each NADES extract. The acquisition system was controlled by OPUS software (Bruker Optics, Milan, Italy).

5. Identification and quantification of bioactive compounds

5.1. Phenolic compounds

5.1.1. Total Flavonoids

The total flavonoid content was made according to the method proposed by Zhishen et al (1999) with some modifications. A $100\text{ }\mu\text{L}$ aliquot of the samples was mixed with $1088\text{ }\mu\text{L}$ of ethanol (30% v/v) and $48\text{ }\mu\text{L}$ of sodium nitrite (0.5 mol/L) and then mixed with vortex. After 5 minutes of reaction, $48\text{ }\mu\text{L}$ of aluminum chloride (0.3 mL/L) was added and vortexed, after another 5 minutes of reaction, $320\text{ }\mu\text{L}$ of sodium hydroxide (1 mL/L) was added and vortex again.

Immediately, the absorbance was measured at a wavelength of 510 nm (Perkin Elmer ® Massachusetts, USA). At the same time, a calibration curve is carried out using catechin as a standard (2 mg/mL), and it is analyzed in the same way as the samples. Results were expressed as mgCAE/mL of sample.

5.1.2. Total polyphenol content

The total polyphenol content (TPC) were analyzed using the method described by Singleton & Rossi (1965) with modifications. A 100 µL aliquot of the sample was mixed with 3 mL of sodium carbonate (2% w/v) and 100 µL of the Folin-Ciocalteu reagent (1:1, v/v). The samples were incubated in the dark for one hour; then, the absorbance was measured at a wavelength of 765 nm (Perkin Elmer®, Massachusetts, USA). At the same time, a gallic acid calibration curve (0.1 – 0.6 mg/mL) was carried out, and it was analyzed and treated in the same way as the samples. Results were given as mgGAE/mL of sample.

5.1.3. Phenolic compounds by liquid chromatography.

5.1.3.1. *Clean-up* solid phase extraction

To achieve optimal chromatographic separation of bioactive compounds, a solid-phase extraction (*clean-up*) is performed before the analysis.

The clean-up was carried out following the methodology proposed by Gonzales et al (2015) with the modifications described by Anticono et al. (2022). Briefly, C18 (Phenomenex, Torrance, USA) pre-filled cartridges were pre-conditioned by adding 3 mL of Milli-Q water twice and 3 mL of methanol twice. Solvents and samples were allowed to sit in the cartridges for two minutes before loading. At the same time, 5 mL of the sample was diluted with 15 mL of 0.1% formic acid in MilliQ-Water (v/v). The samples were passed through the cartridges and then

cleaned with 5 mL of 0.1% formic acid in MilliQ-Water (v/v). For recovery, 3 mL of 0.1% formic acid in methanol (v/v) was passed through the column until everything was recovered.

After evaporation to dry matter the samples using N₂, were reconstituted with 1 mL of Methanol:HCl (0.6 mol/L) (75:25, v/v), and ready to inject.

5.1.3.2. Determination of phenolic compounds by HPLC-UV

Chromatographic analyses were performed using a column of C18 (250 × 4.6 mm, 5 µm particle size; Luna PFP2, Phenomenex, Torrance, USA) and an HPLC system (Agilent Technologies 1120 Compact LC) with an ultraviolet light detector, as described by Anticono et al. (2022). Mobile phase A consists of 5% formic acid in MilliQ Water (v/v), and phase B consists of 40% A phase in acetonitrile (v/v). Mobile phases were used to create the following elution gradient: 0 – 10 min, 100% A; 10 – 20 min, 85% A; 20 – 50 min, 82% A; 50 – 75 min, 0% A; 75 – 80 min, 100% A. The flow rate remained constant (0.8 mL/min) with an injection volume of 20 µL. Chromatograms were acquired at a wavelength of $\lambda = 280$ nm. All samples were filtered with nylon filters (0.22 µm) before injection.

Simultaneously, the calibration lines of 16 standards (Table 6) of phenolic compounds were analyzed under the same conditions as the samples at concentrations between 0 – 50 µg/mL.

5.1.3.3. Phenolic compounds determination by UPLC-qTOF-MS/MS

For the separation of phenolic compounds, confirmatory studies were performed using an LC/MS/MS TripleTOFTM 5600 (SCIEX) system with an ACQUITY UPLC BEHC18 column (2.1 50 mm I.D., 1.7 m; Waters, Milford, USA).

The following binary elution gradient is used with a flow rate of 0.4 mL.min⁻¹: 0-5 min, 70% isocratic solvent A (water/formic acid 99.9/0.1 (v/v)) and solvent B 30% (methanol/formic acid, 99.9/0.1(v/v)); 5-12 min, linear 70 to 5% A; 12-18 min, isocratic 5% A; 18-18.5 min, linear from 5 to 70% A; and 18.5-25 min, isocratic 70% A. They are injected into the 5 μ L system. The instrument settings employed for data acquisition in negative mode within a mass/charge range of 100-950m/z included the following: ion source gases 1 and 2, set at 50 psi; curtain gas 1, set at 25 psi; 450°C; and ion spray voltage, adjusted to -4500 V. For the purpose of external calibration, polyphenol standards with concentrations ranging from 1 to 5 g/mL were utilized. In instances where the sample concentration exceeded that of the calibration curve, a process of sample dilution was employed. The mass spectrometer (MS) employs the information-dependent acquisition (IDA) method with the sounding sweep type (TOF-MS) and the scan-dependent type (production) using a collision energy of 30 V. The PeakViewTM 2.2 program is used to qualitatively analyze the data, and the MultiquantTM-3.0.3 program is used to quantify the phenolic chemicals found. The results are reported in μ g/mL of extract.

Table 6. Phenolic compounds tested and their characteristics

Analytes	Molecular formula	m/z	[m-H]	Error (ppm)
3,4-dihydroxybenzoic acid	C ₇ H ₆ O ₃	138,03169	137,02442	-0,8
catechin	C ₁₅ H ₁₄ O ₆	290,07904	289,07176	-1,8
caffeic acid	C ₉ H ₈ O ₄	180,04226	179,03498	1,1
vanillic acid	C ₈ H ₈ O ₄	168,04226	167,03498	1,8
quercetin	C ₁₅ H ₁₀ O ₇	302,04265	301,03538	0,4
chlorogenic acid	C ₁₆ H ₁₈ O ₉	354,09508	353,08781	-0,7
p-coumaric acid	C ₉ H ₈ O ₃	164,04734	163,04007	1,8
rutin	C ₂₇ H ₃₀ O ₁₆	610,15339	609,14611	-0,9
ferulic acid	C ₁₀ H ₁₀ O ₄	194,05791	193,05063	-0,2
narirutin	C ₂₇ H ₃₂ O ₁₄	580,17921	579,17193	-1,2
naringin	C ₂₇ H ₃₂ O ₁₄	580,17921	579,17193	-1,2
hesperidin	C ₂₈ H ₃₄ O ₁₅	610,18977	609,18249	-0,9
trans-cinnamic acid	C ₉ H ₈ O ₂	148,05243	147,04515	0,6
naringenin	C ₁₅ H ₁₂ O ₅	272,06847	271,0612	0,7
apigenin	C ₁₅ H ₁₀ O ₅	270,05282	269,04555	1,1
hesperetin	C ₁₆ H ₁₄ O ₆	302,07904	301,07176	-0,9

5.2. Determination of Ascorbic Acid by HPLC-UV

The AA was quantified using an Agilent 1120 Compact HPLC-UV/VIS chromatograph (Agilent Technologies, CA, USA), equipped with a UV/VIS detector and a Luna PFP2 C18 column (250 × 4.6 mm, particle size 5 µm, Phenomenex). 5% formic acid in Milli-Q water (v/v) (solvent A) and 40% solvent A in acetonitrile (solvent B) were used in isocratic mode: 60% A and 40% B. The flow rate was 0.5 ml/min, and the injection volume was 1 µl. The chromatograms were acquired at 280 nm. The AA calibration curve was done in the 0–200 µg/ml range under the same conditions as the samples.

The efficiency of the HPLC-UV procedure for AA analysis from orange peel extracts was assessed based on the validation parameters. For the AA standard, linearity was assessed by constructing calibration curves ($n = 5$ calibration points) between 0 and 1000 $\mu\text{g/ml}$. Chromatographic peak areas were analyzed by least squares linear regression, and linearity was evaluated in terms of R^2 values ($R^2 = 0.9991$). The limit of detection (LOD) and the limit of quantification (LOQ) were assessed based on a signal-to-noise (S/N) ratio equal to or higher than 3 and 10, respectively. The LOD (5 ng/ml) and LOQ (18 ng/ml) ($n = 10$) were determined from the lowest concentration level (ng/ml) necessary to identify and quantify AA. The intra-day ($n = 5$) % RSD (0.4494%) and the inter-day ($n = 5$) % RSD (3.2443%) were lower than 5%, suggesting that this HPLC-UV is a reproducible, robust method to determine ascorbic acid compounds from orange peel.

6. Antioxidant capacity methods

6.1. ORAC

ORAC assay was determined using the method described by Zulueta et al (2009). It was carried out using the Wallac 1420 VICTOR2 multilabel counter equipment (PerkinElmer, MA, USA) with fluorescence filters, at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. The measurements were made on white plates of 96 flat-bottomed wells (Thermofisher®). The reaction was carried out at 37 °C, where the thermolysis of AAPH started, in 75 mM phosphate buffer (pH 7.0) The fluorescence measurement was carried out immediately after the addition of the reactants, and the measurements were carried out in 5-min cycles until the relative fluorescence intensity decreased by more than 95% of the initial value. ORAC values expressed in μM Trolox equivalents ($\mu\text{M TE}$) were calculated by applying the Equation (3) shown below:

$$\text{Equation 3: } \text{ORAC } (\mu\text{M TE}) = \frac{C_{\text{Trolox}} \cdot (\text{AUC sample} - \text{AUC Blank}) \cdot k}{(\text{AUC Trolox} - \text{AUC Blank})} \quad (3)$$

Where, CTrolox is the concentration of Trolox (μM), k is the dilution factor of the sample, and AUC is the area under the fluorescence decay curve, sample, target, and Trolox standard respectively, and is calculated by applying the equation below:

$$\text{Equation 4: } \text{AUC} = 0.5 + \frac{f_5}{f_0} + \frac{f_0}{f_0} + \dots + f_n + 5/f_0 \cdot 5 \quad (4)$$

Where f_0 is the initial fluorescence and f_n is the fluorescence at time n.

6.2. FRAP

The FRAP assay is performed following the method described by Gardeli et al (2008) with some modifications. The principle of this method was based on the reduction of a ferric-tripyrrolyltri-*z*-ine complex to its colored ferrous form in the presence of antioxidants. The FRAP reagent in solution was prepared daily by mixing the TPTZ radical solution (10 mM in 40 mM HCl), ferric chloride hexahydrate solution (20 mM), and acetate buffer (300 mM, pH 3.6) in the ratio 1:1:10 (v/v). The operating model consists of a mixture of 900 μL of FRAP solution, 90 μL of distilled water, and 30 μL of suitably diluted sample for incubation in amber glass tubes at 37 °C. Absorbance is determined on a Lambda 365 spectrophotometer (Perkin Elmer ® Massachusetts, USA). The calibration curve of the FRAP standard is performed under the same conditions as the samples, in a concentration range of 0 – 250 μM .

6.3. TEAC

Trolox equivalent antioxidant capacity (TEAC) is measured using the method described by Re et al (1999). The ABTS radical ($\text{ABTS}^{\bullet+}$) (7 mM) is generated

with 440 μL of potassium persulfate (140 mM) and stored in the dark at room temperature for 12 – 16 h. The solution is diluted with ethanol to an absorbance of 0.70 ± 0.02 at 734 nm. The absorbance of 2 mL of ABTS•+ formed is taken, and 100 μL of samples are added and diluted if necessary. The reaction is then incubated at 30 °C for 3 min and measured. A Trolox standard in the range of 0 – 250 μM was used for the calibration curve. Results were expressed as NADES $\mu\text{M TE/mL}$. The percentage of inhibition (% I) was calculated using the following equation:

$$\text{Equation 5:} \quad \% \text{ inhibition} = \frac{A_1}{A_0} \times 100 \quad (5)$$

Where A_0 is the absorbance of the control (ABTS•+) and A_1 is the absorbance in the presence of the sample.

6.4. DPPH

The assay was carried out following the method described by Brand-Williams et al (1995). 50 μL of adequate sample dilution at 1.45 mL DPPH (0.06 mM) is added. The sample is incubated for 30 min at room temperature. Absorbance is measured at the wavelength of 515 nm in a Lambda 365 spectrophotometer (Perkin Elmer ® Massachusetts, USA). The %I is calculated using the Trolox standard (Equation 5) prepared in the range of 0 - 500 μM for the calibration curve and the results are expressed as $\mu\text{M TE/mL}$ of NADES.

7. Analysis and characterization of volatile compounds

The volatile molecule profiles were detected by GC/MS/SPME technique, as described by Gottardi et al (2021). A CAR/PDMS, 75 μm fiber (SUPELCO, Bellefonte, PA, USA) was used to perform the solid-phase microextraction. The samples (3 mL) were placed in vials and incubated for 10 min at 45°C. Then the

fiber was exposed to the vial headspace for 30 min at 45 °C. The volatile molecules adsorbed were desorbed in the gas chromatograph (GC) injector port in splitless mode at 250°C for 10 min. The headspace of the volatile compounds was analyzed using GC 6890N, Network GC System with mass spectrometry (MS) 5970 MSD (Agilent Hewlett–Packard, Geneva, Switzerland). The column used was J & W CP-Wax 52 CB (50 m x 320 μ m x 1.2 μ m). The initial temperature was 40 °C for 1 min and then was increased by 4.5 °C/min up to 65 °C. The temperature was then increased by 10 °C/min up to 230 °C and remained at this temperature for 17 min. Compounds were identified by comparison using a NIST 11 (National Institute of Standards and Technology) database. The gas carrier was helium at 1.0 mL/min flow.

8. Gastrointestinal and Skin parallel artificial membrane permeability assay (PAMPA)

Skin–PAMPA was performed following the method described by Soriano-Meseguer et al (2023), and the Gastrointestinal (GI)–PAMPA following the analysis described by Bélair et al (2021). To predict the passive human gastrointestinal and skin absorption of ascorbic acid, the ascorbic acid standard (0.2 mg/mL) was added to different NADES systems. In this test, a hydrophobic polyvinylidene fluoride multi-screen permeability plate assembly with 96-well filtration plate (Merk, Millipore, MA, USA) was used as a carrier of the artificial membrane and as the receptor plate. The filter material of each well in the filtration (donor) plate was coated with 15 μ L of solution (L- α -phosphatidylcholine, 1% (w/v) dodecane). The donor plate was placed onto the receptor plate. For the passive gastrointestinal assay, the receptor plate was filled with 200 μ L of phosphate buffer solution pH 5.5 (for simulating the large intestine segment), while for the skin assay the receptor place was previously filled with 200 μ L of

phosphate buffer saline solution pH 7.4 (to stimulate the epithelial barrier). Subsequently, 125 μ l of the sample was added to each well of the donor plate. The plate system was closed and incubated in darkness 6 h at room temperature. After incubation, the concentration of ascorbic acid of the receiving and donor plate was determined by HPLC-UV as previously described in section 5.2. The permeability (Pe) was calculated following the equations 6 and 7.

$$\text{Equation 6: } P_e = -\frac{2.303 V_D}{A(t-t_{ss}) \cdot \epsilon_a} \cdot \left(\frac{1}{1-r_a}\right) \cdot \log_{10} \left[-r_a + \left(\frac{1+r_a}{1-R_M}\right) \cdot \frac{C_D(t)}{C_D(0)}\right] \quad (6)$$

$$\text{Equation 7: } R_M = 1 - \frac{C_D(t)}{C_D(0)} - \frac{V_A \cdot C_A(t)}{V_D \cdot C_D(0)} \quad (7)$$

Where Pe: permeability coefficient (cm/s), V_D and V_A : volume of the solution in the donor side and acceptor side, A: membrane area (0.3 cm²), t: incubation time (s), t_{ss} : lag time (s) [$t_{ss} = (54 \cdot R_M + 1) \cdot 60$ s], ϵ_a : apparent membrane porosity (0.76), $C_D(t)$: concentration in the donor side at time t, $C_D(0)$: initial concentration in the donor side, $C_A(t)$: concentration in the acceptor side at time t, R_M : membrane retention. r_a : the sink asymmetry ratio.

9. *In-silico* simulation of the solubility of bioactive compounds in different NADES

To predict the solubility of the different bioactive compounds in different NADES, the molecules were optimized geometrically and energetically using the BIOVIA TmoleX19 software version 2023 (Dassault System, Paris, France).

COSMOtherm software was used to model the solubility of bioactive compounds in different NADES. The discrete Fourier transform was used to optimize the geometry and density of molecules in the initial stage. To do this, each molecule is optimized using the COSMO-BP-TZVP template of the software package. Next, the COSMO calculations were performed with the

BP_TZVP_C30_19.CTD parameterization using BIOVIA COSMOtherm 2023 version 20.0.0. The COSMOtherm software was used as a tool to predict the coefficient of activity of bioactive compounds in 100%, 85%, 75% of NADES as well as to simulate the solubility of ascorbic acid and hesperidin in the different NADES.

10. Green metric analysis

Sustainability analyses are performed by the Analytical Greenness Calculator (AGREE) described by Pena-Pereira et al (2020). This program analyzes each step of the chemical processes following the 12 principles of green chemistry, which are transformed on a scale of 0 to 1, with 1 being the most sustainable method. The scale considers different parameters, such as the use of organic solvents, the number of residues, and the analytical method.

11. Digestive enzyme activity

The activities of pepsin, trypsin, and chymotrypsin are determined according to Borgonovi et al (2023) with slight modifications. Extracts corresponding to 0.2 mM (final concentration, f.c.) of hesperidin equivalent to 400 μ L hemoglobin (f.c. 2.5% w/v in water, pH 2 for pepsin and pH 7 for trypsin and chymotrypsin) are added. The reaction is initiated with 100 μ L of an appropriate enzyme solution (30 μ g/mL at 0.15 M NaCl, 0.0115 M CaCl_2 , pH 2 for pepsin and pH 7 for trypsin and chymotrypsin) in 2 mL plastic tubes. After 5 to 10 min, the reaction is stopped by adding 1 mL of 20% trichloroacetic acid (TCA) (w/v). Untransformed proteins are separated by centrifugation (12,000 $\times$ g for 10 min), and TCA-soluble peptides released from the substrate are detected spectrophotometrically at 280 nm. Alpha-amylase activity is assessed using the alpha-amylase assay kit (Megazyme, Wicklow, Ireland), with slight modifications.

Extracts corresponding to an equivalent of 0.2 mM (f.c.) hesperidin are added to 200 µl of substrate solution containing blocked p-nitrophenyl maltoheptaoside and excessive levels of amyloglucosidase and alpha-glucosidase. The reaction is initiated with the addition of 0.1 mL of alpha-amylase (30 µg/mL at 0.15 M NaCl, 0.0115 M CaCl₂, pH 7). After 10 min at 40°C, the reaction is stopped by adding 3 mL of the stop solution provided by the kit, and the absorbance is read at 400 nm. In all cases, one unit of enzyme corresponds to an absorbance change of 0.001/min. Activity data are normalized for the amount of enzyme (mg) in each assay.

12. Antimicrobial activity

12.1. Agar well diffusion assay

Antimicrobial activity is determined by an agar well diffusion assay against foodborne bacteria responsible for pathogenicity or spoilage (*Listeria monocytogenes* Scott A; *Escherichia coli* 555, and *Staphylococcus aureus* 231, and bacteria associated with human skin contamination, *Staphylococcus epidermidis* E6), according to Siroli et al (2023). The extracts were tested as were or after diluting them to 50% in sterile distilled water. Antimicrobial activity is based on the observed diameter of the growth inhibition areas: «-», < 8 mm; "+", 8 - 11 mm; "++", 11 - 14 mm; "+++", 14 - 18 mm; "++++", > 18 mm.

12.2. Plate counting method

During the storage period, cell loads of mesophilic aerobic bacteria, lactic acid bacteria, yeasts, and molds, and aerobic spore-forming bacteria were detected by plate counting, after appropriate dilution of samples in sterile saline (0.9% NaCl), on plate counting agar (PCAg) (Oxoid Ltd., Basingstoke, UK), Basingstoke, UK), of Man Rogosa and Sharpe (MRS) (Oxoid Ltd., Basingstoke,

United Kingdom), Yeast Extract Peptone Dextrose (YPD) (Oxoid Ltd., Basingstoke, United Kingdom) and PCA respectively according to Siroli et al (2023). For the determination of aerobic spore-forming bacteria, the samples were previously subjected to heat treatment at 80°C for 10 min. All agar plates were incubated at 30 °C for 48 h before colony counting.

13. Sensorial profile: electronic nose and tongue

The aromatic footprint of the samples was evaluated using the commercial e-nose (PEN3; Win Muster Air sense Analytics Inc., Schwerin, Germany) composed of ten semiconductor metal oxide (MOS) sensors Buratti et al (2022). For analysis, 3 mL of samples were placed in 20 mL Pyrex® vials fitted with a perforable silicone/Teflon disc in the lid. To develop headspace, samples were kept for 1 hour at room temperature. During measurement, the headspace is pumped over the sensor surfaces for 60 s (injection time) at a flow rate of 300 mL/min, and the sensor signals are taken after 50 s. After each determination, the sensors are purged for 180 s with filtered air, and the sensor baselines were reset. Each sample was evaluated in triplicate; the average values were collected and subjected to statistical processing.

For taste evaluation, analyses were performed with the SA 402B flavor detection system (Intelligent Sensor Technology Co., Ltd., Atsugi, Japan) (Kobayashi et al., 2010) i.e. e-tongue. The detection sensors applied were CT0 and C00 for bitterness and bitter aftertaste, CA0 for sour, and AE1 for astringency and astringent aftertaste. Fifteen grams of the samples were added to 100 ml of distilled water, filtered, and analyzed with the e-tongue, applying the measurement process described by (Laureati et al., 2010). Each sample was evaluated in triplicate, and the sensor outputs were converted into "flavor

values" using appropriate coefficients based on the Weber-Fechner law (Kobayashi et al., 2010). Statistical processing is applied to average values.

14. Challenge test in the presence of spoiling microorganisms

A challenge test evaluated the extracts' effects on specific spoiling microorganisms in the orange juice. The strains used were *Lactiplantibacillus plantarum* 82 and *Saccharomyces cerevisiae* SPA both belong to the Department of Agricultural and Food Sciences (DISTAL, University of Bologna) microbial collection. These strains were isolated from spoiled juices (Patrignani et al., 2020b). Before being used, the strains were cultured twice in MRS broth and YPD broth at 30 °C for 24 h respectively for *L. plantarum* and *S. cerevisiae*. To avoid the presence of interfering microflora, the juice (4.0L) was initially pasteurized (90 °C, 10 min). Then the microbial species were inoculated in the orange juice at a level of about 3.0 log CFU/mL. As reported for the shelf-life trial, the inoculated juice was portioned in nine batches (400mL) and each one was added with the different NADES extracts (E1-4) or only NADES (N1-4). The batch of pure orange juice inoculated with the target microorganisms represented the control. As for shelf-life trials, the nine batches were aliquoted (10 mL) in 30 sterile tubes. For each batch, half of the tubes were stored at 4°C and half at 8°C. During the product storage, samples were collected after 0, 2, 4, 7, and 9 days, for samples storage at 8 °C, and until day 11 for samples storage at 4 °C. The analysis was conducted for the cell loads of the inoculated *L. plantarum* and *S. cerevisiae* determined by sampling on different selective media such as MRS for *L. plantarum* and YPD for *S. cerevisiae*. The plates were incubated at 30 °C for 48 h for both *S. cerevisiae* and *L. plantarum*.

15. Statistical analysis

In the present doctoral thesis, all analyses were performed in triplicate to ensure the reproducibility of the assays. Analysis of variance (ANOVA) was employed to evaluate differences in means with a threshold significance of 0.05, and Tukey post hoc analysis ($p < 0.05$) was utilized to ascertain the level differences for parameters with more than two levels and significant differences. Both statistical analyses were carried out using the SPSS 26.0.0® statistical program (SPSS Inc., Chicago, USA).

The e-nose and e-tongue data were processed by K-mean partition clustering and by principal component analysis (PCA). K-mean clustering was performed using Minitab v 17.1 (Minitab Inc., State College, PA, USA), PCA was performed using XLSTAT v. 3.1 statistical software (Addinsoft 2021, New York, NY, USA).

Heat-map *descriptive plots* were performed with the *ggplot2* statistical RStudio package, and *heatmaply*, correlation analyses were performed with RStudio's *ggplot statistical package (Integrated Development for R (RStudio, PBC, Boston, MA) after data normalization.*

The other graphs were created using Excel spreadsheets, version 16.91 (Microsoft, Albuquerque, United States).

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RESULTADOS

CHAPTERS

CHAPTER I

Natural deep eutectic solvents: A paradigm of stability and permeability in the design of new ingredients



Ascorbic acid is a crucial component in the food industry, serving as both a quality and safety criterion. It is used to fortify foods due to its significant role in human health, acting as an antioxidant. Its benefits extend to both product quality and consumer health in the food and cosmetic industry. Due to their physicochemical properties, Natural Deep Eutectic Solvents (NADES) have the potential to be used for the extraction and stabilization of bioactive compounds. The objective of this study is to characterize various NADES, simulate the solubility of AA, evaluate the antioxidant and protective capacity of NADES, and investigate the permeability of this vitamin through the skin and gastrointestinal membrane.

1. Results and discussion

Many bioactive compounds, such as ascorbic acid in fruits, contain polar functional groups that interact favorably with polar solvents through hydrogen bonds or other intermolecular forces (Wang et al., 2023) the solubility and bioavailability of bioactive compounds can be increased by having a similar polarity between the solvent and the compound (Recharla et al., 2017). In this case, NADES were characterized, and the results are presented in Table 1. It was observed that NADES based on organic acids, were more acidic than those based on sugars and that NADES based on polyalcohols were more polar than those based on acids, matching the results with other studies (Bakirtzi et al., 2016; Farooq et al., 2020; Mohd Fuad & Mohd Nadzir, 2022). To be able to fully characterize each of the NADES, FT-IR spectra of each component and their eutectic mixtures were recorded at room temperature. In Figure S1 (Annex 1) the FT-IR spectra between all the components and the eutectic mixture are presented, as can be observed, new interactions and the formation of new hydrogen bonds appeared when the two compounds were forming the eutectic

mixture when compared with single compounds. An upward shift of the stretching vibration band VC-O in the eutectic mixture was observed from 1200 to 1300 cm^{-1} , indicative of the appearance of a hydrogen bond which can indicate the formation of the eutectic system (Santana et al, 2019).

Table 1. NADES characterization.

Acronym	Hydrogen bond acceptor	Hydrogen bond donor	Molar ratio	Conductivity (mScm ⁻¹)	pH	Polarity (E _{NR})	Density (g/cm ³)
ChChl:Glu		Glucose	2:1	7.80 ± 0.05 ^d	4.04 ± 0.02 ^{b*}	49.93 ± 0.02 ^c	1.190 ± 0.01
ChChl:Fru		Fructose	1.9:1	10.50 ± 0.06 ^c	4.02 ± 0.01 ^{c*}	53.15 ± 0.01 ^a	1.190 ± 0.01
ChChl:Xyl		Xylose	2:1	13.50 ± 0.25 ^{ab}	4.00 ± 0.01 ^{d*}	50.46 ± 0.01 ^b	1.210 ± 0.03
ChChl:Gly		Glycerol	1:2	12.67 ± 0.18 ^b	5.00 ± 0.00 ^{a*}	48.85 ± 0.02 ^d	1.120 ± 0.01
ChChl:MA	Choline chloride	Malic acid	1:1	5.68 ± 0.00 ^e	1.06 ± 0.03 ⁱ	47.86 ± 0.00 ^e	1.190 ± 0.02
ChChl:TA		Tartaric acid	2:1	9.19 ± 0.70 ^d	1.01 ± 0.02 ^l	47.89 ± 0.06 ^e	1.160 ± 0.02
ChChl:LA		Lactic acid	1:3	14.14 ± 0.19 ^a	1.03 ± 0.02 ^j	47.97 ± 0.03 ^e	1.130 ± 0.03
ChChl:CA		Citric acid	2:1	3.76 ± 0.22 ^f	1.02 ± 0.01 ^k	47.81 ± 0.03 ^e	1.220 ± 0.01
ChChl:LP:MA		L-Proline:Malic acid	1:1:1	1.60 ± 0.08 ^{gh}	2.06 ± 0.00 ^e	49.18 ± 0.01 ^c	1.210 ± 0.01
LA:Glu	Lactic acid	Glucose	5:1	2.21 ± 0.01 ^{gh}	1.03 ± 0.04 ^j	47.89 ± 0.01 ^e	1.260 ± 0.02
MA:Glu	Malic acid	Glucose	1:1	1.18 ± 0.08 ^{gh}	1.00 ± 0.02 ^m	46.56 ± 0.03 ^f	1.420 ± 0.01
LP:MA	L-Proline	Malic acid	1:1	2.59 ± 0.12 ^{fg}	2.01 ± 0.01 ^g	48.32 ± 0.03 ^d	1.300 ± 0.02
Bet:CA	Betaine	Citric acid	1:1	2.09 ± 0.22 ^{gh}	2.02 ± 0.00 ^f	48.00 ± 0.04 ^{de}	1.240 ± 0.01
Bet:MA	Betaine	Malic acid	1:1	1.13 ± 0.00 ^h	2.00 ± 0.01 ^h	48.71 ± 0.01 ^d	1.280 ± 0.01

(a-h): different letters indicate statistically significant differences. Values were expressed as the means (n=3) ± SD. The asterisks show the pH values higher than 4. ChChl: Choline chloride, CA: Citric acid, LA: lactic acid, LP: L-Proline, MA: Malic acid, Gly: Glycerol, Glu: Glucose, TA: Tartaric acid, Bet: Betaine, Xyl: Xylose, Fru: Fructose

1.1. Antioxidant activity

The antioxidant capacity of food by-products has been extensively researched (da Silva et al., 2020; Sir Elkhatim et al., 2018; Vicente et al., 2021; Wojeicchowski et al., 2021). However, there is a lack of information on the antioxidant capacity of NADES, so examining their antioxidant capacity is a crucial parameter to understand the protective role of bioactive compounds when used as solvents.

Table 2 shows the determinations of TEAC, ORAC, and DPPH methods for antioxidant capacity in NADES. In this instance, the TEAC method generated values that ranged from 17.7 to 2600 $\mu\text{M TE}$, with LP:MA yielding the highest output, and ChChl:TA resulting in the lowest.

Table 2. Antioxidant capacity of fourteen NADES

NADES	TEAC ($\mu\text{M TE/mL NADES}$)	DPPH ($\mu\text{M TE/mL NADES}$)	ORAC (mM TE/mL NADES)
ChChl:Glu	46.4 $\pm$ 10.1 ^a	9.3 $\pm$ 4.7 ^a	2.4 $\pm$ 1.8 ^a
ChChl:Fruc	56.7 $\pm$ 12.4 ^a	13.6 $\pm$ 0.0 ^a	2.9 $\pm$ 0.4 ^a
ChChl:Xyl	120.3 $\pm$ 8.6 ^b	6.9 $\pm$ 1.3 ^a	6.0 $\pm$ 0.7 ^{ab}
ChChl:Gly*	51.8 $\pm$ 3.6 ^a	10.7 $\pm$ 0.0 ^a	2.2 $\pm$ 1.1 ^a
ChChl:MA*	105.0 $\pm$ 8.0 ^{bd}	424.4 $\pm$ 13.4 ^b	25.7 $\pm$ 2.8 ^h
ChChl:TA	17.7 $\pm$ 5.1 ^c	493.4 $\pm$ 10.1 ^d	7.0 $\pm$ 2.8 ^{abc}
ChChl:LA	21.0 $\pm$ 3.3 ^c	462.5 $\pm$ 6.7 ^{cd}	17.5 $\pm$ 2.8 ^{ef}
ChChl:CA	48.0 $\pm$ 2.6 ^a	455.3 $\pm$ 10.1 ^{bc}	2.4 $\pm$ 0.1 ^a
ChChl:LP:MA	2449.2 $\pm$ 2.7 ^e	298.5 $\pm$ 10.1 ^g	23.7 $\pm$ 2.3 ^{gh}
LA:Glu	24.9 $\pm$ 4.2 ^c	355.5 $\pm$ 3.4 ^e	15.1 $\pm$ 0.6 ^{de}
MA:Glu	55.9 $\pm$ 1.9 ^a	284.2 $\pm$ 16.8 ^{fg}	21.9 $\pm$ 0.2 ^{fg}
LP:MA	2600.7 $\pm$ 3.7 ^f	253.3 $\pm$ 6.7 ^f	11.5 $\pm$ 1.2 ^{cd}
Bet:CA	94.3 $\pm$ 4.0 ^d	27.9 $\pm$ 0.0 ^{ah}	20.5 $\pm$ 0.7 ^{fg}
Bet:MA	118.4 $\pm$ 4.2 ^b	48.8 $\pm$ 0.0 ^h	10.5 $\pm$ 0.9 ^{bcd}

Antioxidant activity was expressed as the means (n=3) $\pm$ SD. Presented values followed by different letters (a-h) are significantly different on the same column ($p < 0.05$) as

measured by Tukey's HSD test. ChCl: Choline chloride, CA: Citric acid, LA: lactic acid, LP: L-Proline, MA: Malic acid, Gly: Glycerol, Glu: Glucose, TA: Tartaric acid, Bet: Betaine, Xyl: Xylose, Fru: Fructose. (*) Results already published on Gómez-Urios et al., (2023)

The DPPH radical is hydrophobic, so it is soluble in organic solvents, in contrast to the ABTS and peroxide radicals. Antioxidants that react rapidly with the latter may be slow or inert toward DPPH due to steric inaccessibility (Prior et al., 2005). Due to steric hindrance, the total activity of antioxidant compounds is lower than that of individual compounds (Schaich et al., 2015). ChCl:TA was the NADES with the highest antioxidant capacity according to the DPPH method ($493.4 \pm 10.1 \mu\text{M TE}$), followed by ChCl:LA ($462.5 \pm 6.7 \mu\text{M TE}$), and the lowest was ChCl:Xyl ($6.9 \pm 1.3 \mu\text{M TE}$). Popović et al (2022) also used ChCl:MA and ChCl:Fru NADES systems and they did not reach IC₅₀ for them using DPPH antiradical capacity method. Our study, confirmed this observation having found just slight antioxidant activity for the mentioned systems, 185.5 296 and 18.1 $\mu\text{M TE}$ of NADES, respectively.

The ORAC method measures the inhibitory capacity against a peroxide radical, which is reflected in a break in the oxidation reaction chain by HAT. According to the ORAC results, ChCl:LP:MA ($25.7 \pm 2.3 \mu\text{M TE}$) was the NADES with the highest antioxidant capacity, while ChCl:Gly ($2.2 \pm 1.2 \mu\text{M TE}$) provided the lowest values. The results obtained by the ORAC method in NADES containing organic acids were similar to those obtained by other authors (Mitar et al., 2019), who observed an important contribution of malic acid and proline in the neutralization of the AAPH radical. However, NADES containing ChCl:CA was the only one containing organic acids that produced a lower relative intensity of fluorescence decay. In another study, Radošević et al (2016) uncovered the antiradical properties of these solvents with organic acids.

The physicochemical properties of NADES may be related to its antioxidant capacity. The antioxidant capacity measurements obtained by the DPPH method and the acidic pH of the solvents were shown to be negatively correlated ($R = -0.8943$, $p < 0.05$). Low pH interferes with the proton transfer mechanism and eliminates the inductive effect of the electron transfer mechanism, as was originally found by Popović et al (2022). In addition, the steric hindrance of the three aromatic rings of the radical, which blocks access to large molecules and slows down or eliminates their contribution, depending on the action of small molecules, can reduce the total antioxidant capacity as determined by the DPPH technique (Schaich et al., 2015).

1.2. Ascorbic acid solubility in NADES

The simulated solubility of AA in fourteen NADES was checked, using the COSMOTherm software. Consequently, to evaluate the accuracy of the software, the model was experimentally validated with the suitable and unsuitable NADES for the solubility of AA. To calculate the option-activity coefficient, $\ln(\gamma)$, the solubility of AA with the NADES has to be simulated. The activity coefficient was calculated using 100%, 85%, and 75% NADES in water (v/v). The findings of the NADES screening are shown in Table 3. When the activity coefficient of the measured NADES is low, there is a higher AA solubility (green and yellow color); while when the activity coefficient is high, there is a poor AA solubility (red color). Furthermore, the projections indicated reduced $\ln(\gamma)$, in NADES based on choline chloride, indicating a low AA solubility, and correlating with higher pH values.

Table 3. Predicted $\ln \gamma_{\text{solutos}}$ for ascorbic acid in 100%, 85% and 75% NADES (v/v) using COSMOtherm

	$\ln (\gamma)$ 100% NADES	$\ln (\gamma)$ 85% NADES	$\ln (\gamma)$ 75% NADES
50% EtOH	-1,6426367		
LP:MA	-2,80775909	-2,54418391	-2,3096089
MA:Glu	-2,72668458	-2,53386698	-2,3613983
LA:Glu	-2,8963338	-2,46788863	-2,2004938
ChChl:LA	-1,36054089	-1,37500952	-1,4366716
ChChl:LP:MA	-1,1873146	-1,28876572	-1,3855881
ChChl:MA	-1,02606524	-1,2042532	-1,3395896
Bet:CA	-0,97814648	-1,19687699	-1,3371742
ChChl:Gly	-0,94756226	-1,08529751	-1,2292576
ChChl:CA	-0,79244385	-1,0031890	-1,1451346
ChChl:Fruc	-0,77475059	-0,97149614	-1,1274976
ChChl:TA	-0,72915462	-0,94439832	-1,0965519
Bet:MA	-0,66448907	-0,87082694	-1,0375337
ChChl:Glu	-0,6065273	-0,81335	-0,9859873
ChChl:Xyl	-0,60466085	-0,78412056	-0,9301183

Gray color, reference; green, better or equivalent solvent than reference; yellow color, slightly worse solvent than reference; red color, worse solvent than reference. ChChl: Choline chloride, CA: Citric acid, LA: lactic acid, LP: L-Proline, MA: Malic acid, Gly: Glycerol, Glu: Glucose, TA: Tartaric acid, Bet: Betaine, Xyl: Xylose, Fru: Fructose. 50% EtOH: Ethanol 50% (v/v).

Polarity is another physicochemical property to consider (Table 1), organic acid-based NADES are more polar than sugar and polyalcohol-based NADES. As a result, NADES with higher polarity performed better with polar substances such as polyphenols, anthocyanins, and flavonoids (Grillo et al., 2020; Silva et al., 2020). Ascorbic acid's polarity enables it to interact with other polar molecules, including NADES. These interactions can impact its antioxidant function, which

typically involves donating electrons to neutralize free radicals (Wang et al., 2024). The polarity of NADES may offer some defense against the oxidation of ascorbic acid. The formation of complexes and solvation in a polar environment may impede the exposure of ascorbic acid to oxidizing agents present in the medium (Biela et al., 2023), other articles have shown that the solubility of AA was increased with water content in lactic acid-based NADES (Obluchinskaya et al., 2021), moreover, not exceeding 30% of water content, to avoid breaking the eutectic complex (Benvenuti et al., 2020).

It is important to calculate the correlation between the physicochemical characteristics of the different NADES and the predicted solubility. For this purpose, correlation matrixes are shown in Annex 1 Figure S2. Solubility is strongly positive correlated with the density in different NADES such as Bet:CA ($R = 0.93$, $p < 0.05$), Bet:MA ($R = 1$, $p < 0.05$), ChChl:LA ($R = 0.89$, $p < 0.05$), and ChChl:Xyl ($R = 0.99$, $p < 0.05$), and strongly negative correlated with ChChl:MA ($R = -0.79$, $p < 0.05$), and ChChl:Gly ($R = -0.89$, $p < 0.05$). As polarity helps the AA interact in one way or another with different NADES, a strong correlation was found between Bet:CA ($R = 1$, $p < 0.05$), ChChl:CA ($R = 0.83$, $p < 0.05$), ChChl:Fruc ($R = 0.9$, $p < 0.05$), ChChl:Xyl ($R = 0.98$, $p < 0.05$). All the components of the NADES were also tested for AA solubility at 75% (v/v) with COSMOTerm software, the results are given in Table S2 (Annex 1). The solubility of AA in the components solution was lower than the reference solvent and was not higher than the NADES. The results may be attributed to the molecular structure of NADES, which are eutectic mixtures that exhibit numerous hydrogen bonds. NADES possess carboxyl groups in acid-based NADES and carbonyl groups in the sugar-based NADES, which enables them to form intermolecular interactions with AA, thereby achieving the highest solubility. At the atomic level, AA and NADES can interact via hydrogen bond interactions.

AA has the capacity to act as either a hydrogen bond donor HBD or HBA, forming hydrogen bonds with NADES. These hydrogen bonds are formed between the hydroxyl groups of AA and the hydroxyl or carboxyl and Cl groups of NADES (Shekaari et al., 2023, Park et al., 2024).

AA was added to the NADES with the highest and lowest $\ln(\gamma)$ values to illustrate the correctness of COSMOtherm software. Following previous research, AA at 0.2 mg/mL was added to 75% of NADES (Gómez-Urios et al., 2023). Furthermore, an AA stability in water was performed as a control to compare the results.

1.3. Ascorbic acid degradation during storage

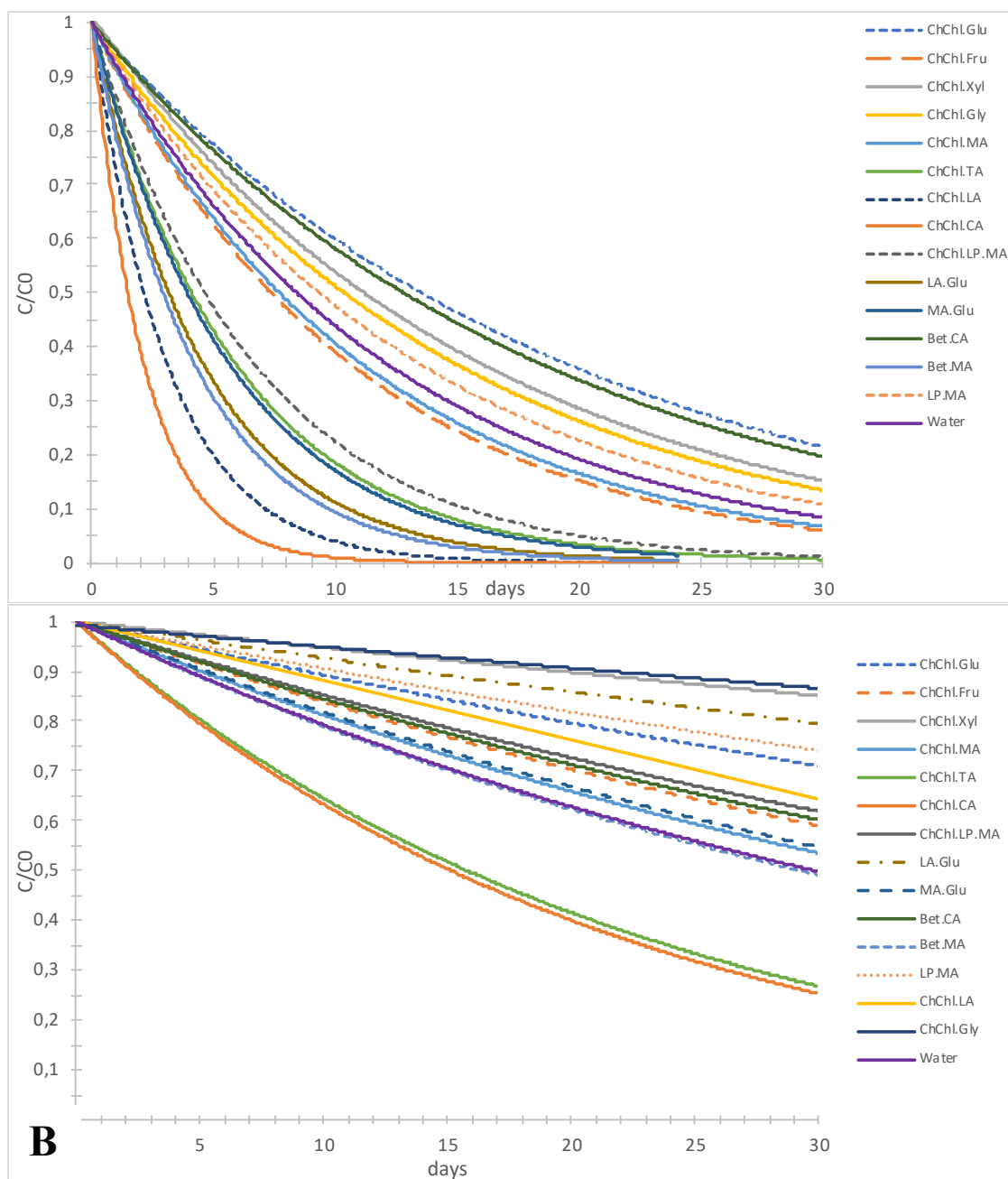
The antioxidant capacity of NADES may contribute to the stability of bioactive compounds after the extraction from plant matrices, playing a protective role in preventing degradation. AA is a molecule that is unstable in aqueous solutions, and the higher the concentration of AA the lower the degradation rate constant (Yin et al., 2022). Although its structure could have helped prevent degradation, the protective role of NADES in preventing bioactive compound degradation has been demonstrated, on different bioactive compounds, such as total phlorotannins content for 360 days (Obluchinskaya et al., 2021), total phenolic content for 30 days (Gómez-Urios, et al 2022), pharmaceutical products for four months (Mustafa, et al 2021), among others. For this purpose, AA was used in the current study as a probe to investigate the protective effect of the different NADES, whose antioxidant capacity has been established. This allowed the researchers to compare its protective role with that of other bioactive chemicals.

AA in NADES degradation kinetics at 4 and 25 °C in complete darkness were monitored for 30 days. The remaining concentration of AA (C/C_0) was plotted

against the storage time for each NADES to evaluate the degradation of AA during the storage period. Figure 1A displays the results at a temperature of 25 °C, while Figure 1B shows the results at a temperature of 4 °C. The fit obtained by applying the least squares method fits the first-order degradation kinetics presented in Table 4 based on equation (1):

Equation 1:
$$\ln(C) = -kt + \ln(C_0) \quad (1)$$

Where C_0 is the initial ascorbic acid content (mg/100 mL), C is the ascorbic acid content (mg/100 mL) at time t , k is the standard degradation rate, and t is the storage time (days).



ChChI: Choline chloride, CA: Citric acid, LA: Lactic acid, LP: L-Proline, MA: Malic acid, Gly: Glycerol, Glu: Glucose, TA: Tartaric acid, Bet: Betaine, Xyl: Xylose, Fru: Fructose.
A: 25 °C. B: 4 °C

Figure 1. Ascorbic acid stability in fourteen natural deep eutectic solvents, at 4 °C, and 25 °C for 30 days.

The degradation coefficient of AA determines the rate of loss of the compound in the matrix. At 4 °C AA is stable in most solvents, but especially the lower rate of degradation is observed in solvents based on sugars and polyalcohols, where the best stability was for ChChl:Xyl followed by ChChl:Gly, and the worst stability was presented by ChChl:CA and ChChl:TA. Then, LP:MA and LA:Glu were the solvents based on organic acids with better results preserving the ascorbic acid content. At 25 °C, NADES showed an increase of about 10 times in the rate of the degradation rate of AA. At this temperature, the degradation showed a similar tendency as at 4 °C, solvents based on sugars and polyalcohols as ChChl:Glu, followed by LP:MA and Bet:CA showed the lowest loss of AA. Moreover, Bet:MA, ChChl:TA and ChChl:CA at 4 °C showed worse results than water, indicating that these three NADES could not been a good alternative to preserve AA.

The half-life time of the AA in NADES was then analyzed, representing the time in days required during storage to reach 50% of the initial concentration. Table 4 shows the half-life time values of AA for the 14 NADES evaluated, at the two temperatures studied. It is known that ascorbic acid is stable at low temperatures and in darkness.

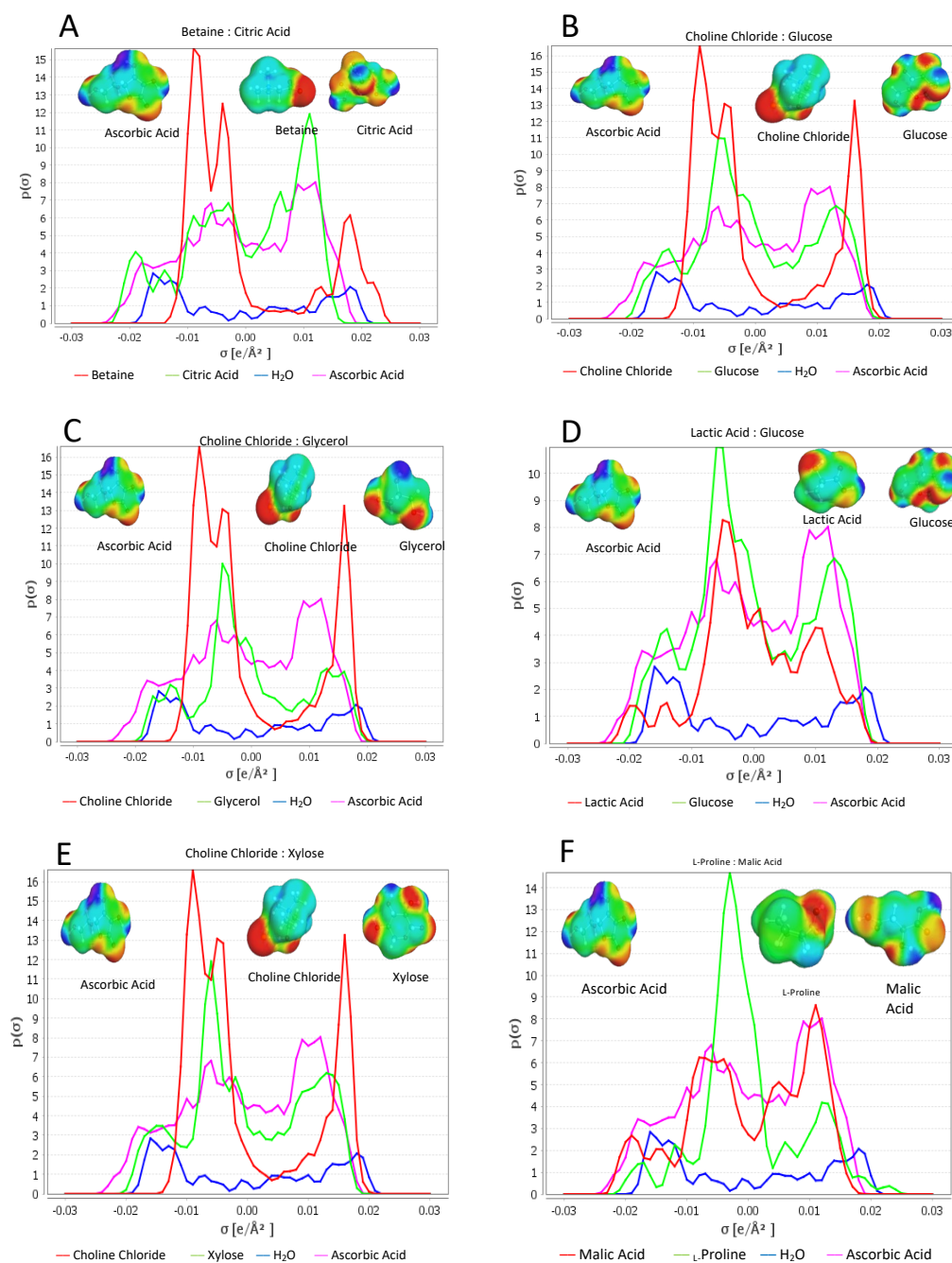
Table 4. Ascorbic acid degradation kinetics and half-life time at 4 °C and 25 °C in fourteen NADES and control in water

NADES	4 °C			25 °C		
	K (days ⁻¹)	R ² (SE)	t ½ (days)	K (days ⁻¹)	R ² (SE)	t ½ (days)
ChChl:Glu	-0.0085	81.9* (0.0351)	28.6	-0.0553	96.4* (0.0929)	6.7
ChChl:Fru	-0.0081	45.6* (0.0781)	12.5	-0.1045	90.0* (0.2985)	4.6
ChChl:Xyl	-0.0027	21.0* (0.0452)	92.6	-0.0633	97.7* (0.0844)	5.0
ChChl:Gly	-0.0048	80.3* (0.0208)	61.5	-0.0854	85.2* (0.3058)	7.3
ChChl:CA	-0.0477	90.1* (0.1383)	6.5	-0.5409	97.7* (0.2634)	1.1
ChChl:MA	-0.0216	97.0* (0.0332)	13.9	-0.0922	96.6* (0.1894)	3.6
ChChl:TA	-0.0457	97.7* (0.0626)	7.1	-0.1882	95.1* (0.3705)	2.3
ChChl:LA	-0.0194	90.2* (0.0561)	19.5	-0.2980	83.8* (0.8883)	0.7
LA:Glu	-0.0061	78.3* (0.0276)	44.7	-0.2991	86.5* (0.8011)	2.3
MA:Glu	-0.0196	93.7* (0.0444)	14.3	-0.2593	87.5* (0.6660)	3.2
ChChl:LP:MA	-0.0111	73.7* (0.0535)	18.5	-0.1946	94.2* (0.4171)	3.5
LP:MA	-0.0041	26.2* (0.0608)	47.2	-0.0716	93.7* (0.1654)	4.8
Bet:MA	-0.0249	98.5* (0.0273)	16.8	-0.2858	88.7* (0.6935)	1.7
Bet:CA	-0.0057	13.1* (0.0273)	12.4	-0.0581	93.7* (0.2217)	6.9
Water	-0.0231	91.1* (0.0416)	13.1	-0.0831	95.3* (0.2329)	3.6

ChChl: Choline chloride, CA: Citric acid, LA: lactic acid, LP: L-Proline, MA: Malic acid, Gly: Glycerol, Glu: Glucose, TA: Tartaric acid, Bet: Betaine, Xyl: Xylose, Fru: Fructose. K: slope. SE: standard error. t ½: Half-life time.

The oxygen is responsible, among other things, for the oxidation in the first reversible case of ascorbic acid (Van Bree et al., 2012). The results showed that the half-life time at 4 °C was 92.6 days for ChChl:Xyl, followed by ChChl:Gly (61.5 days). NADES storage at 25 °C showed a lower half-life time, with Bet:CA (6.9 days) followed by ChChl:Glu (6.7 days) showing the highest results. The spatial arrangement of the chains of simple sugars and polyalcohols that remain free after the formation of the eutectic mixture is an aspect to be considered in explaining the stability of ascorbic acid. The half-life time at 25 °C was doubled for ChChl:Gly (7.3 days) compared to the control (3.6 days), and was also notably increased for Bet:CA and ChChl:Glu. At 4 °C, ChChl:Xyl (92.6 days) provided an extended half-life time seven times higher than water (13.1 days), and ChChl:Gly increased the AA half-life time by 4.7 times. Moreover, ChChl:Glu, ChChl:Xyl, ChChl:Gly, and LP:MA were the more stable in both temperatures studied, and LA:Glu, and Bet:CA for 4 °C and 25 °C respectively.

Being temporarily bound and shielded from oxidation events may promote interactions between the sugars and the target product. Figure 2, shows the solubility of the most suitable NADES for the stability of AA, the profiles are divided into three quadrants based on their corresponding σ values: the HBD, the nonpolar region, and the HBA region. Negative values indicate negative polarity and positive values indicate positive polarity. The peaks from 0.01 to 0.02 e/A² and from -0.01 to -0.02 e/A² indicate the presence of polar regions in this vitamin. It can be observed that the best NADES to dissolve AA are polar ones moreover, the distribution of hydrogen bonds among the molecules in the eutectic mixture should be studied (Barani Pour et al., 2021), for this purpose, Figure 3 indicates where the hydrogen bonds may be formed while the formation of the different NADES.



The different letters indicate the different NADES systems and the solubility simulation of ascorbic acid.

Figure 2. Sigma profile of ascorbic acid (purple) in the most stable natural deep eutectic solvents. Hydrogen bond acceptor (red), hydrogen bond donor (green), and 25% of water (blue)

Lactone ring opening and subsequent decarboxylation are two non-oxidative reactions that can also degrade ascorbic acid, especially in acidic environments (pH 3-4).

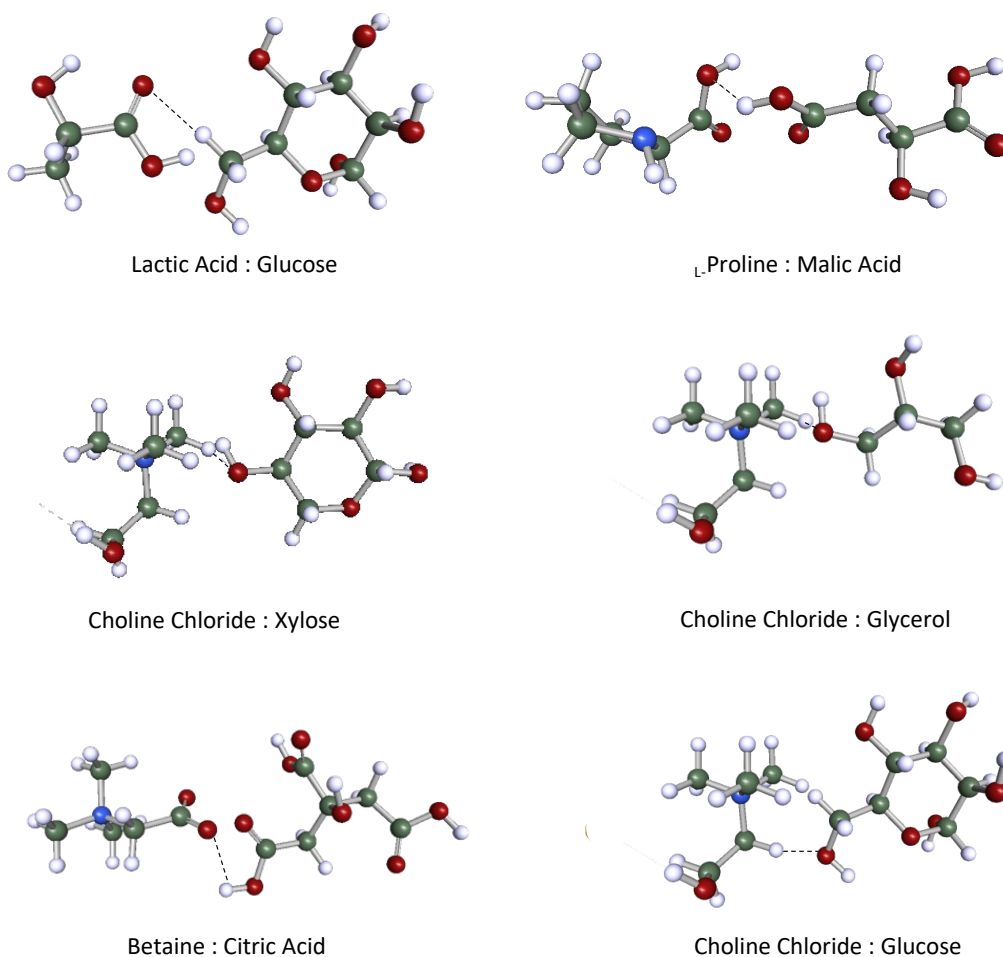


Figure 3. Hydrogen Bond simulation on the formation of most suitable NADES for the stability of ascorbic acid.

The stability of bioactive compounds can also be influenced by the antioxidant capacity of the solvent, and NADES with higher antioxidant capacity also have higher acidity. There is a positive correlation between DPPH and the AA stability at 4 °C ($R = 0.6506$, $p < 0.05$). This is also true for the stability at 25 °C ($R = 0.6816$,

$p < 0.05$). On the other hand, no correlation was found between the other antioxidant capacity methods and the AA stability.

The development of functional foods can be improved by exploring the possibility of incorporating NADES in the formulation of ascorbic acid-fortified foods to increase their stability. The ability of NADES to protect ascorbic acid could improve the shelf life of food products that depend on this vitamin, such as fortified juices or snacks. Additionally, cosmetic products can benefit from the antioxidant and anti-aging properties of ascorbic acid. The addition of natural deep eutectic solvents (NADES) to cosmetic formulations can help maintain the efficacy of ascorbic acid over time. This ensures that products maintain their quality and skin benefits.

1.4. Gastrointestinal and skin PAMPA

Table 5 shows the gastrointestinal and skin permeability assay. In this case, the most used HBA in the formation of NADES, choline chloride, was only tested for gastrointestinal PAMPA because of its prohibition on use in the cosmetics industry (European Commission (2009) in the EU Regulation (EC) 1223/2009 (entry no. 168, Annex II). In the case of betaine, since it has a similar structure to choline chloride can be used as a skin conditioning, and it was also tested for Skin PAMPA. There is a lack of knowledge about the PAMPA essay regarding ascorbic acid membrane permeability, however different studies have studied the skin permeability of other compounds, such as phenolic compounds, and different drugs (Gruber et al., 2023; Rahma et al., 2023; Soriano-Meseguer et al., 2023), at the same time indicating that PAMPA is a good research technique for screening the physical properties of test compounds during the early stages of research. From the data, it can be seen that AA in LA:Glu demonstrated the highest P_e for both gastrointestinal (Log P_e : -4.99) and skin (Log P_e : -4.78). In

the case of MA:Glu, probably due to the high viscosity (Mohd Fuad & Mohd Nadzir, 2022), is the one with the lowest Log Pe value (- 6.9) in GI-PAMPA. In addition, ascorbic acid passes through membranes by simple diffusion (Fraj et al., 2021). Therefore, it is expected that NADES and their components do not cross the skin or GI membranes due to their large size and inability to pass through by simple diffusion, so it could be useful for future studies of green cosmetics formulation, as well as for food fortification. The findings of this study could be applied to the formulation of ascorbic acid-containing drugs, cosmetics, and food products. Component stability is a crucial factor in drug manufacturing, and NADES could offer a solution to improve this stability.

The stability, solubility, and permeability of AA in NADES are crucial for the food and cosmetics industries. AA is a vital ingredient in these sectors, contributing to product stability, shelf-life, and health benefits. It is important to understand its stability in NADES because these solvents offer unique properties that can influence the overall performance of formulations. The food industry aims to preserve the nutritional quality of food products. The NADES results can benefit the development of innovative food products with increased antioxidant qualities and longer shelf lives. The cosmetics sector may also take advantage of these results by using stable AA formulations in skincare and beauty products to guarantee the efficacy and durability of the vitamin's skin-enhancing qualities

Table 5. Gastrointestinal and skin PAMPA

	Gastrointestinal		Skin	
	P_e (cm/s)	Log P_e	P_e (cm/s)	Log P_e
ChChl:Glu	$2.40 \times 10^{-6} \pm 1.29 \times 10^{-7}$	-5.64 ± 0.06	-	-
ChChl:Fru	$2.59 \times 10^{-7} \pm 6.22 \times 10^{-9}$	-6.58 ± 0.01	-	-
ChChl:Xyl	$2.44 \times 10^{-6} \pm 3.27 \times 10^{-7}$	-5.61 ± 0.05	-	-
ChChl:Gly	$4.47 \times 10^{-6} \pm 1.01 \times 10^{-6}$	-5.35 ± 0.10	-	-
ChChl:MA	$2.84 \times 10^{-6} \pm 6.24 \times 10^{-7}$	-5.55 ± 0.09	-	-
ChChl:TA	$3.07 \times 10^{-6} \pm 254 \times 10^{-7}$	-5.51 ± 0.04	-	-
ChChl:LA	$5.87 \times 10^{-6} \pm 1.71 \times 10^{-6}$	-5.24 ± 0.13	-	-
ChChl:CA	$3.13 \times 10^{-6} \pm 4.88 \times 10^{-7}$	-5.50 ± 0.07	-	-
ChChl:LP:MA	$3.67 \times 10^{-6} \pm 5.74 \times 10^{-7}$	-5.43 ± 0.06	-	-
LP:MA	$4.25 \times 10^{-6} \pm 1.25 \times 10^{-6}$	-5.38 ± 0.12	$5.76 \times 10^{-6} \pm 3.03 \times 10^{-7}$	-5.24 ± 0.02
LA:Glu	$1.01 \times 10^{-5} \pm 3.85 \times 10^{-7}$	-4.99 ± 0.02	$1.65 \times 10^{-5} \pm 1.5 \times 10^{-6}$	-4.78 ± 0.04
MA:Glu	$1.29 \times 10^{-7} \pm 3.99 \times 10^{-8}$	-6.90 ± 0.13	$8.51 \times 10^{-6} \pm 7.6 \times 10^{-6}$	-5.18 ± 0.38
Bet:MA	$3.35 \times 10^{-6} \pm 3.50 \times 10^{-7}$	-5.48 ± 0.05	$7.90 \times 10^{-6} \pm 1.1 \times 10^{-6}$	-5.10 ± 0.06
Bet:CA	$3.68 \times 10^{-6} \pm 6.03 \times 10^{-7}$	-5.44 ± 0.07	$1.83 \times 10^{-5} \pm 3.3 \times 10^{-6}$	-4.74 ± 0.08

ChChl: Choline chloride, CA: Citric acid, LA: lactic acid, LP: L-Proline, MA: Malic acid, Gly: Glycerol, Glu: Glucose, TA: Tartaric acid, Bet: Betaine, Xyl: Xylose, Fru: Fructose. P_e (Permeability).

2. Conclusions

In the present study, the antioxidant capacity of fourteen different NADES was quantified by three different antioxidant capacity methods, namely TEAC, ORAC, and DPPH. The solubility of AA in fourteen different NADES was simulated. AA was used as a probe to determine the protective role of NADES

at 4 and 25 °C. In vitro membrane permeability was tested for skin and gastrointestinal membranes, using the PAMPA method. The results showed that NADES have antioxidant properties by themselves, which could be improved when used as an extractant solvent. Regarding AA stability, the half-life time was longer for those NADES stored at 4 °C rather than 25 °C, as expected. While some of the NADES studied provided considerably higher AA half-life time than water, mainly ChChl:Xyl at 4 °C and, ChChl:Gly at 25 °C, offer an extended protective role that could be used in biofunctionalized storage materials. Bet:MA, ChChl:TA, and ChChl:CA, showed worse results than water, which indicates that they could not be a viable alternative to the protection of AA, suggesting that more studies are needed to be able to understand AA stability in other hydrosoluble solvents.

In vitro membrane permeability showed similar results for skin and gastrointestinal membranes. Additionally, choline chloride-based NADES were only tested for GI and showed promising results for future studies of food fortification. Skin-PAMPA showed also interesting results, depending on the NADES used, a different permeability will be obtained, this can help to formulate green cosmetics focusing on the cosmetic industry objective to be achieved.

These results have resulted in a publication:

Gomez-Urios, C., Puchades-Colera, P., Frigola, A., Esteve, M. J., Blesa, J., & Lopez-Malo, D. (2024). Natural deep eutectic solvents: A paradigm of stability and permeability in the design of new ingredients. *Journal of Molecular Liquids*, 412, 125864. <https://doi.org/10.1016/j.molliq.2024.125864>

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CHAPTER II:

Sustainable Development and Storage Stability of Orange By- Products Extract Using Natural Deep Eutectic Solvents



The citrus industry produces large amounts of waste rich in bioactive compounds that have important effects on human health. Their extraction was performed using organic solvents, and a greener alternative to those solvents are natural deep eutectic solvents (NADES). The present study aimed to obtain and optimize extracts rich in polyphenols and flavonoids from orange peels using NADES and monitor polyphenol stability in the extracts for 30 days.

1. Results and discussion

1.1. NADES characterization

The extraction solvent should be selected considering parameters such as safety, price, availability, and affinity with the target compound. Table 1 shows the characteristics of each NADES at 75% mixed with water (75:25, v/v) and the molar ratios used. It is well-studied that NADES physicochemical properties influence the relationship with the target bioactive compounds as reported by Cvjetko Bubalo et al. (2018). The different pH values of NADES may affect the hydrogen bond interactions among solute and NADES. Most of the fourteen NADES studied have acidic pH; moreover, the acidity of each NADES is due to its components. Those solvents made with acidic components (carboxylic group) have lower pH values compared with the ones made with sugar (carbonyl groups), which have the highest pH values. NADES can donate and accept hydrogen bonds and electron pairs, giving the capacity to make hydrogen bonds between NADES and solvents (Doldolova et al., 2021). Polarity and pH are physicochemical characteristics that affect the extraction of bioactive compounds and their solubilizing capabilities, where a high ENR value indicates a low polarity, and acidic pH can be advantageous to extract polar compounds. For example, Dai et al. (2013) found that LP:MA mixed with water (75:25, v/v)

resulted in optimum for the extraction of TPC from *Carthamus tinctorius* L. due to its polarity, which is similar to methanol and ethanol.

Table 1. Used NADES and their physicochemical characteristics

Acronym	Hydrogen bond acceptor	Hydrogen bond donor	Molar ratio	pH	Polarity (kcal/mol)
ChChI: Glu	Choline chloride	Glucose	2:1	4.14	49.79
ChChI: Fruc		Fructose	1.9:1	4.35	51.26
ChChI: Xyl		Xylose	2:1	4.26	49.21
ChChI: Gly		Glycerol	1:2	5.19	49.00
ChChI: MA		Malic acid	1:1	1.62	47.85
ChChI: TA		Tartaric acid	2:1	1.54	47.98
ChChI: LA		Lactic acid	1:3	1.43	47.97
ChChI: CA		Citric acid	2:1	1.45	47.81
ChChI: LP: MA	Lactic acid	L-Proline: Malic acid	1:1:1	2.11	48.87
LA: Glu		Glucose	5:1	1.16	47.89
MA: Glu		Glucose	1:1	1.34	47.57
LP: MA		Malic acid	1:1	2.22	48.30
Bet: CA		Citric acid	1:1	2.36	47.97
Bet: MA		Malic acid	1:1	2.08	48.79

1.2.COSMOtherm Simulation and Model Validation

To design a green and efficient extraction process, the first step is the selection of the most suitable solvent for a target compound, and the second is the optimization of the extraction method (Cvjetko Bubalo et al., 2018). In this way, the process of selection of an optimal solution can be time- and energy-consuming; in consequence, it can increase the costs. For this reason, COSMOtherm software was employed to screen 14 NADES for the extraction of TPC and TFC from orange peels. Hesperidin was selected as the target compound because it is the most abundant flavonoid present in orange peels (Boukroufa et al., 2015; Jokić et al., 2020). Consequently, to evaluate the accuracy of the software, the model was experimentally validated with the suitable and unsuitable NADES for the extraction of hesperidin. One of the outputs that COSMOtherm can create is the sigma profile, and this can help to understand better the polarities and affinity of a molecule for NADES compounds. Figure 1 represents the sigma profile for hesperidin in LA:Glu (Figure 1a) and LP:MA (Figure 1b). The profiles are divided into three quadrants with corresponding σ values: the HBD, the nonpolar region, and the HBA region. Negative values represent positive polarities and vice versa. The peaks around 0 e/A2 show the apolarity of hesperidin, and the peaks from 0.01 to 0.03 and -0.01 to -0.03 show that this polyphenol also includes polar regions. It can be observed that the best NADES to dissolve polyphenols are polar ones. Additionally, when hexane is represented in the sigma profile, the peak is in the non-polar region, reflecting the low affinity for this polyphenol. For the screening of NADES using the COSMOtherm, first, it was necessary to simulate the solubility of hesperidin with the 14 NADES in the software's option-activity coefficient (ln) calculation. The calculation of the activity coefficient was

performed using 100%, 85%, 75%, 50%, 40%, and 30% of NADES in water (v/v). Table 2 shows the results of NADES screening: when there is a low activity coefficient of the tested NADES, there exists a higher hesperidin solubility (green color); in contrast, when the values of activity coefficient are high, there exists a poor hesperidin solubility (orange color).

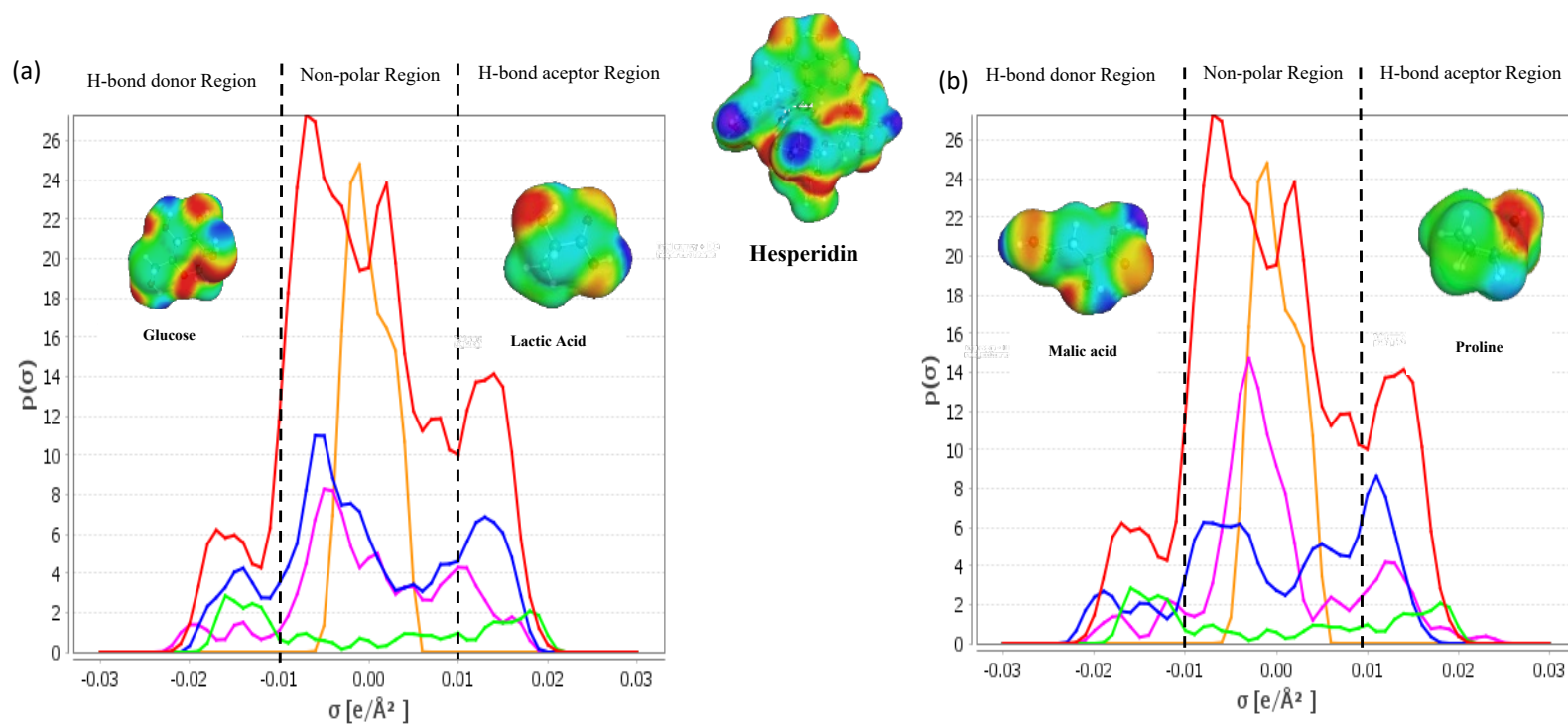


Figure 1. (a): Sigma profile of hesperidin (red), water (green), glucose (blue), lactic acid (pink), and hexane (orange). (b): Sigma profile of hesperidin (red), water (green), malic acid (blue), L-Proline (pink), and hexane (orange) and their sigma surfaces. H-bond: Hydrogen bond

It can be observed that there are higher values of $\ln$ in NADES formed with acid compounds (LA:Glu, LP:MA, and MA:Glu), and for their acidic nature, their pH is also very low. In addition, the predictions showed lower $\ln$ in NADES based on choline chloride, reflecting a low solubility of hesperidin and also corresponding with the higher pH values. In Table 2, it is demonstrated how the solubility of hesperidin can change with different amounts of NADES in water, where the %NADES is proportional to the solubility of hesperidin. The COSMOtherm predictions were in good agreement with other studies, where acid-based NADES had better extraction efficiency than polyphenols (Panić et al., 2021; Radošević et al., 2016). For example, Radošević et al., (2016) reported better extraction of polyphenols from grape skins using acid-based NADES. Another physicochemical property that should be taken into account is polarity, as organic acid-based NADES are more polar than sugar and polyalcohol-based ones. In consequence, NADES with high polarity showed better efficiency with polar compounds such as polyphenols, anthocyanins, and flavonoids (Cvjetko Bubalo et al., 2018; Grillo et al., 2020; Panić et al., 2020). To demonstrate the accuracy of COSMOtherm software and validate the model, extracts were prepared with the NADES with the highest and lowest $\ln$ values. Extractions were prepared with 75% of NADES because the addition of water has been demonstrated to decrease the viscosity of NADES and, in consequence, enhance the mass transfer from plant matrices to the solution, increasing the extraction efficiency (Panić et al., 2019). The selected NADES for the validation were LA:Glu, LP:MA, MA:Glu, and ChChl:Fruc. Furthermore, to compare the results, an extraction with EtOH (50% v/v) was performed as a control. TPC and TFC were measured to validate the model, and the results are shown in Figure 2 and were in good concordance with the prediction of COSMOtherm. There was no significant difference ($p < 0.05$) between LA:Glu (1932 ± 7.83

mgGAE/100 gdw and 82.7 ± 3.01 mg/100 gdw), LP:MA (2164 ± 5.17 mgGAE/100 gdw and 97.0 ± 1.65 mg/100 gdw), and EtOH extract (2164 ± 8.97 mgGAE/100 gdw and 109 ± 5.22 mg/100 gdw) for TPC and TFC, respectively. These results highlight the possibility of NADES replacing conventional solvents such as ethanol for polyphenol and flavonoid extraction. In contrast, the lowest extraction efficiencies were in ChChl:Fruc for TPC and in ChChl:Fru and MA:Glu for TFC. This was also expected because ChChl:Fruc has sugar and belongs to the group of solvents with pH values close to 6, which does not favor the extraction of polar compounds.

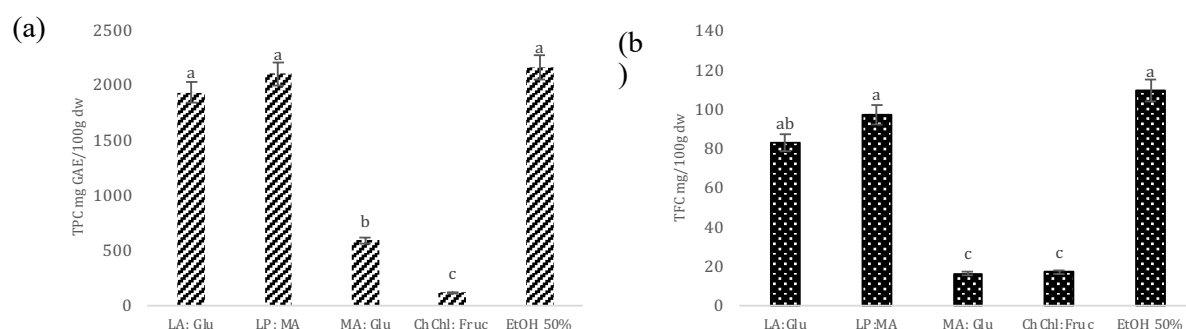
Table 2. Predicted $\ln \gamma_{\text{solutos}}$ for Hesperidin in 70% NADES (v/v) using COSMOtherm

Acronym	$\ln(\gamma)$ 85% NADES	$\ln(\gamma)$ 75% NADES	$\ln(\gamma)$ 50% NADES	$\ln(\gamma)$ 40% NADES	$\ln(\gamma)$ 30% NADES
EtOH	-1.063				
LA: Glu	-4.52	-4.02	-3.66	-3.31	-2.24
LP: MA	-4.73	-3.78	-3.28	-2.83	-1.71
MA: Glu	-4.81	-3.67	-2.94	-2.31	-0.93
ChChl: LA	-2.15	-1.88	-1.60	-1.29	-0.28
Bet: MA	-3.60	-2.12	-1.20	-0.55	0.47
ChChl: LP: MA	-1.44	-1.11	-0.75	-0.30	0.61
ChChl: Xyl	-2.49	-1.73	-0.87	0.00	1.78
Bet: CA	-0.53	-0.26	-0.12	0.06	0.70
ChChl: MA	-1.16	-0.60	-0.07	0.44	1.31
ChChl: TA	-2.14	-1.10	-0.33	0.48	1.74
ChChl: Glu	-2.24	-1.35	-0.34	0.53	2.04
ChChl:CA	-1.52	-0.66	0.02	0.75	1.93
ChChl: Gly	-0.60	-0.21	0.28	0.77	1.62
ChChl: Fruc	-1.01	-0.51	0.11	0.82	1.89

*Gray color: reference; green: better or equivalent solvent than reference; yellow color: slightly worse solvent than reference; red color: worse solvent than reference. ChChl:

Choline chloride, Glu: glucose, Fruc: fructose, Xyl: xylose Gly: glycerol, MA: malic acid, TA tartaric acid, LA: lactic acid, CA: citric acid, LP: L-proline, Bet: betaine

Based on these results, it is possible to conclude that activity coefficient calculation in COSMOtherm software is a good tool for predicting the solubility of hesperidin in NADES. However, another aspect should be considered before the next optimization process. The availability and safety are important for their potential inclusion in the industry. The compounds of NADES are safe, and LA, Glu, LP, and MA are part of our diet and have positive effects on health. Lactic acid, proline, glucose, and malic acid are considered GRAS and are already present in food products. Additionally, these NADES compounds have biological activity by themselves; for example, lactic acid is present in yogurt, cheese, and kefir and can improve gut health. The FDA has approved its use in most products apart from infant foods and formula. In the case of malic acid, there is no scientific basis for suspecting that the amounts of malic acid added to foods would be toxic according to the Select Committee on GRAS Substances (FDA, 2024). Proline is an amino acid and has been also related with positive biological effects as anti-tumor, anti-fungal, and antimalarial (Saharan et al., 2022).



(a) Total polyphenol content (mg GAE/100g_{dw}), b) Total flavonoid content (mg/100g_{dw}). Results are expressed as the means (n=3) $\pm$ DS. Different lower-case letters (a-c) are significantly different ($p < 0.05$) as measured by Tukey's test.

Figure 2. Model validation

1.3. Optimization of extraction conditions by response surface methodology

The optimization process is the second step for an efficient extraction, and for this reason, the most promising NADES selected in the model validation (LA:Glu and LP:MA) were optimized considering TPC and TFC as response variables. The RSM was performed for optimization of the extraction parameters using Design Expert 11.0. Solvent-to-solid ratio (5 to 25 mL), %NADES (85% to 30% (v/v)), and extraction time (5 to 25 min) were the variables considered in the optimization due to their high impact in TPC and TFC; coded levels and independent variables are shown in Table 3. The %NADES (v/v) have a direct impact in the extraction efficiency and, due to the addition of water, can help to reduce the viscosity of NADES and improve the mass transfer to the solvent (Dai et al., 2015). To evaluate the effect of the considered variables in the responses, a Box–Behnken design was used. The experimental values using the presented model considering the two selected NADES for each compound are shown in Annex 2 Table S1. ANOVA was used to calculate the significance of the quadratic model

of all independent variables and is summarized for LA:Glu in Annex 2 Table S2 and for LP:MA Table S3.

Table 3. Coded levels and independent variables

Independent variable		Level				
		$-\infty$	-1	0	+1	$+\infty$
Solid/liquid ratio	X_1	5	10	15	20	25
NADES (% v/v)	X_2	30	40	50	75	85
Extraction time	X_3	5	10	15	20	30

The determination coefficient (R^2) of 0.99 indicates that models provide credible fit values for the extraction of TPC and TFC from orange peels using LA:Glu and LP:MA. Additionally, the lack of fit in the RSM model of the responses was greater than 0.05 (non-significant) in all the cases, which means that the model fits well, and there is a significant effect on output responses. In addition, the coefficients of variation were between 1.78 and 5.85, indicating that the results were reliable and accurate. Then, the values of the coefficients for TPC and TFC for each NADES were used for a final predictive equation. The regression equations were highly significant ($p < 0.05$), and the final polynomials equations in terms of actual factors were:

Lactic Acid:Glucose

$$\text{Equation 1: TPC} = 352.54X_1 + 609.34X_2 + 338.59X_3 - 283.80X_1X_2 + 241.24X_2X_3 - 545X_1^2 - 399.44X_2^2 + 579.71X_3^2 \quad (1)$$

$$\text{Equation 2: TFC} = 127.77 - 15.01X_1 - 58.79X_2 + 68.80X_3 + 14.12X_1X_2 + 9.01X_2X_3 + 15.89X_1^2 + 18.62X_2^2 - 34.19X_3^2 \quad (2)$$

L-Proline:Malic Acid

$$\text{Equation 3: TPC} = 3828.25 + 189.02X_1 - 1895.33X_2 + 1425.09X_3 + 241.16X_1X_2 - 718.06X_1X_3 - 1019.80X_2X_3 - 1610.97X_1^2 + 739.78X_2^2 + 908.10X_3^2 \quad (3)$$

$$\text{Equation 4: TFC} = 137.52 - 44.77X_1 - 66.97X_2 + 80.30X_3 + 8.77X_1X_2 - 38.94X_1X_3 - 34.59X_2X_3 + 9.51X_1^2 + 12.80X_2^2 - 20.81X_3^2 \quad (4)$$

Where X1 is solid/liquid ratio, X2 is %NADES (v/v), X3 extraction time.

According to the p-values, which were <5 for all variables, they had a statistically significant influence on TPC and TFC, and this demonstrated the significance of the presented models. Response surface plots were created to show just the significative interactions between two variables on the response values. In Figure 3 shows the 3D plots using LA:Glu as solvent for TPC and TFC. In Figure 2a are presented the response surfaces for TPC generated first by the %NADES and solid/liquid, and it was observed that by increasing the % of NADES and decreasing the ratio, a higher yield of TPC is achieved. This can be explained because when higher % of NADES exist, the hydrogen bond interaction between the target compound and solvent are stronger, and the addition of water in more than 50% may be considered an aqueous solution, and the components can be dissociated (Panić et al., 2021).

In the second interaction for TPC, it is clear that longer extraction time and high %NADES can increase the extraction yields. A short extraction time can be insufficient for some bioactive compounds, but a longer extraction time can produce degradation of the extracted compounds (Ozturk et al., 2019). In other studies, it has been reported that extraction yield can increase with longer extraction time but also can decrease after long periods (Pal & Jadeja, 2019). Moreover, long periods extraction can contribute to the degradation of bio compounds, and a high solvent-to-solid ratio can increase the dispersion of the solvent in the sample, reducing the extraction performance (Gullón et al., 2020; Manuel Pinelo et al., 2005). In Figure 4b are presented three response surfaces for TFC, and as was expected, they showed the same tendency as TPC, where

higher %NADES and solid:liquid ratio increased the extraction yields, and the interaction between %NADES and extraction time was directly proportional. In Figure 4 are shown the 3D plots using LP:MA as solvent for the optimization of TPC and TFC, and in this case, all the interactions between the variables were significant ($p < 0.05$)

Figure 4a shows the response surfaces for TPC, where the tendency was different when compared with LA:Glu. A lower %NADES showed better extraction yields, and when the extraction time increased, %NADES also became higher. This can be explained due to the high viscosity of LP:MA, which can reduce the extraction efficiency, and when the extraction time is longer, the viscosity can be reduced (Mitar et al., 2019).

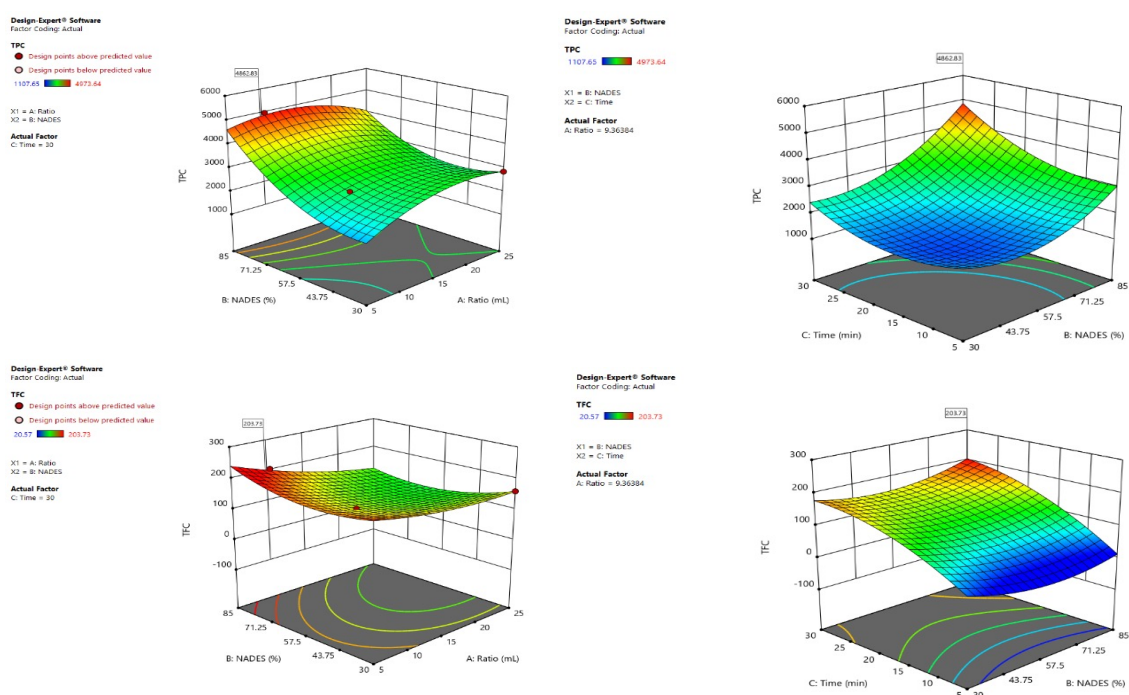


Figure 3. Response Surface plots showing combined significant effects on TPC (a) and TFC (b) using LA: Glu as solvent

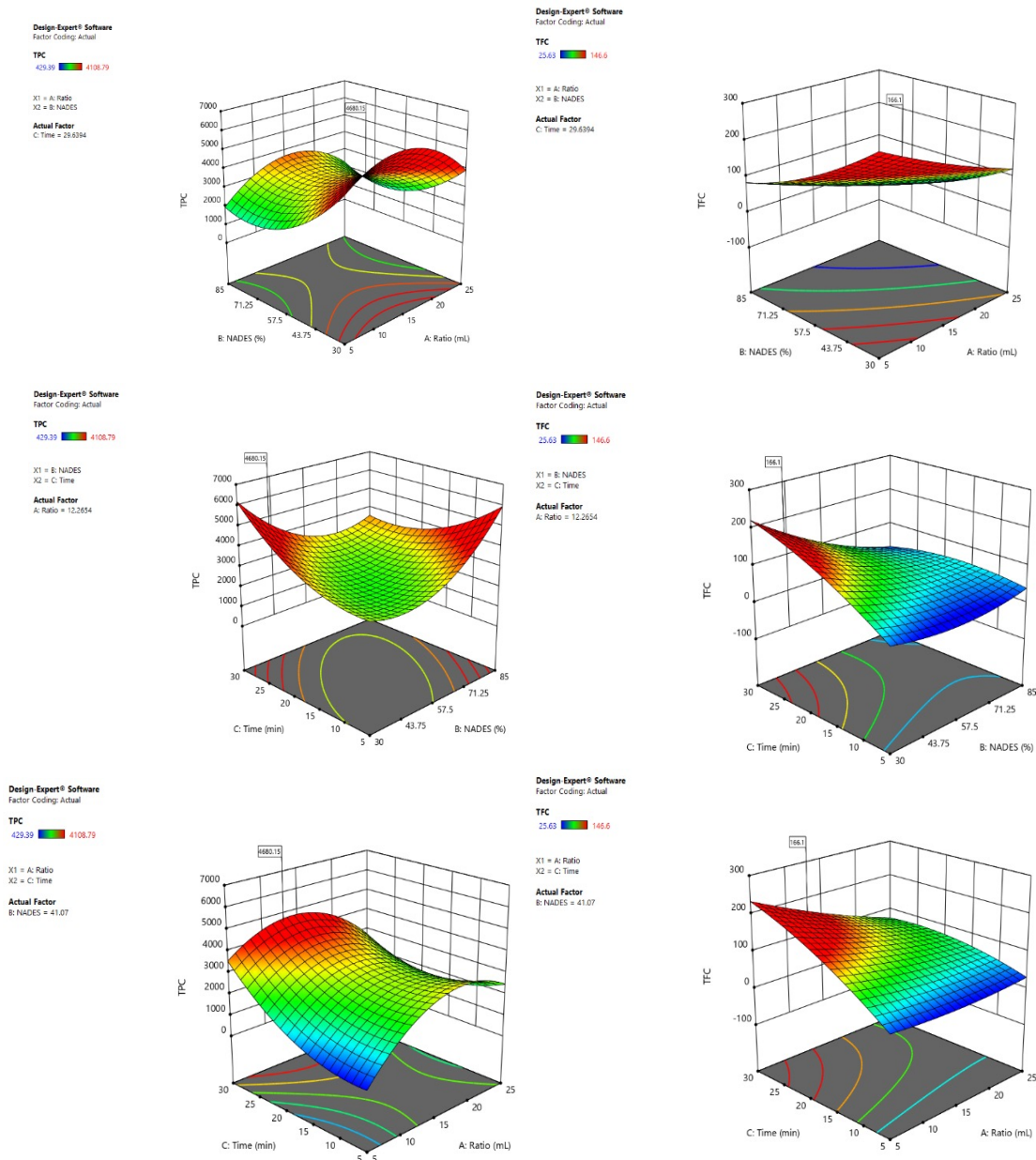


Figure 4. Response surface plots showing combined effects of solid-liquid ratio, %NADES and extraction time for TPC (a) and TFC (b) LP: MA as solvent

The final goal of RSM was the process optimization where the developed models can be used to predict the TPC and TFC. By applying the desirability function and considering the maximum responses, the optimal conditions for the extraction of TPC and TFC simultaneously from orange peels were calculated using LA:Glu and LP:MA. In the first case, the optimal conditions are represented

in Figure 5 and were 9.36 mL/g, 85% of NADES, and 30 min of extraction time. Under these optimal conditions, the extraction yield of TPC was 4862 mg GAE/100 gdw, and in this case, this NADES extracted a higher concentration of TPC than in other similar studies (Dai et al., 2016) and for TFC 203 mg/100 gdw. For the second optimization using LP:MA, results are presented in Figure 5, where the optimal conditions were a solid:liquid ratio of 12.2 mL, %NADES of 41.1, and extraction time of 29.6 min; the extraction yields in these conditions were 4680 mg GAE/100 gdw for TPC and 166.1 mg/100 gdw for TFC. Considering these results, it can be observed that optimal parameters can change in different solvents, and it is associated with their physicochemical properties. LP:MA has higher viscosity (0.0150 Pas) (Panić, Gunjević, et al., 2019); for this reason, it is expected that higher % of NADES in water can reduce the extraction yields.

In addition, in both solvent the extraction, the time was around 30 min, which is in accordance to other studies that suggest that optimum time for the extraction of polyphenolic compounds should be between 30 to 180 min, but it is dependent on the characteristics of the samples (Ozturk et al., 2018). However, extraction time is an important parameter for the industrial application, and due to prolonged operation, they are difficult to be applied on a large scale and could lead to polyphenols oxidation. Rebocho et al (2022) that extraction time affected the extraction yield of TPC. Finally, the solvent:solid ratio was slightly low, which can be explained by the mass transfer principles. It can be concluded with the results presented above that the considered conditions for the optimization in the present study had a strong effect in the extraction of TPC and TPFC. The validation of the suggested extraction process and the optimal conditions were tested using the same methodology as optimization samples and was confirmed with a deviation of ± 5 .

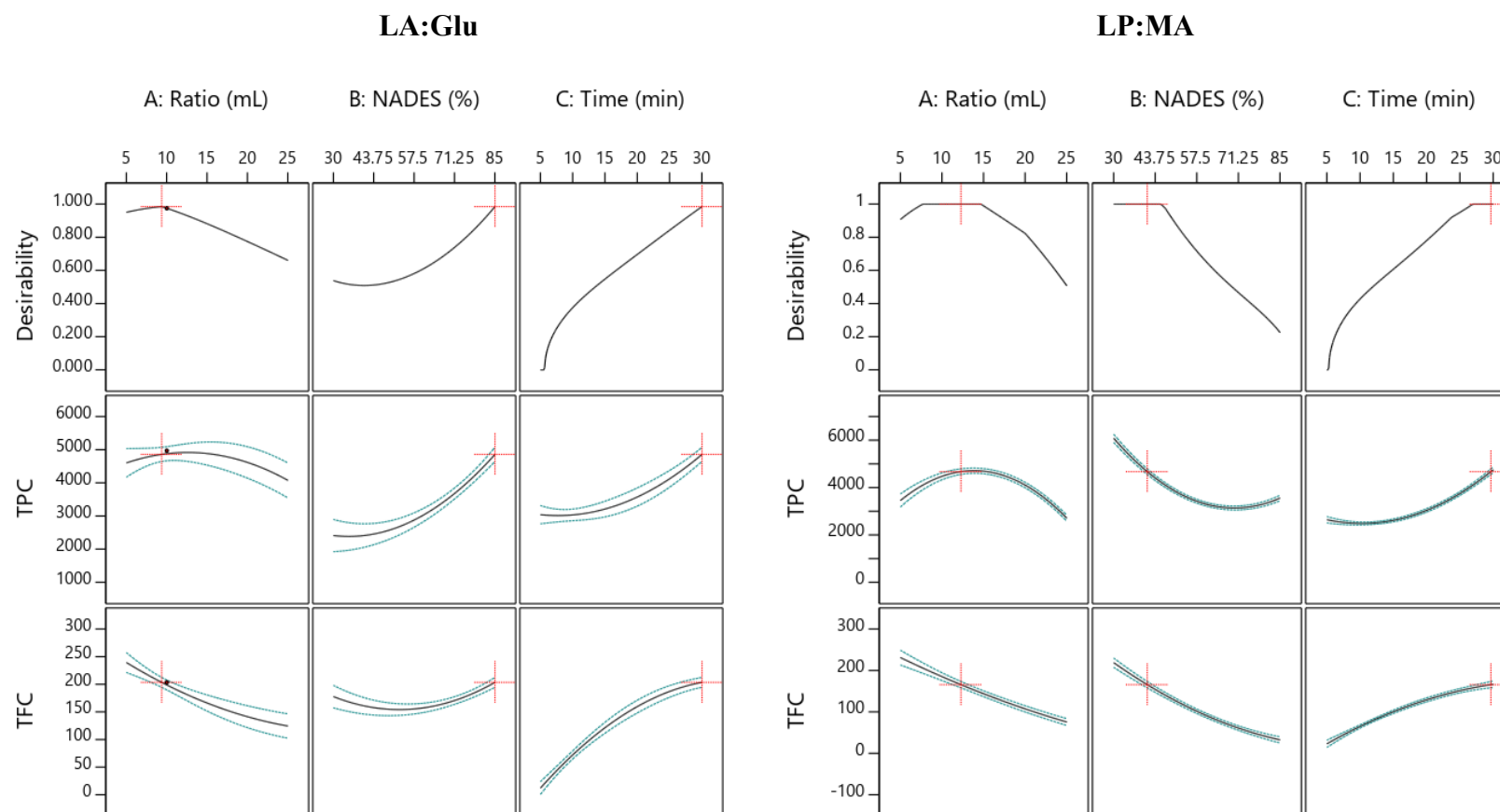


Figure 5. Optimal conditions for the extraction of TPC and TFC simultaneously using LA:Glu and LP:MA as solvent. A: mL NADES / g Sample

1.4. Total polyphenol stability

One important aspect in the selection of the most appropriate solvent is the stability of the extracted compound in the solvent, and this parameter is an important aspect if the final application includes these extracts in food formulation. The stability was monitored in LP:MA and LA:Glu and also with the extraction performed using ethanol (50% v/v). Figure 6 presents the results for TPC during storage for 30 days in different conditions: at 25 °C and 4 °C. The results demonstrated decreased values of TPC in all NADES extracts at room temperature, where TPC was more stable in LP:MA extracts, with 70% of degradation. Then, at low temperatures, TPC in LA:Glu showed degradation of 15% at 4 °C and LP:MA 35% after 30 days. In all the storage conditions, DES extracts showed better stability of polyphenols when compared with ethanol extract. Other studies have reported that DES contributes to the stabilization of phenolic compounds. Panić et al. (2019) reported that extracts with choline chloride: citric acid enhances the stability of 70% of anthocyanins at 4 °C. Additionally, Dai et al. (2016) reported that LA:Glu systems provide better stability of cyanidin when compared to ethanol extractions. These results can be explained due to the interaction between the phenolic compounds and the components of NADES mixtures, which decrease the movement of solute molecules and reduce their contact time with oxygen, and in consequence, the oxidative degradation is reduced (Dai et al., 2016; Rebocho et al., 2022). The enhanced stability of TPC in NADES extracts indicates their potential application in the fortification of food products, and this characteristic can contribute to extending the shelf life of the enriched products.

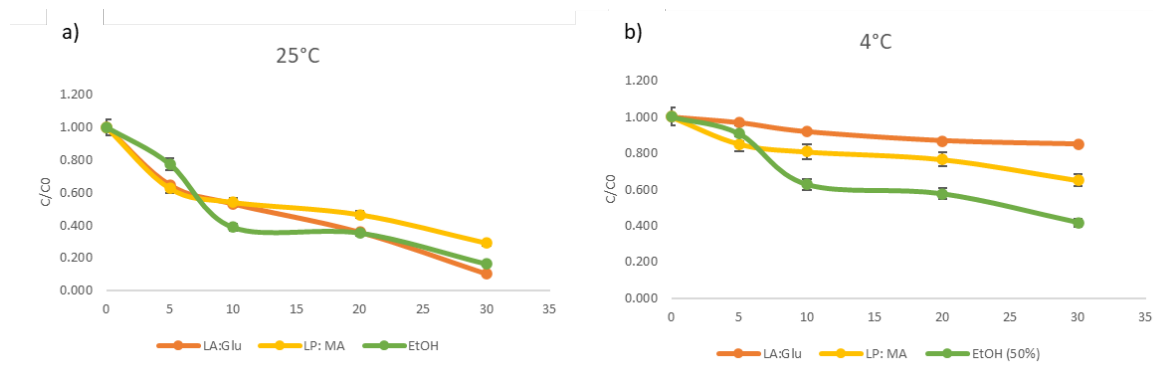


Figure 6. Stability of phenols in NADES (LA:Glu, LP:MA) and Ethanol (50% v/v) extracts at 25°C (a) and 4°C (b) during storage for 30 days. Results were expressed as the means ($n=3$) $\pm$ SD.

2. Conclusions

In this study, green extracts rich in bioactive compounds (polyphenols and flavonoids) from orange peel were obtained following the principles of green chemistry using sustainable solvents. The screening of 14 NADES using COSMOtherm software was performed to select the most suitable solvent for TPC and TFC extraction from orange peel. The experimental validation corroborated that the software is a good tool to predict the solubilization of bioactive compound in NADES. LA:Glu and LP:MA showed the most promising results for TPC and TFC extraction and the process was optimized considering solid/liquid ratio, %NADES and extraction time as variables. The optimization results showed that the considered variables have a strong effect in the extraction of TPC and TFC, but they can change depending on the solvent. Additionally, NADES demonstrated to enhance polyphenol stability during 30 days of storage. In this study the NADES studied might be considered as a good alternative to replace other organic solvents for bioactive compounds extraction from orange peel. In addition, the proposed extracts have the potential to be

included in industrialized food products due to their natural components and safe consumption. Future research should be directed in the study in the physicochemical characteristics of the final food products with the inclusion of NADES extracts.

These results have resulted in a publication:

Gómez-Urios, C., Viñas-Ospino, A., Puchades-Colera, P., López-Malo, D., Frígola, A., Esteve, M. J., & Blesa, J. (2022). Sustainable Development and Storage Stability of Orange By-Products Extract Using Natural Deep Eutectic Solvents. *Foods*, 11(16), 2457. <https://doi.org/10.3390/foods11162457>

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CHAPTER III:

Choline chloride-based natural deep eutectic solvents for the extraction and stability of phenolic compounds, ascorbic acid, and antioxidant capacity from *Citrus sinensis* peel



Orange peel is a great source of bioactive compounds like phenolic compounds and ascorbic acid, which have high antioxidant capacity. New techniques and methodologies have called attention for their extraction employing non-conventional and greener solvents, as natural deep eutectic solvents (NADES). Although, there is a lack of information about the protective role and stability of bioactive compounds in NADES. This study aims to find the most suitable NADES for the extraction of bioactive compounds from orange peel. Table 1 shows the characteristics of the NADES used on this chapter

Table 1. Characteristics of natural deep eutectic solvents used on this chapter

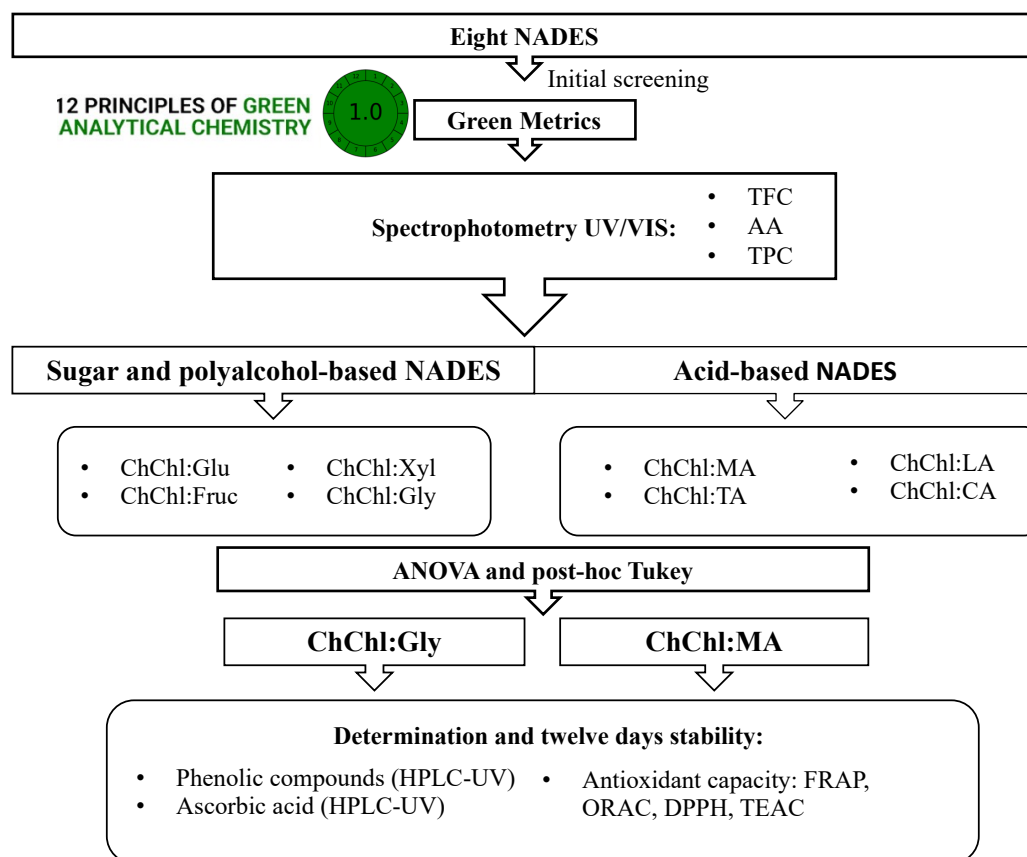
	Acronym	Hydrogen bond acceptor	Hydrogen bond donor
Sugar and polyalcohol-based	ChChl:Glu	Choline chloride	Glucose
	ChChl:Fruc		Fructose
	ChChl:Xyl		Xylose
	ChChl:Gly		Glycerol
Acid-based	ChChl:MA		Malic acid
	ChChl:TA		Tartaric acid
	ChChl:LA		Lactic acid
	ChChl:CA		Citric acid

1. Results and discussion

1.1. Initial Screening and physicochemical properties

In Figure 1, it is shown the experimental procedure followed. To establish the most sustainable method, the AGREE program was used, and the analysis of the 12 principles of green chemistry was transformed into a 0–1 scale. In this case,

UV/VIS spectrophotometry method and HPLC-UV/VIS method were compared, to establish the greenness of the methods used to screen the NADES extracts. The AGREE tool scale determined that the spectrophotometric method was the closest to the green chemistry principles (0.66), using a lower volume of organic solvents and it is represented in Figure 2.



ChChl: choline chloride, Glu: glucose, Fruc: fructose, Xyl: xylose, Gly: glycerol, TA: tartaric acid, MA: malic acid, CA: citric acid, LA: lactic acid. TFC: total flavonoid content. AA: ascorbic acid. TPC: total polyphenol content. ORAC: oxygen radical absorbance capacity assay, FRAP: ferric reducing-antioxidant

Figure 1. Procedure flux diagram.

power, TEAC: Trolox equivalent antioxidant capacity. DPPH: 2,2-diphenyl-1-picrylhydrazyl.

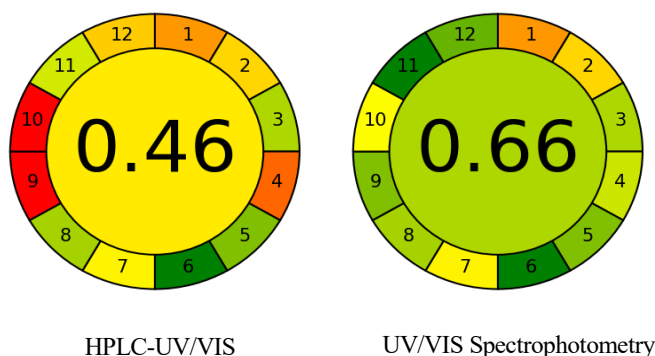
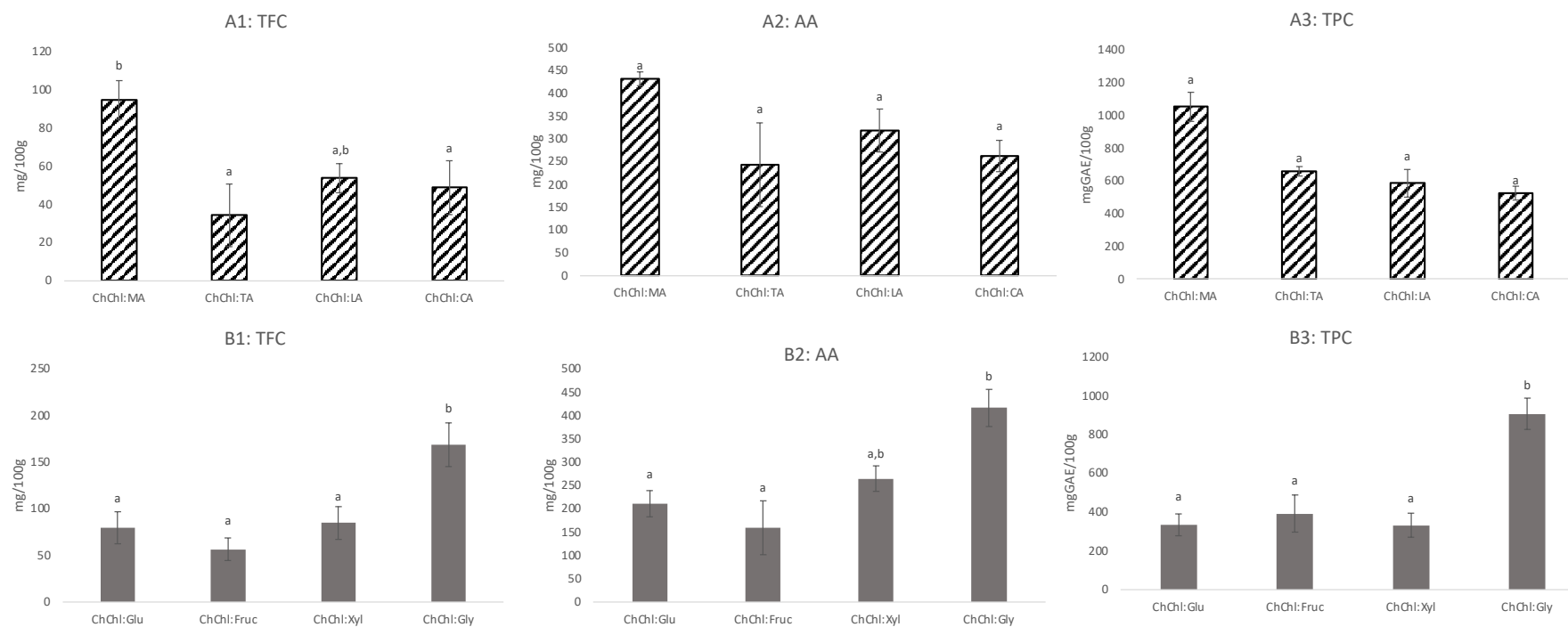


Figure 2. Sustainability of the extraction process, according to the Analytical Greenness Calculator (AGREE)

This work investigated three classes of NADES, including 3 sugar, 1 polyalcohol, and 4 carboxylic acid-based NADES. Under the same conditions (75% of NADES (v/v); solid-liquid ratio, 1:10 g/ml; extraction time (20 min); temperature, 40 ± 5 °C). The results were grouped according to the components of NADES, sugar, polyalcohol-based NADES, and acid-based NADES (Table 1). It is interesting to study all bioactive compounds together due to their characteristics, phenolic compounds and ascorbic acid are all of them water-soluble and have AC (Apraj & Pandita, 2016) and are present in high concentrations in orange peel. For this reason, the extraction of bioactive compounds was carried out with eight NADES, and the highest extraction yield was considered. Figure 3 showed the concentration of each bioactive compound in sugar and polyalcohol-based and acid-based NADES. ChChl:Gly and ChChl:MA are the NADES that have the highest extraction yield. Statistical analysis (ANOVA, and Poshoc Tukey $p < 0.05$) results indicated that the extraction efficiency of total bioactive compounds from orange peel largely depended on the type of NADES used for extraction (Annex 3 Table S1).



A: Acid-based NADES, B: sugar and polyalcohol-based NADES. ChChl: choline chloride, Glu: glucose, Fruc: fructose, Xyl: xylose, Gly: glycerol, TA: tartaric acid, MA: malic acid, CA: citric acid, LA: lactic acid. GAE: gallic acid equivalent. a, b: different letters indicate statistical differences ($p < 0.05$)

Figure 3. Extraction yield of total flavonoid content, ascorbic acid, and total polyphenol content in eight NADES

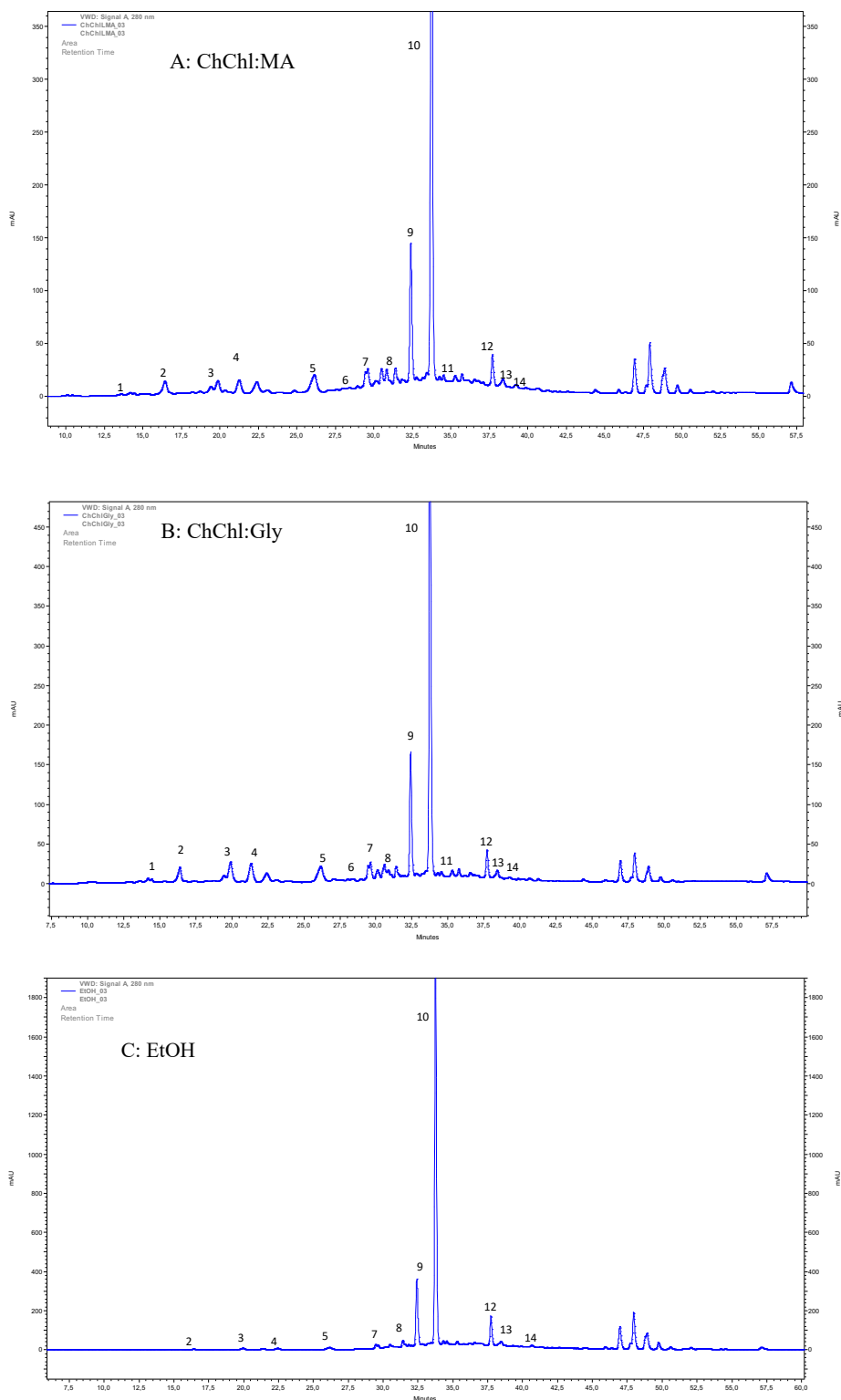
Viscosity is an important parameter to take into account, so that, with lower water content, the viscosity of NADES, helps the process of extraction and diffusion of bioactive compounds from the food matrix to the solvent (Panić et al., 2021). Benvenuti et al (2020), stated that high amounts of water do not prevent NADES from being good solvents for the extraction of bioactive compounds and would reduce costs, but they will not be considered eutectic mixtures. NADES polarity also affects their extraction capacity, the tunability of NADES polarity helps to selectively extract target compounds. Statistical analysis ANOVA was performed to know if there are statistical differences. After the statistical analysis, one NADES of each group was selected focusing on the extraction yield of total bioactive compounds, total polyphenol content, total flavonoid content, and ascorbic acid, ChChl:Gly 75% (v/v) for the sugar-based ($p < 0.05$) and ChChl:MA 75% (v/v) for the acid-based NADES ($p < 0.05$). The extraction yields of these NADES are shown in Figure 3, even though, ChChl:Gly and ChChl:MA are not the best NADES for the extraction of TPC, they are the best for the total bioactive compounds.

1.2. Phenolic compounds and ascorbic acid by HPLC-UV

Based on the results of the screening, the selected NADES were then analyzed chromatographically. The polyphenolic profile changes depending on the NADES used for extraction. In this case, ChChl:MA and ChChl:Gly extracts have different concentrations of each polyphenol. The polarity of each NADES can favor the extraction of bioactive compounds, for example, ChChl:Gly with a polarity of 49.00 ± 0.17 kcal/mol (Gómez-Urios et al., 2022) is more apolar than ChChl:MA (Enr: 47.85 ± 0.05 kcal/mol), NADES based on organic acids being the most polar, due to their ability to form hydrogen bonds (Fuad et al., 2021), this physicochemical property affects the extraction of hydrosoluble bioactive

compounds and their solubilizing capabilities (Huang et al., 2017). NADES composed of organic acids are more polar even than water (Enr: 51.98 kcal/mol) (Farooq, Abbasi & Anderson, 2020), this allows the hydrogen bond formation with phenolic compounds which increases their dissolving capacities (Da Silva et al., 2020; Grillo et al., 2020). In Figure 4, the HPLC-UV chromatograms, the differences between the polyphenolic profile in NADES and EtOH as a control are shown. Both NADES extracted more of 4-hydroxybenzoic, vanillic acid, chlorogenic acid, ferulic acid, catechin, p-coumaric acid, and caffeic acid than EtOH, this could be explained by the EtOH polarity (Enr: 50.83 kcal/mol), so NADES would have more affinity to those compounds than EtOH.

The most abundant phenolic compound in orange peel is hesperidin. In a study made by Irkin et al. (2015) they demonstrated a connection between orange peel's high hesperidin concentration and its strong AC. ChChI is commonly employed in NADES as a non-toxic quaternary ammonium salt (Doldolova et al., 2021; Panić et al., 2021), it has an ideal behavior that allows for effectively reducing the melting temperature of the eutectic mixture due to its low enthalpy of fusion (1.03 Kcal /mol) and the formation of strong specific molecular interactions (Martins, Pinho, & Couthino, 2018).



A: ChChl: Choline chloride, Gly :glycerol, MA: malic acid. C: EtOH, Ethanol 50%.
 1: 4-hydroxybenzoic acid. 2: Catechin. 3: Caffeic acid. 4: Vanillic acid. 5: Chlorogenic acid. 6: Ferulic acid. 7: p-Coumaric acid. 8: Rutin. 9: Naringin. 10: Hesperidin. 11: Quercetin. 12: Naringenin. 13: Hesperetin. 14: Apigenin

Figure 4. Phenolic compounds chromatograms

Regarding AA, this vitamin plays an important role in human health, and it is a bioactive compound present in high concentrations in orange peel (Anticono et al., 2020). Our results show that both ChChl:Gly (4.57 mg/100 ml) and ChChl:MA (4.69 mg/100 ml) provided a higher yield extraction than 50% (v/v) EtOH (2.92 mg/100 ml). In this case, both NADES had similar extraction yields, so both could be good solvents for the extraction of AA, even though, the result is lower than in other studies (Montero-Calderon et al., 2019), this could be due to the extraction method.

1.3. Antioxidant capacity

Antioxidants are especially abundant components in foods of plant origin, being or not nutrients, that present in small amounts in the food, and can slow or prevent the oxidation of other molecules (Shahidi & Zhong, 2018). Due to the different mechanisms of action and the nature of antioxidant and oxidizing substances, there is no single method of determining AC that reflects the effect of all antioxidants present in a complex mixture of bioactive substances, so a proper selection of methods is required for the determination of total AC, based on the reaction characteristics of the compounds. The AC of fruit by-products extracts has been determined before by different methods (Reis et al., 2020; Trych et al., 2020; Jurić et al., 2021; Martínez-Linda et al., 2023; Viñas-Ospino et al., 2023), according to Viñas-Ospino et al, (2023), ChChl:Gly was the choline chloride based-NADES that presented higher ORAC values. In this case, the AC of ChChl:Gly and ChChl:MA is shown in Table 2.

Table 2. Antioxidant capacity of NADES using different antioxidant methods

NADES	TEAC ($\mu\text{M TE}$)	DPPH ($\mu\text{M TE}$)	ORAC (mM TE)	FRAP ($\mu\text{M TE}$)
ChChl:Gly	51.8 ± 3.6^a	10.7 ± 0.0^a	2.2 ± 1.1^a	24.0 ± 1.9^a
ChChl:MA	105.0 ± 8.0^b	424.4 ± 13.4^b	25.7 ± 2.8^b	33.7 ± 1.8^a

ChChl: Choline chloride, Gly :glycerol, MA: malic acid. TEAC: Trolox equivalent antioxidant capacity, DPPH: 2,2-diphenyl-1-picrylhydrazyl, ORAC: oxygen radical antioxidant capacity, FRAP: Ferric reducing-antioxidant power. (a-b): Different letters indicate significantly different values on the same column ($p < 0.05$); the same letter means no difference.

The results show that ChChl:Gly has a lower AC than ChChl:MA in the four methods studied. Moreover, has more AC by the TEAC method ($51.8 \pm 3.6 \mu\text{M}$ Trolox), this could be due to its mechanism. The AC obtained by the TEAC method is predictable by the structure of the antioxidant component. Monophenol compounds react quickly and in the presence of polyhydroxylated rings or adducts of aromatic rings, the reaction is initiated and takes place slowly (Schaich et al., 2015). And for ChChl:MA, the results obtained by DPPH method were the best. DPPH method is based on a non-competitive anti-radical reaction where the loss of coloration may be due to HAT (hydrogen atom transference) and SET (single electron transference) or causes unrelated to the above mechanisms. The DPPH radical is hydrophobic, and soluble in organic solvents such as methanol, unlike the ABTS radical and peroxide radicals (Prior et al., 2005). AC stability was measured in the extract and compared with the AC in NADES components (Table 3), and EtOH (50% v/v) was measured in parallel as a control. Even in the study of the AC of the NADES, as far as the extracts AC is concerned, better results were observed using ChChl:Gly than ChChl:MA in the FRAP method.

1.4 Stability of bioactive compounds

The stability of polyphenols is a topic of interest in the food industry and a way to take advantage of resources. Different studies have been carried out in which the ability to preserve NADES extracts to different storage conditions has been tested. Jeong et al (2017) showed that the polyphenolic compounds are stable in NADES-based in sugars, polyalcohol, betaine, and lactic acid. The results of polyphenol stability are shown in Figure 5, where 4-hydroxybenzoic acid was the least detected, presenting a concentration of $2.07 \pm 0.44 \mu\text{g}/100 \text{ mL}$ in ChChl:MA and ChChl:Gly of $13.13 \pm 0.21 \mu\text{g}/100 \text{ mL}$ and not detectable in EtOH (Annex 3 Table S2), and it was not detected after storage. Polyphenolic compounds presented great stability in NADES based on sugars, glycerol, betaine, and lactic acid, possibly due to the formation of hydrogen bonds with the solute (Jeong et al., 2017). In turn, Zannou & Koca (2020) evaluated the thermal stability of extracts from *Alkanna tinctoria* rich in flavonoids, whose antioxidant property values are maintained at 40°C , but greater degradation is observed between $60 - 80^\circ\text{C}$ and 120 minutes of heating, and the degradation ratios obtained were lower than those previously observed using water, ethanol, or hexane:acetone mixtures. Hesperidin is the most abundant flavonoid present in orange peel, in this case, in both NADES, hesperidin was not stable, for ChChl:Gly, with an initial concentration of $326.11 \pm 11.87 \text{ mg}/100 \text{ mL}$ which was reduced to a third on the twelfth day ($101.30 \pm 0.21 \text{ mg}/100 \text{ mL}$). For ChChl:MA, hesperidin had an initial concentration of $299.96 \pm 10.32 \text{ mg}/100 \text{ mL}$ at the beginning, which decreased to $54.16 \pm 3.39 \text{ mg}/100 \text{ mL}$, this data indicates that ChChl:Gly would be a better choice in terms of hesperidin. Moreover, hesperidin was the only polyphenol and flavonoid that were detectable during the 12 days of storage in addition to chlorogenic acid, ferulic acid, naringin, and naringenin.

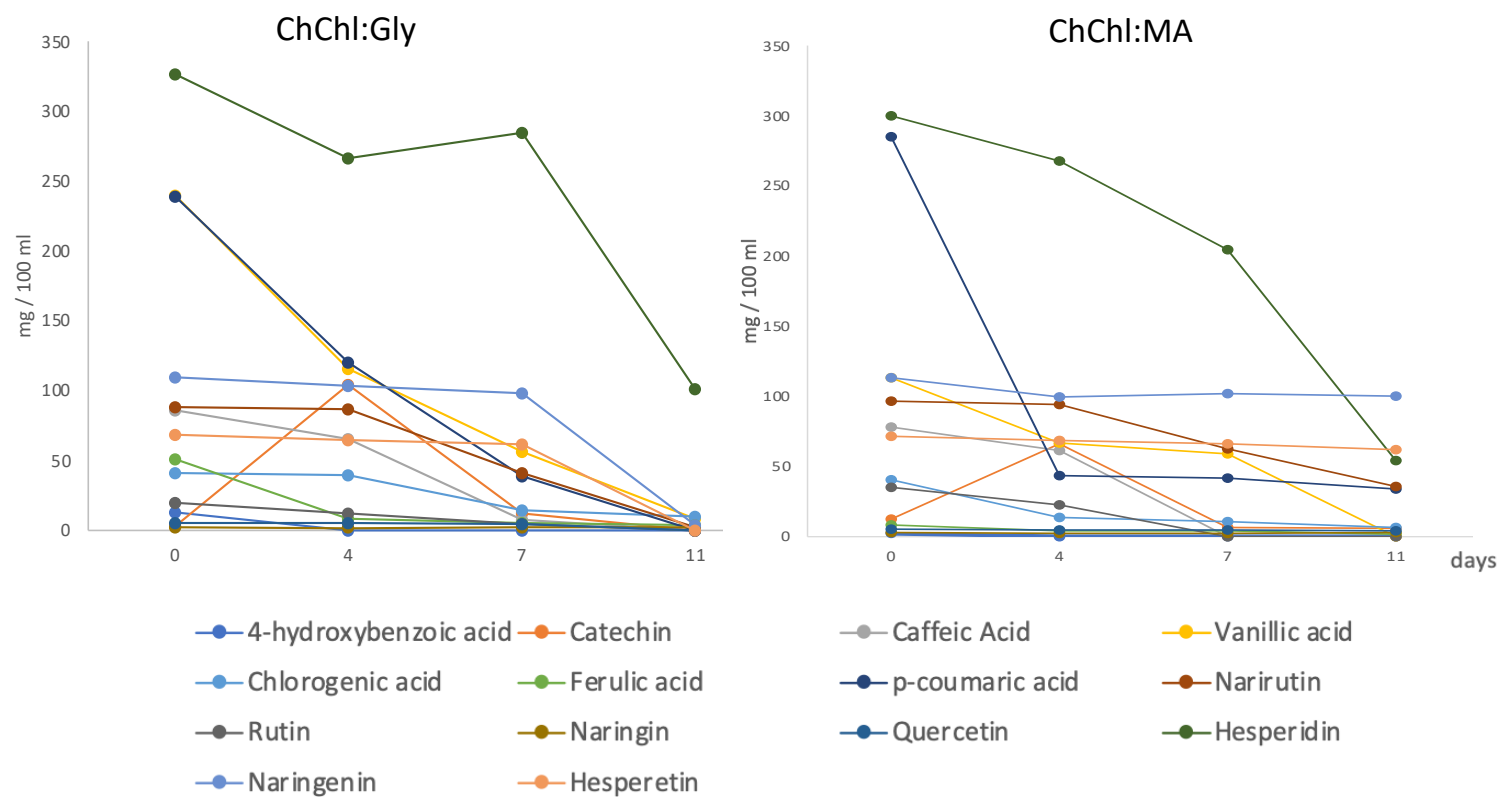
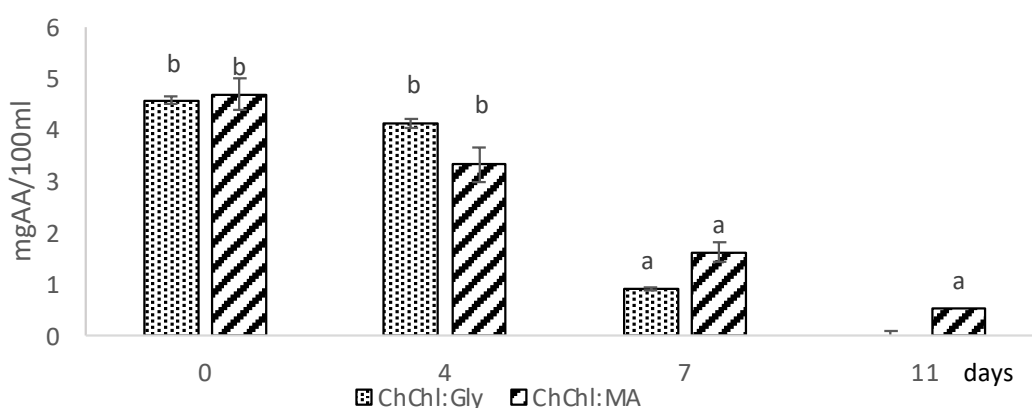


Figure 5. Stability of polyphenols in choline chloride: malic acid (ChChI:MA) and choline chloride: glycerol (ChChI:Gly).

AA is an essential component of some foods, since, in addition to being a vitamin, it also has an anti- and pro-oxidant character (Van Boekel & Roux, 2022), and is used in the food industry as a parameter of quality and stability as it is a photosensitive and thermolabile molecules (Gómez-Ruiz et al., 2018). The degradation of AA, in this case, resulted in a degradation without the presence of oxygen (anaerobic reaction), so in this case, AA degrades without being oxidized, and dehydroascorbic acid is not formed (Giannakourou & Taoukis, 2021). The stability of AA in NADES extracts is shown in Figure 6, extracts with ChChl:MA (89.2%) and ChChl:Gly (100%) show more degradation after twelve days of storage, even though, there is no statistical difference between the NADES studied ($p > 0.05$).



a, b: Different letters indicate statistical differences ($p < 0.05$).

Figure 6. Ascorbic acid (AA) stability during eleven days in choline chloride: glycerol (ChChl:Gly), and choline chloride: malic acid (ChChl:MA).

The pH is a parameter with great influence on the stability of the AA, the NADES used for extraction have pH of 5.10 and 1.06 for ChChl:Gly and ChChl:MA

respectively. Jeney-Nagymate & Fodor (2008) analyzed the degradation of AA in different beverages, resulting in the fact that after storage the degradation was lower in the case of pH 1.99, stating that with pH > 4 the concentration of AA will decrease. In this case, the AA extracted with ChChl:MA was more stable than ChChl:Gly extracts, but the extraction yield of AA on day zero was similar between the two NADES.

Regarding the stability of the AC, it can be observed (Table 3) that the FRAP method presents a linear degradation in all cases, as well as the ORAC method, presenting point 0 for ChChl:MA ($11376 \pm 902 \mu\text{M TE}$) greater amounts than extracts with EtOH ($9559 \pm 967 \mu\text{M TE}$). FRAP method acts as transferring electrons. The reaction of the antioxidant compound against the ferric complex appears to be related to the degree of hydroxylation and conjugation of the polyphenolic compounds. There is a study made with peppermint leaves, that shows a clear correlation between flavonoid content and FRAP antioxidant method (Jurić et al., 2021).

Our results are in close agreement with those reported in the literature. To the best of our knowledge, no previous results for the AC characterization by three different methods for orange peel extracts using NADES have been reported in the literature, as well as bioactive compounds stability in the studied NADES.

Table 3. Antioxidant capacity of extracts stored at 25 °C for 12 days.

	Day	FRAP ($\mu\text{M TE}$)	ORAC (mM TE)	DPPH ($\mu\text{M TE}$)	TEAC ($\mu\text{M TE}$)
ChChl:MA	0	474 $\pm$ 0.01 ^d	11.4 $\pm$ 0.90 ^b	1065 $\pm$ 109 ^b	978 $\pm$ 156 ^c
	4	422 $\pm$ 0.01 ^c	9.13 $\pm$ 0.73 ^a	261 $\pm$ 19.1 ^a	13.70 $\pm$ 2.8 ^b
	7	320 $\pm$ 0.01 ^b	8.08 $\pm$ 0.89 ^a	260 $\pm$ 15.6 ^a	9.55 $\pm$ 2.1 ^a
	11	276 $\pm$ 0.01 ^a	6.81 $\pm$ 0.86 ^a	222 $\pm$ 10.6 ^a	ND
ChChl:Gly	0	664 $\pm$ 0.00 ^c	7.60 $\pm$ 0.97 ^b	909 $\pm$ 21.3 ^b	439 $\pm$ 42.20 ^c
	4	530 $\pm$ 0.02 ^b	5.01 $\pm$ 0.98 ^{ab}	33.1 $\pm$ 15.9 ^a	22.6 $\pm$ 4.40 ^b
	7	430 $\pm$ 0.02 ^a	3.90 $\pm$ 1.00 ^a	ND	11.0 $\pm$ 2.21 ^a
	11	372 $\pm$ 0.01 ^a	2.58 $\pm$ 0.68 ^a	ND	ND
EtOH 50%	0	981 $\pm$ 0.02 ^d	9.56 $\pm$ 0.96 ^c	1037 $\pm$ 137 ^b	718 $\pm$ 175 ^c
	4	805 $\pm$ 0.01 ^c	8.46 $\pm$ 0.96 ^c	118 $\pm$ 57.6 ^a	50.10 $\pm$ 6.40 ^b
	7	560 $\pm$ 0.01 ^b	6.29 $\pm$ 0.88 ^b	106 $\pm$ 36.1 ^a	17.10 $\pm$ 1.96 ^a
	11	507 $\pm$ 0.01 ^a	3.05 $\pm$ 0.44 ^a	ND	ND

ChChl: Choline chloride, Gly :glycerol, MA: malic acid. ND: not detected. TEAC: Trolox equivalent antioxidant capacity, DPPH: 2,2-diphenyl-1-picrylhydrazyl, ORAC: oxygen radical antioxidant capacity, FRAP: ferric reducing-antioxidant power). (a-b): different letters indicate significantly different values ($p < 0.05$); the same letter means no difference.

In summary, the screening technique, UV/VIS spectrophotometry, was efficient and obtained the most sustainable score in AGREE method, allowing us to rationalize the use of organic solvents in HPLC-UV. Both studied NADES extraction yields were higher than ethanol 50% in the case of AA; and among phenolic compounds: 4-hydroxybenzoic acid, vanillic acid, chlorogenic acid, ferulic acid, catechin, p-coumaric acid, and caffeic acid. These results suggest that NADES could be used in the food industry as an alternative to organic solvents in the extraction process. The studied NADES presented AC, ChChl:MA showed the highest values accordingly to the four antioxidant methods assayed.

According to the results, both NADES provided extended protection for the bioactive compounds in the orange peel extracts.

2. Conclusion

In the present study extracts rich in the bioactive compound were obtained according to the principles of green chemistry, using alternative solvents to organics, and employing by-products resources. The best extraction yields of bioactive compounds were achieved by ChChl:MA and ChChl:Gly with low water content (75% of NADES) considering them as eutectic mixtures. These NADES extracts demonstrated to have more AC than ethanol extracts, whose values could differ depending on the method used. Also, the results showed that the AC can enhance the stability of the extracts during storage for twelve days. Additionally, phenolic compounds and ascorbic acid were more stable in ChChl:MA than ChChl:Gly, moreover, hesperidin (majoritarian polyphenol in the orange peel) presented more extraction yield and was more stable in ChChl:Gly extracts. These findings highlight the potential for using NADES extracts from orange peels to enrich food formulations that could be used for human consumption due to the low toxicity of their components. This indicates that extracts with NADES are a good alternative to conventional solvents, both for extraction and for their protective role in the stability of phenolic compounds and ascorbic acid. Although, there is a lack of information about the protective role of these solvents on the bioactive compounds and future works should focus on this topic.

These results have resulted in a publication:

Gómez-Urios, C., Viñas-Ospino, A., Puchades-Colera, P., Blesa, J., López-Malo, D., Frígola, A., & Esteve, M. J. (2023). Choline chloride-based natural deep eutectic solvents for the extraction and stability of phenolic compounds, ascorbic acid, and antioxidant capacity from *Citrus sinensis* peel. *LWT*, 177, 114595. <https://doi.org/10.1016/j.lwt.2023.114595>

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CHAPTER IV

Unlocking Value from Waste: A Comparative Study of Orange Peel Extraction Methods Using Choline Chloride-Based Natural Deep Eutectic Solvents



Green chemistry is a fundamental aspect of the food industry in the extraction of bioactive compounds from plant byproducts, through the utilization of sustainable methodologies, such as ultrasound-assisted extraction, enhancing food safety. Conventional solvents are being replaced by green alternatives, including natural deep eutectic solvents, which are nontoxic and biodegradable. The objective of this study is to compare UAE with heating-stirring methods for the extraction of bioactive compounds from orange peels using NADES. The antioxidant capacity of the extracts will be compared by three methods [oxygen radical absorbance capacity (ORAC), DPPH, and ferric ion reducing antioxidant power (FRAP)], also the phenolic profile will be studied, and the stability of the extracted compounds will also be assessed.

1. Results and discussion

The extraction was analyzed with four different NADES (Table 1) to compare the two extraction methods and find the most suitable one. The UAE is a flexible technique that can be used to extract biofunctional chemicals as well as to preserve food, maintaining the organoleptic properties of the product (Guntero et al., 2018). This method aims to speed up the processing while using less energy than conventional methods (Anticono et al., 2021). In contrast, HS allows bioactive compounds to diffuse into the solvent.

Table 1. Composition of studied Natural Deep Eutectic Solvents

Acronym	Hydrogen bond acceptor	Hydrogen bond donor	Molar Ratio
ChChl:Fruc	Choline chloride	Fructose	1.9:1
ChChl:LP:MA		Malic acid: L-Proline	1:1:1
ChChl:LA		Lactic acid	1:3
ChChl:Gly		Glycerol	1:2

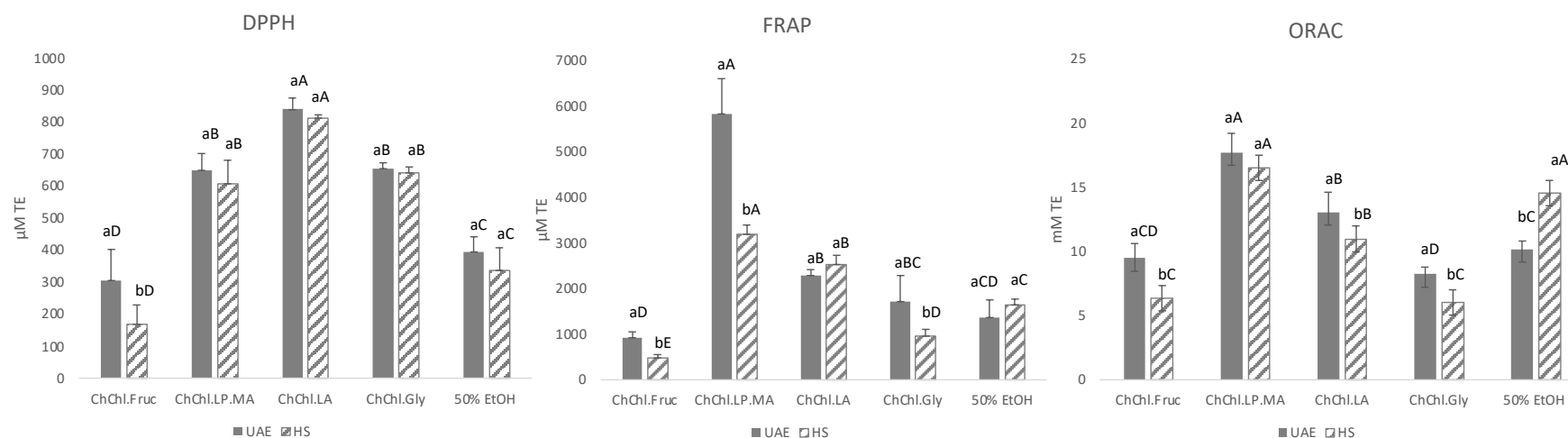
1.1. Antioxidant capacity

In order to compare the extraction methods, and to find the most suitable one, three antioxidant capacity methods were employed (Figure 1, extraction yield is shown in Table S1). The DPPH results show that the four NADES extracts from the UAE are more active than those obtained from HS. Specifically, for ChChl:Fru, the UAE extraction ($305.9 \pm 116.57 \mu\text{M TE}$) double the extraction from HS ($168.7 \pm 60.0 \mu\text{M TE}$). This difference may be attributed to the UAE's ability to break down cell walls, facilitating the transfer of bioactive compounds to the solvents (Anticono et al., 2021). ChChl:LP:MA, ChChl:Gly, and ChChl:LA showed better results than EtOH 50% in both extraction methods, showing their extraction capacity.

The ChChl:LA produced the highest DPPH value, with $812.8 \pm 10.0 \mu\text{M TE}$ for HS and $840.5 \pm 34.5 \mu\text{M TE}$ for UAE. In contrast, ChChl:Gly, though noted to have the lowest antioxidant capacity according to Gómez-Urios et al. (2023), ranked as the second most effective extract for antioxidant capacity after HS, with no statistical differences from ChChl:LP:MA.

The DPPH method generated the lowest value for ChChl:Fru in both extraction methods, showing this sample worse results than EtOH 50%. These findings differ from those of Mir-Cerdà et al. (2023) who obtained better DPPH results using the HS method with ChChl:Gly and ChChl:LA, possibly due to the distinct UAE parameters.

In the FRAP analysis, significant changes in values were observed among NADES employed by HS. ChChl:Fru ($483.0 \pm 63.0 \mu\text{M TE}$) and ChChl:LP:MA ($3194 \pm 193 \mu\text{M TE}$) were identified as the lower and higher values, respectively, for reducing the potential of the ferric complex present.



ChChI: choline chloride. DPPH: 2,2-diphenyl-1-picrylhydrazyl. EtOH: ethanol. FRAP: Ferric ion reducing antioxidant power. Fruc: fructose. Gly: glycerol. HS: heating-stirring extraction. LA: lactic acid. LP: proline. MA: malic acid. ORAC: Oxygen radical absorbance capacity. UAE: ultrasound-assisted extraction. Different letters (a-b) indicate statistical differences between the different extraction methods for the same NADES. Different letters (A-B) indicate statistical differences between NADES for the same method

Figure 1. Antioxidant capacity in three different methods

The differences in the values obtained from the DPPH and FRAP methods suggest that the compounds extracted from the orange peel have a greater ability to reduce Fe^{3+} .

Jurić et al (2021) found a correlation between the FRAP method's evaluation of antioxidant capacity and the extracted content of flavonoids, their glycosylated forms, and phenolic compounds. FRAP results for ChChl:LP:MA displayed significant differences in comparison to both methods, while ChChl:Fruc showed the worst results for FRAP, as well as with DPPH method.

After extraction by HS using the ORAC method, it was observed that there were no statistically significant differences in antioxidant capacity between ChChl:Fru and ChChl:Gly, both NADES showed worse results than the control. However, ChChl:LP:MA was found to be the NADES with the highest antioxidant capacity, followed by ChChl:LA. Recent studies have reported that ChChl:Gly exhibited the highest antioxidant capacity among NADES (Viñas-Ospino et al., 2023). However, our present study contradicts these findings, which could be explained by a study conducted by Gómez-Urios et al. (2023). In their study, the antioxidant capacity of NADES was measured and ChChl:MA (25.7 ± 2.8 mM TE) was found to have a higher ORAC method value than ChChl:Gly (2.2 ± 1.1 mM TE).

1.2. Phenolic compounds

Ultrasonic-assisted extraction (UAE) has been found to disrupt plant cell walls, facilitating the release of bioactive compounds into solvents without requiring sample heating, thus preventing degradation (Anticono et al., 2021). Compared to heat-assisted extraction (HS), which relies on diffusion, UAE is expected to extract a greater number of bioactive compounds. Using UAE technique, and ChChl:Fruc, the extraction yield of TPC was the highest (6.0 ± 0.14 mg GAE/100

mL). However, as Table 2 shows, ChChl:LA extracted over double of phenolic compounds when compared to UAE (UAE: 5.7 ± 0.5 mg/100 mL; HS: 2.34 ± 0.20 mg/100 mL). ChChl:LP:MA was the NADES that extracted the lowest amount using both methods.

Table 2. Total polyphenol content extracted with four different NADES and the two different extraction techniques

NADES	TPC (mg GAE/100 mL)	
	Heating-Stirring	Ultrasound-assisted
ChChl:Fruc	5.3 ± 0.2^{bB}	6.1 ± 0.1^{aB}
ChChl:LP:MA	0.6 ± 0.1^{bD}	1.4 ± 0.1^{abD}
ChChl:LA	2.3 ± 0.2^{bC}	5.7 ± 0.1^{aB}
ChChl:Gly	3.5 ± 0.2^{aC}	3.6 ± 0.1^{aC}
50% EtOH	7.6 ± 0.2^{aA}	8.1 ± 0.1^{aA}

ChChl: choline chloride. EtOH: ethanol. Fruc: fructose. GAE: Gallic acid equivalent. Gly: glycerol. LA: lactic acid. LP: proline. MA: malic acid. NADES: Natural Deep Eutectic Solvent. TPC: total polyphenol content. (a-b) Different letters indicate statistical differences between methods. (A-C) Different letters indicate statistical differences between different NADES for the same method.

For each phenolic compound identified through HPLC-UV analysis, Figure 2 illustrates the variances between the UAE and HS methods, while Table S2 (Annex 4) displays the corresponding concentrations, and statistical analysis determined the more influential extraction method for each compound.

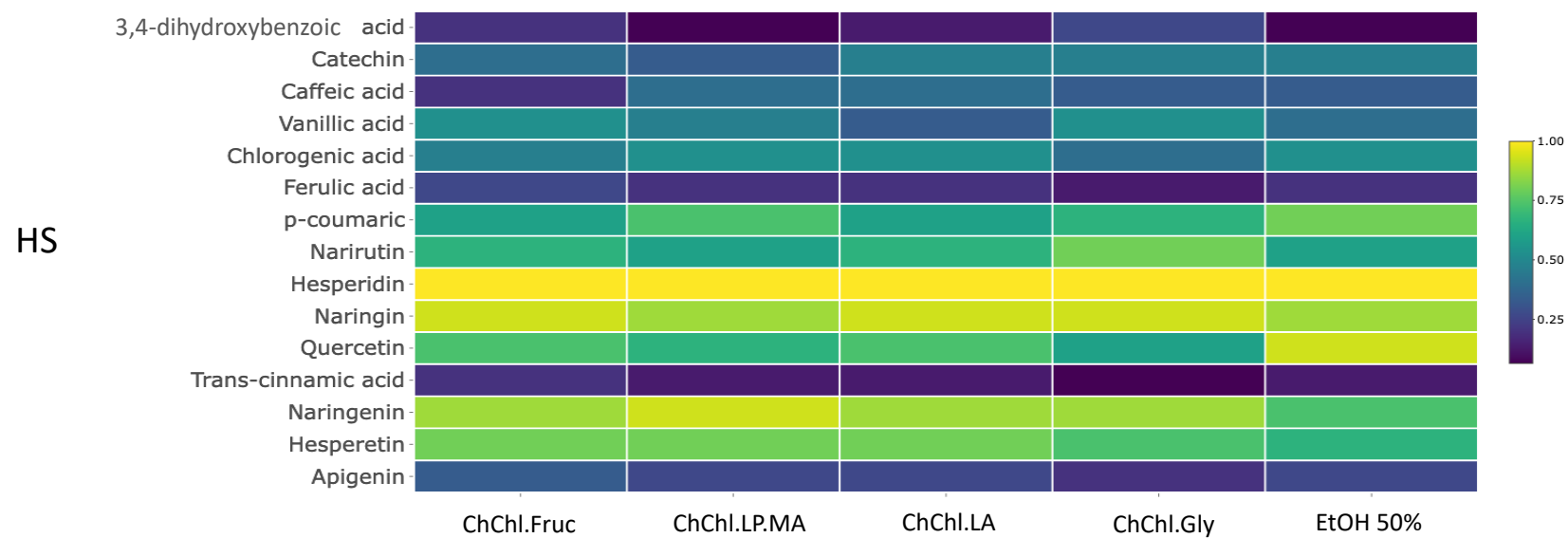
3,4-dihydroxybenzoic acid was not detectable in ChChl:LP:MA and ChChl:LA with 50% EtOH for the UAE. For HS, ChChl:Gly was the only solvent that extracted this phenolic acid. This could be attributed to the viscosity of the NADES, which interferes with the extraction capacity at higher viscosity levels

and permits the diffusion of bioactive compounds from the matrix at lower viscosity levels (Yang et al., 2021).

ChChl:Fruc gives an extraction of caffeic acid ($4.38 \pm 0.05 \mu\text{g/mL}$) via UAE, while the HS method provides results below the detection limit, and the other NADES show no statistical differences, indicating that the results are similar between them, different from the results obtained by Ünlü (2021), where ChChl:Fruc did not extract caffeic acid from olive leaves. Airouyuwa et al. (2022) conducted a study on the extraction of vanillic acid and found that ChChl:Gly performed better than UAE with a yield of $18.05 \pm 1.52 \mu\text{g/mL}$ compared to $8.90 \pm 0.25 \mu\text{g/mL}$. Additionally, ChChl:LA was identified as the most effective NADES for the extraction of vanillic acid, outperforming NADES containing sugars and polyalcohol. Moreover, ChChl:LA was the solvents that presented the lowest extraction yield for UAE method. Extraction yields for chlorogenic acid, ferulic acid, and apigenin were similar for both extraction methods. In terms of hesperidin, the major phenolic compound found in orange peel, UAE had better results for ChChl:Fruc ($493.05 \pm 42.49 \mu\text{g/mL}$) compared to HS ($355.55 \pm 4.97 \mu\text{g/mL}$), ChChl:LP:MA (UAE: $364.21 \pm 30.69 \mu\text{g/mL}$, HS: $267.75 \pm 29.24 \mu\text{g/mL}$), and ChChl:LA (UAE: $305.01 \pm 5.70 \mu\text{g/mL}$, HS: $196.69 \pm 2.50 \mu\text{g/mL}$) had also better results for UAE. However, ChChl:Gly exhibited better results with UAE showing $492.65 \pm 2.49 \mu\text{g/mL}$ and HS showing $567.45 \pm 16.09 \mu\text{g/mL}$. Additionally, EtOH 50% showed improved results with UAE demonstrating $841.31 \pm 15.83 \mu\text{g/mL}$ and HS showing $974.31 \pm 31.67 \mu\text{g/mL}$. In UAE, testing showed naringin ($539.52 \pm 18.08 \mu\text{g/mL}$), vanillic acid ($11.08 \pm 0.84 \mu\text{g/mL}$), and quercetin ($83.26 \pm 4.89 \mu\text{g/mL}$). The extraction yield using ChChl:Fruc is at least 40% higher than that of EtOH 50%, with values of $343.19 \pm 29.73 \mu\text{g/mL}$, $5.23 \pm 0.16 \mu\text{g/mL}$, and $45.56 \pm 0.11 \mu\text{g/mL}$, respectively. This difference can be

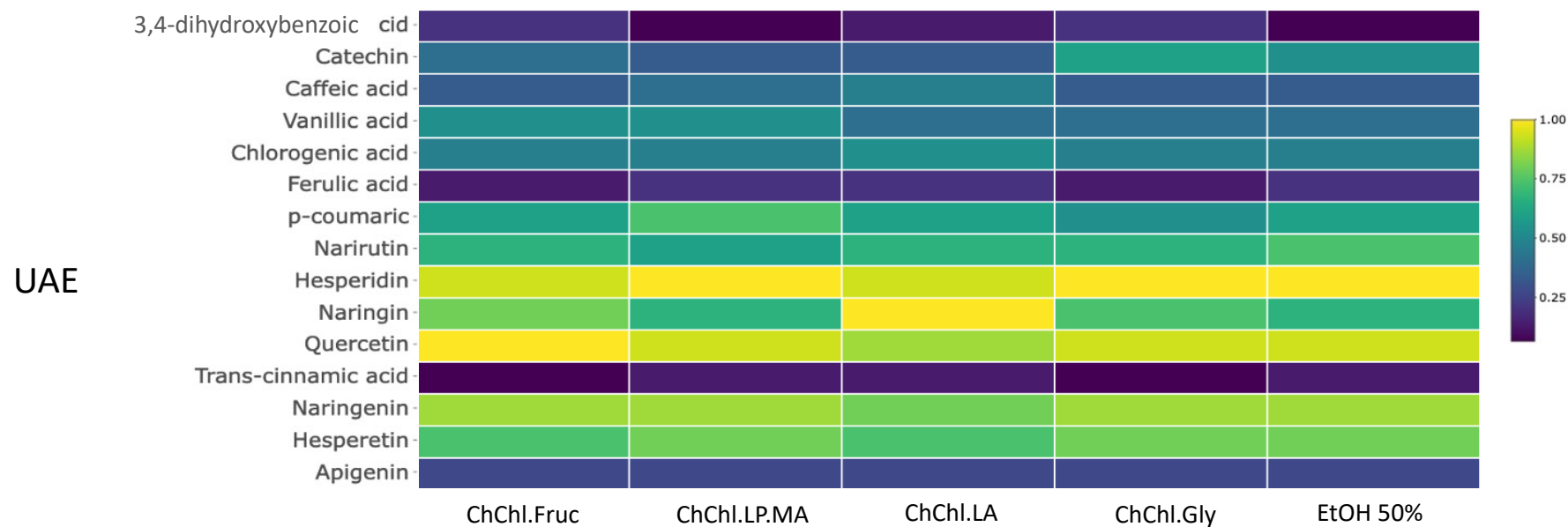
attributed to the polarity of the NADES, which permits the extraction of either compound (Panić et al., 2021).

Airouyuwa et al (2022) conducted an analysis of various NADES for the extraction of TPC and found that ChChl:LA was the most effective NADES for TPC and caffeic acid. This study yielded similar results.



ChChl: choline chloride. EtOH: ethanol. Fruc: fructose. GAE: Gallic Acid Equivalent. Gly: glycerol. LA: lactic acid. LP: proline. MA: malic acid. HS: Heating-stirring extraction method

Figure 2. Heatmap of different extraction techniques



ChChl: choline chloride. EtOH: ethanol. Fruc: fructose. GAE: Gallic Acid Equivalent. Gly: glycerol. LA: lactic acid. LP: proline. MA: malic acid. UAE: ultrasound-assisted extraction method.

Figure 2. Heatmap of different extraction techniques

1.3. Stability and degradation rate of extracts

The stability of extracted compounds is a critical factor when selecting a method. Stability evaluation includes assessing antioxidant capacity and phenolic compound integrity after storage. The antioxidant capacity of NADES is a physical property of great importance, as it improves the stability of bioactive compounds (Panić et al., 2019).

The stability of HS and UAE antioxidant capacity over three days is shown in Table 3, the extraction yield is shown in Table S3. The extracts obtained through HS proved to be more stable compared to those extracted through UAE, except for ChChl:Fruc. On day 0, the HS yielded the lowest value in ChChl:Fruc ($168.7 \pm 60.0 \mu\text{M TE}$), which was approximately ten times higher than the blank's value ($13.6 \pm 0.0 \mu\text{M TE}$).

After three days of storage at 25 °C for HS, the DPPH values decreased more in ChChl:Gly than in ChChl:Fruc. ChChl:LA had the highest antioxidant capacity in this case ($291.5 \pm 24.6 \mu\text{M TE}$), representing 35.8% of the initial antioxidant capacity value. However, the process control, which is the 50% EtOH (v/v) extract, showed a decrease to 53.7% of its initial value. This rapid decline in the antioxidant capacity during storage can be attributed to the ascorbic acid present in the extract (Anticono, et al., 2022). After three days of storage, the antioxidant capacity of ChChl:LA decreased to 30.7% of its initial value. Moreover, the extract with 50% EtOH (v/v) retained only 20.3% of its initial antioxidant capacity.

Table 3. Degradation percentage of antioxidant capacity in 3 study days, on different NADES and two extraction techniques.

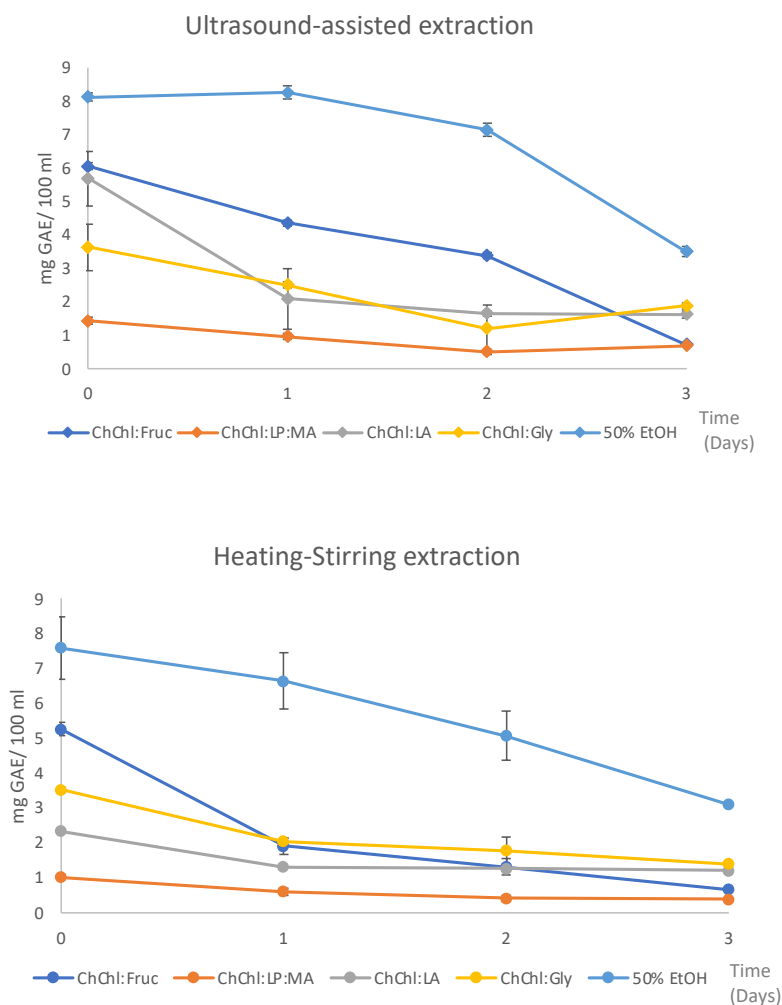
		ChChl:Fruc	ChChl:LP:MA	ChChl:LA	ChChl:Gly	EtOH
DPPH (μ M TE)	HS	71.6 $\pm$ 1.7 ^b	69.0 $\pm$ 2.3 ^{bc}	64.2 $\pm$ 3.8 ^c	89.8 $\pm$ 2.9 ^a	46.3 $\pm$ 3.6 ^d
	UAE	65.7 $\pm$ 1.3 ^d	73.4 $\pm$ 2.0 ^{bc}	69.3 $\pm$ 2.5 ^{cd}	93.4 $\pm$ 2.1 ^a	79.7 $\pm$ 1.3 ^b
FRAP (μ M TE)	HS	61.7 $\pm$ 3.6 ^c	96.9 $\pm$ 4.5 ^a	86.2 $\pm$ 2.8 ^b	89.1 $\pm$ 2.4 ^{ab}	93.5 $\pm$ 1.9 ^a
	UAE	73.2 $\pm$ 4.1 ^b	99.0 $\pm$ 3.9 ^a	90.2 $\pm$ 3.6 ^a	91.4 $\pm$ 4.2 ^a	99.2 $\pm$ 5.1 ^a
ORAC (mM TE)	HS	66.7 $\pm$ 3.1 ^b	53.8 $\pm$ 0.9 ^d	75.5 $\pm$ 2.1 ^b	N.D.	83.6 $\pm$ 1.8 ^a
	UAE	71.6 $\pm$ 3.1 ^b	57.2 $\pm$ 1.4 ^d	66.9 $\pm$ 4.1 ^{bc}	80.5 $\pm$ 0.8 ^a	64.4 ^c

ChChl: choline chloride. DPPH: 2,2-diphenyl-1-picrylhydrazyl. EtOH: ethanol. FRAP: ferric ion-reducing antioxidant power. Fruc: fructose. Gly: glycerol. HS: heating-stirring extraction. LA: lactic acid. LP: proline. MA: malic acid. ORAC: Oxygen radical absorbance capacity. UAE: ultrasound-assisted extraction. N.D. Not detectable. Different letters (a-e) indicate statistical differences on the same row.

Regarding the FRAP method with HS, the antioxidant capacity exhibited a decrease of less than 10% of the initial value. However, the ChChl:Fruc extract (184.6 $\pm$ 33.8 μ M TE) stood out and retained 38.3% of the original value. This result can be explained by the interaction and protection against oxidizing agents that may be favored by the analogous polarities of sugars and phenols (Gómez-Urios et al. 2022).

Using the ORAC assay and HS, the antioxidant capacity of the extract containing ChChl:Gly was not detected after three days, while ChChl:LP:MA (7.4 $\pm$ 1.8 mM TE) exhibited the maximum antioxidant capacity which represents 46.2% of the initial value. The reduced antioxidant capacity observed during UAE may be attributed to the rupturing of cell vacuoles caused by ultrasound treatment, which results in the release of polyphenol oxidase.

TPC stability was monitored using the two extraction techniques proposed over three days. The results are presented in Figure 3. For ChChl:Fruc both extraction techniques showed a degradation of 12% and 13%, respectively on day 3. Similarly, for 50% EtOH, both methods yielded similar values with degradation of 43% and 41% for UAE and HS, respectively, on day 3.



ChChl: choline chloride. GAE: Gallic Acid Equivalent. LP: proline. MA: malic acid. Fruc: fructose. EtOH: ethanol. LA: lactic acid. Gly: glycerol.

Figure 3. Total stability of polyphenols over a 3-day period, using two different extraction methods.

The TPC in ChChl:LP:MA and ChChl:Gly was found to be more stable using the UAE method rather than the HS method. The polyphenols in the ChChl:LA extract with the HS method were the most stable, retaining 52% of the initial value. The increased stability of ChChl:LA and ChChl:Gly in comparison to 50% EtOH can be attributed to the interaction between phenolic compounds and components of NADES, which hinder the movement of solute molecules and delay oxidative decay (Rebocho et al. 2022).

The study aimed to identify the optimal approach for extracting phenolic compounds from orange peel, by comparing the UAE and HS methods. Different solvents based on choline chloride-based NADES were utilized while employing 50% EtOH as a control.

The selection of the extraction procedure, whether UAE or HS, is of importance in determining the efficacy of NADES. For example, UAE has been shown to yield higher concentrations of bioactive compounds due to its capacity to disrupt cell walls without the application of heat, which otherwise leads to compound degradation. However, HS frequently results in greater stability of these compounds over time, particularly in maintaining antioxidant capacity. The comparison demonstrates that while NADES are effective green solvents, their performance is not uniform across all methods. While UAE may be the optimal choice for maximizing immediate yields, HS may be the preferred method when long-term stability is of paramount importance. This highlights the necessity of meticulous selection of both the NADES and the extraction method to achieve an optimal equilibrium between efficiency and stability. By focusing on the comparative effectiveness of these extraction procedures, it is possible to better benefit the potential of NADES as green solvents, thereby optimizing their use for specific applications in a sustainable manner.

2. Conclusion

In conclusion, it is crucial to consider the phenolic compounds of interest, as well as the stability of the extract components and their antioxidant capacity when selecting the extraction technique for the final application. NADES have demonstrated of being considerable potential as environmentally friendly solvents in this context, offering a more sustainable alternative to traditional organic solvents. Their non-toxic and biodegradable nature, coupled with their capacity to enhance the extraction of bioactive compounds, renders them particularly valuable. However, while NADES can enhance the yield and stability of specific compounds, their efficacy is contingent upon the particular extraction methodology employed. In conclusion, NADES represent a significant advancement in green chemistry. However, their effectiveness may depend on the careful selection of the appropriate NADES and extraction technique for each specific application.

These results have resulted in a publication:

Gomez-Urios, C., Puchades-Colera, P., Frigola, A., Lopez-Malo, D., Esteve, M. J., & Blesa, J. (2024). *Unlocking Value from Waste: a Comparative Study of Orange Peel Extraction Methods Using Choline Chloride-Based Natural Deep Eutectic Solvents.* ACS Food Science & Technology. <https://doi.org/10.1021/ACSFOODSCITECH.4C00476>

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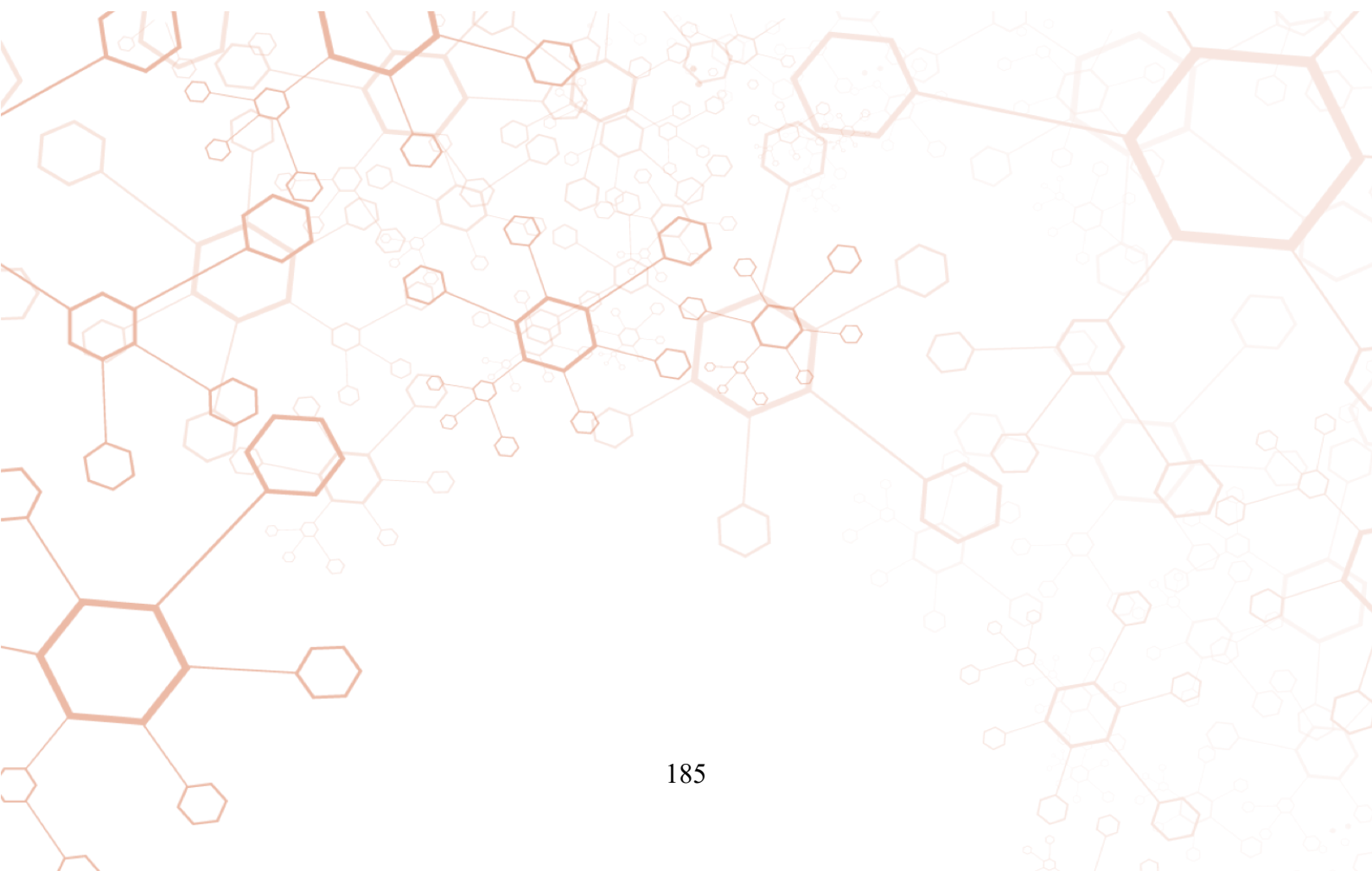
CHAPTER V:

Biological effect and food products application of choline chloride-based NADES extract.



PART I

Valorization of food byproducts: green solvents for bioactive extraction and biological applications of citrus sinensis peel.



Orange is one of the most consumed fruits worldwide, generating significant byproducts and wastes. The reuse of orange byproducts may be recovered sustainably by following the principles of green chemistry. In this context, natural deep eutectic solvents (NADES) have emerged as a promising alternative. The present study aims to analyze the physicochemical characteristics and biological effects of orange peel extracts obtained with choline chloride-based NADES. On Table 1 it is shown the NADES used on this chapter.

Table 1. Natural deep eutectic solvents and control used, and their characteristics

Acronym	Hydrogen bond acceptor	Hydrogen bond donor	Molar ratio	Water content	Apparience
ChChl:Fruc	Choline Chloride	Fructose	1.9:1	25% (w/w)	Transparent colorless liquid
ChChl:Xyl		Xylose	2:1		
ChChl:Gly		Glycerol	1:2		
ChChl:Gly:CA		Glycerol: citric acid	1:1:1		

1. Results and discussion

1.1 Polyphenols profile, content, and antioxidant activity

Orange peel is a rich source of phenolic compounds, including flavonoids, phenolic acids, tannins, and coumarins (Gómez-Urios et al., 2022). Table 2 summarizes the most abundant phenolics found in various extracts from orange peel. Phenolic compounds identified by LC/MS are grouped into two classes (two phenolic acids and three flavanones). Flavanones were the most representative free phenolic class, accounting for approximately 96-99% of total free phenolic content. Hesperidin is the most abundant free polyphenol, accounting for 88-94% of total polyphenols, followed by narirutin (4-8%) and rutin (2-4%).

Table 2. Phenols content in orange peel extracts

Compounds	ChChl:Fru	ChChl:Xyl	ChChl:Gly	ChChl:Gly:CA	50% EtOH
Phenolic acids					
p-Coumaric acid	16.4±1.1 ^a	13.4±0.0 ^{ab}	11.8±1.4 ^b	6.31±0.2 ^c	5.11±0.0 ^c
Ferulic acid	118.0±1.0 ^a	53.7±1.2 ^c	91.5±0.4 ^b	29.1±0.0 ^d	29.2±0.2 ^d
Total phenolic acids	134.4±0.1 ^a	67.1±1.2 ^c	103.3±1.8 ^b	35.4±0.2 ^d	34.4±0.2 ^d
Flavanones					
Hesperidin	2827±25.5 ^b	3388±3.1 ^a	3259±137.5 ^a	3442±54.6 ^a	3205±143.0 ^a
Rutin	2.46±0.03 ^a	1.45±0.02 ^c	1.93±0.02 ^b	0.87±0.00 ^d	1.98±0.00 ^b
Narirutin	242.7±1.2 ^{ab}	251.0±6.0 ^a	225.3±0.1 ^{bc}	221.0±5.6 ^c	160.5±6.4 ^d
Total flavanones	3072±22.3 ^c	3641±7.3 ^a	3487±137.4 ^b	3664±60.2 ^a	3367±149.4 ^{bc}
Total phenolic compounds	3207±22.2 ^c	3708±8.6 ^a	3590±139.2 ^b	3700±60.1 ^a	3402±149.2 ^{bc}

Data are expressed as µg/mL extract and are mean ± SD of three chromatographic analyses. Statistical analysis was by one-way ANOVA (all: $p < 0.005$) with Tukey's post hoc test (different letters indicate significant differences, on the same row). CA: citric acid; ChChl: choline chloride; Et-OH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose

Table 2 makes it evident that the total extraction yield (of the compounds analyzed) and levels of the various phenolic classes and of individual compounds depends on the type of extractant. ChChl:Xyl and ChChl:Gly:CA resulted in the highest extraction of total phenolic compounds and flavanones, whereas ChChl:Fru gave a good extraction yield for phenolic acids, but a modest one for hesperidin, that was most effectively extracted by ChChl:Gly and by 50% EtOH.

NADES selectivity was reported in the literature (Grisales-Mejía et al., 2024; Panić et al., 2021; Picchio et al., 2022; Rocha De Oliveira et al., 2022), and related

to the physicochemical properties of these solvents. Each NADES possesses distinctive characteristics, including polarity, pH, and conductivity (Gomez-Urios et al., 2024; Jurić et al., 2021; Lefebvre et al., 2021), all playing an important role in the extraction yield achieved. It is also possible to modify the NADES formulation to obtain a solvent system specifically tailored to the extraction of a specific compound (Dai et al., 2015). Water addition helps to decrease viscosity, moreover, with a water content of less than 50%, NADES are still considered eutectic mixtures, which help maintain their extractive properties (Chen et al., 2024; Martins & Ferreira, 2017)

All the phenolics identified in these extracts are known to have antioxidant activity (Banjarnahor & Artanti, 2014), reportedly exerted through a variety of mechanisms (Sakurai et al., 2022). Table 3 compares the antioxidant activity of the various NADES extracts, as evaluated by four distinct methodologies. Water/ethanol extracts had the highest antioxidant capacity, as determined by the ABTS, FRAP, and ORAC assays. ChCl:Gly:CA extracts had the highest antioxidant capacity in the DPPH and ORAC assays, but the lowest in FRAP assays. These discrepancies are quite common and have been related to the different nature of the chemical reactions in each assay, as well as to electron transfer kinetic issues and the solubility of individual phenols in each reaction medium (Johnsen et al., 2023).

Table 3. Total antioxidant capacity of orange peel extracts.

	ChChl:Fru	ChChl:Xyl	ChChl:Gly	ChChl:Gly:CA	50% EtOH
ABTS	2.13±0.01 ^c	2.32±0.01 ^b	1.84±0.01 ^d	1.06±0.02 ^e	2.76±0.05 ^a
DPPH	1.04±0.03 ^b	0.73±0.12 ^c	0.57±0.01 ^d	1.44±0.02 ^a	0.81±0.05 ^c
FRAP	2.03±0.02 ^c	2.29±0.04 ^b	1.97±0.07 ^c	0.39±0.02 ^d	3.20±0.09 ^a
ORAC	11341±1052 ^c	8072±457 ^c	18293±488 ^b	21314±2104 ^a	25422±4559 ^a

Data are expressed as mmol TE/mL extract and are means ± SD of three determinations. Statistical analysis was by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences). CA: citric acid; ChChl: choline chloride; Et-OH: ethanol; FRAP: ferric reducing antioxidant power; Fru: fructose; Gly: glycerol; ORAC: oxygen radical antioxidant capacity; Xyl: xylose.

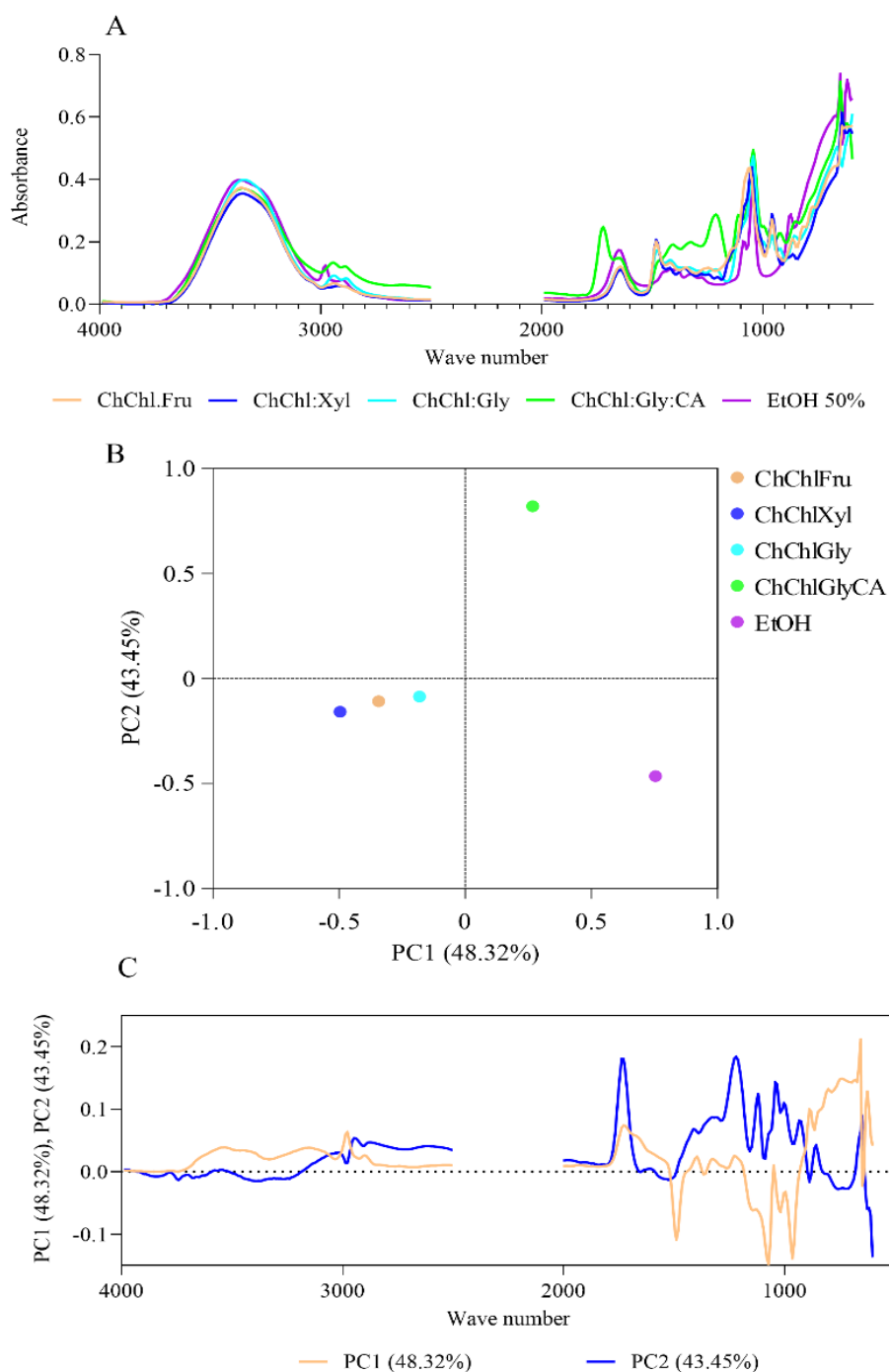
For instance, FRAP cannot detect species that act by a radical quenching mechanism (Malta & Liu, 2014), whereas the antioxidant behavior in the ORAC assay is dominated by the number and type of substituents and not by the Bors criteria, at least in the absence of steric hindrance effects (Platzer et al., 2022). These results can be compared with previous results, where, Gomez-Urios, et al (2024) studied the different antioxidant capacities of fourteen NADES systems, showing different results depending on the radical used. Unlike the peroxide and ABTS radicals, the DPPH radical is soluble in organic solvents because it is hydrophobic. Due to steric inaccessibility, antioxidants that react quickly with the latter may be slow or inactive toward DPPH (Prior et al., 2005). The overall activity of antioxidant compounds is lower than that of individual compounds because of steric hindrance (Schaich et al., 2015).

1.2. FT-IR

As reported in Figure 1A, FTIR spectra show distinctive absorption peaks of ChChl:Fru, ChChl:Xyl, ChChl:Gly, ChChl:Gly:CA, and 50% EtOH extracts corresponding to the functional groups present. Notably, the broad intense absorption band between 3000 and 3600 cm^{-1} , present for all the extracts, can be associated with the oscillation of the $-\text{OH}$ groups of water with slight differences among the extracts due to the formation of intermolecular hydrogen bonds in the NADES mixtures (Jurić et al., 2021). In the region 2873-2972 cm^{-1} the extracts differed in peaks: ChChl:Gly, ChChl:Gly:CA showed two peaks at 2950 and 2890 cm^{-1} , resulting in unique broadband for ChChl:Xyl and one visible peak at 2950 cm^{-1} for ChChl:Fru which can be assigned to $\nu\text{C-H}$ ($-\text{H}_2$) vibrations (Wang et al., 2024).

At 1480 cm^{-1} , the CH_2 oscillation mode band can be noticed, characteristic for ChChl:Fru, ChChl:Xyl, ChChl:Gly, ChChl:Gly:CA (Wiercigroch et al., 2017). The ChChl:Gly has a similar profile to the one recorded by Wang et al (2024). The ChChl:Gly:CA extract showed the highest absorbance at the two peaks at 2950 and 2890 cm^{-1} as well as in the region 1500-1150 cm^{-1} , which might be related to the highest presence of total phenolic compounds ($3700 \pm 60 \mu\text{g/mL}$) in the extract. The 50% EtOH showed a different profile all along the IR region with characteristic peaks linked to ethanol at around: 3400 cm^{-1} due to O-H bond stretching, 2980 cm^{-1} due to C-H bond stretching, from 1350 to 1260 cm^{-1} due to O-H bending deformation band, at around 1055-1100 cm^{-1} , with two peaks linked to C-O bond stretching.

The fingerprint region (1100-850 cm^{-1}) is more complex to be specifically described for each extract as it is typical for the signal related to the combination of both NADES and extracted phenolic compounds..



Data are expressed as absorbance and are mean of three spectroscopic analyses. CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose.

Figure 1. Representative FT-NIR spectrum (A), PCA scores plot (B), and loadings plot (C) on the whole spectra dataset collected in orange peel NADES and 50% EtOH extracts

A PCA was performed to explore the complexity of the spectra, thus highlighting the relation among the sample and the influence of the variables. The scores plot of PC1 (explained variance: 48.32%) vs PC2 (explained variance: 43.45%) (Figure 1B) confirmed the observed spectral differences for ChChl:Gly:CA and 50% EtOH concerning ChChl:Fru, ChChl:Xyl, ChChl:Gly. In detail the ChChl:Gly:CA showed positive PC1 and PC2 scores, the 50% EtOH extract is located in the IV quadrant, and ChChl:Fru, ChChl:Xyl, ChChl:Gly are grouped in the III quadrant. The differences along PC1 (Figure 1C) are linked to the positive loadings of PC1 at 2978 cm^{-1} , characteristic of 50% EtOH extract, together with 1720, 1390, 1211, 1219, and 1040 cm^{-1} which is characteristic of citric acid extract. Furthermore, ChChl:Fru, ChChl:Xyl, ChChl:Gly differed from ChChl:Gly:CA along PC2 due to the signals in the region 1500 – 1000 cm^{-1} , linked to fructose, xylose, and glycerol absorbance.

1.3. Volatile compounds profile and relative content

This study identified 50 volatile compounds in orange peel extracts. Table 4 summarises the volatile compounds, divided into six classes: terpenes (13), aldehydes (10), alcohols (18), ketones (5), organic acids (2), and esters (2). As for phenolics, the levels of the volatile classes and individual components were found to depend on the type of extraction solvent. In NADES extracts, the most representative classes were alcohols, aldehydes, and terpenes, which accounted for approximately 95% of the total volatile compound content. In contrast, terpenes were the most representative class in 50% EtOH extracts, accounting for about 99% of the total volatile compounds. The profile of volatile molecules in the extracts is mainly related to the polarity of the solvent. Choline chloride-based NADES have a hydrophilic nature and exhibit higher polarity than water (Negi et al., 2024). This leads to greater extraction of polar and high molecular

weight molecules concerning more hydrophobic terpenes, which were most abundant in extracts using 50% EtOH, which has lower polarity (González-Mas et al., 2019).

Table 4. Volatile compounds relative content in orange peel extracts

Compounds	ChChl:Fruc	ChChl:Xyl	ChChl:Gly	ChChl:Gly:CA	50% EtOH
Terpenes					
α -Thujene	n.d.	n.d.	n.d.	n.d.	0.15 ± 0.05^a
β -Thujene	n.d.	n.d.	n.d.	n.d.	1.31 ± 0.35^a
3-Carene	n.d.	n.d.	n.d.	n.d.	0.35 ± 0.07^a
β -Myrcene	0.59 ± 0.09^{bc}	0.51 ± 0.08^{cd}	0.40 ± 0.01^d	0.79 ± 0.13^b	5.82 ± 0.76^a
Isocineole	n.d.	n.d.	n.d.	0.53 ± 0.14^a	n.d.
Limonene	17.23 ± 0.66^c	16.43 ± 0.84^c	17.27 ± 0.46^c	38.15 ± 1.87^b	88.38 ± 2.12^a
β -Phellandrene	n.d.	n.d.	n.d.	n.d.	1.12 ± 0.36^a
β -Cimene	n.d.	n.d.	n.d.	0.80 ± 0.08^a	0.06 ± 0.02^b
Terpinolen	n.d.	n.d.	n.d.	2.16 ± 0.12^a	0.14 ± 0.04^b
α -Pinene	0.43 ± 0.04^{ab}	0.36 ± 0.06^b	0.49 ± 0.06^{ab}	0.50 ± 0.05^a	n.d.
Carvone	0.51 ± 0.05^a	0.49 ± 0.07^a	0.49 ± 0.05^a	0.42 ± 0.07^a	n.d.
Dimethylstyrene	n.d.	n.d.	n.d.	2.87 ± 0.31^a	n.d.
Total terpenes	18.76 ± 0.76^c	17.79 ± 0.75^c	18.65 ± 0.36^c	46.22 ± 1.51^b	98.70 ± 0.28^a
Aldehydes					
Hexanal	5.31 ± 0.55^b	6.95 ± 0.94^a	4.17 ± 0.29^c	0.76 ± 0.12^d	n.d.
Octanal	4.21 ± 0.32^b	4.98 ± 0.77^b	15.35 ± 1.09^a	2.14 ± 0.14^c	0.44 ± 0.10^d
Nonanal	n.d.	0.29 ± 0.08^b	0.91 ± 0.10^a	0.47 ± 0.12^b	0.06 ± 0.01^c
Decanal	n.d.	0.22 ± 0.03^c	0.97 ± 0.15^a	0.48 ± 0.07^b	0.47 ± 0.09^b
2-Hexenal	5.68 ± 0.53^a	5.92 ± 0.46^a	3.78 ± 0.27^b	3.12 ± 0.42^b	n.d.
Butanal	0.19 ± 0.02^a	0.18 ± 0.03^a	n.d.	n.d.	n.d.
Neral	2.05 ± 0.16^a	1.77 ± 0.31^a	2.20 ± 0.19^a	n.d.	0.04 ± 0.02^b
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Geranial	1.67 ± 0.13 ^b	1.78 ± 0.34 ^{ab}	2.30 ± 0.32 ^a	n.d.	0.05 ± 0.02 ^c
Perillal	0.29 ± 0.06 ^b	0.29 ± 0.08 ^b	0.37 ± 0.11 ^{ab}	0.50 ± 0.10 ^a	n.d.
Benzaldehyde	0.23 ± 0.03 ^a	n.d.	n.d.	n.d.	n.d.
Total aldehydes	19.62 ± 0.27 ^c	22.38 ± 1.04 ^b	30.05 ± 1.40 ^a	7.46 ± 0.70 ^d	1.06 ± 0.22 ^e
Alcoholes					
Eucalyptol	n.d.	n.d.	0.31 ± 0.09 ^b	2.06 ± 0.18 ^a	n.d.
trans-2-Hexenol	1.91 ± 0.13 ^a	1.53 ± 0.45 ^{ab}	0.55 ± 0.07 ^c	1.04 ± 0.17 ^b	n.d.
2-Buten-1-ol, 3-methyl-	n.d.	n.d.	n.d.	0.58 ± 0.05 ^a	n.d.
1-Hexanol	2.78 ± 0.16 ^a	2.59 ± 0.42 ^a	0.95 ± 0.10 ^c	1.41 ± 0.13 ^b	n.d.
3-Hexen-1-ol, (Z)-	0.71 ± 0.10 ^a	0.41 ± 0.06 ^b	0.28 ± 0.05 ^c	0.31 ± 0.02 ^c	n.d.
1-Hexanol, 2-ethyl-	0.45 ± 0.09 ^a	0.32 ± 0.10 ^a	n.d.	n.d.	n.d.
Linalol	29.41 ± 0.66 ^a	27.83 ± 1.62 ^a	30.25 ± 0.87 ^a	1.77 ± 0.61 ^b	0.16 ± 0.03 ^c
1-Octanol	9.23 ± 0.35 ^b	10.07 ± 0.87 ^{ab}	9.19 ± 0.37 ^b	10.67 ± 0.62 ^a	0.04 ± 0.02 ^c
4-Terpineol	1.47 ± 0.12 ^b	1.47 ± 0.19 ^b	0.97 ± 0.15 ^c	2.64 ± 0.10 ^a	n.d.
Verbenol	0.49 ± 0.04 ^b	0.57 ± 0.27 ^{abc}	0.28 ± 0.05 ^c	0.93 ± 0.09 ^a	n.d.
Menthol	n.d.	n.d.	n.d.	2.07 ± 0.18 ^a	n.d.
α-Terpineol	5.97 ± 0.16 ^b	4.60 ± 0.39 ^c	3.28 ± 0.33 ^d	17.90 ± 1.69 ^a	0.03 ± 0.02 ^e
β-Citronellolo	0.65 ± 0.10 ^a	0.71 ± 0.27	0.56 ± 0.12 ^a	n.d.	n.d.
Nerol	1.56 ± 0.15 ^a	1.26 ± 0.15 ^a	0.91 ± 0.07 ^b	n.d.	n.d.
2-Cyclohexen-1-ol, 1-methyl-4-(1-methylethenyl)-, trans-	0.26 ± 0.01 ^a	0.28 ± 0.05 ^a	n.d.	n.d.	n.d.
cis-Carveol	0.36 ± 0.05 ^a	0.38 ± 0.15 ^a	n.d.	n.d.	n.d.
Geraniol	0.69 ± 0.08 ^a	0.89 ± 0.22 ^a	0.28 ± 0.10 ^b	n.d.	n.d.

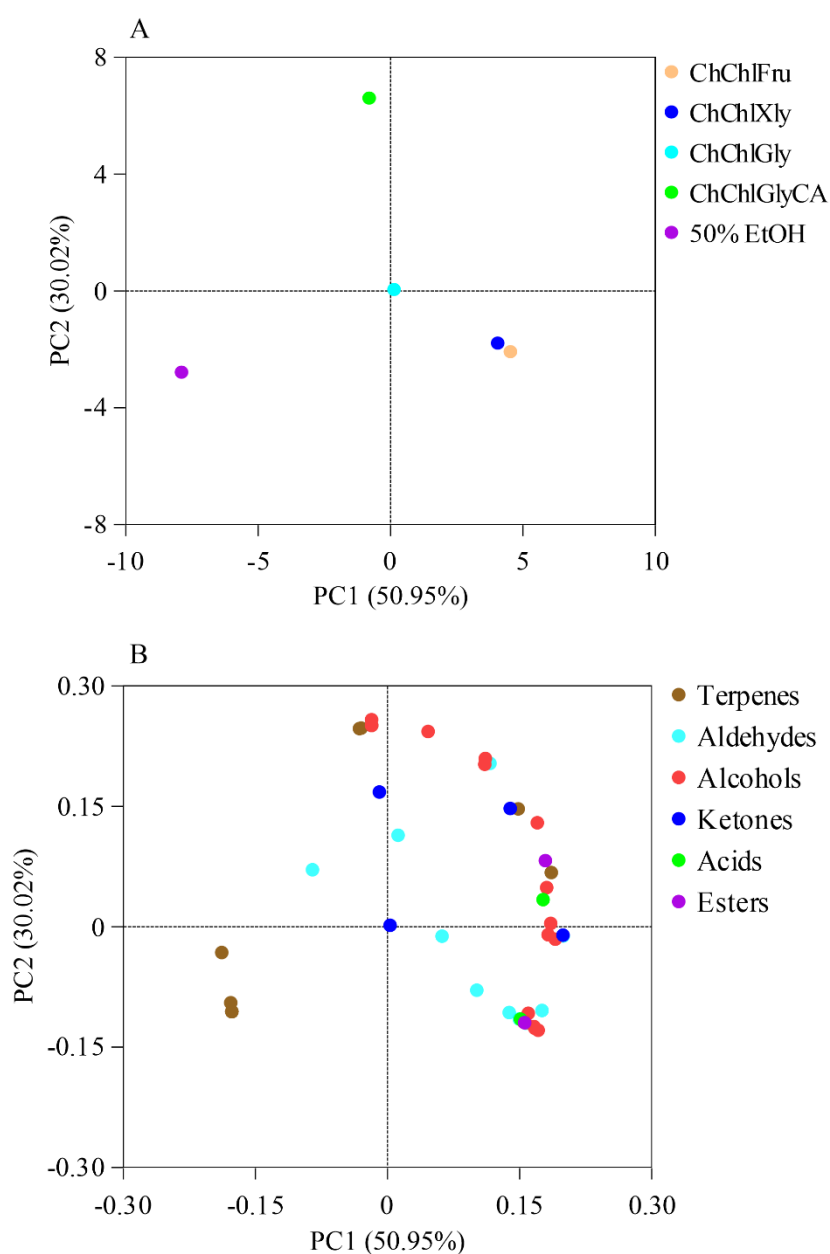
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p-Mentha-1(7),8(10)-dien-9-ol	0.61 ± 0.05 ^a	0.38 ± 0.03 ^b	0.26 ± 0.04 ^c	0.39 ± 0.12 ^{bc}	n.d.
Total alcohols	56.54 ± 0.06 ^a	53.30 ± 0.79 ^b	48.07 ± 2.03 ^c	41.76 ± 0.21 ^d	0.24 ± 0.06 ^e
Ketones					
2-Propanone, 1-Hydroxy	n.d.	n.d.	0.69 ± 0.13 ^a	0.49 ± 0.06 ^a	n.d.
Camphor	n.d.	n.d.	0.19 ± 0.02 ^a	n.d.	n.d.
Cyclopentanone, 2-ethyl	0.64 ± 0.11 ^a	0.57 ± 0.15 ^{ab}	0.36 ± 0.05 ^b	0.33 ± 0.08 ^b	n.d.
Isopiperitenon	0.99 ± 0.06 ^a	0.90 ± 0.08 ^a	0.29 ± 0.04 ^b	1.23 ± 0.28 ^a	n.d.
Total ketones	1.63 ± 0.17 ^{ab}	1.47 ± 0.23 ^b	1.52 ± 0.09 ^b	2.05 ± 0.30 ^a	n.d.
Organic acids					
Glycolic Acid	2.24 ± 0.41 ^b	3.64 ± 0.58 ^a	1.41 ± 0.19 ^c	2.08 ± 0.25 ^b	n.d.
Fumaramic acid	0.40 ± 0.03 ^a	0.58 ± 0.16 ^a	n.d.	n.d.	n.d.
Total organic acids	2.64 ± 0.38 ^b	4.22 ± 0.74 ^a	1.41 ± 0.19 ^c	2.08 ± 0.25 ^b	n.d.
Esters					
Methoxyacetic acid, cyclopentylethyl ester	0.39 ± 0.12 ^a	0.30 ± 0.09 ^a	n.d.	n.d.	n.d.
Butanoic acid, 3-methylphenyl ester	0.40 ± 0.08 ^{ab}	0.56 ± 0.10 ^a	0.30 ± 0.01 ^b	0.43 ± 0.05 ^a	n.d.
Total esters	0.79 ± 0.20 ^a	0.85 ± 0.02 ^a	0.30 ± 0.01 ^c	0.43 ± 0.05 ^b	n.d.

Data are expressed as relative percentage and are means ± SD of three chromatographic analyses. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences on the same row). CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; n.d.: not detected; Xyl: xylose.

As for individual volatile components, the most typical compounds in ChChl:Fru, ChChl:Xyl, and ChChl:Gly were linalool > limonene > 1-octanol, which accounted for nearly 54% of the total compounds. In ChChl:Gly:CA, the most characteristic volatile compounds were limonene > α -terpineol > 1-octanol, accounting for approximately 55% of compounds. In the 50% EtOH extract, the most prominent volatile compounds were limonene >> β -myrcene, which together accounted for almost 94% of the total. Ethanol's lower polarity compared to the other solvent mixtures likely favors the extraction of more hydrophobic terpenes, such as limonene and β -myrcene (González-Hernández et al., 2024), whereas the presence of both polar and moderately hydrophobic volatile compounds in NADES extracts indicates a balance in extracting a range of volatiles with varying polarities.

All the major volatile compounds detected in this study have been reported to be components of citrus essential oil (Angane et al., 2022). However, ChChl:Gly extract was different from those obtained using ChChl:Fru and ChChl:Xyl, which showed fairly similar flavor profiles. The extract obtained using ChChl:Gly:CA was very different from the others, including ChChl:Gly, likely because of the acidic pH of the extractant containing citric acid, which could lead to the conversion of some terpenoids. For example, the conversion of nerol and geraniol to α -terpineol has been found to occur in an exceedingly acidic environment (Dziadas & Jeleń, 2016). Literature data indicate that terpenoids such as α -terpineol and linalool are more effective antimicrobials compared to terpenes such as limonene and β -myrcene (Masyita et al., 2022).



CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose

Figure 2. Representative PCA scores (A) and loadings plot (B) of the whole volatile in the various orange peel extracts

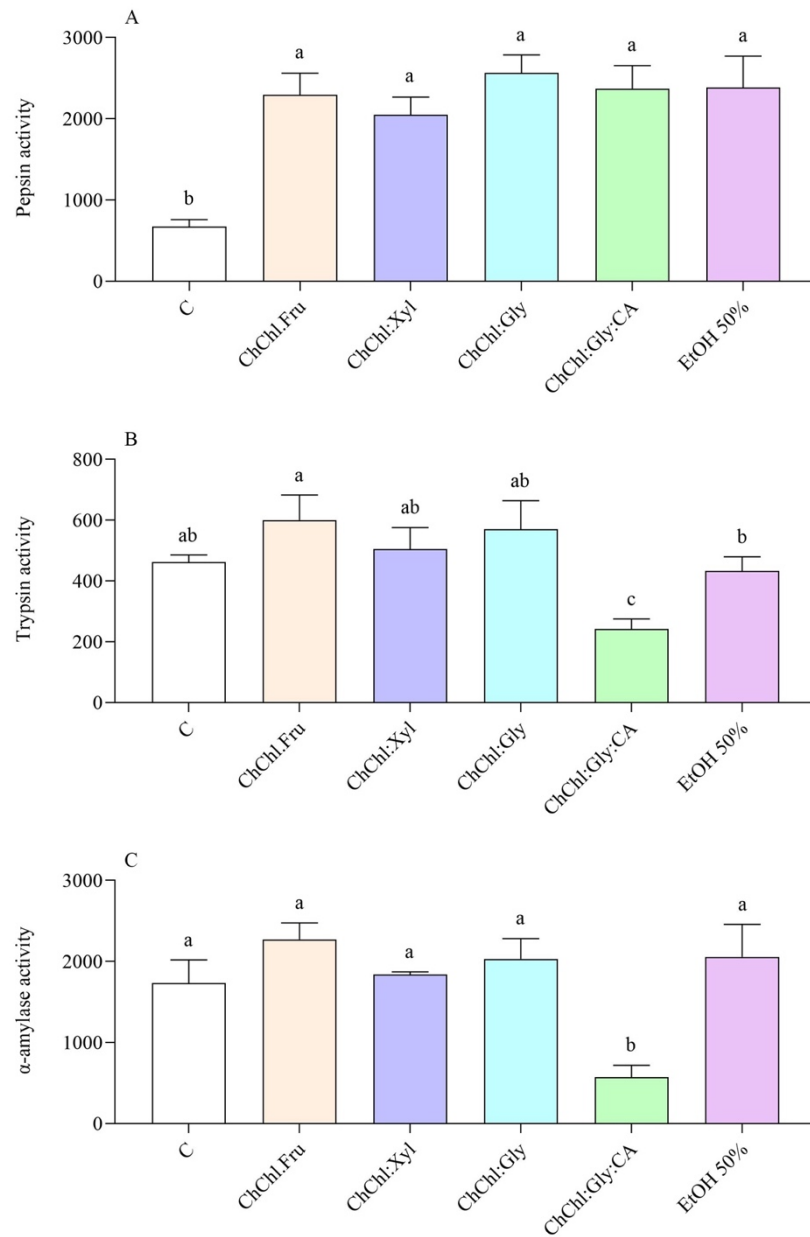
As shown in Figure 2, the PCA scores of the entire volatile profile of orange peel NADES and 50% EtOH extracts displayed a clear separation of the NADES extract in the factor plane, except for the extracts obtained with ChChl:Fru and ChChl:Xyl, which clustered together. The latter were well separated from ChChl:Gly and from the 50% EtOH extract mainly along PC1, which explains 50.95% of the total variance. On the contrary, ChChl:CA extracts were well separated from the ChChl:Fru and ChChl:Xyl ones and from the 50% EtOH extract both along PC1 and PC2 (which explain 30.02% of the total variance) and from the ChChl:Gly extract mainly along PC2.

1.4. Effect on modulation of digestive enzymes.

The effect of orange peel NADES and ethanolic extracts on the activity of pepsin, trypsin, and α -amylase is reported in Figure 3 A-C, respectively. In these assays, the final concentration of substrate and polyphenols was selected according to the standardized static in vitro digestion INFOGEST protocol (Minekus et al., 2014) and to the predicted gut polyphenols concentration (Williamson, 2013).

All the extracts were able to increase pepsin activity concerning the control condition (absence of extract) (Figure 3A), but none of them affected chymotrypsin activity (data not shown). The ChCh:Gly:CA extract was found to decrease significantly the activity of trypsin and amylase (Figure 3B and 3C, respectively). While we are not aware of any studies that have evaluated the effect of the NADES on digestive enzymes activity, there seems to be some conflicting evidence on the impact of polyphenols and polyphenol-rich foodstuffs on it. Polyphenol-rich balsamic vinegar was found to decrease pepsin activity, with no effects on trypsin (Urbinati et al., 2021). Flavonoids and stilbenes have been shown to improve pepsin activity by increasing the enzyme V_{max}

(Tagliazucchi et al., 2005). Similarly, Tantoush et al. (2012) reported that pepsin-resistant proteins, such as β -lactoglobulin and Ara h 1 and Ara h 2, degrade faster in the presence of catechin-enriched green tea polyphenols. Although polyphenols may contribute to blood glucose control through various mechanisms, including the reduction of dietary sugars in the intestine (Iametti et al., 2022), numerous studies have demonstrated that this class of bioactive compounds can inhibit pancreatic amylases and glucosidase activity through a structure-dependent effect (Gong et al., 2020; Sun et al., 2019). The diverse effects may stem from the binding of polyphenols to either the enzyme or its substrate(s), as dictated by their structure. Enzymes can also be inhibited when their structure is modified by ligands that affect the correct positioning and orientation of substrate and/or catalytic residues (Cirkovic Velickovic & Stanic-Vucinic, 2018). In contrast, polyphenols may act as allosteric effectors or modify enzyme conformation (stabilization) to improve the protein/substrate interaction (Shahidi & Dissanayaka, 2023).



Data are expressed as U/mg enzymes and are means $\pm$ SD of at least three replicates. Statistical analysis was by the one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test. Different letters in the same row indicate significant difference (at least $p < 0.05$). CA: citric acid; ChCl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose

Figure 3. Pepsin (A), trypsin (B), and α -amylase (C) activity in the absence/presence of orange peel NADES and 50% EtOH extracts.

1.5. Antimicrobial activity

Table 5 compares the antimicrobial activity of NADES and ethanol extracts. The antimicrobial activity of the extracts was found to be similar to that of ethanolic extracts against selected strains of common foodborne pathogens, namely *E. coli*, *S. aureus*, and *S. epidermidis*. None of the extracts had an inhibitory effect on the growth of *Listeria*. An example of the original results of inhibition studies is provided in the Annex 5 (Figure S1).

Regarding antimicrobial activity of both NADES and NADES-based extracts it is reported that it can be highly variable, depending on the NADES used, extraction matrix, the target microorganism, and the method employed to assess antimicrobial activity (Bedair et al., 2024).

However, extracts obtained with ChChl:Gly:CA had the highest antimicrobial activity towards sensitive species, and retained it after 1/1 dilution of the extract in water (at difference with most other NADES extracts). Noteworthy, the antimicrobial activity of the ChChl:Gly:CA extract was retained after correcting the pH of the original undiluted extract (slightly above 1) to a value close to that of the other NADES extracts (pH 4.2).

This is in agreement with literature data indicating that the antimicrobial activity of NADES and related extracts is greater and more efficient when NADES are based on organic acids, particularly citric acid (Bedair et al., 2024; Mouffok et al., 2023).

Extracts are rich in terpenes and terpenoids, including limonene, linalool, and alpha-terpineol, which are known for their high antimicrobial efficacy (Bukvicki et al., 2023), but also in polyphenols, which are present in high concentrations in

the NADES extracts and reportedly exhibit notable antimicrobial activity (Manso et al., 2021).

Most literature data on the antimicrobial activity of fruit extracts deal with ethanolic and methanolic extracts (Hanafy et al., 2021). This study found the antimicrobial efficacy of NADES extracts similar to that of 50% EtOH extracts, despite the differing composition of the extracts. The available literature data regarding the comparison between NADES and ethanolic extracts are quite variable. For instance, García-Roldán et al (2023) demonstrated greater antimicrobial activity in NADES extracts from spent coffee grounds compared to ethanolic extracts. However, the observed higher antimicrobial activity was attributed more to the composition of the NADES themselves rather than the phenolic compounds extracted. Conversely, other authors have reported greater antimicrobial activity in ethanolic extracts compared to NADES-based ones (Jenny et al., 2024).

The sensitivity of microorganisms to natural antimicrobials is reported to be strain-dependent (Wiktorczyk-Kapischke et al., 2021). This finding is corroborated by the results of the present study, which demonstrated that the strain of *L. monocytogenes* used exhibited resistance to all the antimicrobial solutions tested. In contrast, the other strains, comprising both Gram-positive and Gram-negative bacteria, displayed comparable sensitivity to all the antimicrobials tested.

Table 5. Antimicrobial activity of orange peel extracts as determined by the agar well diffusion method.

NADES	pH	<i>L. monocytogenes</i>		<i>E. coli</i>	<i>S. aureus</i>	<i>S. epidermidis</i>
		Conc.	Inhibition	Inhibition	Inhibition	Inhibition
ChChl:Fruc	4.2	100%	-	+	+	+
		50%	-	+	+	+
ChChl:Xyl	4.01	100%	-	+	+	+
		50%	-	+	+	+
ChChl:Gly	4.28	100%	-	+	-	+
		50%	-	+	-	+
ChChl:Gly:CA	1.03	100%	++++	++++	++++	++++
		50%	+++	++++	++++	+++
	4.03	100%	+	++	++	++
		50%	-	+	+	+
EtOH 50%	4.98	100%	-	+	+	+
		50%	-	+	+	+

Scores are assigned on the basis of the diameter of the inhibition halo: "-", < 8mm; "+", 8-11 mm; "++", 11-14 mm; "+++", 14-18mm; "++++", > 18mm. CA: citric acid; ChChl: choline chloride; EtOH: ethanol; Fru: fructose; Gly: glycerol; Xyl: xylose.

2. Conclusions

This study confirms the promising potential of NADES based on choline chloride as solvents for the extraction of biologically active constituents from citrus peel by-products in comparison with traditional organic solvents. NADES-based extracts exhibited a high phenolic content and a distinctive volatile. Additionally, NADES extracts could modulate digestive enzyme activity, with ChChl:Gly:CA exhibiting notable inhibition of trypsin and amylase activities. ChChl:Gly:CA also had the most pronounced antimicrobial activity against a range of foodborne pathogens.

In conclusion, the utilization of choline chloride-based NADES for the extraction of bioactive compounds from orange peel not only aligns with the principles of green chemistry but also provides a sustainable method for the valorization of orange by-products. This approach has the potential to boost the development of natural antioxidants and antimicrobials for a variety of applications, promoting both environmental sustainability and economic efficiency. However, further research should be conducted to optimize NADES compositions and to investigate (and possibly to exploit) any synergistic effects with specific bioactives and/or their potential for application in industrial-scale processes

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PART II

Improving the shelf-life and functionality of orange juice by the addition of polyphenol-enriched extracts obtained using natural deep eutectic solvents



This study aims to develop a novel orange juice (OJ) formulated using different NADES extracts from orange by-products. An electronic nose and tongue were employed in the formulation of the novel juice. The formulated juice was characterized (color, pH, and phenolic profile content). A shelf-life study was conducted, monitoring the microbiological spoilage, volatile profile, and color over thirteen days at two different storage temperatures (4°C and 8°C), additionally, a challenge test was performed against *S. cerevisiae* and *L. plantarum*, using pure OJ as a control.

For the shelf-life study the new orange juices formulated was used without any thermal treatments. To elucidate the potential antimicrobial effect of the vehicle, orange juices with NADES without any prior orange peel extraction were formulated. Half of the samples were stored at 4 °C and half at 8 °C simulating a thermal abuse condition. During thirteen days of storage, the samples were analyzed at days 0, 3, 6, 9, and 13 for microbiological analysis, and at days 0, 6, and 13 for the color and volatile molecule profiles. The analyses were performed after 0, 6, and 13 days of both storage temperatures.

Table 1. Natural deep eutectic solvents used, molar ratio, appearance, and batch composition.

NADES	Extract	HBA	HBD	Molar Ratio	Appearance
N1	E1	Choline Chloride	Glycerol	1:2	Transparent, colorless liquid
N2	E2		Glycerol: Citric acid	1:1:1	Transparent, colorless, viscous liquid
N3	E3		Fructose	1.9:1	Transparent, slightly white colored liquid
N4	E4		Xylose	1:2	Transparent, colorless liquid

HBA: hydrogen bond acceptor; HBD: hydrogen bond donor

These NADES were selected for their hydrogen bond donor (HBD) components, which are sugar-based, and with a pH above 4, trying not to change much of the organoleptic properties of the newly formulated juice.

1. Results and discussion

1.1. Sensory profile assessed by e-nose and e-tongue

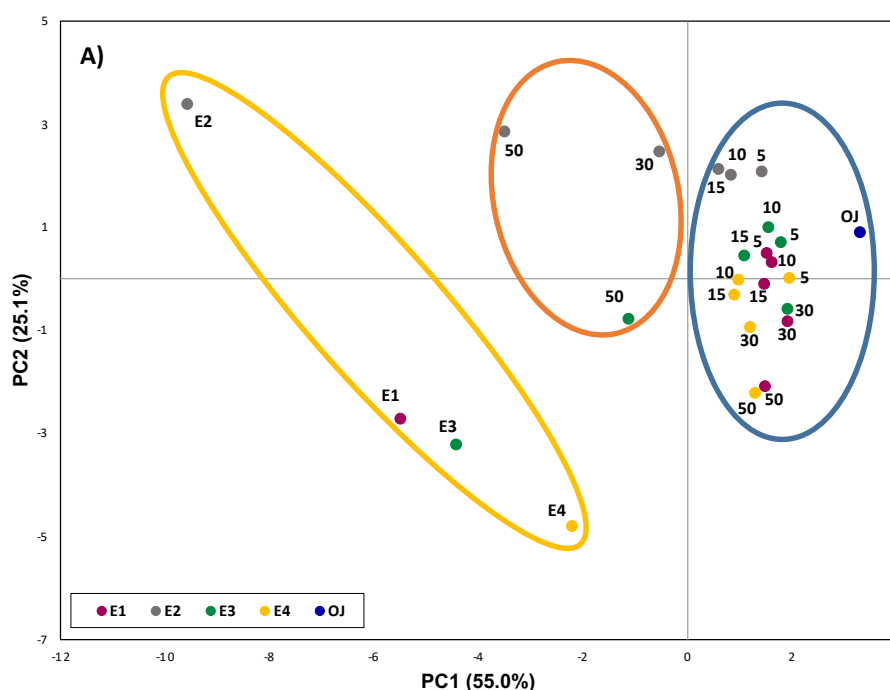
E-tongue and e-nose analyses were performed to study the sensory profile of the orange juice samples enriched with different percentage of NADES extracts (from 5% to 50%) and to classify them according to their sensory properties.

As first, e-nose and e-tongue data were processed by the K-means method in order to identify clusters among samples. By selecting a K value of 3, it was possible to discriminate three clusters in which the samples were grouped based on their sensory properties.

In particular, the first group included the four orange peel NADES extract (E1-E4), the second group was composed of orange juice added with 50% and 30%

of E2 and with 50% of E3. All the other orange juice samples enriched with NADES extracts were grouped in the same cluster together with freshly squeezed OJ.

In order to better characterize the sensory profile of the analyzed samples, e-nose and e-tongue data were processed by PCA and in Figure 1 are reported the score plot (Figure 1A) and the loading plot (Figure 1B) in the plane defined by the first two principal component (PCs) explaining 80.1% of the total variance.



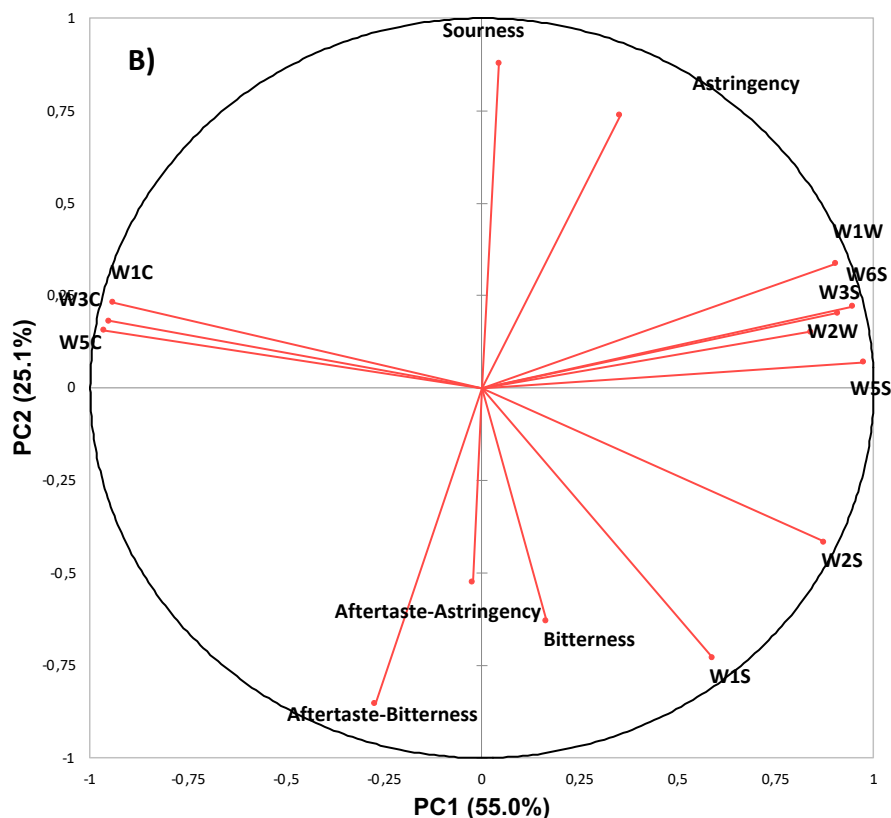
The three clusters (yellow, orange, and blue) refer to the groups defined by K-means clustering. OJ: orange juice; E1: orange peel ChChl:Gly extract; E2: orange peel ChChl:Gly:CA extract; E3: orange peel ChChl:Fru extract; E4: orange peel ChChl:Gly extract. Numbers indicate concentration of the orange peel NADES extracts in orange juice., W3S: Methane.

Figure 1A. PCA score plot and loading plot (B) of newly orange juice formulated with orange peel NADES extract at different concentration

In the score plot samples are clustered into three groups as defined by the K-means method and are distributed on PC1 (55.0% of explained variance), from positive to negative scores, on the basis of the percentage of NADES extracts (5% to 50%); furthermore, the freshly squeezed orange juice sample (OJ) is positioned on the far right of the score plot, while the four orange peel NADES extracts (E1-E4) are located on the left.

Considering the loading plot, it can be noted that all the samples positioned in the positive part of PC1 and clustered in the blue group, have a sensory profile similar to that of freshly squeezed orange juice and are characterized by astringency and bitterness, and by WS (mainly of broad range sensitivity) and WW (specific for sulphur organic compounds) e-nose sensors. NADES extracts all grouped in the yellow cluster in the negative part of PC1, are mainly characterized by WC e-nose sensors, specific for alkanes and aromatic compounds, and are perceived less astringent and bitter. The sensory profile of the three samples grouped in the orange cluster is recognized as different from that of the orange juice and the samples added with E2 extract (50% and 30%) were more characterized by sourness and astringency.

Following the e-nose and e-tongue characterization, the percentages of NADES extracts that resembled pure orange juice (blue cluster) were taken into account and the highest percentage common to all extracts was chosen (15%) to develop new drinks.



W1C: aromatic compounds, W5C: Nitrous oxide, low specificity, W3C: Ammonium, aromatic compounds, W6C: Hydrogen, W5C: Alkanes, aromatic compounds, less polar compounds, W1S: Methane, low specificity, W1W: Sulfur compounds, terpenes, limonene, pyrazines, W2S: Alcohol, partially aromatic compounds, low specificity, W2W: Aromatic compounds, sulfur compounds, organic sulfur compounds, W3S: Methane.

Figure 1B. PCA of loading plot (B) of newly orange juice formulated with orange peel NADES extract at different concentration

1.2. Characterization of formulated orange juice

The newly formulated orange juice based on 15% of NADES extract, was characterized by measuring the color, the pH, and the phenolic profile. Characterizing the phenolic profile in the beverage is important due to its

antioxidant and health-promoting properties. In orange juice, specific phenolic compounds such as hesperidin, naringin, and ferulic acid have already been studied (Irkin et al., 2015; Multari et al., 2020), most probably because of their protective effects against oxidative stress and inflammation (da Silva et al., 2020). The phenolic profile can influence the color and the taste of the juice. In the present study, sixteen phenolic compounds were identified, but only the major compounds were analyzed, as shown in Table 2. The concentration of each phenolic compound would depend on the extract used for the formulation. Hesperidin presents a higher concentration when measuring OJ+E1 ($87.49 \pm 0.55 \mu\text{g/mL}$), and lower when using OJ+E2 ($39.37 \pm 2.22 \mu\text{g/mL}$). In the case of rutin, no statistical differences were found between the different formulations. The difference in the phenolic compound concentration between the various formulations would depend on the NADES used for the initial orange peel extraction, due to the affinity of each NADES for a target compound (Gómez-Uríos et al., 2022).

One of the most important factors in determining the consumer acceptability of a product, like orange juice, is its color (Steur et al., 2023). This factor can even affect how the consumer perceives the beverage's flavor (Spence, 2019). As shown in Table S1, color parameters (L^* , a^* and b^*) of all the samples showed differences with the control, moreover, the results indicate a more yellow tendency, which can verify a first visual acceptance by the consumer of this formulated beverage. Also, for the parameter b^* , the control presented slightly more color yellow than the others, with no statistical differences between them

Table 2. Phenolic compound composition, color, and pH of the different samples studied.

	Phenolic compounds (µg/mL)					
	Ferulic acid	p-coumaric acid	Rutin	Narirutin	Hesperidin	Total phenolic content
OJ	0.82 ± 0.11 ^b	2.18 ± 0.26 ^{bc}	1.12 ± 0.03 ^{ab}	34.29 ± 2.11 ^{bc}	70.04 ± 0.53 ^b	109.52 ± 0.7 ^b
OJ+E1	1.01 ± 0.05 ^a	2.70 ± 0.12 ^a	1.09 ± 0.06 ^{ab}	39.79 ± 1.56 ^{ab}	87.49 ± 0.45 ^a	132.05 ± 1.8 ^a
OJ+E2	0.42 ± 0.12 ^c	1.03 ± 0.15 ^d	0.63 ± 0.05 ^c	29.23 ± 4.14 ^c	39.87 ± 1.81 ^d	70.21 ± 7.5 ^d
OJ+E3	0.75 ± 0.11 ^b	2.40 ± 0.29 ^{ab}	1.48 ± 0.27 ^a	32.00 ± 1.32 ^c	62.18 ± 2.87 ^c	97.54 ± 1.1 ^c
OJ+E4	0.52 ± 0.09 ^c	1.78 ± 0.11 ^c	0.83 ± 0.06 ^{bc}	41.63 ± 1.63 ^a	62.08 ± 3.29 ^c	106.33 ± 0.7 ^b

The data is expressed as mean ± standard deviation, of three replicate samples. The data is presented in µg/mL. Different letters (a-d) indicated statistical differences between the different samples ($p < 0.05$). OJ: pure orange juice, OJ+E1: orange juice with choline chloride:glycerol, OJ+E2: orange juice with choline chloride:glycerol:citric acid, OJ+E3: orange juice with choline chloride:fructose, OJ+E4: orange juice with choline chloride:xylose.

1.3.Shelf-life assessment of an orange juice enriched by polyphenol-enriched extract using NADES

1.3.1.Microbiological stability

Microbiological stability was assessed in all samples over 13 days of storage at two different temperatures (4 °C and 8 °C). Monitoring included total mesophilic aerobic bacteria (TMAB), yeast and molds (YM), and lactic acid bacteria (LAB). Citrus beverages are characterized by an acidic pH, generally ranging between 3-4 and a high sugar content. In these conditions, lactic acid bacteria, molds, and yeasts are the primary cause of spoilage (Aneja et al., 2014; Arias et al., 2002).

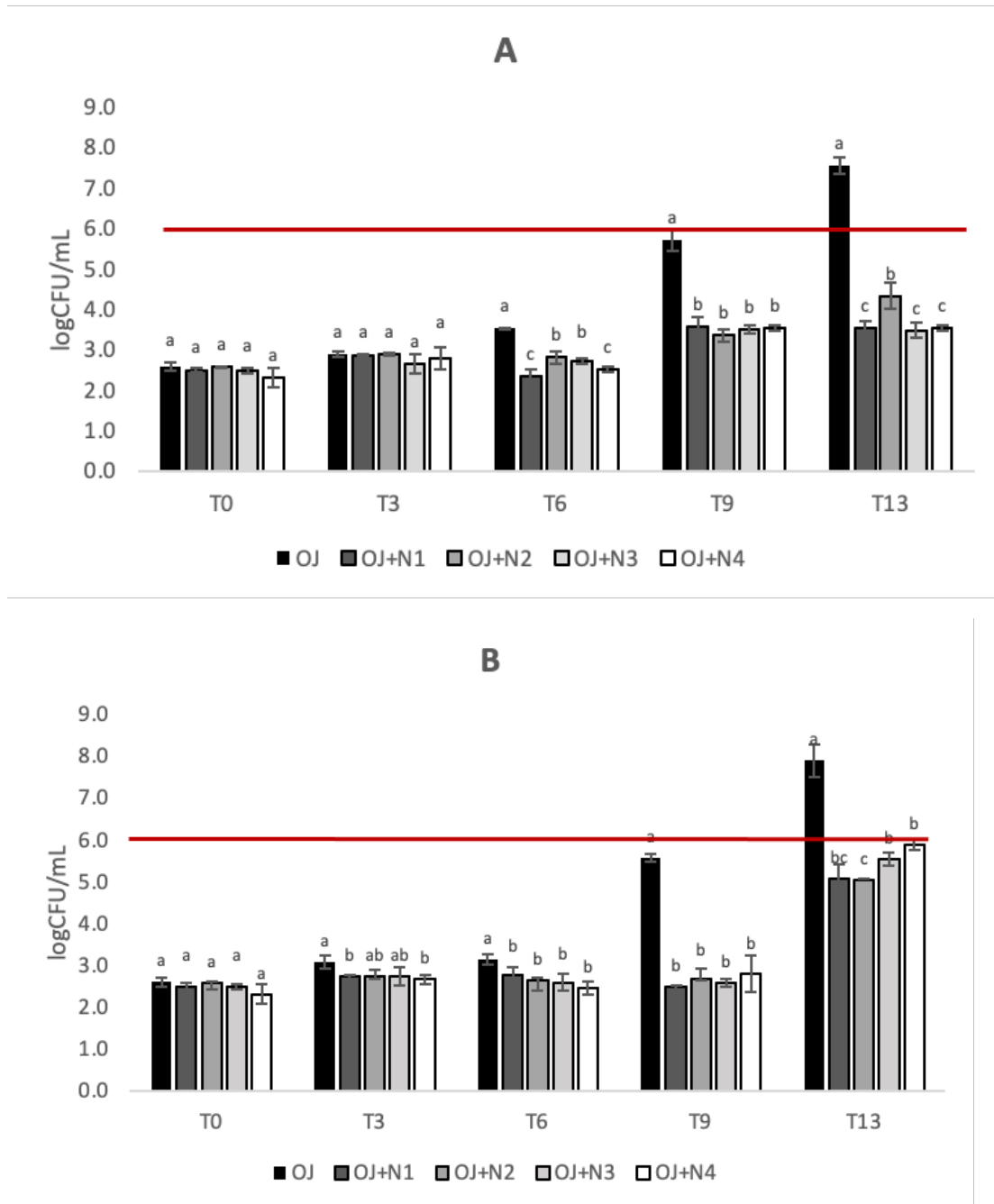
As shown in Figure 2, the initial load of TMAB ranged between 2.42 and 2.76 log CFU/mL while YM ranged between 2.31 and 2.86 log CFU/mL regardless of the sample. Yeasts represented the predominant microbial group in all the samples throughout the entire shelf-life period showing similar or higher loads than TMAB. Lactic acid bacteria were found to be present at concentrations below 2.0 log CFU/mL, regardless of sample type, sampling time, and storage temperature. The growth kinetics of yeasts and TMAB were found to be dependent on both the sample type and the storage temperature.

At the temperature of 4°C both yeast and TMAB cell loads showed a slight increase over time in the samples supplemented with extracts, with no significant differences observed among samples E1-E4 at the same time of storage. However, after 13 days, samples OJ+E1, OJ+E3, and OJ+E4 had a yeast load of about 3.5 log CFU/mL, while sample OJ+E2 showed a load of 4.3 log CFU/mL. In contrast, the control samples already displayed a significantly higher yeast cell load than the other samples after just 6 days. After 9 days, the yeast

load reached 5.7 log CFU/mL, and after 13 days, it was approximately 7.5 log CFU/mL. At 8°C the yeast loads in the control samples during storage were very similar to those observed at 4°C reaching a concentration of 7.90 log CFU/mL at the end of storage. In contrast, the samples supplemented with extracts (E1-E4) showed yeast loads below 3.0 log CFU/mL up to the 9th day of storage, after which it increased by more than 2 logarithmic cycles by the end of the shelf life (ranging between 5.0 and 5.9 log CFU/mL). Even though the trend of the extracts was similar, extracts OJ+E1 and OJ+E2 appear to be the ones most capable of slowing down yeast proliferation at 8°C. The addition of only NADES (OJ+N1-OJ+4) (Annex 6 Tables S2 and S3) also reduced the growth kinetics of yeasts at 4°C and 8°C compared to the controls, showing cell loads similar to those of the samples added with the extracts up to the 9th day of storage. However, by the end of the shelf life, the cell load in the juice samples supplemented with NADES was about one logarithmic cycle higher than that observed in the samples supplemented with the extracts.

The results obtained showed a good stability of the juices despite not undergoing thermal treatments for the whole period of storage. The threshold for yeast spoilage in juices is considered 6.0 log CFU/ml (Krisch et al., 2016) and only the control exceeded this limit after 13 days of storage, regardless of the storage temperature. On the other hand, the low storage temperatures, the antimicrobial activity of the added extracts, and the low pH values of the product reduced the microbial groups that could potentially grow on the juice and slowed their growth kinetics. The pH of the juice with added extracts or NADES was around 3 and remained more or less constant throughout the shelf-life period, regardless of the storage temperature. The only exceptions were the control samples, which showed a significantly higher pH of around 3.5, and the OJ+E2 sample, which, on the contrary, showed a significantly lower pH of

around 2 (Annex 6). Such low pH values strongly affect the microbial population able to survive and grow, definitely favoring yeasts due to their ability to thrive in highly acidic environments (Sourri et al., 2022; Tournas et al., 2006). In addition, the antimicrobial effect of the extracts used in this study aligns with previously data Gomez-Urios et al. (2024) demonstrated the effectiveness of these citrus-based extracts, which contain terpenoids (limonene, linalool, and alpha-terpineol) and polyphenols with proven antimicrobial properties also against yeasts (Mahizan et al., 2019; Scariot et al., 2021). These compounds likely contributed to the reduced growth kinetics of spoilage organisms in the supplemented samples. While the addition of NADES alone showed a reduction of microbial grow compared to the control, probably due to the lower pH of these samples, it was less effective than the extracts in maintaining microbial stability by the end of the storage period. This suggests that the combined presence of terpenoids and polyphenols in the extracts plays a crucial role in inhibiting microbial proliferation, possibly by reducing functionality of cell membranes or interfering with microbial metabolism (Álvarez-Martínez et al., 2021; Hochma et al., 2021).



Results are given by the statistic mean and SD of three different replicates. (a-d): Different letters indicate statistical differences between different storage times ($p < 0.05$). OJ: pure orange juice, OJ+E1: orange juice with choline chloride:glycerol, OJ+E2: orange juice with choline chloride:glycerol:citric acid, OJ+E3: orange juice with choline chloride:fructose, OJ+E4: orange juice with choline chloride:xylose. A: 4 °C. B: 8 °C. Red line: threshold yeast spoilage (6.0 log CFU/mL).

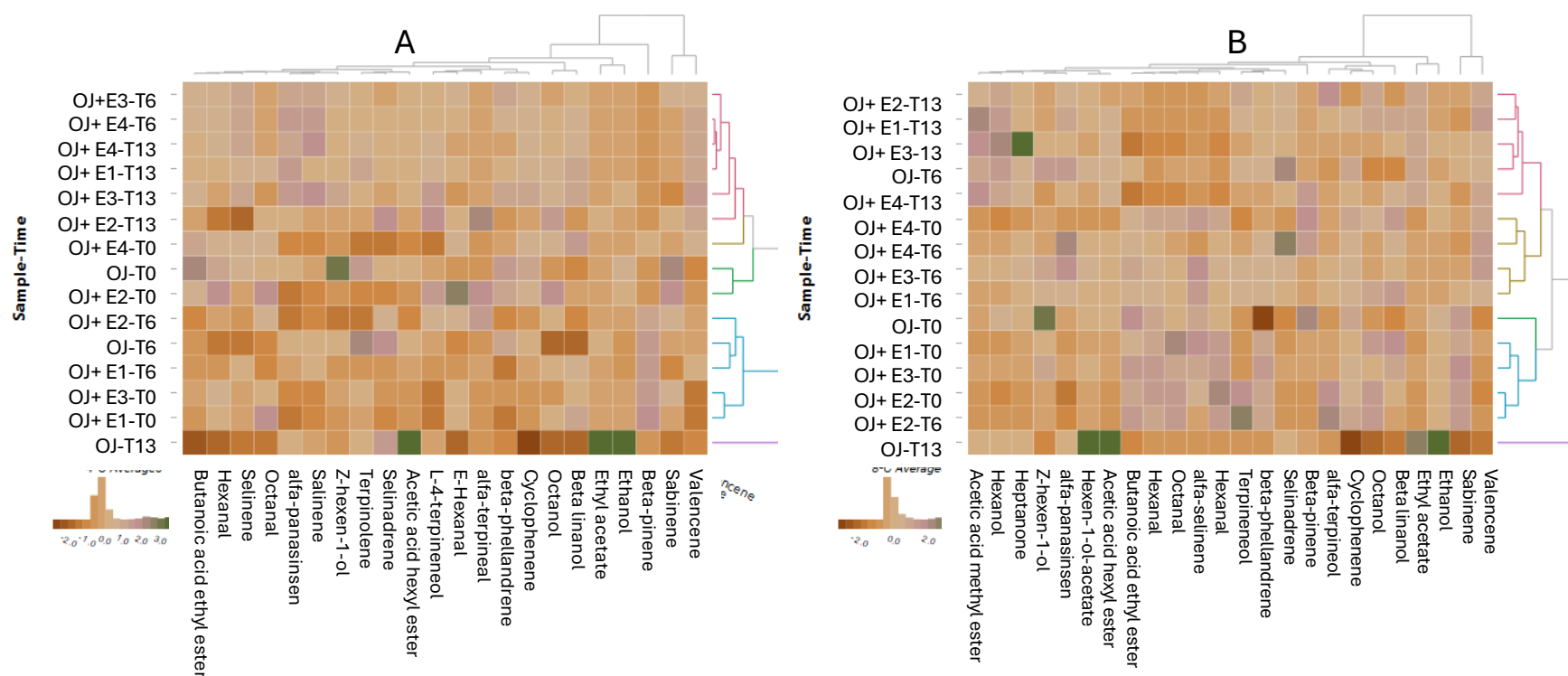
Figure 2. Cell load of yeast and molds, expressed as log CFU/mL, of the different juice samples added with extracts stored at 4°C (A) and 8°C (B)

1.3.2. Volatile and color stability

The volatile profile is an important parameter to monitor the stability and shelf-life of a beverage. Some microorganism like yeasts generates some metabolites, easily monitored by the volatile profile, which can negatively affect the sensorial properties of the product (Rahmat & Kang, 2020).

The volatile profile of samples during storage at 4 °C and 8°C (Annex 6 Table S5) showed some notable differences due to sample type and storage time. A total of 154 volatile molecules were detected in the different samples, belonging to the chemical classes of alcohols, aldehydes, esters, ketones, acids, and terpenes. Limonene was the characteristic molecule of the juices, representing about 75% of the volatile components in the controls and about 70% in the samples with added extracts (OJ+E1–OJ+E4). On the other hand, limonene represents a key contributor to the citrus aroma (Qiao et al., 2008). The relative abundance of limonene decreased over time in all samples, and by the end of the shelf life, it represented 63-66% in the control samples and ranged between 66% and 72% in the samples with added extracts. Other significant detected terpenes were sabinene and valencene, which were present at a relative abundance ranging between 5-6% in all the samples. The juices added with extracts showed a higher abundance of terpenoids (mainly β -Linalool) and some aldehydes (hexanal and 2-E-hexenal) compared to the controls and samples added with NADES for the whole period of storage. In particular, terpenoids were present at a relative percentage higher than 3-4% while most of the other samples showed a relative abundance above 2%. Thus, at both storage temperatures, the control samples showed the greatest variations in the volatile molecule profile during storage in particular after 13 days of storage. The higher terpenoid content in the extract-supplemented samples may have contributed

to maintaining a more stable aroma profile throughout storage. β -linalool, for instance, is known for its antimicrobial activity (Guo et al., 2021) and could have limited microbial growth. This higher stability suggests that the extracts played a protective role, not only by limiting microbial spoilage but also by preserving the sensory quality of the juice over the storage period. In contrast, the control samples and to a minor extent samples containing only NADES showed significant changes in their volatile profile over time, with a noticeable increase in alcohols, particularly ethanol, and esters, such as ethyl acetate. These volatile compounds are well-known byproducts of yeast metabolism (Fleet, 2011) confirming the yeast proliferation observed in the microbiological data. This was also confirmed by the heat map reported in Figure 3, in which it is evident that samples, independent of the storage temperatures clustered mainly based on the storage time, and only the control samples at day 13 showed a completely altered volatile molecule profile due to the increase of esters and alcohols.



The analysis was done by normalizing the samples. The data considered were all the samples higher than 0.5% except for limonene. A: 4 °C storage temperature. B: 8 °C storage temperatures. OJ: pure orange juice, OJ+E1: orange juice with choline chloride:glycerol, OJ+E2: orange juice with choline chloride:glycerol:citric acid, OJ+E3: orange juice with choline chloride:fructose, OJ+E4: orange juice with choline chloride:xylose. T0: Day 0. T6: day 6. T13: day

Figure 3. Volatile compounds over 13 days of storage at two different temperatures

Color is one of the first attributes consumers notice, influencing their perception of quality and freshness. Color stability is an important factor in determining the shelf life of orange juice. By monitoring how the color changes over time, it can be estimated the product's longevity and ensure it remains visually appealing and safe to consume within its intended shelf life, the variations of color might indicate oxidation or microbial growth (Giménez et al., 2012). Table 3 shows the trend of the samples' L^* , a^* , and b^* parameters analyzed over time at different storage temperatures. In this instance, the L^* value indicates the sample's brightness (a large value indicates the sample is very bright), the a^* value indicates green and red (low a^* values indicates green and high a^* values indicates red color), the b^* value indicates yellow and blue (low b^* values indicates blue and high b^* values indicates yellow). The ΔE^* value indicates the color difference between the orange juice samples' pre and post-storage (if the ΔE^* value is high, the sample's color is significantly different from the initial value) (Sirichan et al., 2022). L^* resulted higher in control samples, however in all the samples, an increase of L^* was observed during storage, which means the juice became slightly brighter than initially, this increase is positive since it means that no browning or oxidation of the juice happened (Pokhrel et al., 2022). Also, a^* and b^* increased in all the samples during storage, leading to less green and more yellow juice, in this case, a negative a^* value could indicate microbial spoilage or degradation of pigments (Pokhrel et al., 2022). ΔE is the color change indicator (Annex 6 Table S6), where values above 5 are color change indicators detectable as different from the initial (Wibowo et al., 2015). At the end of shelf-life, all samples underwent color changes compared to the same initial day. However, for samples stored at 4°C and on day 6, samples control, E3, and E4 would not have undergone fully detectable color changes.

Table 3. Evolution of color parameters (L*, a*, b*) of different juice samples during 13 days at 4 °C and 8 °C

	4 °C								
	L*			a*			b*		
	T0	T6	T13	T0	T6	T13	T0	T6	T13
OJ	51.52 ± 2.4 ^a	53.71 ± 1.6 ^a	58.87 ± 0.8 ^a	1.95 ± 1.9 ^a	1.38 ± 0.3 ^b	2.88 ± 0.6 ^a	50.92 ± 4.5 ^a	51.02 ± 3.5 ^a	56.96 ± 1.6 ^a
OJ+E1	41.27 ± 0.4 ^b	51.19 ± 0.7 ^{ab}	50.85 ± 1.9 ^b	-1.68 ± 0.1 ^e	2.51 ± 0.7 ^a	1.36 ± 0.2 ^c	40.09 ± 0.1 ^c	53.85 ± 1.7 ^a	51.55 ± 3.4 ^{ab}
OJ+E2	41.52 ± 0.6 ^b	46.07 ± 1.2 ^c	50.02 ± 0.4 ^b	-0.96 ± 0.2 ^d	0.25 ± 0.1 ^c	2.01 ± 0.3 ^b	42.80 ± 2.3 ^{bc}	44.18 ± 1.3 ^b	49.99 ± 0.7 ^b
OJ+E3	44.16 ± 0.6 ^b	47.18 ± 0.4 ^b	48.95 ± 0.3 ^b	0.25 ± 0.1 ^b	-0.23 ± 0.0 ^d	0.64 ± 0.1 ^d	47.43 ± 0.1 ^{ab}	47 ± 1.1 ^{ab}	49.62 ± 0.5 ^b
OJ+E4	43.09 ± 0.6 ^b	49.01 ± 0.3 ^b	50.90 ± 0.8 ^b	-0.44 ± 0.0 ^c	0.31 ± 0.0 ^c	1.08 ± 0.2 ^c	45.27 ± 1.8 ^b	50.39 ± 0.6 ^a	50.95 ± 0.5 ^b
	8 °C								
	L*			a*			b*		
	T0	T6	T13	T0	T6	T13	T0	T6	T13
OJ	51.52 ± 2.4 ^a	56.91 ± 0.3 ^a	57.87 ± 0.3 ^a	1.95 ± 1.9 ^a	4.37 ± 0.2 ^a	1.98 ± 0.2 ^a	50.92 ± 4.5 ^a	57.96 ± 0.4 ^a	54.54 ± 0.5 ^a
OJ+E1	41.27 ± 0.4 ^b	50.55 ± 0.1 ^b	51.11 ± 0.1 ^b	-1.68 ± 0.1 ^e	1.62 ± 0.1 ^c	1.745 ± 0.1 ^a	40.09 ± 0.1 ^c	52.42 ± 0.0 ^b	52.21 ± 0.3 ^{ab}
OJ+E2	41.52 ± 0.6 ^b	49.98 ± 1.1 ^{bc}	48.87 ± 0.5 ^c	-0.96 ± 0.2 ^d	2.50 ± 0.3 ^b	1.55 ± 0.0 ^a	42.80 ± 2.3 ^{bc}	52.1 ± 2.1 ^b	48.60 ± 0.0 ^c
OJ+E3	44.16 ± 0.6 ^b	51.70 ± 0.9 ^b	50.61 ± 0.5 ^b	0.25 ± 0.1 ^b	2.20 ± 0.4 ^b	0.985 ± 0.1 ^b	47.43 ± 0.1 ^{ab}	53.95 ± 1.4 ^{ab}	50.65 ± 0.2 ^b
OJ+E4	43.09 ± 0.6 ^b	48.79 ± 0.7 ^c	51.38 ± 0.4 ^b	-0.44 ± 0.0 ^c	2.18 ± 0.4 ^b	1.65 ± 0.4 ^a	45.27 ± 1.8 ^b	49.65 ± 0.4 ^b	51.925 ± 0.4 ^b

Data are expressed as µg/mL extract and are mean ± SD of three independent analyses. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (a-d: different letters indicate significant differences between storage time, OJ: indicate statistical differences between samples). Control: pure orange juice. OJ+E1: orange juice with choline chloride:glycerol extract. OJ+E2: orange juice with choline chloride:glycerol:citric acid extract. OJ+E3: orange juice with choline chloride:fructose extract. OJ+E4: orange juice with choline chloride:xylose extract. T0-T13: study days

1.4. Challenge test

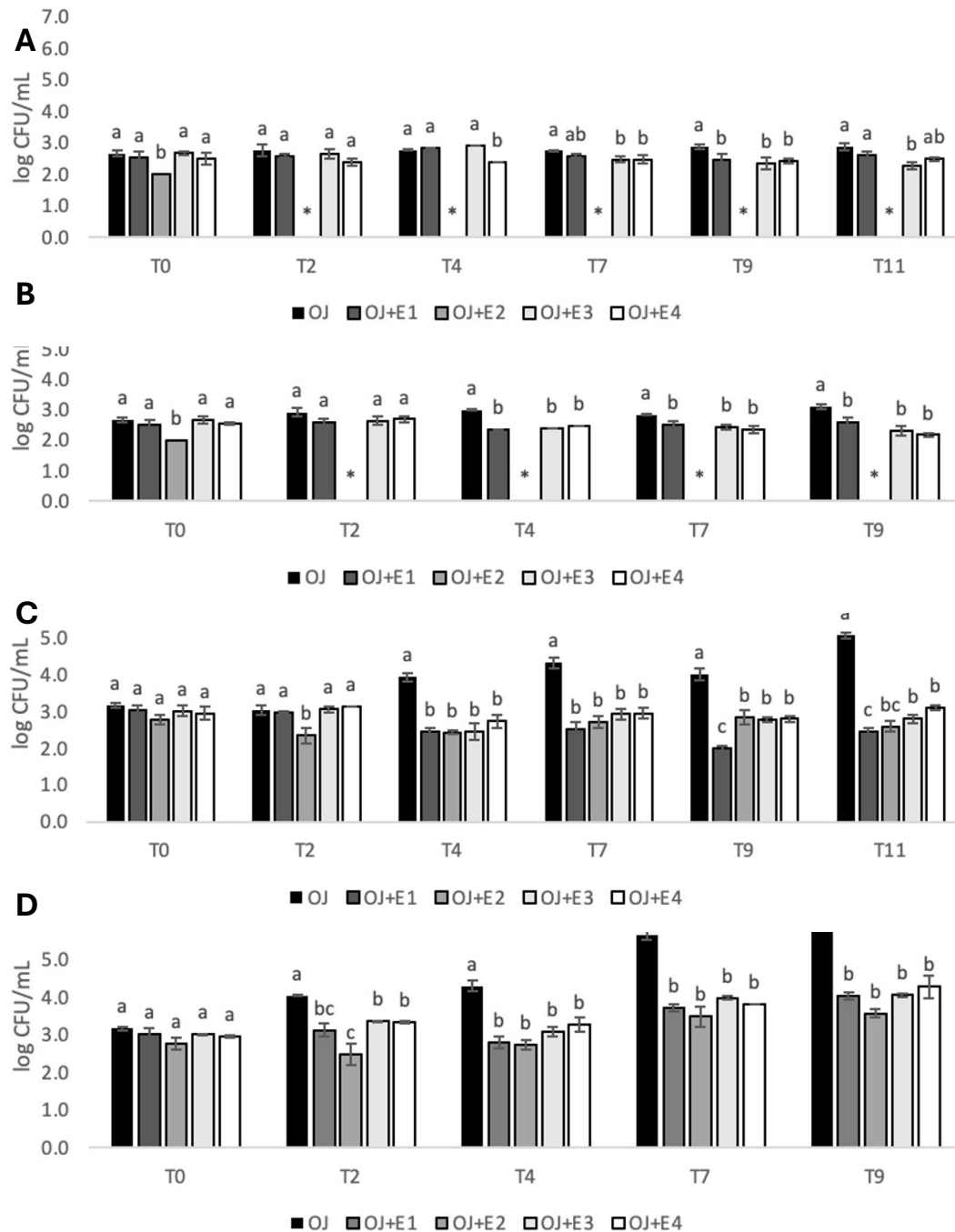
To evaluate the effect of the presence of the extracts on a known microbial population, a challenge test was performed following the inoculation in the juice of *Lactiplantibacillus plantarum* and *Saccharomyces cerevisiae* at a cell load of approximately 3.0 log CFU/mL. These microorganisms have been chosen because they were isolated from spoiling beverages (Patrignani et al., 2020).

The juices added or not with the extracts or the NADES and inoculated with the microorganisms were stored at 4°C and 8°C for 11 and 9 days respectively (Tables S6-S7). As is shown in Figure 4A, *L. plantarum*, at both storage temperatures, showed an initial cell load of about 2.7 log CFU/mL, regardless of the samples. A slight increase of the cell load was observed in control samples that showed significantly higher loads than the other samples starting from day 9 at 4°C and from day 4 at 8°C. Samples OJ+E1, OJ+E3, and OJ+E4, showed very similar loads of *L. plantarum* throughout the entire storage period (11 days at 4°C and 9 days at 8°C). The only exception was the OJ+E2 samples, in which *L. plantarum* was already below the detection limit (0.5 log CFU/mL) after just 2 days of storage at both storage temperatures.

On the other hand, these samples were characterized by the lowest pH, around 2.0, a pH level at which *L. plantarum* was not able to survive. However, it is important to note that in all the other samples with higher pH levels, around 3.0, *L. plantarum* could survive but not grow. On the other hand, other authors indicate a pH_{min} for this species ranging between 3.2 and 3.5 (Aryani et al., 2016). In the case of juices inoculated with *S. cerevisiae* at a level of approximately 3.0 log CFU/mL and stored at 4°C, the cell load remained more or less similar throughout the whole storage period in the samples added with extracts (OJ+E1- OJ+E4).

In contrast, in the control sample, a significant increase in yeast cell load was observed starting from the fourth day of storage, and after 11 days of storage, the load exceeded 5.0 log CFU/mL. In the case of juices stored at 8°C, the growth kinetics of *S. cerevisiae* were faster in the control samples compared to 4°C. Control samples showed a significant increase in yeast cell load after just two days of storage. The control samples were already spoiled after 7 days of storage, as *S. cerevisiae* reached a cell load of about 6.0 log CFU/mL which is considered the spoiling threshold for this microorganism (Krisch et al., 2016). In contrast, none of the samples with added extracts was spoiled after 9 days of storage at 8°C, except samples OJ+N3 (Table S8). All these samples showed a similar growth kinetics of *S. cerevisiae*; a significant increase in cell load was observed starting from the seventh day of storage. However, after 9 days of storage, the yeast load in all samples with added extracts did not exceed 4.0 log CFU/mL.

The results of the challenge test are in agreement with those observed in the shelf-life trials and indicate that the addition of extracts with antimicrobial activity is capable of preventing the growth of spoilage bacteria and yeasts, allowing an extension of the shelf life of orange juices supplemented with these extracts.



Results are given by the statistic mean and SD of three different replicates. (a-d): Different letters indicate statistical differences between the storage times ($p < 0.05$). OJ: pure orange juice, OJ+E1: orange juice with choline chloride:glycerol, OJ+E2: orange juice with choline chloride:glycerol:citric acid, OJ+E3: orange juice with choline chloride:fructose, OJ+E4: orange juice with choline chloride:xylose. A: *L. plantarum* 4 °C. B: *L. plantarum* 8 °C. C: *S. cerevisiae* 4°C. D: *S. cerevisiae* 8 °C. (*) under detection limit (0.5 log CFU/mL).

Figure 4. *L. plantarum* and *S. cerevisiae* cell loads (log CFU/mL) of the different juice samples stored at 4°C and 8°C

The incorporation of NADES into the juice matrix has had a significant impact on extending the shelf life of the product while preserving its sensory attributes. Juice spoilage is primarily caused by microbial growth and enzymatic activity, which were suppressed by the protective environment created by NADES, showing a significant reduction in spoilage rates when compared to control samples. The juice formulation proved to be a successful way to extend the shelf life of the product, opening up a potential new direction for the development of more useful and stable beverages having many potential applications in the food industry. This application can be very helpful in the production of functional foods, nutraceuticals and fortified beverages, where the increasing of the shelf-life is critical. The use of NADES provides manufacturers with creative ways to improve product quality, shelf life and utility, while satisfying consumer demand for natural, sustainable ingredients.

2. Conclusion

The present study formulated an orange juice using NADES extracts as ingredients. Showing that, the addition of these extracts increased the shelf-life of the juice just by the addition of this ingredients without any thermal treatment, at the same time that is able to maintain the color and pH properties of the newly formulated orange juice.

The results are promising, for improving the shelf-life of orange juice, without changing the organoleptic properties. The use of NADES extracts as a strategy to enhance the microbiological stability and shelf life of an orange juice, combining the benefits of natural preservation techniques with the potential to enrich the nutritional value of the control, NADES have been shown to maintain the quality and safety of this orange juice.

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DISCUSIÓN GENERAL

GENERAL DISCUSSION

La presente tesis doctoral se centra en el uso de los NADES como alternativas no tóxicas, sostenibles y verdes, a los solventes orgánicos convencionales para la extracción, caracterización y estabilización de los compuestos bioactivos obtenidos a partir de subproductos de la industria citrícola así como en el uso de técnicas de extracción que se enmarcan en los principios de la química verde y en sus aplicaciones tecnológicas en la industria alimentaria.

Para la realización de una discusión general más detallada, se han estructurado los diferentes capítulos en tres grandes bloques:

- Caracterización y formulación de los NADES, y extracción de compuestos bioactivos de los subproductos de matrices vegetales.
- Metodologías verdes y no convencionales de extracción y recuperación de compuestos bioactivos.
- Aplicaciones tecnológicas en la industria alimentaria de los extractos obtenidos con NADES.

CARACTERIZACIÓN Y FORMULACIÓN DE LOS NADES, Y EXTRACCIÓN DE COMPUESTOS BIOACTIVOS DE LOS SUBPRODUCTOS DE MATRICES VEGETALES

Los NADES vienen utilizándose desde hace varios años como una nueva clase de solventes verdes y sostenibles para la extracción de compuestos bioactivos de matrices vegetales. Estos solventes, han sido estudiados y caracterizados en diferentes proporciones molares de sus componentes.

Dai et al. (2013) propusieron por primera vez el uso de NADES para la extracción de los compuestos bioactivos. También observaron que su mezcla con agua en diferentes porcentajes, útil para disminuir su viscosidad y aumentar su aplicabilidad, resulta en un factor limitante para la mezcla eutéctica, siendo un

50% de agua (v/v), la cantidad máxima aceptada para poder considerarse una mezcla eutéctica (Benvenuti et al., 2020). Los resultados obtenidos por Dai et al. (2015) muestran que las conexiones intramoleculares que se forman en la mezcla eutéctica confieren unas propiedades únicas de polaridad, solubilidad, densidad, viscosidad, etc. Esto demuestra que los NADES, en función de sus componentes, su proporción molar y contenido de agua, pueden ser diseñados a medida para aplicaciones experimentales específicas, especialmente en la extracción de diversos componentes de la matriz estudiada.

Las limitaciones de los NADES engloban, su escalabilidad industrial, ya que esto requeriría una síntesis y una integración en los procesos industriales rentables. Además, dependería de la infraestructura y procesos más sostenibles, así como de una inversión en la modificación de los procesos para adaptarlos a las características de los NADES. También, se necesitarían la aprobación completa de los organismos competentes de cada país en cuanto a la evaluación del impacto sobre la salud de las personas a largo plazo (Alirezai et al., 2015; Benlebna et al., 2018; Silva et al., 2020), sin embargo los componentes de los NADES si están aceptados como uso alimentario (FDA, 2024).

La creciente demanda de tecnologías sostenibles y ecológicas en la industria alimentaria, farmacéutica y cosmética ha impulsado la búsqueda y utilización de alternativas a los solventes convencionales. Estudios recientes han demostrado la eficacia de los NADES para la extracción de compuestos bioactivos de diferentes matrices vegetales. Debido a su baja toxicidad, biodegradabilidad y capacidad para actuar como solventes de compuestos polares y no polares se alinean con los principios de la química verde (Grisales-Mejía et al., 2024; Morgana et al., 2022; Nicolae et al., 2019; Vieira et al., 2022).

El uso de herramientas computacionales como COSMOtherm facilita la preselección de NADES basada en predicciones de solubilidad. Esto reduce el

ensayo y error experimental y ajusta las propiedades del solvente con las características del compuesto bioactivo diana (Panić et al., 2021; Zurob et al., 2020). Esta herramienta permite identificar las mejores características de los NADES para la extracción o uso específico.

Esta selección de los NADES más apropiados en función del compuesto bioactivo objeto de estudio o grupo de compuestos bioactivos, podría favorecer la solubilidad y la biodisponibilidad de los mismos, al tener una polaridad similar entre el solvente y el compuesto (Recharla et al., 2017).

La caracterización de los NADES estudiados constata que los basados en ácidos orgánicos, son más ácidos que los basados en azúcares y que aquellos con polialcoholes son más polares que los formados por ácidos, coincidiendo estos resultados con los obtenidos en otros estudios (Bakirtzi et al., 2016; Farooq et al., 2020; Fuad & Mohd Nadzir, 2022). Para poder caracterizar cada uno de los NADES objeto de este estudio, se registran los espectros FT-IR de cada componente y sus mezclas eutécticas a temperatura ambiente, los resultados revelan nuevas interacciones y la formación de enlaces de hidrógeno adicionales cuando los dos compuestos forman la mezcla eutéctica, en comparación con los compuestos por separado. Se evalúa la capacidad antioxidante de los NADES y de los extractos obtenidos, parámetro crucial para comprender el papel protector de estos solventes sobre los compuestos bioactivos. Los resultados obtenidos muestran que los NADES por si solos tienen capacidad antioxidante y los resultados difieren según el método de determinación aplicado, posiblemente debido al diferente fundamento de los métodos. La mezcla ChChl:TA mostró la mayor capacidad antioxidante según el método DPPH, seguida de ChChl:LA, mientras que ChChl:Xyl presentó el valor más bajo. Al aplicar el método ORAC, ChChl:LP:MA fue el NADES con mayor capacidad antioxidante, mientras que ChChl:Gly proporcionó los valores más bajos. Los

resultados obtenidos por el método ORAC en NADES que contienen ácidos orgánicos fueron similares a los obtenidos por otros autores (Mitar et al., 2019), quienes observaron un importante aporte del ácido málico y la prolina en la neutralización del radical AAPH. Además, en el presente trabajo se ha demostrado que las propiedades fisicoquímicas de los NADES pueden estar relacionadas con su capacidad antioxidante.

Recientemente se ha demostrado la eficacia de los NADES para la extracción de compuestos fenólicos de los residuos del aguacate (Grisales-Mejía et al., 2024), del hollejo de cereza (Popovic et al., 2022), de la piel de las avellanas (Fanali et al., 2021), del hollejo de uva (Panić et al., 2019), piel de naranja (Ozturk et al., 2018; Panić et al., 2021; Shehata et al., 2021; Viñas-Ospino et al., 2023), subproductos de las cebollas (Pal & Jadeja, 2019), piel de uva (Cvjetko Bubalo et al., 2016), aceite de oliva (Bonacci et al., 2020; Buldo et al., 2019; Carmona et al., 2023; Chanioti & Tzia, 2018; García et al., 2016; Morgana et al., 2022) entre otras matrices vegetales. Dichos estudios ponen de manifiesto el gran poder extractante de los NADES y la afinidad por los compuestos fenólicos.

Por ello, en la presente tesis doctoral se realiza una optimización de las mejores condiciones de extracción para TPC y TFC mediante la metodología de respuesta de superficie, donde se optimizan los distintos parámetros (%NADES (v/v), ratio de extracción (p/v) y el tiempo de extracción (min), de los dos mejores NADES obtenidos con el software COSMOTherm (LP:MA y LA:Glu) para la solubilidad de la hesperidina. Para poder evaluar el efecto de las variables consideradas en las respuestas, se utiliza un diseño de Box-Behnken, este diseño ha sido ampliamente utilizado en otros estudios con NADES (Argun et al., 2022; Chen et al., 2021; Santos-Martín et al., 2023; Wang et al., 2024).

Una ventaja fundamental de los NADES es su capacidad para estabilizar compuestos bioactivos. Por ejemplo, el ácido ascórbico y los compuestos

fenólicos, que son propensos a la oxidación y degradación, muestran una mayor estabilidad cuando se disuelven en NADES. Este efecto de estabilización se puede atribuir a la capacidad de los NADES para formar enlaces de hidrógeno e interacciones con los compuestos bioactivos, reduciendo su exposición a entornos oxidativos.

Se ha demostrado que la capacidad antioxidante de los NADES puede contribuir a la estabilidad de los compuestos bioactivos después de la extracción de las matrices vegetales, desempeñando un papel protector en la prevención de la degradación. Para ello, la presente tesis doctoral ha estudiado la estabilidad del ácido ascórbico en catorce NADES diferentes. Los resultados muestran que a 4 °C el AA es estable en la mayoría de los solventes. En particular, la menor tasa de degradación se observa en los solventes a base de azúcares y polialcoholes, donde el AA es más estable en ChChl:Xyl, seguido de ChChl:Gly. La degradación es mayor en ChChl:CA y ChChl:TA. Además, LP:MA y LA:Glu son los solventes basados en ácidos orgánicos que mostraron los mejores resultados en la preservación del contenido de ácido ascórbico, siendo también los mejores NADES para la extracción de la hesperidina predichos *in silico* por el COSMOtherm. A 25 °C, el control (estabilidad del AA en Agua MilliQ) mostró un aumento de aproximadamente 10 veces en la tasa de degradación de AA, respecto a los otros NADES estudiados. Esto demuestra, que los NADES pueden ser incorporados en la formulación de alimentos para aumentar su estabilidad, aumentando su vida útil. Otros estudios han demostrado el mecanismo protector de los NADES, donde estos mejoran eficazmente la actividad antioxidante de los compuestos extraídos (Dai et al., 2016; Delgado-Mellado et al., 2018; Fu et al., 2021; Grillo et al., 2020; Mustafa et al., 2021).

También se ha estudiado la estabilidad de diferentes compuestos fenólicos (ácido 4-hidroxibenzoico, ácido clorogénico, rutina, naringenina, catequina, ácido ferúlico, naringina, hesperidina, hesperitina, ácido cafeico, ácido p-cumárico, ácido vanílico, narirutina y quercitina) en ChChl:MA y ChChl:Gly, para ello se almacenan a temperatura ambiente durante 12 días y se demuestra que los compuestos fenólicos son más estables en ChChl:MA, posiblemente debido a la formación de enlaces de hidrógeno con el soluto (Jeong et al., 2017) y a su afinidad. Solamente, la hesperidina, el ácido clorogénico, el ácido ferúlico, la naringina y la naringenina fueron detectables hasta los 12 días de almacenamiento.

El presente trabajo ha demostrado que los rendimientos de extracción de los NADES estudiados son superiores al etanol en un 50% en el caso del AA y de compuestos fenólicos como el ácido 4-hidroxibenzoico, ácido vainílico, ácido clorogénico, ácido ferúlico, ácido p-cumárico, ácido cafeico y catequina. Estos resultados sugieren que estos NADES podría utilizarse en la industria alimentaria como alternativa a los disolventes orgánicos en el proceso de extracción.

METODOLOGÍAS VERDES Y NO CONVENCIONALES DE EXTRACCIÓN Y RECUPERACIÓN DE COMPUESTOS BIOACTIVOS

Las técnicas de extracción no convencionales surgieron para abordar los desafíos ambientales y económicos asociados con los métodos tradicionales, como el uso de tecnologías térmicas con alto gasto de energía.

La UAE, la MAE y las HPP han demostrado, en diferentes estudios, tener mayor rendimiento de extracción que los métodos térmicos convencionales, como la extracción por HS. Diversos estudios demuestran la eficacia de los métodos no convencionales en la extracción de compuestos fenólicos de matrices vegetales, como de los subproductos de granadas (Kaderides et al., 2015), tomates (Lianfu

& Zelong, 2008), cítricos (Anticono et al., 2021; Montero-Calderon et al., 2019.), mango (Lanjekar et al., 2022; Plaza et al., 2021) y de la industria vinícola (Xue et al., 2020), entre otros. Esto demuestra la gran eficacia de los métodos no convencionales, no térmicos y sostenibles para la extracción de flavonoides, polifenoles y antocianinas. Además, estas técnicas pueden ser utilizadas conjuntamente con los NADES para realizar el proceso más eficiente, pudiendo aplicarse en las industrias alimentaria, cosmética y farmacéutica, permitiendo la recuperación eficiente y sostenible de compuestos bioactivos ofreciendo un equilibrio entre la eficiencia de los procesos, la sostenibilidad y su posterior utilización sin necesidad de purificar los extractos (da Silva et al., 2021; Panić et al., 2019). Los resultados obtenidos en la presente tesis doctoral concuerdan con los expuestos anteriormente, donde la UAE, demuestra mayor extracción que las técnicas convencionales, además de la posibilidad de combinar su uso con distintos NADES, de manera que el proceso resulte totalmente sostenible.

La UAE altera las paredes celulares de las plantas, facilitando la liberación de compuestos bioactivos en los solventes sin necesidad de un calentamiento adicional de la muestra, evitando su degradación. Se espera que la UAE extraiga un mayor número de compuestos bioactivos que al aplicar HS, debido a su capacidad para romper las paredes celulares por cavitación. Utilizando UAE y ChChl:Fruc el rendimiento de extracción de TPC fue el más alto. Por otra parte, ChChl:LA extrajo más del doble de compuestos fenólicos por UAE que por HS. En cuanto a la hesperidina, el principal compuesto fenólico que se encuentra en la piel de naranja, la UAE obtiene mejores resultados con ChChl:Fruc, ChChl:LP:MA y ChChl:LA, en comparación con HS. Sin embargo, la extracción con ChChl:Gly es mayor con HS.

En el presente estudio se demuestra que la selección del método de extracción ya sea UAE o HS, es importante para determinar la eficacia de NADES. Por

ejemplo, con la UAE la concentración de compuestos bioactivos es mayor (Anticono et al., 2021; Cao et al., 2023; Obluchinskaya et al., 2023). Sin embargo, la estabilidad de los compuestos bioactivos a lo largo del tiempo es mayor al realizar la extracción con HS, particularmente en el mantenimiento de la capacidad antioxidante. La comparación demuestra que si bien los NADES son solventes verdes eficaces, su rendimiento depende del método aplicado. Si la UAE puede ser la opción óptima para maximizar el rendimiento de la extracción de los compuestos bioactivos, la aplicación de HS puede ser el mejor método para garantizar la estabilidad a largo plazo. Esto pone de manifiesto la necesidad de la selección tanto del NADES como del método de extracción para lograr un equilibrio óptimo entre eficiencia y estabilidad.

APLICACIONES TECNOLÓGICAS EN INDUSTRIA ALIMENTARIA DE LOS EXTRACTOS OBTENIDOS CON NADES

El uso de NADES en la obtención de extractos bioactivos ha abierto nuevos horizontes para la industria alimentaria. Estos solventes verdes permiten la extracción, estabilización e integración de compuestos como polifenoles, flavonoides y vitaminas, ofreciendo tanto sostenibilidad como funcionalidad.

Para poder conocer si los NADES son aptos para ser añadidos a una matriz alimentaria, aumentando sus propiedades y vida útil, se analiza la actividad antimicrobiana de cuatro extractos de NADES basados en cloruro de colina (ChChl:Fruc, ChChl:Xyl, ChChl:Gly, ChChl:Gly:CA) y se compara con la actividad antimicrobiana del extracto etanólico (EtOH 50%, v/v). Los resultados muestran que la actividad antimicrobiana de los extractos se limita a las cepas de patógenos comunes transmitidos por los alimentos (*E. coli*, *S. aureus* y *S. epidermidis*). Sin embargo, ninguno de los extractos tuvo un efecto inhibitor sobre el crecimiento de *L. monocytogenes*. No obstante, los extractos obtenidos con ChChl:Gly:CA tuvieron la mayor actividad antimicrobiana hacia

las especies sensibles, que se mantuvo después de diluir el extracto en agua a partes iguales. La eficacia antimicrobiana de los extractos de NADES es similar a la de los extractos de EtOH (50%, v/v). La sensibilidad de los microorganismos a los antimicrobianos naturales dependen de la cepa (Wiktorczyk-Kapischke et al., 2021), esto se corrobora con los resultados de este estudio, donde se muestra la resistencia de la cepa de *L. monocytogenes* a los extractos de NADES utilizados, sin embargo las otras cepas (*E. coli*, *S. aureus* y *S. epidermidis*), que comprenden bacterias Gram-positivas y Gram-negativas, mostraron una sensibilidad comparable a todos los antimicrobianos probados.

Se procede a la formulación de un zumo de naranja, adicionado con extracto de piel de naranja obtenido utilizando estos cuatro NADES. Para estudiar el perfil sensorial de las muestras de zumo de naranja enriquecidas con diferentes porcentajes de extractos de NADES (del 5% al 50%), se utilizan la lengua y la nariz electrónicas, lo que permite clasificarlas según sus propiedades sensoriales. Considerando las características del zumo de naranja, se elige el porcentaje más alto de extracto (15%) presente en todos los zumos adicionados estudiados. Aunque algunos zumos adicionados pueden tener porcentajes más altos con características similares al zumo de naranja, se selecciona el 15% por ser el porcentaje coincidente más alto. Se caracteriza (color y pH) el zumo de naranja adicionado con un 15% de extracto de NADES. Uno de los factores más importantes para determinar la aceptabilidad de un producto, como el zumo de naranja, por parte de los consumidores es su color (Steur et al., 2023). Este factor puede incluso afectar la forma en que el consumidor percibe el sabor de la bebida. Se observan cambios de color notables durante el almacenamiento en todas las muestras analizadas (Wibowo et al., 2015), sin embargo, todas las muestras presentaron tonalidades hacia el amarillo, lo que indicaría una aceptabilidad por parte de los consumidores (Spence, 2019).

Además, se analiza el perfil fenólico, ya que puede influir en el color y el sabor del zumo. La concentración de cada compuesto fenólico depende del extracto utilizado para la formulación. La hesperidina presenta una mayor concentración en el zumo de naranja adicionado con el extracto de ChChl:Gly, y menor con ChChl:Gly:CA. Sin embargo, no se encuentran diferencias estadísticamente significativas en la concentración de rutina presente en las diferentes formulaciones.

Para conocer la estabilidad microbiológica del zumo de naranja formulado con un 15% de extracto de NADES, se evalúa la estabilidad de todas las muestras durante 13 días de almacenamiento a dos temperaturas diferentes (4 °C y 8 °C como temperatura de abuso térmico). El monitoreo incluye bacterias aeróbicas mesófilas totales, levaduras y mohos y bacterias lácticas. Las levaduras representan el grupo microbiano predominante en todas las muestras a lo largo de todo el período de vida útil, mostrando cargas similares o superiores a las de bacterias aeróbicas mesófilas totales. En el caso de bacterias lácticas, estas están presentes en concentraciones inferiores a 2,0 log UFC/mL, independientemente del tipo de muestra, el tiempo de muestreo y la temperatura de almacenamiento. Las cargas de levadura en las muestras de control (zum de naranja puro) durante el almacenamiento han sido muy similares en ambas temperaturas, alcanzando una concentración de 7,90 log UFC/mL al final del almacenamiento. Por el contrario, las muestras adicionadas con los extractos muestran cargas de levadura inferiores a 3,0 log UFC/mL hasta el 9° día de almacenamiento, y de entre 5,0 y 5,9 log UFC/mL a los 13 días. Se ha demostrado que la cinética de crecimiento de las levaduras y el bacterias aeróbicas mesófilas totales depende tanto del tipo de extracto de NADES como de la temperatura de almacenamiento.

Además, se realiza una prueba challenge test (prueba de exposición), inoculando en el zumo *L. plantarum* y *S. cerevisiae* a una carga celular de aproximadamente 3,0 log UFC/mL. Se eligen estos microorganismos porque han sido aislados a partir de bebidas en mal estado (Patrignani et al., 2020).

Los resultados del challenge test concuerdan con los observados en los ensayos de vida útil e indican que la adición de extractos con actividad antimicrobiana es capaz de prevenir el crecimiento de bacterias y levaduras de deterioro, permitiendo una extensión de la vida útil de los zumos de naranja adicionados con estos extractos.

Los resultados demuestran que la adición de un 15% de extracto de NADES al zumo de naranja, tiene un impacto significativo en la extensión de la vida útil del producto, al tiempo que preserva sus cualidades sensoriales. Por lo tanto, la aplicación de los extractos con NADES puede resultar en una nueva oportunidad para la industria alimentaria en la producción de alimentos funcionales, nutracéuticos y bebidas fortificadas.

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CONCLUSIONES GENERALES

GENERAL CONCLUSIONS

Del estudio realizado se pueden establecer las siguientes conclusiones:

1. Al estudiar el papel protector de los NADES frente a la degradación de los compuestos bioactivos y evaluar su eficacia como vehículo para la liberación de compuestos de interés a través de membranas biológicas, se demuestra que:
 - Los NADES presentan capacidad antioxidante y extienden el tiempo de vida media del ácido ascórbico frente a un control con agua, siendo a 4 °C siete veces mayor en cloruro de colina:xilosa y a 25 °C el doble en cloruro de colina:glicerol.
 - El ácido ascórbico en ácido láctico:glucosa presenta el mayor coeficiente de permeabilidad, tanto en la barrera gastrointestinal (LogP_e : -4.99) como en la epitelial (LogP_e : -4.78).
2. Al optimizar los extractos obtenidos con diferentes NADES de la piel de naranja y valorar la estabilidad de los polifenoles durante 30 días se obtiene que:
 - Mediante el uso del COSMOtherm y la validación experimental, se selecciona el NADES ácido láctico:glucosa, optimizando las condiciones de extracción en 30 min, con un 80% de NADES y un ratio de 1 g de piel de naranja en 9,36 mL de NADES, obteniendo 4862 mgGAE/100g **p.s.** de fenoles solubles totales. Por otra parte, el ensayo de estabilidad muestra que, a los 30 días de almacenamiento, se mantiene un 85% del valor inicial de fenoles solubles totales.
3. Al aplicar los principios de la química verde, a ocho NADES seleccionados basados en cloruro de colina y determinar los compuestos fenólicos y el ácido ascórbico, así como su posterior estudio de estabilidad, se concluye que:

- El método más sostenible para un primer cribado es el método espectrofotométrico, debido a un menor uso de solventes.
- Entre los NADES basados en cloruro de colina con azúcares o polialcoholes y los basados en cloruro de colina con ácidos, el cloruro de colina:glicerol y el cloruro de colina:ácido málico son los que muestran el mejor rendimiento de extracción y la mayor capacidad antioxidante.
- La estabilidad de los compuestos fenólicos y del ácido ascórbico es mayor en el cloruro de colina:ácido málico que en el cloruro de colina:glicerol, observándose a partir del 7º día una disminución significativa de las concentraciones en los extractos con cloruro de colina:glicerol.

4. Al comparar los dos métodos de extracción (ultrasonidos y agitación en placa) utilizados en piel de naranja y NADES basados en cloruro de colina, se obtiene que:

- El NADES que presenta mayor rendimiento de extracción, con diferencias estadísticamente significativas entre ambos métodos, es el cloruro de colina:fructosa ($5,3 \pm 0,2$ y $6,1 \pm 0,1$ mgGAE/100mL, para agitación en placa y ultrasonidos, respectivamente). Si bien los ultrasonidos maximizan los rendimientos de extracción, con agitación en placa se obtienen extractos que presentan mayor estabilidad a largo plazo. El extracto más estable es el de cloruro de colina:ácido láctico obtenido con agitación en placa, con un 52% de fenoles solubles totales remanentes.
- Además, en la mayoría de los casos, la estabilidad de la capacidad antioxidante es mayor en los extractos obtenidos por agitación en placa, mostrando diferencias según el método de determinación: se retiene un 35,8% en cloruro de colina:ácido láctico por DPPH, un 38,3% en cloruro

de colina:fructosa por FRAP y un 46,2% en cloruro de colina:prolina:ácido málico por ORAC.

5. Al analizar la actividad biológica y el efecto antimicrobiano de extractos de piel de naranja extraídos con NADES basados en cloruro de colina, se encuentra que:

- Todos los extractos aumentan la actividad de la pepsina en comparación con el control, pero ninguno afecta a la actividad de la quimiotripsina. El extracto con cloruro de colina:glicerol:ácido cítrico, disminuye significativamente la actividad de la tripsina y de la amilasa.
- Los extractos presentan actividad antimicrobiana frente a *E. coli*, *S. aureus* y *S. epidermidis*, teniendo el mayor efecto inhibidor el extracto con cloruro de colina:glicerol:ácido cítrico. Sin embargo, al utilizar cloruro de colina:glicerol no se inhibe el crecimiento de *S. aureus*. Por otra parte, ninguno de los extractos tiene un efecto inhibidor sobre el crecimiento de *L. monocytogenes*.

6. Al formular una bebida a base de zumo de naranja con la adición de extractos de piel de naranja, obtenidos con NADES basados en cloruro de colina y estudiar su vida útil, se constata que:

- La adición de un 15% de extracto de NADES al zumo de naranja, aumenta la vida útil del producto, al tiempo que preserva sus cualidades sensoriales. Los zumos adicionados que presentan mayor y menor concentración de hesperidina son los formulados con colina:glicerol y colina:glicerol:ácido cítrico, respectivamente.
- Las cargas de levaduras en el zumo de naranja son muy similares 4 °C y 8 °C, alcanzando una concentración de 7,90 log UFC/mL al final del almacenamiento. Por otra parte, los zumos adicionados con los extractos

muestran cargas de levaduras inferiores a 3,0 log UFC/mL hasta el 9º día de almacenamiento, y de entre 5,0 y 5,9 log UFC/mL hasta el día 13. Las bacterias lácticas están presentes en concentraciones inferiores a 2,0 log UFC/mL, en todas las muestras.

- Durante el almacenamiento, la cantidad de etanol y acetyl acetato (productos del metabolismo de las levaduras) aumenta significativamente en el zumo de naranja, en comparación con los zumos adicionados. Además, se observan cambios de color en todas las muestras analizadas, presentando tonalidades hacia el amarillo.

From the conducted study, the following conclusions can be drawn:

1. Protective Role of NADES Against Degradation of Bioactive Compounds and Efficacy as a Delivery Vehicle Across Biological Membranes

- NADES exhibit antioxidant capacity and extend the half-life of ascorbic acid compared to water as a control, with an extension of sevenfold at 4°C in choline chloride:xylose and double at 25°C in choline chloride:glycerol.
- Ascorbic acid in lactic acid:glucose demonstrates the highest permeability coefficient, both in the gastrointestinal barrier (LogPe: -4.99) and the epithelial barrier (LogPe: -4.78).

2. Optimization of NADES-Extracted Orange Peel Extracts and Stability of Polyphenols Over 30 Days

- Using COSMOtherm and experimental validation, lactic acid:glucose NADES is selected, optimizing extraction conditions to 30 minutes with 80% NADES and a ratio of 1 g orange peel in 9.36 mL NADES, yielding 4862 mg GAE/100g dry weight of total soluble phenols. Stability tests indicate that 85% of the initial total soluble phenols remain after 30 days of storage.

3. Green Chemistry Principles in NADES-Based Phenolic and Ascorbic Acid Extraction and Stability Studies

- The most sustainable initial screening method is spectrophotometry due to reduced solvent usage.
- Among choline chloride-based NADES with sugars or polyalcohols versus acids, choline chloride:glycerol and choline chloride:malic acid demonstrate the best extraction yields and highest antioxidant capacity.

- The stability of phenolic compounds and ascorbic acid is higher in choline chloride:malic acid than in choline chloride:glycerol, with significant reductions in concentrations observed after day 7 in choline chloride:glycerol extracts.

4. Comparison of Extraction Methods (Ultrasound vs. Plate Shaking) for Orange Peel and Choline Chloride-Based NADES

- Choline chloride:fructose shows the highest extraction yield, with significant statistical differences between methods (5.3 ± 0.2 and 6.1 ± 0.1 mg GAE/100mL for plate shaking and ultrasound, respectively). Ultrasound maximizes extraction yields, while plate shaking produces extracts with greater long-term stability. The most stable extract is choline chloride:lactic acid via plate shaking, retaining 52% of total soluble phenols.
- Antioxidant stability is generally higher in extracts obtained via plate shaking, with differences observed depending on the determination method: 35.8% retention in choline chloride:lactic acid by DPPH, 38.3% in choline chloride:fructose by FRAP, and 46.2% in choline chloride:proline:malic acid by ORAC.

5. Biological Activity and Antimicrobial Effects of NADES-Based Orange Peel Extracts

- All extracts enhance pepsin activity compared to the control, but none affect chymotrypsin activity. The choline chloride:glycerol:citric acid extract significantly reduces trypsin and amylase activities.
- Extracts exhibit antimicrobial activity against *E. coli*, *S. aureus*, and *S. epidermidis*, with the highest inhibitory effect from the choline chloride:glycerol:citric acid extract. However, choline chloride:glycerol

does not inhibit *S. aureus* growth, and none of the extracts inhibit *L. monocytogenes* growth.

7. Formulation of Orange Juice Beverages with NADES-Based Orange Peel Extracts and Shelf-Life Study

- Adding 15% NADES extract to orange juice extends the product's shelf life while preserving sensory qualities. Juices with the highest and lowest hesperidin concentrations are formulated with choline:glycerol and choline:glycerol:citric acid, respectively.
- Yeast loads in orange juice are similar at 4°C and 8°C, reaching 7.90 log CFU/mL by the end of storage. Juices with extracts show yeast loads below 3.0 log CFU/mL until day 9 and between 5.0 and 5.9 log CFU/mL until day 13. Lactic acid bacteria remain below 2.0 log CFU/mL in all samples.
- During storage, ethanol and acetyl acetate levels (byproducts of yeast metabolism) increase significantly in orange juice compared to juice with extracts. Color changes are observed in all samples, with hues shifting towards yellow.

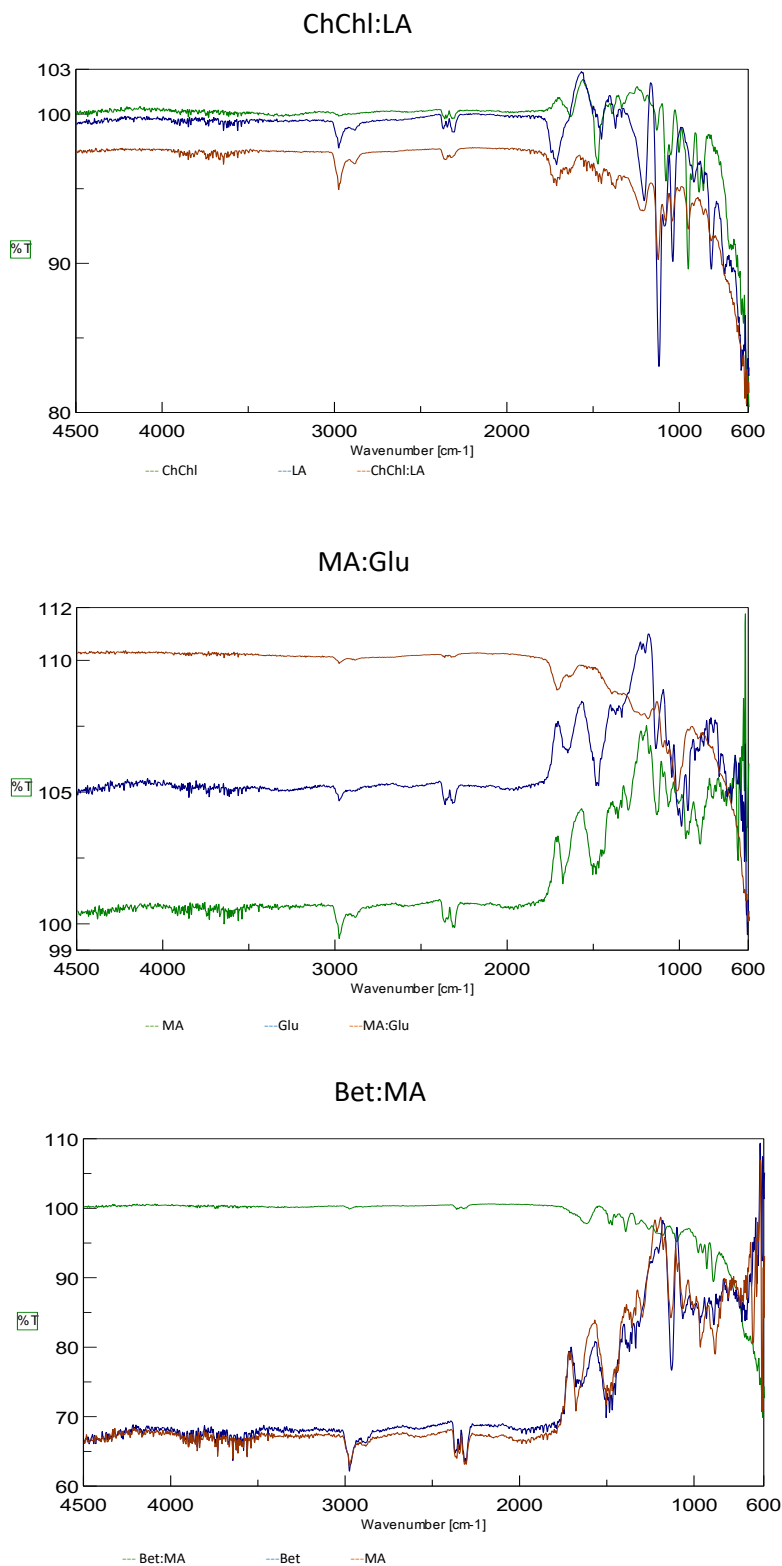


ANEXOS

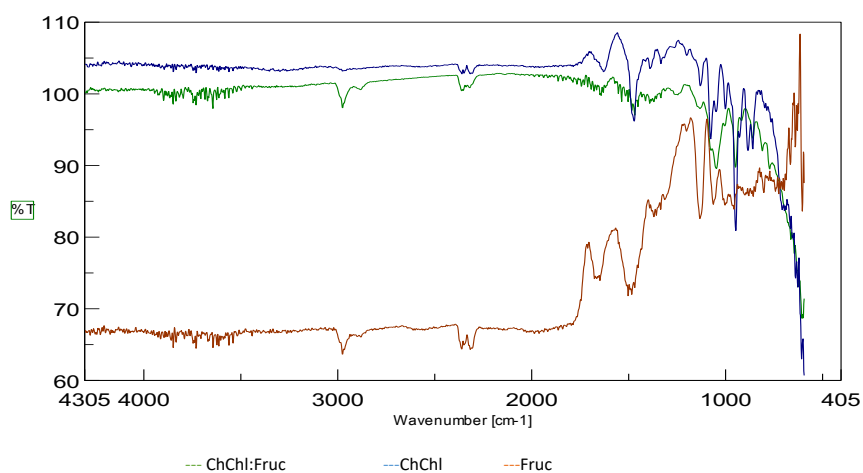
ANNEXES

Anexo I: Supplementary Material Chapter I

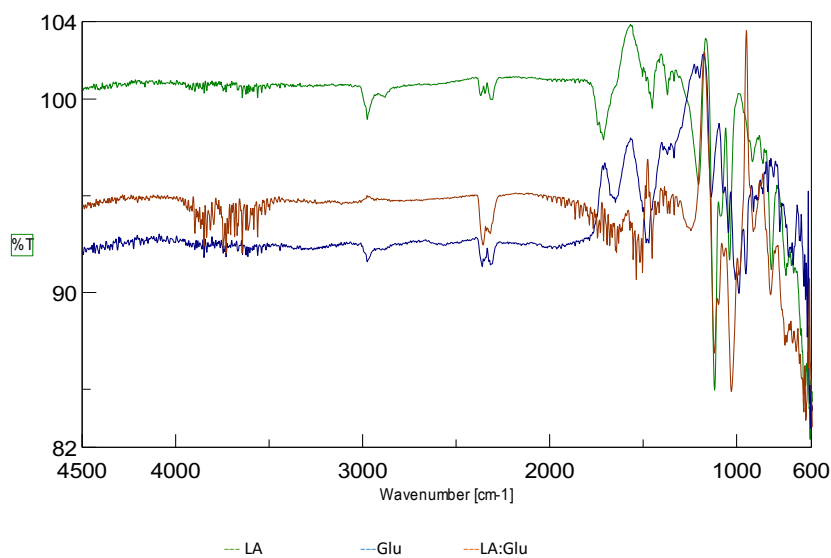
Figure S1. FTIR analysis of fourteen NADES studied and their components.



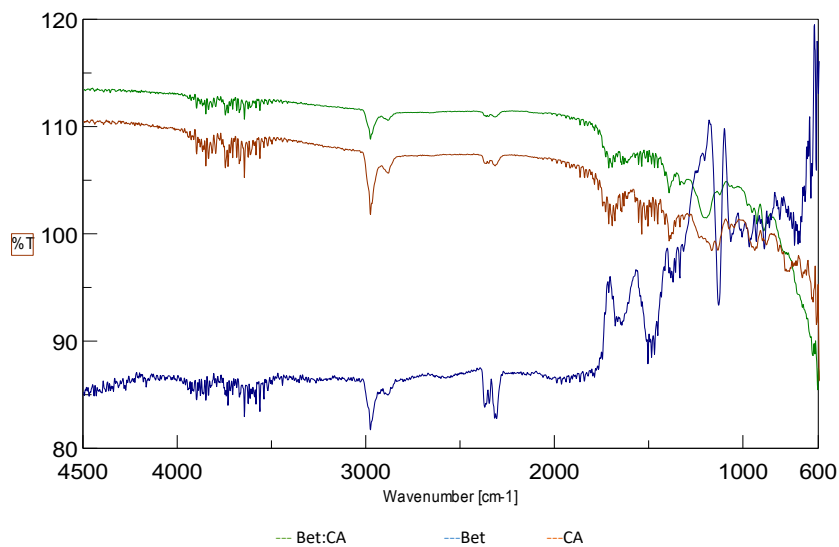
ChChl:Fruc



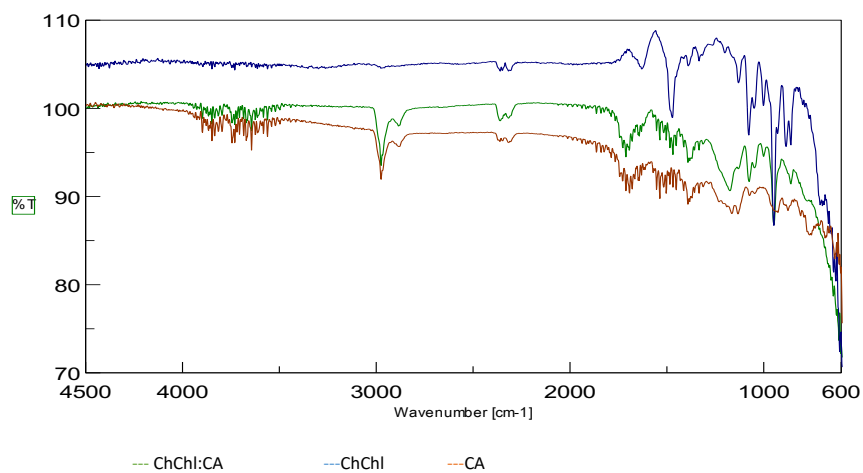
LA:Glu



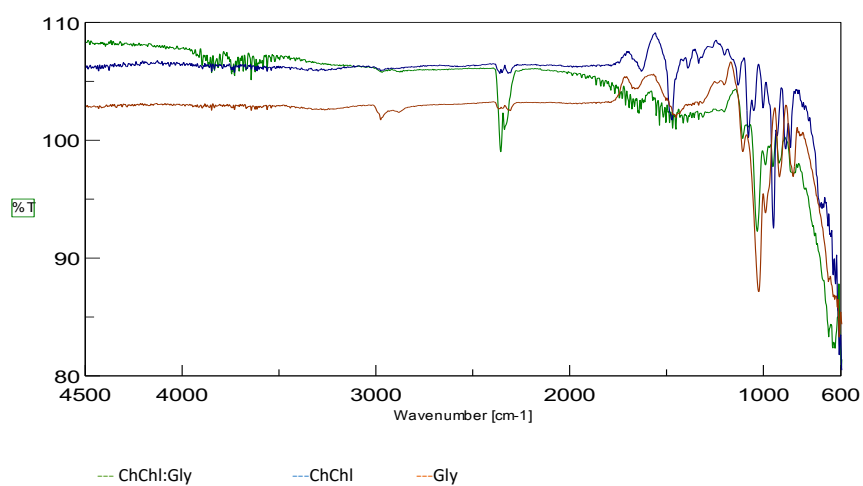
Bet:CA



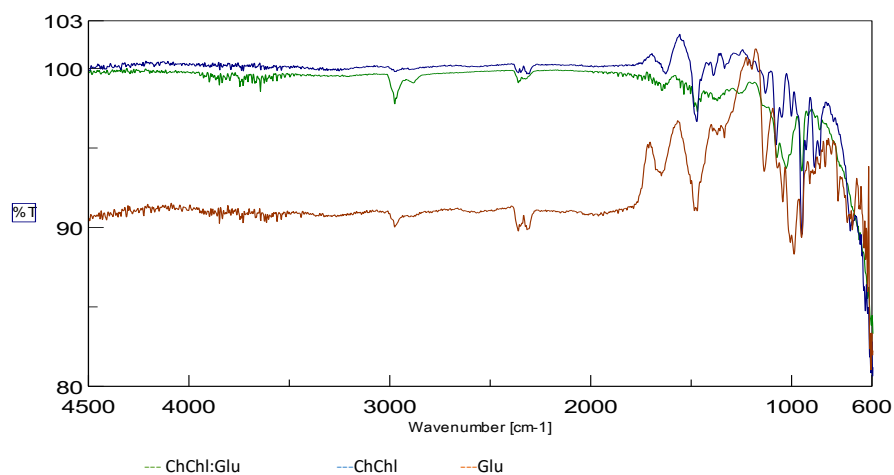
ChChI:CA



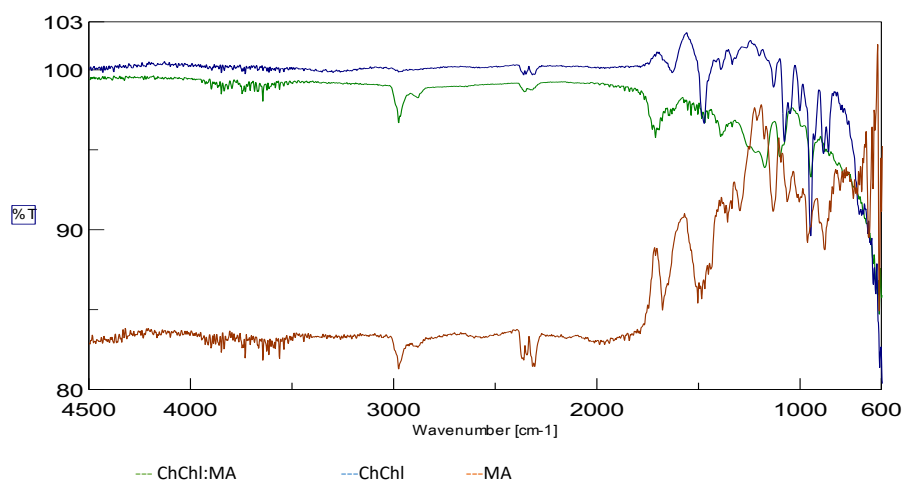
ChChI:Gly



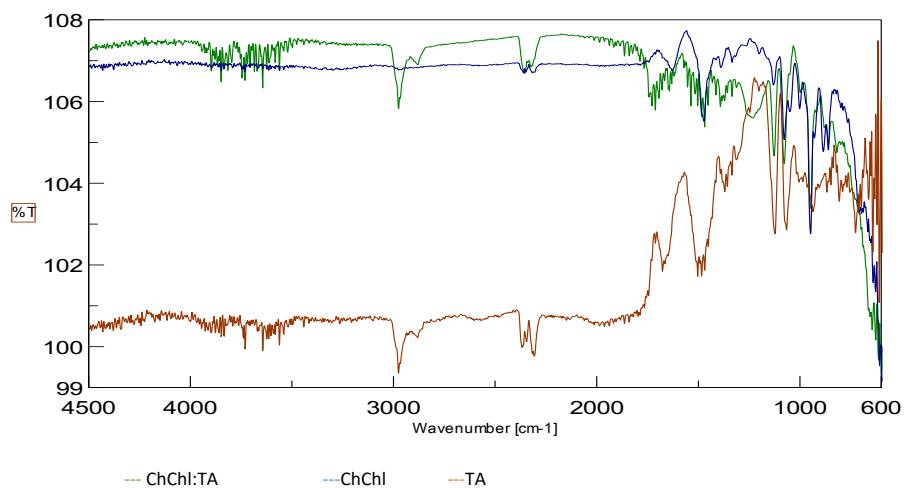
ChChI:Glu



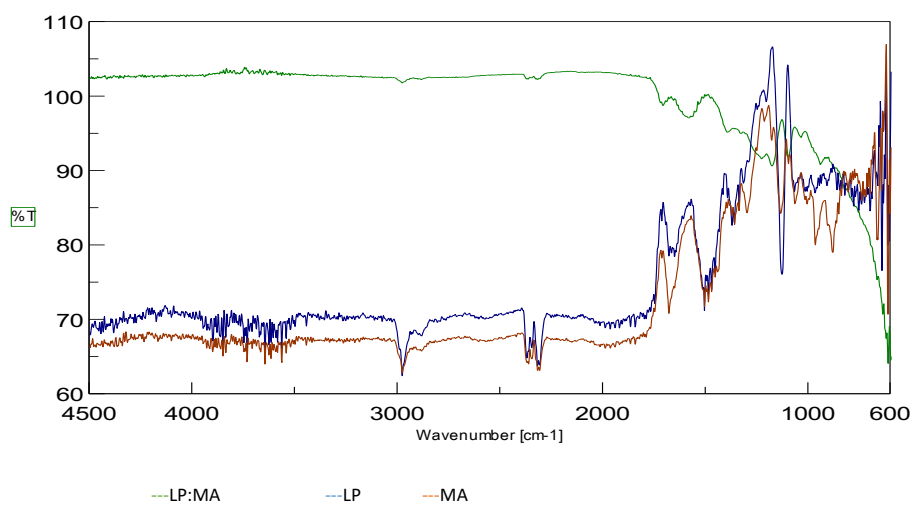
ChChI:MA



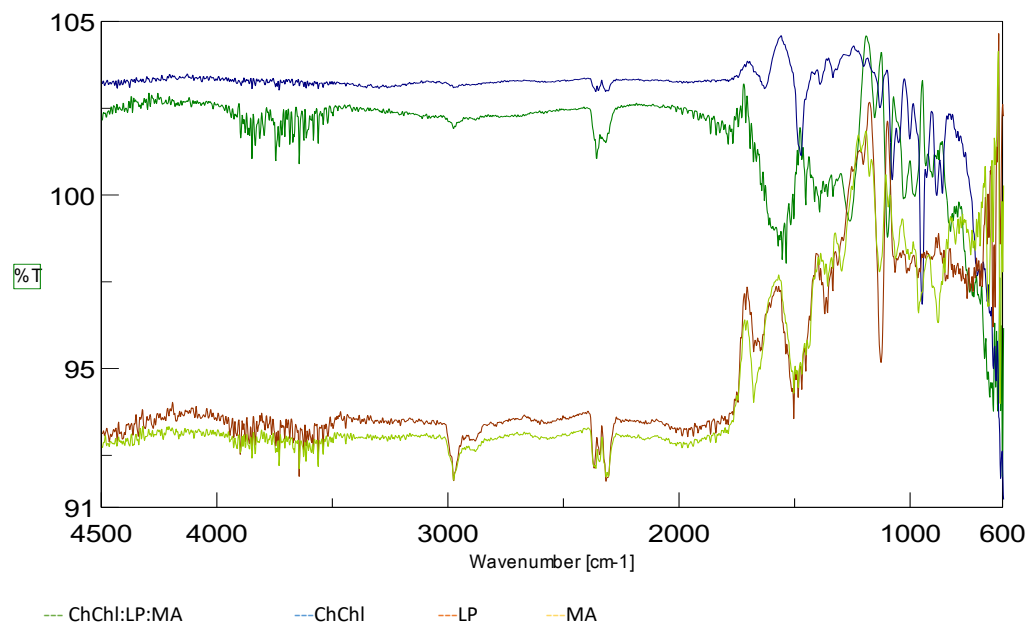
ChChI:TA



LP:MA



ChChI:LP:MA



ChChI:Xyl

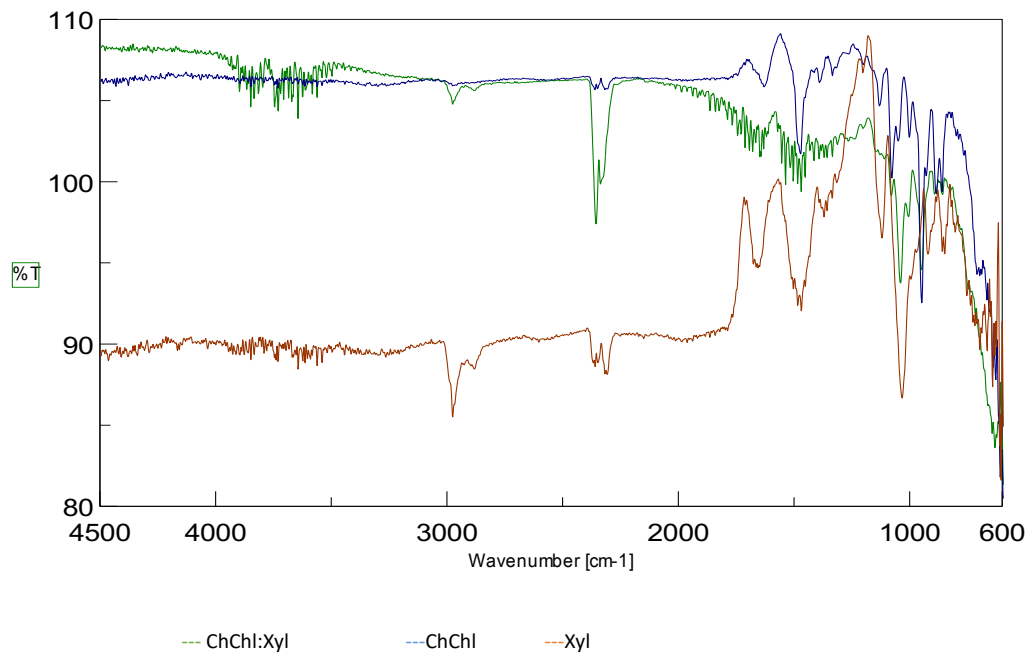
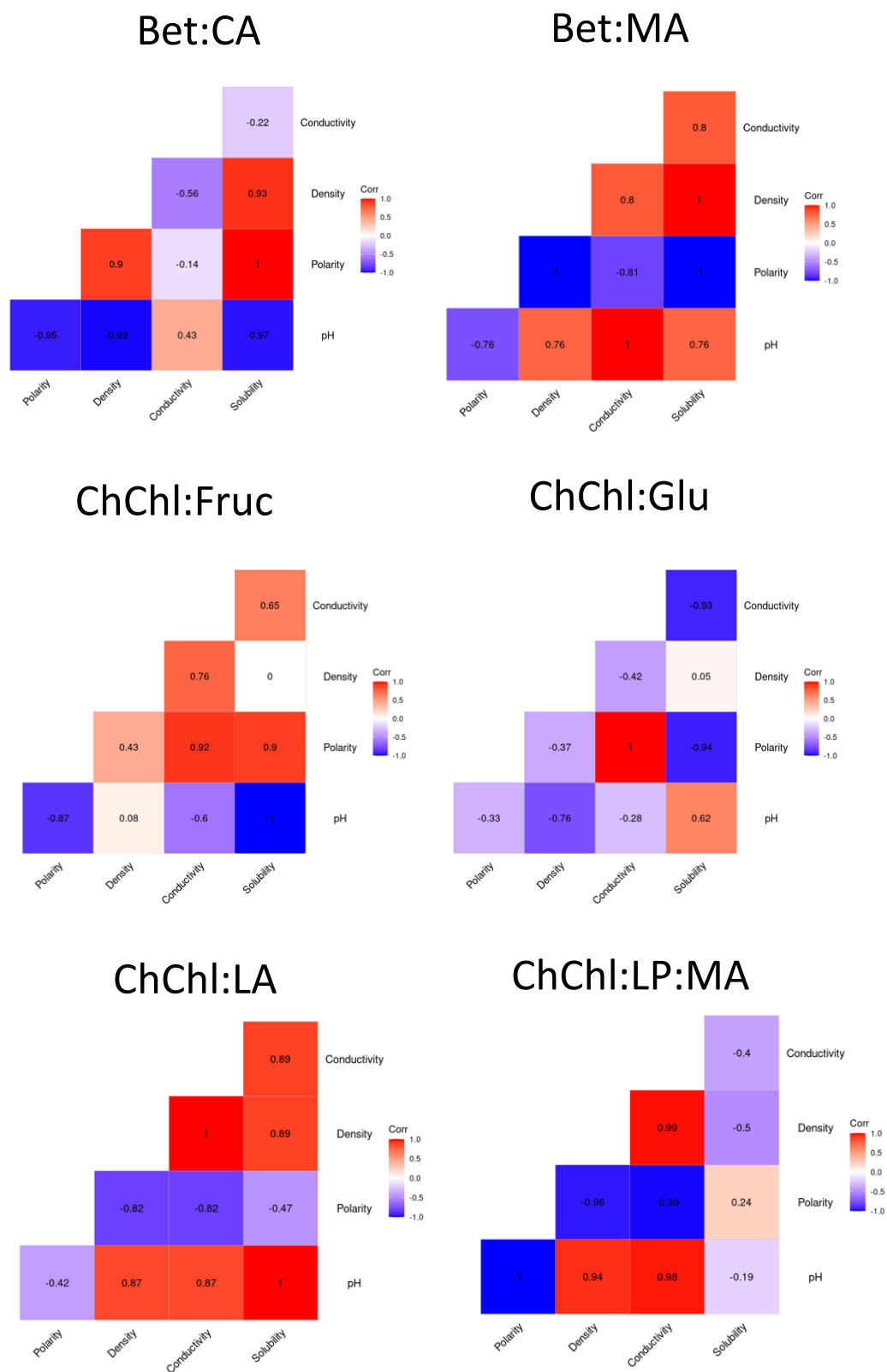


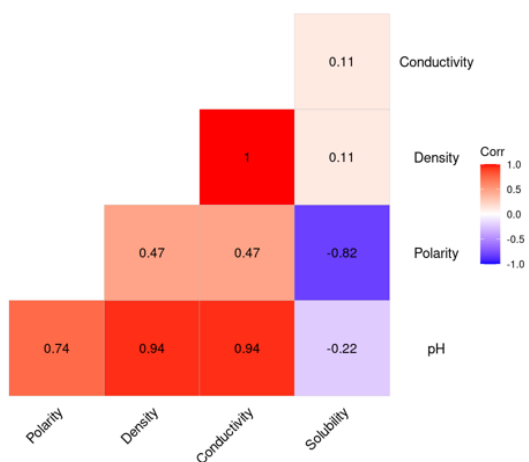
Figure S2. Correlation matrixes between the different physicochemical properties and the predicted solubility of ascorbic acid in different NADES



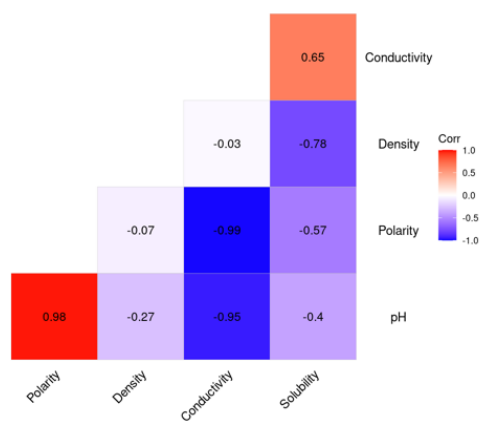
Continúa

Continuación

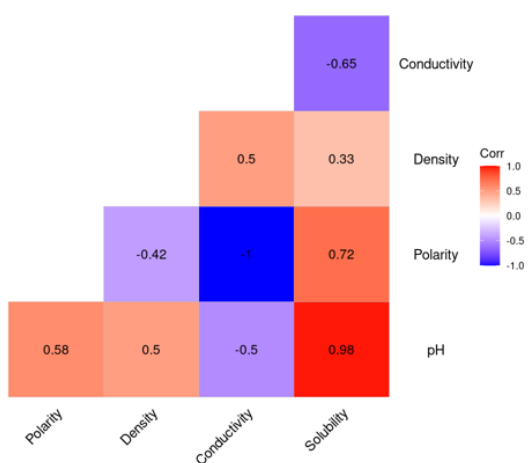
LA:Glu



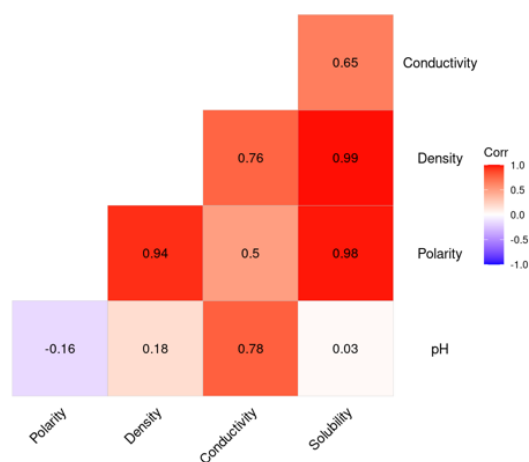
ChChl:MA



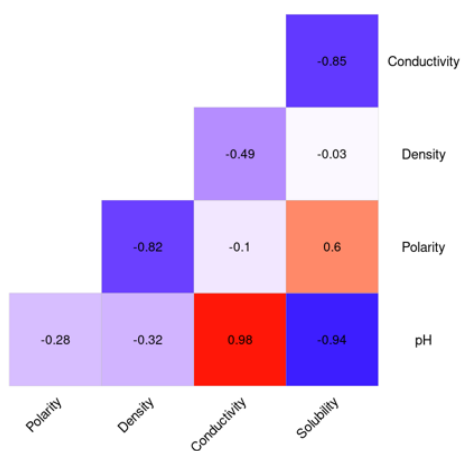
ChChl:TA



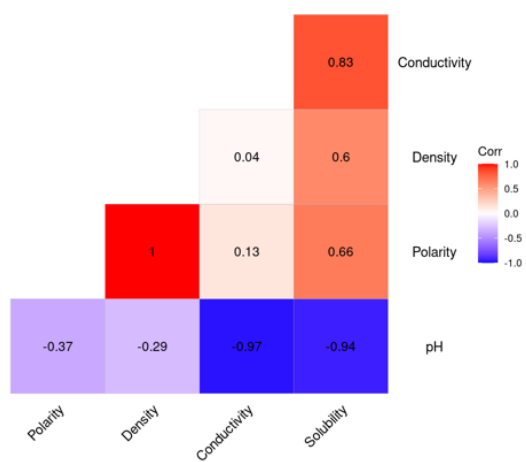
ChChl:Xyl



LP:MA



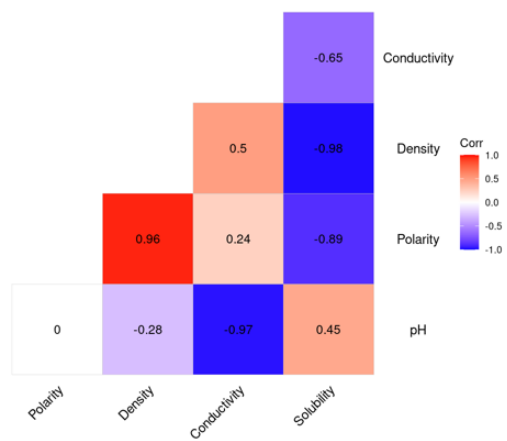
MA:Glu



Continúa

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ChChI:Gly



ChChI:CA

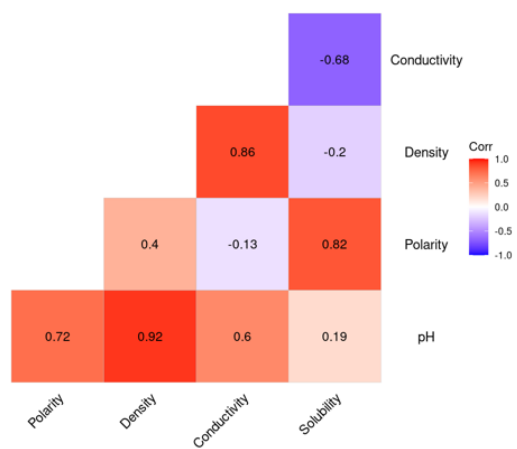


Table S1. Predicted $\ln \gamma_{\text{solute}}$ for ascorbic acid in 75% component (v/v) using COSMOtherm

Natural Component	$\ln (\gamma)$ 75% component
EtOH	-1,6426367
Xylose	-1,1697506
Citric Acid	-0,805363
Tartaric Acid	-0,7811088
Malic Acid	-0,7558087
Glucose	-0,7550654
Fructose	-0,6807057
Glycerol	-0,6804946
Lactic Acid	-0,6098188
L-Proline	-0,4945931
Choline Chloride	0,08185497
Betaine	0,65926947

Gray color, reference; yellow color, slightly worse solvent than reference; red color, worse solvent than reference.

Anexo II: Supplementary Material Chapter II

Table S1. Box-Behnken design with the independent variables and response data of the optimization for the suitable NADES for each bioactive compound

Run	Ratio (mL) X ₁	NADES(%) X ₂	Time (min) X ₃	LA:Glu		LP:MA	
				TPC (mgGAE/100g _{dw})	TFC (mg/100g _{dw})	TPC (mgGAE/100g _{dw})	TFC (mg/100g _{dw})
1	15	50	10	1520	31.1	2804	46.8
2	25	75	15	1740	38.8	2824	42.7
3	5	50	5	1398	23.7	429	28.5
4	15	30	15	1925	113.3	3582	120.2
5	5	85	5	2956	46.0	3381	33.0
6	25	30	20	1942	28.9	2569	41.8
7	10	50	30	2687	153.2	3712	146.6
8	15	30	15	1920	109.2	3580	120.5
9	15	30	15	1921	116.3	3578	121.3
10	10	75	30	3873	167.3	2990	70.0
11	20	30	5	1753	29.9	2645	39.4
12	25	85	15	2001	52.4	3895	51.1
13	10	85	30	4973	203.7	3232	45.3
14	5	75	5	2124	22.7	1954	25.6
15	5	40	20	ND	ND	ND	ND
16	25	30	30	2852	159.7	4021	123.1
17	25	50	20	1107	56.5	1561	50.9
18	15	75	10	2360	20.6	4108	41.2
19	15	30	15	1922	112.6	3577	124.0
20	5	30	5	1435	47.4	786	47.45

X₁: solid/liquid ratio, X₂: %NADES, X₃: extraction time, TPC: Total phenolic content, TFC: Total flavonoid content, ND: Not detected, Glu: glucose, MA: malic acid, LA: lactic acid, LP: L-proli

Table S2: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of TPC and TFC using LA: Glu as solvent

LA:Glu						
Source	SS	TPC		TFC		
		F-value	p-value	SS	F-value	p-value
Model	1533.71	133.91	< 0.0001*	61492.02	306.60	< 0.0001*
X ₁ -Ratio	1741.50	13.69	0.0049*	315.53	14.16	0.0045*
X ₂ -NADES	3723.52	29.28	0.0004*	3465.88	155.53	< 0.0001*
X ₃ -Time	2037.53	16.02	0.0031*	8410.14	377.40	< 0.0001*
X ₁ X ₂	4626.54	36.38	0.0002*	1144.33	51.35	< 0.0001**
X ₁ X ₃	37855.13	2.98	0.1186 ^{ns}	18.56	0.8330	0.3852 ^{ns}
X ₂ X ₃	2253.52	17.71	0.0023*	314.33	14.11	0.0045*
X ₁ ²	2577.57	20.27	0.0015*	218.35	9.80	0.0121*
X ₂ ²	1317.60	103.60	< 0.0001*	2862.31	128.44	< 0.0001*
X ₃ ²	2650.51	20.84	0.0014*	921.90	41.37	0.0001*
Residual	1145.54			200.56		
Lack of Fit	96524.88	4.31	0.0911 ^{ns}	174.50	5.36	0.0645 ^{ns}
Pure Error	17930.91			26.06		
C.V. %	5.05			5.85		
R ²	0.99			0.99		
Adjusted R ²	0.98			0.99		

TPC: Total phenolic content, TFC: Total flavonoid content, Glu: glucose, LA: lactic acid. Significant at $p < 0.05$, ns Not significant at $p > 0.05$. C.V: Coefficient of variation, SS: Sum of squares

Table S3: Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of TPC and TFC using LP:MA as solvent of squares.

LP:MA						
Source	TPC			TFC		
	SS	F-value	p-value	SS	F-value	p-value
Model	1999.70	826.03	< 0.0001*	29938.42	293.83	< 0.0001*
X ₁ -Ratio	50035.89	18.61	0.0020*	2807.40	247.98	< 0.0001*
X ₂ -NADES	36020.60	1339.90	< 0.0001*	4497.46	397.26	< 0.0001*
X ₃ -Time	3609.60	1342.27	< 0.0001*	11458.21	1012.11	< 0.0001*
X ₁ X ₂	3340.50	124.24	< 0.0001*	441.28	38.98	0.0002*
X ₁ X ₃	7233.50	269.03	< 0.0001*	2127.45	187.92	< 0.0001*
X ₂ X ₃	4025.60	1497.23	< 0.0001*	4631.05	409.06	< 0.0001*
X ₁ ²	2245.60	834.95	< 0.0001*	78.23	6.91	0.0274 ^{ns}
X ₂ ²	4519.60	1680.77	< 0.0001*	1352.80	119.49	< 0.0001*
X ₃ ²	6505.50	241.95	< 0.0001*	341.76	30.19	0.0004*
Residual	24197.37			101.89		
Lack of Fit	21241.88	5.75	0.0575 ^{ns}	90.07	6.10	0.0522 ^{ns}
Pure Error	2955.49			11.82		
C.V. %	1.78			4.84		
R ²	0.99			0.99		
Adjusted R ²	0.99			0.99		

TPC: Total phenolic content, TFC: Total flavonoid content, LP: L-proline, MA: malic acid. Significant at $p < 0.05$, ns Not significant at $p > 0.05$. C.V: Coefficient of variation, SS: Sum

Anexo III: Supplementary Material Chapter III

Table S1: Initial screening of NADES

	TFC (mg/100 ml)	AA (mg/100 ml)	TPC (mg/100 ml)
ChChl.MA	94.67 ± 10.18 ^b	430.71 ± 15.41 ^b	1053.97 ± 85.56 ^c
ChChl.TA	34.23 ± 16.50 ^a	243.45 ± 91.61 ^{ab}	658.86 ± 28.39 ^{abc}
ChChl.LA	53.64 ± 7.53 ^{ab}	317.82 ± 147.30 ^{ab}	586.79 ± 83.98 ^{ab}
ChChl.CA	48.67 ± 13.95 ^{ab}	262.75 ± 34.26 ^{ab}	525.05 ± 44.18 ^{ab}
ChChl.Glu	79.63 ± 17.07 ^{ab}	210.15 ± 27.75 ^{ab}	332.92 ± 57.55 ^a
ChChl.Fruc	56.45 ± 11.79 ^{ab}	159.08 ± 57.96 ^a	390.17 ± 94.98 ^a
ChChl.Xyl	84.69 ± 17.37 ^b	263.97 ± 87.15 ^{ab}	330.57 ± 61.26 ^a
ChChl.Gly	168.55 ± 23.52 ^c	416.33 ± 40.01 ^b	903.84 ± 80.79 ^{bc}

TFC: total flavonoid content. AA: ascorbic acid. TPC: total polyphenol content. ChChl: choline chloride. MA: malic acid. TA: tartaric acid. LA: lactic acid. CA: citric acid. Glu: glucose. Fruc: fructose. Xyl: xylose. Gly: glycerol. (a-c): Different letters indicate statistical differences.

Table S2: Phenolic compounds stability during twelve days by HPLC-UV ($\mu\text{g}/100\text{ml}$).

	ChCh/MA				ChCh/Gly				EtOH			
	Days				Days				Days			
	0	4	7	11	0	4	7	11	0	4	7	11
4-hydroxybenzoic acid	2.07 $\pm$ 0.44	ND	ND	ND	13.13 $\pm$ 0.21 ^a	ND	ND	ND	ND	ND	ND	ND
Catechin	66.25 $\pm$ 2.07 ^b	12.25 $\pm$ 1.14 ^a	6.30 $\pm$ 4.21 ^a	6.01 $\pm$ 0.54 ^a	104.22 $\pm$ 12.17 ^b	11.90 $\pm$ 0.76 ^a	3.31 $\pm$ 0.25 ^a	ND	63.26 $\pm$ 5.63 ^c	12.61 $\pm$ 1.49 ^b	11.64 $\pm$ 0.87 ^b	2.34 $\pm$ 0.12 ^a
Caffeic acid	78.25 $\pm$ 5.61 ^b	61.11 $\pm$ 0.29 ^a	ND	ND	86.17 $\pm$ 1.67 ^c	65.85 $\pm$ 0.75 ^b	7.43 $\pm$ 0.53 ^a	ND	61.96 $\pm$ 0.3 ^b	46.26 $\pm$ 0.91 ^a	ND	ND
Vanillic acid	113.49 $\pm$ 9.99 ^b	66.80 $\pm$ 2.95 ^a	59.04 $\pm$ 2.28 ^a	ND	239.44 $\pm$ 17.22 ^c	115.46 $\pm$ 35.51 ^b	56.70 $\pm$ 6.65 ^a	8.47 $\pm$ 0.83 ^a	82.44 $\pm$ 1.13 ^c	63.89 $\pm$ 8.20 ^b	31.35 $\pm$ 4.03 ^a	ND
Chlorogenic acid	40.24 $\pm$ 3.74 ^c	13.49 $\pm$ 0.63 ^b	10.6 $\pm$ 0.42 ^{ab}	6.17 $\pm$ 0.10 ^a	40.96 $\pm$ 3.45 ^b	39.85 $\pm$ 2.01 ^b	14.65 $\pm$ 0.93 ^a	10.19 $\pm$ 0.48 ^a	11.76 $\pm$ 2.79 ^a	7.11 $\pm$ 0.42 ^a	8.09 $\pm$ 3.17 ^a	5.77 $\pm$ 0.15 ^a
Ferulic acid	8.27 $\pm$ 0.81 ^b	4.01 $\pm$ 0.72 ^a	4.15 $\pm$ 1.73 ^a	1.84 $\pm$ 0.61 ^a	50.88 $\pm$ 3.87 ^b	8.21 $\pm$ 0.68 ^a	5.30 $\pm$ 0.55 ^a	3.80 $\pm$ 0.41 ^a	11.27 $\pm$ 1.07 ^b	5.07 $\pm$ 0.43 ^a	4.27 $\pm$ 0.63 ^a	4.0 $\pm$ 1.42 ^a
p-coumaric acid	285.32 $\pm$ 49.96 ^b	43.52 $\pm$ 2.58 ^a	41.31 $\pm$ 3.36 ^a	33.70 $\pm$ 1.31 ^a	239.03 $\pm$ 24.8 ^c	120.51 $\pm$ 12.53 ^b	39.24 $\pm$ 2.29 ^a	ND	244.24 $\pm$ 21.22 ^b	59.27 $\pm$ 6.4 ^a	57.57 $\pm$ 8.82 ^a	30.46 $\pm$ 0.04 ^a
Narirutin	96.29 $\pm$ 4.54 ^c	94.21 $\pm$ 5.88 ^c	62.65 $\pm$ 0.82 ^b	35.77 $\pm$ 1.09 ^a	88.67 $\pm$ 2.85 ^c	86.80 $\pm$ 3.65 ^c	41.34 $\pm$ 1.51 ^b	2.28 $\pm$ 0.35 ^a	154.38 $\pm$ 7.13 ^b	138.25 $\pm$ 12.05 ^b	46.17 $\pm$ 3.04 ^a	46.01 $\pm$ 6.82 ^a
Rutin	35.04 $\pm$ 3.6 ^b	22.44 $\pm$ 3.71 ^a	ND	ND	19.79 $\pm$ 1.01 ^b	11.91 $\pm$ 1.93 ^{ab}	4.30 $\pm$ 0.87 ^a	ND	15.44 $\pm$ 5.23 ^b	13.52 $\pm$ 1.99 ^{ab}	9.05 $\pm$ 2.71 ^a	ND
Naringin	2.53 $\pm$ 0.1 ^b	2.02 $\pm$ 0.05 ^a	2.39 $\pm$ 0.17 ^b	2.59 $\pm$ 0.03 ^b	2.68 $\pm$ 0.15 ^c	2.17 $\pm$ 0.12 ^a	2.56 $\pm$ 0.04 ^{ab}	2.31 $\pm$ 0.32 ^{ab}	5.27 $\pm$ 0.68 ^b	3.80 $\pm$ 1.58 ^{ab}	3.60 $\pm$ 0.42 ^{ab}	2.33 $\pm$ 0.57 ^a
Quercetin	4.97 $\pm$ 0.15 ^b	4.80 $\pm$ 0.21 ^b	4.76 $\pm$ 0.33 ^b	3.92 $\pm$ 0.10 ^a	5.60 $\pm$ 0.27 ^b	5.25 $\pm$ 0.41 ^{ab}	4.57 $\pm$ 0.13 ^a	ND	16.91 $\pm$ 0.20 ^c	12.84 $\pm$ 4.98 ^{bc}	7.61 $\pm$ 0.39 ^{ab}	3.77 $\pm$ 2.44 ^a
Hesperidin	299.96 $\pm$ 10.32 ^d	268.01 $\pm$ 17.37 ^c	204.7 $\pm$ 8.29 ^b	54.16 $\pm$ 3.39 ^a	326.11 $\pm$ 11.87 ^b	266.31 $\pm$ 43.74 ^b	284.65 $\pm$ 12.05 ^b	101.3 $\pm$ 0.21 ^a	1215.69 $\pm$ 54.64	1021.8 $\pm$ 138.0 ^c	783.27 $\pm$ 64.33 ^b	105.37 $\pm$ 11.87 ^a
Naringenin	113.26 $\pm$ 7.49 ^b	99.69 $\pm$ 1.45 ^a	101.9 $\pm$ 3.92 ^a	100.2 $\pm$ 0.34 ^a	109.52 $\pm$ 6.16 ^c	103.49 $\pm$ 1.53 ^{bc}	98.55 $\pm$ 0.14 ^b	3.52 $\pm$ 0.91 ^a	135.15 $\pm$ 24.27 ^a	119.48 $\pm$ 30.45 ^a	119.56 $\pm$ 4.13 ^a	106.95 $\pm$ 2.81 ^a
Hesperetin	71.15 $\pm$ 0.8 ^b	68.20 $\pm$ 1.02 ^{ab}	65.9 $\pm$ 5.14 ^{ab}	61.73 $\pm$ 0.33 ^a	68.66 $\pm$ 0.16 ^a	64.75 $\pm$ 5.05 ^a	61.92 $\pm$ 0.04 ^a	ND	103.31 $\pm$ 2.52 ^b	94.92 $\pm$ 5.78 ^b	86.66 $\pm$ 19.15 ^{ab}	65.72 $\pm$ 2.75 ^a
Apigenin	5.74 $\pm$ 0.48 ^d	3.98 $\pm$ 0.19 ^c	2.60 $\pm$ 0.87 ^b	1.71 $\pm$ 0.07 ^a	2.77 $\pm$ 0.61 ^b	2.49 $\pm$ 0.27 ^{ab}	1.87 $\pm$ 0.03 ^a	ND	6.54 $\pm$ 0.86 ^b	3.10 $\pm$ 0.23 ^a	2.94 $\pm$ 0.90 ^a	2.47 $\pm$ 0.46 ^a

MA: malic acid. Gly: glycerol. (a-b): Different letters indicate statistical significative differences. ND: not detected.

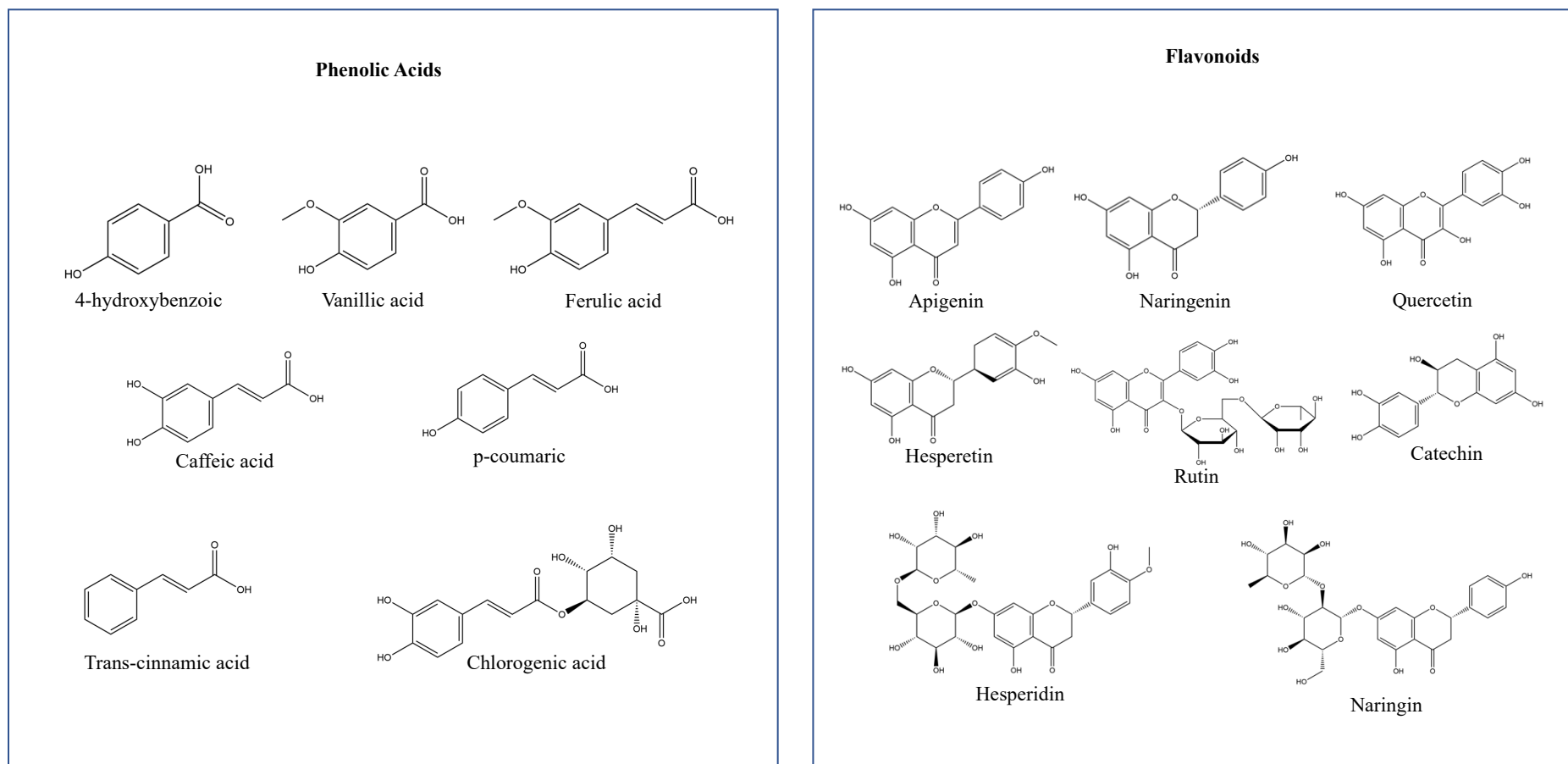
Figure S1. Molecular structure of the bioactive compounds

Table S3: Water solubility of the phenolic compounds studied at 25 °C unless stated otherwise.

Phenolic Compound	Water solubility (g/L)	References
4-hydroxybenzoic acid	6.1 ± 0.1	(Gracin & Rasmuson, 2002)
Catechin	0.64	(Human Metabolome Database, 2022)
Caffeic acid	0.98 ± 0.02	(Mota et al., 2008)
Vanillic acid	1.53	(Noubigh et al., 2008)
Chlorogenic acid	40	(Zhou et al., 2016)
Ferulic acid	0.78 ± 0.01	(Mota et al., 2008)
p-coumaric acid	18.3	(TGSC Information System, 2021)
Narirutin	2.369	(TGSC Information System, 2021)
Rutin	0.125	(EFSA, 2014)
Naringin	0.5 (at 20 °C)	(EFSA, 2010)
Quercetin	0.06 (at 16 °C)	(Human Metabolome Database, 2022)
Hesperidin	2.125	(EFSA, 2014)
Naringenin	0.04383 ± 0.000039	(Semalty et al., 2010)
Hesperetin	0.2725	(EFSA, 2010)
Apigenin	0.1825	(TGSC Information System , 2021)

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Anexo IV: Supplementary Material Chapter IV

Table S1. Antioxidant capacity of four NADES and 50% Ethanol in different extraction methods

	NADES	ORAC ($\mu\text{M TE}$)	FRAP ($\mu\text{M TE}$)	DPPH ($\mu\text{M TE}$)
UAE	ChChl.Fruc	9484.18 $\pm$ 1161.74	<u>922.78</u> $\pm$ 43.20	<u>305.92</u> $\pm$ 116.57
	ChChl.LP.MA	17723.09 $\pm$ 1531.32	5824.52 $\pm$ 773.02	650.31 $\pm$ 51.76
	ChChl.LA	13084.91 $\pm$ 1543.62	2282.12 $\pm$ 138.92	840.53 $\pm$ 34.58
	ChChl.Gly	<u>8240.18</u> $\pm$ 553.22	1715.45 $\pm$ 774.64	655.31 $\pm$ 17.09
	50% EtOH	10171.74 $\pm$ 676.51	1364.48 $\pm$ 391.76	394.42 $\pm$ 46.87
HS	ChChl.Fruc	6336.46 $\pm$ 559.43	<u>483.56</u> $\pm$ 63.12	<u>168.71</u> $\pm$ 60.03
	ChChl.LP.MA	16533.47 $\pm$ 3383.47	3193.57 $\pm$ 193.15	607.78 $\pm$ 72.66
	ChChl.LA	10974.63 $\pm$ 856.07	2530.61 $\pm$ 203.38	812.80 $\pm$ 9.99
	ChChl.Gly	<u>6028.65</u> $\pm$ 776.44	960.91 $\pm$ 149.21	641.45 $\pm$ 18.44
	50% EtOH	14547.57 $\pm$ 1116.91	1632.44 $\pm$ 342.62	337.48 $\pm$ 69.89

ChChl: choline chloride. EtOH: ethanol. Fruc: Fructose. Gly: glycerol. HS: heating-stirring extraction. LA: lactic acid. MA: malic acid. LP: proline. UAE: ultrasound-assisted extraction. In bold: higher extraction yield per antioxidant capacity methods. Underlined: lower extraction yield per antioxidant capacity methods.

Table S2. Extraction yield of the phenolic compounds in two different extraction methods.

	Ultrasound-assisted extraction					Heating-Stirring extraction				
	ChChl.Fruc	ChChl.LP.MA	ChChl.LA	ChChl.Gly	EtOH 50%	ChChl.Fruc	ChChl.LP.MA	ChChl.LA	ChChl.Gly	EtOH 50%
3,4-dihydroxybenzoic acid	1.53 ± 0.53 ^b	< LD	< LD	0.53 ± 0.20 ^a	< LD	< LD	< LD	< LD	4.35 ± 0.50 ^a	< LD
Catechin	4.41 ± 2.01 ^a	3.65 ± 0.39 ^a	2.22 ± 0.09 ^a	36.73 ± 3.02 ^c	13.35 ± 0.49 ^b	3.02 ± 0.33 ^a	3.39 ± 0.20 ^a	5.25 ± 0.29 ^b	7.77 ± 0.49 ^d	6.41 ± 0.08 ^c
Caffeic acid	4.38 ± 0.05 ^a	4.32 ± 0.32 ^a	4.05 ± 0.00 ^a	4.83 ± 0.55 ^a	4.25 ± 0.00 ^a	< LD	4.14 ± 0.08 ^a	4.15 ± 0.00 ^a	4.71 ± 0.59 ^a	4.29 ± 0.06 ^a
Vanillic acid	11.08 ± 0.84 ^d	5.66 ± 1.81 ^b	3.43 ± 0.00 ^a	8.90 ± 0.25 ^c	5.23 ± 0.16 ^b	7.90 ± 0.74 ^c	4.83 ± 0.16 ^{ab}	3.38 ± 0.45 ^a	18.05 ± 1.52 ^d	5.77 ± 0.23 ^{bc}
Chlorogenic acid	7.85 ± 1.10 ^{bc}	5.87 ± 0.12 ^a	6.02 ± 0.04 ^a	8.91 ± 0.14 ^c	6.49 ± 0.66 ^{ab}	7.75 ± 0.31 ^b	6.17 ± 0.23 ^a	6.77 ± 0.38 ^a	6.23 ± 0.30 ^a	8.24 ± 0.31 ^b
Ferulic acid	0.14 ± 0.03 ^a	0.49 ± 0.12 ^a	0.14 ± 0.04 ^a	0.30 ± 0.12 ^a	0.44 ± 0.14 ^a	0.11 ± 0.01 ^a	0.32 ± 0.06 ^a	0.15 ± 0.10 ^a	1.01 ± 0.23 ^b	0.83 ± 0.06 ^b
p-coumaric acid	38.90 ± 1.04 ^{ab}	48.76 ± 4.21 ^b	36.95 ± 2.96 ^a	33.70 ± 1.65 ^a	37.08 ± 2.66 ^a	36.72 ± 0.83 ^a	47.79 ± 2.54 ^{ab}	35.52 ± 6.46 ^a	54.10 ± 0.70 ^b	111.86 ± 7.94 ^c
Narirutin	41.94 ± 2.55 ^a	43.63 ± 4.39 ^a	39.74 ± 3.67 ^a	39.41 ± 0.55 ^a	46.71 ± 2.17 ^a	38.59 ± 1.88 ^b	36.83 ± 0.98 ^b	41.19 ± 2.35 ^b	66.85 ± 4.57 ^c	29.01 ± 0.74 ^a
Hesperidin	493.0 ± 42.5 ^c	364.21 ± 30.7 ^b	305.0 ± 5.70 ^b	492.65 ± 2.49 ^c	841.3 ± 15.8 ^d	355.5 ± 4.9 ^c	267.75 ± 29.24 ^b	196.7 ± 2.50 ^a	567.4 ± 16.0 ^d	974.31 ± 31.67 ^e
Naringin	539.5 ± 18.0 ^c	300.12 ± 24.5 ^b	163.9 ± 34.3 ^a	360.99 ± 16.56 ^b	343.2 ± 29.7 ^b	328.7 ± 17.6 ^d	89.91 ± 3.14 ^a	391.5 ± 2.50 ^e	167.2 ± 28.3 ^b	256.15 ± 28.48 ^c
Quercetin	83.26 ± 4.89 ^b	46.01 ± 0.67 ^a	75.07 ± 19.45 ^b	48.58 ± 2.42 ^a	45.56 ± 0.11 ^a	45.55 ± 0.03 ^a	45.77 ± 0.21 ^a	47.10 ± 0.0 ^a	44.75 ± 1.37 ^a	67.89 ± 16.56 ^b
trans-cinnamic acid	0.03 ± 0.02 ^a	0.10 ± 0.07 ^b	< LD	< LD	0.14 ± 0.07 ^b	< LD	0.05 ± 0.00 ^a	< LD	< LD	0.19 ± 0.09 ^b
Naringenin	98.82 ± 0.76 ^a	101.83 ± 1.69 ^a	100.91 ± 0.67 ^a	98.44 ± 0.33 ^a	110.40 ± 6.93 ^b	99.93 ± 0.39 ^a	101.04 ± 1.14 ^a	113.43 ± 1.98 ^b	99.78 ± 0.72 ^a	103.33 ± 1.70 ^a
Hesperetin	63.10 ± 0.62 ^{ab}	62.36 ± 0.34 ^a	70.47 ± 1.02 ^c	62.29 ± 0.16 ^a	65.24 ± 1.13 ^b	61.80 ± 0.2 ^a	61.57 ± 0.16 ^a	62.04 ± 1.17 ^a	66.41 ± 0.67 ^a	97.59 ± 5.34 ^b
Apigenin	2.34 ± 0.49 ^a	1.52 ± 0.14 ^a	2.12 ± 0.11 ^a	2.30 ± 0.22 ^a	2.44 ± 0.65 ^a	1.37 ± 0.01 ^a	1.39 ± 0.02 ^a	1.98 ± 0.48 ^a	2.92 ± 0.25 ^b	3.71 ± 0.34 ^c

ChChl: choline chloride. EtOH: ethanol. Fruc: Fructose. Gly: glycerol. LA: lactic acid. MA: malic acid. LP: proline. Different letters (a-d) indicate statistical significance differences. In bold: higher extraction yield and statistical differences. Values are expressed in ppm (µg/mL).

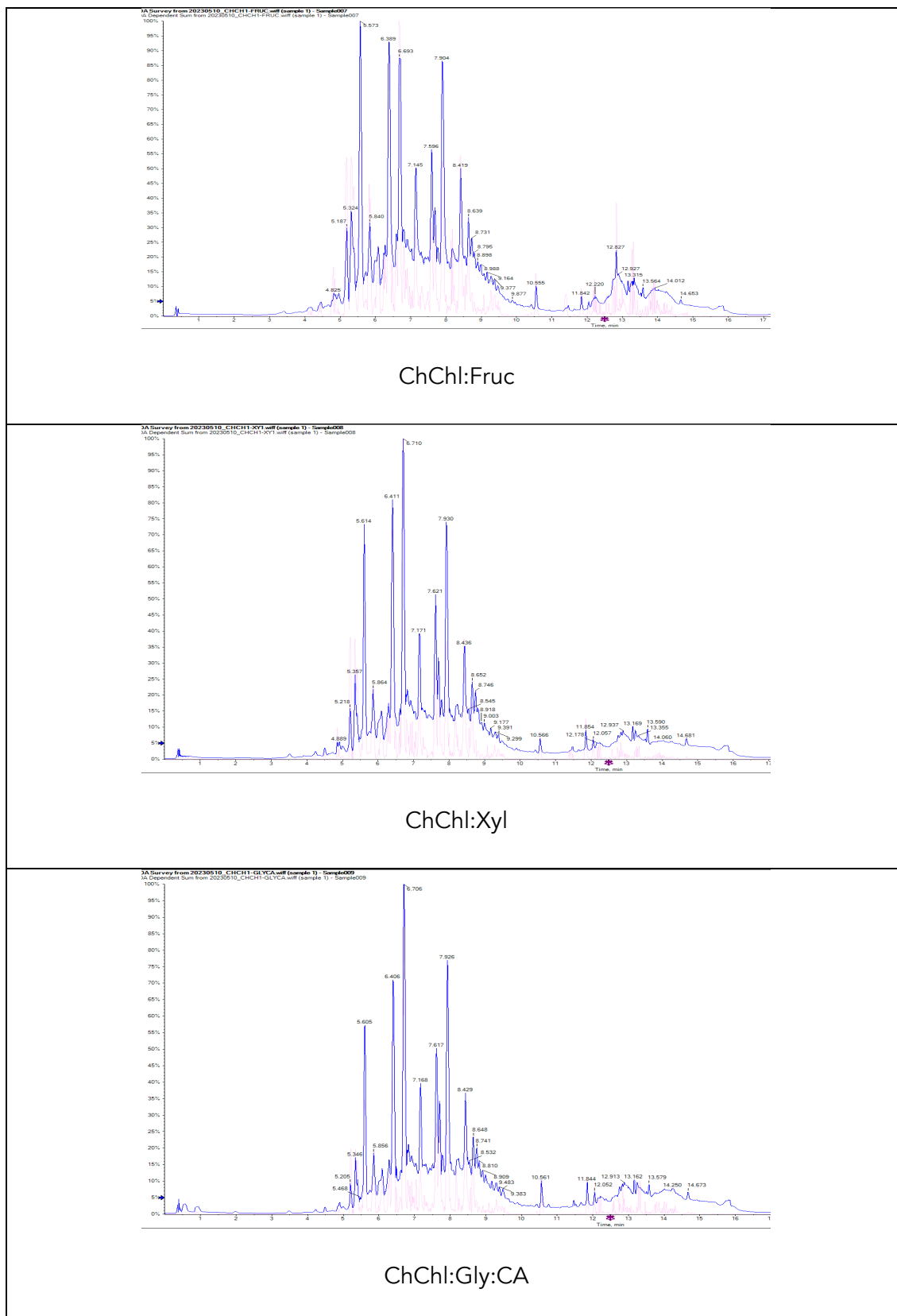
Table S3. DPPH, ORAC, and FRAP stability by ultrasound-assisted extraction and heating-stirring extraction for three days.

Days		ChChl:Fruc		ChChl:LP:MA		ChChl:LA		ChChl:Gly		EtOH	
		0	3	0	3	0	3	0	3	0	3
DPPH ($\mu\text{M TE}$)	HS	169 $\pm$ 60	48 $\pm$ 29	607 $\pm$ 73	188 $\pm$ 29	812 $\pm$ 10	291 $\pm$ 24	645 $\pm$ 18	66 $\pm$ 17	337 $\pm$ 70	181 $\pm$ 23
	UAE	306 $\pm$ 116	105 $\pm$ 17	650 $\pm$ 52	173 $\pm$ 13	840 $\pm$ 34	258 $\pm$ 24	655 $\pm$ 17	43 $\pm$ 26	394 $\pm$ 47	80 $\pm$ 18
ORAC (mM TE)	HS	6.3 $\pm$ 0.6	2.1 $\pm$ 0.6	16 $\pm$ 3	7.4 $\pm$ 1.8	11 $\pm$ 0.9	2.7 $\pm$ 0.4	6.0 $\pm$ 0.8	N.D.	14 $\pm$ 1.1	2.3 $\pm$ 1.3
	UAE	9.5 $\pm$ 1.2	2.7 $\pm$ 0.7	18 $\pm$ 1.5	7.7 $\pm$ 1.1	13 $\pm$ 1.5	4.3 $\pm$ 0.4	8.2 $\pm$ 0.5	1.6 $\pm$ 0.5	10.1 $\pm$ 0.7	3.6 $\pm$ 0.2
FRAP ($\mu\text{M TE}$)	HS	483 $\pm$ 63	185 $\pm$ 34	3194 $\pm$ 193	98 $\pm$ 17	2530 $\pm$ 203	349 $\pm$ 74	960 $\pm$ 249	105 $\pm$ 6	1632 $\pm$ 343	106 $\pm$ 5
	UAE	923 $\pm$ 430	247 $\pm$ 74	5824 $\pm$ 773	59 $\pm$ 15	2282 $\pm$ 139	224 $\pm$ 33	1716 $\pm$ 775	147 $\pm$ 34	13645 $\pm$ 392	108 $\pm$ 22

ChChl: choline chloride. EtOH: ethanol. Fruc: Fructose. Gly: glycerol. HS: heating-stirring extraction. LA: lactic acid. MA: malic acid. LP: proline. UAE: ultrasound-assisted extraction. N.D.: Not detecta

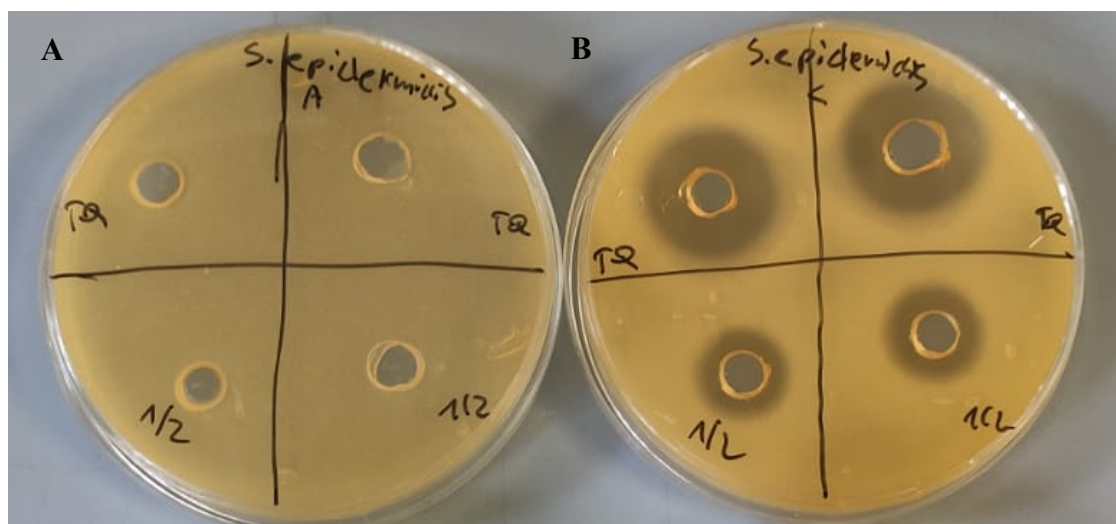
Anexo V: Supplementary Material Chapter V part I

Figure S1. Chromatograms of the best NADES for phenolic acids and flavanones.



CA: citric acid; ChCl: choline chloride; Fru: fructose; Xyl: xylose

Figure S2. Example of agar spot test with wells containing 50 μ L of *Staphylococcus epidermidis* and observation of inhibition diameters after 24 hours of incubation.



A: 50% EtOH extract. B: ChCl:Gly:CA extract. TQ: 100% extract. 1/2: 50% Extract

Anexo VI: Supplementary Material Chapter V part II

Table S1. Color and pH of the newly formulated orange juice

	Color			pH
	L*	a*	b*	
OJ	51.08 ± 1.84 ^a	3.06 ± 0.42 ^a	50.72 ± 3.21 ^a	3.46 ± 0.05 ^a
OJ+E1	41.35 ± 0.25 ^c	-1.68 ± 0.23 ^d	40.87 ± 1.36 ^c	3.09 ± 0.06 ^b
OJ+E2	41.37 ± 0.49 ^b	-1.07 ± 0.39 ^{cd}	42.24 ± 1.91 ^c	2.07 ± 0.09 ^d
OJ+E3	44.29 ± 0.47 ^c	0.22 ± 0.07 ^b	47.40 ± 0.06 ^{ab}	3.00 ± 0.08 ^c
OJ+E4	43.22 ± 0.49 ^{bc}	-0.55 ± 0.11 ^c	45.27 ± 3.89 ^{bc}	3.08 ± 0.01 ^{bc}

The data is expressed as mean of three independent replicates. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences between samples). OJ: pure orange juice. OJ+E1: orange juice with choline chloride:glycerol extract. OJ+E2: orange juice with choline chloride:glycerol:citric acid extract. OJ+E3: orange juice with choline chloride:fructose extract. OJ+E4: orange juice with choline chloride:xylose extract

Table S2 Total mesophilic aerobic bacteria expressed as log CFU/mL of the different juice samples stored for 13 days at 4 °C and 8 °C

	4 °C					8 °C				
	T0	T3	T6	T9	T13	T0	T3	T6	T9	T13
OJ	2.54 ± 0.18	2.78 ± 0.11	3.51 ± 0.02 ^a	5.82 ± 0.25 ^a	7.69 ± 0.11 ^a	2.54 ± 0.18	3.02 ± 0.10 ^a	4.49 ± 0.06 ^a	6.43 ± 0.16 ^a	7.43 ± 0.15 ^a
OJ+E1	2.53 ± 0.03	2.66 ± 0.05	2.60 ± 0.08 ^b	2.52 ± 0.02 ^b	2.69 ± 0.09 ^d	2.52 ± 0.03	2.54 ± 0.09 ^b	2.85 ± 0.06 ^b	2.57 ± 0.10 ^c	5.65 ± 0.19 ^b
OJ+E2	2.51 ± 0.04	2.61 ± 0.13	2.61 ± 0.28 ^b	2.58 ± 0.17 ^b	3.51 ± 0.09 ^c	2.51 ± 0.15	2.92 ± 0.12 ^a	2.49 ± 0.20 ^c	2.81 ± 0.16 ^c	5.56 ± 0.07 ^b
OJ+E3	2.49 ± 0.09	2.62 ± 0.14	2.70 ± 0.19 ^b	2.65 ± 0.12 ^b	3.05 ± 0.11 ^d	2.49 ± 0.09	2.80 ± 0.10 ^{ab}	2.75 ± 0.18 ^b	3.27 ± 0.03 ^b	5.98 ± 0.11 ^b
OJ+E4	2.42 ± 0.15	2.78 ± 0.17	2.65 ± 0.34 ^b	2.64 ± 0.09 ^b	2.92 ± 0.16	2.42 ± 0.15	2.59 ± 0.16 ^b	3.03 ± 0.01 ^b	3.33 ± 0.13 ^b	6.17 ± 0.14 ^{ab}
OJ+N1	2.76 ± 0.05	2.60 ± 0.04	2.93 ± 0.14 ^b	2.72 ± 0.04 ^b	4.24 ± 0.14 ^b	2.76 ± 0.06	2.70 ± 0.06 ^b	2.78 ± 0.06	3.48 ± 0.06 ^b	7.18 ± 0.06 ^a
OJ+N2	2.65 ± 0.15	2.85 ± 0.05	2.73 ± 0.05 ^b	2.63 ± 0.05 ^b	3.76 ± 0.05 ^c	2.64 ± 0.15	2.95 ± 0.05 ^a	2.78 ± 0.05 ^b	3.44 ± 0.05 ^b	7.08 ± 0.15 ^a
OJ+N3	2.57 ± 0.04	2.93 ± 0.12	2.83 ± 0.12 ^b	2.83 ± 0.12 ^b	4.34 ± 0.12 ^b	2.57 ± 0.14	2.44 ± 0.04 ^b	2.95 ± 0.04 ^b	3.69 ± 0.14 ^b	7.04 ± 0.04 ^a
OJ+N4	2.65 ± 0.12	2.60 ± 0.14	2.63 ± 0.03 ^b	2.80 ± 0.04 ^b	3.92 ± 0.13 ^{bc}	2.65 ± 0.15	2.48 ± 0.05 ^b	3.08 ± 0.05 ^b	2.73 ± 0.15 ^c	7.00 ± 0.05 ^a

The data is expressed as mean ± SD of three independent replicates. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences). Control: pure orange juice. E1: orange juice with choline chloride:glycerol extract. E2: orange juice with choline chloride:glycerol:citric acid extract. E3: orange juice with choline chloride:fructose extract. E4: orange juice with choline chloride:xylose extract. N1: orange juice with choline chloride:glycerol NADES. N2: orange juice with choline chloride:glycerol:citric acid NADES. N3: orange juice with choline chloride:fructose NADES. N4: orange juice with choline chloride:xylose NADES.

Table S3. Yeast and molds expressed as log CFU/mL of the different juice samples stored for 13 days at 4°C and 8°C.

	4°C					8°C				
	T0	T3	T6	T9	T13	T0	T3	T6	T9	T13
OJ	2.60 ± 0.10	2.89 ± 0.06	3.53 ± 0.02 ^a	5.72 ± 0.29 ^a	7.56 ± 0.19 ^a	2.61 ± 0.11	3.07 ± 0.16	3.14 ± 0.11 ^a	5.56 ± 0.09 ^a	7.90 ± 0.30 ^a
OJ+E1	2.50 ± 0.07	2.87 ± 0.03	2.36 ± 0.17 ^b	3.57 ± 0.26 ^b	3.53 ± 0.17 ^c	2.51 ± 0.07	2.73 ± 0.05	2.78 ± 0.18 ^{ab}	2.47 ± 0.06 ^c	5.08 ± 0.34 ^c
OJ+E2	2.58 ± 0.02	2.89 ± 0.04	2.81 ± 0.14 ^b	3.36 ± 0.16 ^b	4.34 ± 0.33 ^b	2.59 ± 0.03	2.73 ± 0.15	2.63 ± 0.06 ^b	2.67 ± 0.23 ^c	5.03 ± 0.04 ^c
OJ+E3	2.48 ± 0.06	2.66 ± 0.20	2.74 ± 0.07 ^b	3.51 ± 0.09 ^b	3.48 ± 0.18 ^c	2.50 ± 0.07	2.73 ± 0.22	2.59 ± 0.19 ^b	2.58 ± 0.09 ^c	5.54 ± 0.16 ^c
OJ+E4	2.31 ± 0.24	2.80 ± 0.28	2.51 ± 0.07 ^b	3.54 ± 0.08 ^b	3.54 ± 0.08 ^c	2.31 ± 0.08	2.66 ± 0.10	2.46 ± 0.15 ^b	2.81 ± 0.39 ^{bc}	5.88 ± 0.11 ^{bc}
OJ+N1	2.67 ± 0.10	2.48 ± 0.01	2.30 ± 0.02 ^b	3.50 ± 0.07 ^b	3.97 ± 0.01 ^{bc}	2.68 ± 0.10	2.70 ± 0.01	2.85 ± 0.02 ^{ab}	3.05 ± 0.07 ^{bc}	6.70 ± 0.01 ^b
OJ+N2	2.86 ± 0.12	2.91 ± 0.13	2.20 ± 0.05 ^b	3.68 ± 0.08 ^b	4.16 ± 0.18 ^b	2.87 ± 0.12	2.70 ± 0.13	2.95 ± 0.05 ^a	3.21 ± 0.08 ^b	6.78 ± 0.18 ^b
OJ+N3	2.49 ± 0.08	2.83 ± 0.06	2.35 ± 0.14 ^b	3.66 ± 0.16 ^b	4.26 ± 0.21 ^b	2.49 ± 0.08	2.95 ± 0.06	2.85 ± 0.14 ^{ab}	2.43 ± 0.16 ^c	6.85 ± 0.21 ^b
OJ+N4	2.83 ± 0.07	2.79 ± 0.08	2.86 ± 0.12 ^b	3.63 ± 0.02 ^b	3.84 ± 0.09 ^c	2.83 ± 0.07	2.95 ± 0.08	2.76 ± 0.12 ^b	2.45 ± 0.02 ^c	6.48 ± 0.09 ^b

The data is expressed as mean ± SD of three independent replicates. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences). S1: pure orange juice. S2: orange juice with choline chloride:glycerol extract. S3: orange juice with choline chloride:glycerol:citric acid extract. S4: orange juice with choline chloride:fructose extract. S5: orange juice with choline chloride:xylose extract. N2: orange juice with choline chloride:glycerol NADES. N3: orange juice with choline chloride:glycerol:citric acid NADES. N4: orange juice with choline chloride:fructose NADES. N5: orange juice with choline chloride:xylose NADES.

Table S4. Average pH values during 13 days at 4 °C and 8 °C storage temperature

	4 °C			8 °C		
	T0	T6	T13	T0	T6	T13
OJ	3.46 ± 0.06 ^a	3.52 ± 0.04 ^a	3.41 ± 0.02 ^a	3.46 ± 0.06 ^a	3.53 ± 0.01 ^a	3.47 ± 0.02 ^a
OJ+E1	3.09 ± 0.07 ^b	2.99 ± 0.04 ^b	2.92 ± 0.07 ^b	3.09 ± 0.07 ^b	3.02 ± 0.02 ^b	2.98 ± 0.01 ^b
OJ+E2	2.07 ± 0.09 ^c	2.01 ± 0.03 ^b	1.96 ± 0.02 ^c	2.07 ± 0.09 ^c	2.00 ± 0.00 ^c	1.97 ± 0.01 ^c
OJ+E3	3.00 ± 0.09 ^b	2.79 ± 0.04 ^b	2.94 ± 0.02 ^b	3.00 ± 0.09 ^b	2.94 ± 0.01 ^b	2.94 ± 0.03 ^b
OJ+E4	3.08 ± 0.02 ^b	2.84 ± 0.03 ^b	2.88 ± 0.02 ^b	3.08 ± 0.02 ^b	2.89 ± 0.00 ^b	2.93 ± 0.04 ^b

The data is expressed as mean ± SD of three independent replicates. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences). S1: pure orange juice. S2: orange juice with choline chloride:glycerol. S3: orange juice with choline chloride:glycerol:citric acid. S4: orange juice with choline chloride:fructose. : orange juice with choline chloride:xylose.

Table S5. Percentage of total volatile compounds

Sample	Temperature	Time	Total Terpenes	Total Terpenoids	Total Alcohols	Total aldehydes	Total esters	Total ketones	Total acids
OJ	4 °C	T0	91.91	1.46	3.01	1.80	1.00	0.70	0.12
		T6	92.76	1.64	2.39	0.89	1.38	0.94	0.00
		T13	81.83	1.46	5.90	0.48	9.35	0.76	0.22
	8 °C	T0	91.89	1.35	3.31	1.74	1.03	0.68	0.00
		T6	90.61	1.95	2.69	0.12	3.62	0.91	0.09
		T13	78.28	1.33	8.39	0.04	11.28	0.32	0.35
OJ+E1	4 °C	T0	88.48	3.66	3.26	2.90	1.05	0.65	0.00
		T6	89.57	3.97	3.19	1.42	0.84	0.96	0.05
		T13	90.21	3.67	2.95	1.40	0.81	0.90	0.06
	8 °C	T0	87.92	3.50	3.57	3.20	1.13	0.69	0.00
		T6	88.62	4.30	3.64	1.63	0.99	0.76	0.05
		T13	87.31	4.24	3.53	0.20	3.79	0.76	0.16
OJ+E2	4 °C	T0	87.49	3.80	3.39	3.30	1.24	0.77	0.00
		T6	88.26	4.21	2.98	2.63	1.10	0.82	0.00
		T13	87.60	4.36	3.71	1.66	1.91	0.77	0.00
	8 °C	T0	87.90	3.60	3.41	3.20	1.11	0.77	0.00
		T6	87.72	4.25	3.39	2.83	1.05	0.76	0.00
		T13	86.31	4.31	3.81	0.61	4.26	0.71	0.00
OJ+E3	4 °C	T0	89.23	3.27	3.18	2.26	1.16	0.78	0.12
		T6	89.76	3.36	3.06	1.85	0.97	0.92	0.09
		T13	90.03	3.58	3.07	1.26	0.86	1.08	0.11

OJ+E4	8 °C	T0	88.70	3.30	3.42	2.59	1.20	0.72	0.07
		T6	90.17	3.27	3.02	1.74	0.87	0.83	0.10
		T13	87.57	3.15	3.64	0.05	4.06	1.32	0.20
	4 °C	T0	88.41	3.49	3.10	2.93	1.27	0.80	0.00
		T6	89.93	3.59	2.79	1.71	0.93	1.00	0.05
		T13	90.42	3.51	2.69	1.60	0.82	0.86	0.11
	8 °C	T0	88.99	3.27	3.14	2.89	0.98	0.74	0.00
		T6	91.00	3.39	2.59	1.44	0.78	0.75	0.05
		T13	87.55	3.88	3.48	0.05	4.02	0.84	0.18
OJ+N1	4 °C	T0	90.29	1.61	3.06	2.89	1.41	0.73	0.00
		T6	92.38	1.76	2.39	1.61	1.05	0.80	0.00
		T13	92.74	1.88	2.53	0.68	1.12	1.05	0.00
	8 °C	T0	90.40	1.62	3.07	2.91	1.32	0.68	0.00
		T6	92.99	1.78	2.29	1.28	0.88	0.77	0.00
		T13	79.52	1.54	5.69	0.11	12.29	0.44	0.41
	4 °C	T0	90.60	1.56	2.53	3.06	1.30	0.88	0.06
		T6	92.06	1.79	2.10	2.11	0.97	0.96	0.00
		T13	92.21	2.08	2.49	0.80	1.61	0.80	0.00
OJ+N2	8 °C	T0	90.75	1.57	2.54	3.08	1.18	0.89	0.00
		T6	91.26	1.96	2.24	2.74	1.02	0.73	0.05
		T13	81.43	2.10	4.34	0.04	11.52	0.37	0.20
	4 °C	T0	88.00	3.12	3.69	3.02	1.34	0.77	0.06
		T6	92.02	1.91	2.63	1.57	0.96	0.84	0.07
		T13	89.93	1.88	3.91	0.41	2.88	1.00	0.00
	8 °C	T0	88.00	3.08	3.71	3.03	1.22	0.90	0.06

OJ+N4	4 °C	T6	92.70	1.65	2.35	1.47	1.04	0.79	0.00
		T13	85.13	1.80	3.86	0.00	8.64	0.35	0.23
		T0	90.55	1.56	2.74	2.91	1.34	0.85	0.06
		T6	91.94	1.84	2.45	1.97	0.97	0.83	0.00
		T13	91.69	2.27	2.71	0.99	1.25	1.09	0.00
		T0	90.97	1.57	2.76	2.60	1.30	0.81	0.00
	8 °C	T6	93.13	1.68	2.33	1.23	0.88	0.75	0.00
		T13	89.55	1.72	3.32	0.00	4.68	0.54	0.19

The data is expressed as a percentage of three independent replicates.

Table S6. Color stability (ΔE) during storage at 4 °C and 8 °C

	4 °C		8 °C	
	T6	T13	T6	T13
OJ	2.27	9.56	9.19	7.31
OJ+E1	11.23	11.44	15.78	15.98
OJ+E2	4.90	11.52	13.04	9.7
OJ+E3	3.09	5.29	10.161	7.258
OJ+E4	7.87	9.78	7.65	10.841

The data is expressed as the ΔE . S1: pure orange juice. S2: orange juice with choline chloride:glycerol. S3: orange juice with choline chloride:glycerol:citric acid. S4: orange juice with choline chloride:fructose: orange juice with choline chloride:xylose. T6: Stability at day 6. T13: Stability on day 13.

Table S7. *L. plantarum* cell loads (log CFU/mL) of the different juice samples stored at 4 °C and 8 °C

4 °C						
	T0	T2	T4	T7	T9	T11
OJ	2.67 ± 0.08 ^a	2.78 ± 0.20 ^a	2.77 ± 0.07 ^a	2.75 ± 0.06 ^a	2.88 ± 0.19 ^a	2.88 ± 0.04 ^a
OJ+E1	2.52 ± 0.19 ^a	2.59 ± 0.07 ^a	2.85 ± 0.07 ^a	2.60 ± 0.15 ^{ab}	2.46 ± 0.12 ^b	2.63 ± 0.05 ^a
OJ+E2	2.00 ± 0.02 ^b	ND	ND	ND	ND	ND
OJ+E3	2.68 ± 0.02 ^a	2.65 ± 0.05 ^a	2.92 ± 0.16 ^a	2.47 ± 0.09 ^b	2.36 ± 0.13 ^b	2.29 ± 0.10 ^b
OJ+E4	2.51 ± 0.08 ^a	2.39 ± 0.21 ^a	2.39 ± 0.22 ^b	2.48 ± 0.19 ^b	2.44 ± 0.07 ^b	2.49 ± 0.08 ^{ab}
OJ+N1	2.43 ± 0.10 ^a	2.75 ± 0.10 ^a	2.65 ± 0.15 ^{ab}	2.42 ± 0.12 ^b	2.73 ± 0.05 ^a	2.41 ± 0.02 ^{ab}
OJ+N2	2.66 ± 0.02 ^a	ND	ND	ND	ND	ND
OJ+N3	2.65 ± 0.02 ^a	2.44 ± 0.06 ^a	3.09 ± 0.01 ^a	2.41 ± 0.11 ^b	2.59 ± 0.08 ^b	1.97 ± 0.04 ^c
OJ+N4	2.85 ± 0.02 ^a	2.53 ± 0.20 ^a	2.30 ± 0.12 ^b	2.29 ± 0.13 ^b	2.12 ± 0.05 ^c	2.23 ± 0.02 ^b
8 °C						
	T0	T2	T4	T7	T9	
OJ	2.67 ± 0.08 ^a	2.93 ± 0.17 ^a	3.00 ± 0.00 ^a	2.84 ± 0.13 ^{ab}	3.12 ± 0.05 ^a	
OJ+E1	2.52 ± 0.19 ^a	2.60 ± 0.10 ^a	2.36 ± 0.10	2.53 ± 0.15 ^b	2.60 ± 0.12 ^b	
OJ+E2	2.00 ± 0.02 ^b	ND	ND	ND	ND	
OJ+E3	2.68 ± 0.02 ^a	2.66 ± 0.10 ^a	2.39 ± 0.36 ^b	2.43 ± 0.09 ^b	2.32 ± 0.12 ^b	
OJ+E4	2.51 ± 0.08 ^a	2.70 ± 0.15 ^a	2.48 ± 0.31 ^b	2.37 ± 0.16 ^b	2.18 ± 0.07 ^b	
OJ+N1	2.43 ± 0.10 ^a	2.60 ± 0.03 ^a	2.35 ± 0.04 ^b	2.64 ± 0.12 ^b	2.32 ± 0.06 ^b	
OJ+N2	2.66 ± 0.02 ^a	ND	ND	ND	ND	
OJ+N3	2.65 ± 0.02 ^a	2.74 ± 0.03 ^a	2.32 ± 0.04 ^b	2.56 ± 0.12 ^b	2.57 ± 0.06 ^b	
OJ+N4	2.85 ± 0.02 ^a	2.64 ± 0.10 ^a	2.85 ± 0.10 ^{ab}	3.04 ± 0.15 ^a	2.74 ± 0.12 ^{ab}	

The data is expressed as **mean ± SD** of three independent replicates. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences). OJ: pure orange juice. OJ+E1: orange juice

with choline chloride:glycerol extract. OJ+E2: orange juice with choline chloride:glycerol:citric acid extract. OJ+E3: orange juice with choline chloride:fructose extract. OJ+E4: orange juice with choline chloride:xylose extract. OJ+N1: orange juice with choline chloride:glycerol NADES. OJ+N2: orange juice with choline chloride:glycerol:citric acid NADES. OJ+N3: orange juice with choline chloride:fructose NADES. OJ+N4: orange juice with choline chloride:xylose NADES.

Table S8. *S. cerevisiae* cell loads (log CFU/mL) of the different juice samples stored at 4 °C and 8 °C

4 °C						
	T0	T2	T4	T7	T9	T11
OJ	3.17 ± 0.06 ^a	3.05 ± 0.13 ^a	3.94 ± 0.12 ^a	4.32 ± 0.15 ^a	4.02 ± 0.17 ^a	5.07 ± 0.07 ^a
OJ+E1	3.04 ± 0.01 ^a	2.98 ± 0.02 ^a	2.46 ± 0.21 ^c	2.53 ± 0.07 ^d	2.00 ± 0.00 ^d	2.46 ± 0.22 ^e
OJ+E2	2.00 ± 0.15 ^a	2.36 ± 0.10 ^c	2.44 ± 0.04 ^c	2.72 ± 0.22 ^d	2.85 ± 0.17 ^c	2.59 ± 0.16 ^e
OJ+E3	3.02 ± 0.13 ^a	3.06 ± 0.18 ^a	2.46 ± 0.15 ^c	2.93 ± 0.16 ^d	2.77 ± 0.15 ^c	2.81 ± 0.07 ^e
OJ+E4	2.96 ± 0.05 ^a	3.14 ± 0.07 ^a	2.74 ± 0.19 ^c	2.96 ± 0.07 ^d	2.80 ± 0.07 ^c	3.11 ± 0.07 ^{de}
OJ+N1	3.13 ± 0.10 ^a	3.06 ± 0.10 ^a	3.18 ± 0.15 ^b	3.28 ± 0.12 ^d	3.86 ± 0.05 ^b	4.44 ± 0.02 ^c
OJ+N2	3.07 ± 0.02 ^a	2.69 ± 0.06 ^b	2.50 ± 0.06	2.76 ± 0.02 ^c	2.83 ± 0.00 ^c	3.40 ± 0.00 ^d
OJ+N3	3.03 ± 0.03 ^a	3.16 ± 0.06 ^a	3.67 ± 0.01 ^{ab}	3.73 ± 0.11 ^b	4.18 ± 0.08 ^a	4.39 ± 0.04 ^c
OJ+N4	3.16 ± 0.06 ^a	3.11 ± 0.20 ^a	3.34 ± 0.00 ^b	3.77 ± 0.13 ^b	3.86 ± 0.05 ^b	4.68 ± 0.02 ^b
8 °C						
	T0	T2	T4	T7	T9	
OJ	3.17 ± 0.06 ^a	4.05 ± 0.13 ^a	4.31 ± 0.15 ^a	5.65 ± 0.01 ^a	6.56 ± 0.02 ^a	
OJ+E1	3.04 ± 0.01 ^a	3.13 ± 0.18 ^{bc}	2.80 ± 0.28 ^e	3.72 ± 0.01 ^d	4.04 ± 0.03 ^d	
OJ+E2	2.78 ± 0.15 ^a	2.49 ± 0.17 ^c	2.73 ± 0.12 ^e	3.49 ± 0.12 ^d	3.58 ± 0.19 ^d	
OJ+E3	3.02 ± 0.13 ^a	3.37 ± 0.08 ^b	3.09 ± 0.09 ^e	3.99 ± 0.04 ^d	4.06 ± 0.00 ^d	
OJ+E4	2.96 ± 0.05 ^a	3.35 ± 0.09 ^b	3.27 ± 0.10 ^d	3.83 ± 0.05 ^d	4.29 ± 0.29 ^{cd}	
OJ+N1	3.13 ± 0.10 ^a	3.32 ± 0.03 ^b	3.71 ± 0.03 ^c	4.13 ± 0.12 ^c	4.84 ± 0.06 ^{bc}	
OJ+N2	3.07 ± 0.02 ^a	3.57 ± 0.00 ^b	3.63 ± 0.02 ^c	4.22 ± 0.01 ^c	4.66 ± 0.01 ^c	
OJ+N3	3.03 ± 0.03 ^a	3.62 ± 0.03 ^b	3.94 ± 0.04 ^b	4.88 ± 0.12 ^b	5.08 ± 0.06 ^b	
OJ+N4	3.16 ± 0.06 ^a	3.86 ± 0.10 ^{ab}	4.31 ± 0.10 ^a	5.32 ± 0.15 ^a	6.27 ± 0.12 ^a	

The data is expressed as **mean ± SD** of three independent replicates. Statistical analysis was done by one-way ANOVA (all: $p < 0.05$) with Tukey's post hoc test (different letters indicate significant differences). OJ: pure orange juice. OJ+E1: orange

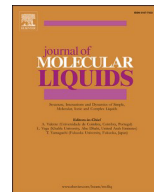
juice with choline chloride:glycerol extract. OJ+E2: orange juice with choline chloride:glycerol:citric acid extract. OJ+E3: orange juice with choline chloride:fructose extract. OJ+E4: orange juice with choline chloride:xylose extract. OJ+N1: orange juice with choline chloride:glycerol NADES. OJ+N2: orange juice with choline chloride:glycerol:citric acid NADES. OJ+N3: orange juice with choline chloride:fructose NADES. OJ+N4: orange juice with choline chloride:xylose NADES..

Anexo XII: Publicaciones



Contents lists available at ScienceDirect

Journal of Molecular Liquids

journal homepage: www.elsevier.com/locate/molliq

Natural deep eutectic solvents: A paradigm of stability and permeability in the design of new ingredients

Clara Gomez-Urios^a, Pablo Puchades-Colera^a, Ana Frigola^a, Maria J. Esteve^{a,*}, Jesus Blesa^a, Daniel Lopez-Malo^b

^a Nutrition and Food Science Area, Faculty of Pharmacy and Food Science, University of Valencia, Avda. Vicent Andrés Estellés, s/n, 46100 Burjassot (Valencia), Spain

^b Faculty of Health Sciences, Universidad Europea de Valencia, Pg. de l'Albereda, 7, 46010 Valencia, Spain

ARTICLE INFO

Keywords:

Antioxidant capacity
Membrane permeability
Ascorbic acid
Natural deep eutectic solvents
Degradation kinetics
Solubility

ABSTRACT

Ascorbic Acid (AA) is a crucial component in the food industry, serving as both a quality and safety criterion. It is used to fortify foods due to its significant role in human health, acting as an antioxidant. Its benefits extend to both product quality and consumer health in the food and cosmetic industry. Due to their physicochemical properties, Natural Deep Eutectic Solvents (NADES) have the potential to be used for the extraction and stabilization of bioactive compounds. The objective of this study is to characterize various NADES, simulate the solubility of AA, evaluate the antioxidant and protective capacity of NADES, and investigate the permeability of this vitamin through the skin and gastrointestinal membrane. Antioxidant capacity was measured using three methods: TEAC, DPPH, and ORAC. The solubility of AA in NADES systems was simulated using the COSMO-Therm software. The degradation of AA was monitored by HPLC/UV-Vis for 30 days at two different storage temperatures. Additionally, the membrane solubility was measured using the PAMPA method in both skin and gastrointestinal. The results indicate that organic acid-based NADES are more polar than sugar and polyalcohol-based NADES. The solubility prediction shows that reduced $\ln(\gamma)$ in Choline chloride-based NADES results in low AA solubility, which positively correlates with higher pH values. AA degradation increased at lower pH, and its half-life time was longer at 4 °C, being the best choline chloride (ChChl):xylose. Moreover, betaine:malic acid, ChChl:tartaric acid, and ChChl:lactic acid gave worse stabilizing results than control. According to the PAMPA study, AA in lactic acid:glucose had the highest P_e (permeability coefficient) for both gastrointestinal ($\log P_e$: 4.99) and skin ($\log P_e$: 4.78). Malic acid:glucose, on the other hand, had the lowest $\log P_e$ value (-6.9). In this study, some NADES can play a protective role in the preservation of ascorbic acid. This statement could be extended to other bioactive compounds found in NADES extracts that may be impacted by oxidative processes. Moreover, not all NADES stabilized equally AA. These findings could be applied to the formulation of ascorbic acid-containing drugs, cosmetics, and food products.

1. Introduction

One of the most important challenges facing the chemical industry is green chemistry, which strives to reduce harmful impacts on human health while minimizing environmental damage [11]. In recent years, it has become increasingly important to replace hazardous solvents in the extraction of natural chemicals from plant matrices with innovative alternatives [21]. Natural Deep Eutectic Solvents (NADES) are a good choice, due to their structural diversity and physicochemical properties,

to extract bioactive compounds from food and plant matrices [27,31].

NADES are a mixture of compounds whose melting points are decreased when combined in the appropriate molar ratios. These solvents consist of a hydrogen bond acceptor (HBA), which is commonly a quaternary ammonium salt like choline chloride, and a hydrogen bond donor (HBD) based on amines, sugars, alcohols, polyols, or organic acids [13]. NADES also has many other advantageous properties, such as being nonflammable, recyclable, biodegradable, and having low toxicological effects on the environment and human health [23].

Abbreviations: NADES, Natural Deep eutectic solvents; AA, Ascorbic acid; PAMPA, parallel artificial membrane permeability assay; ChChl, Choline chloride; CA, Citric acid; LA, lactic acid; LP, L-Proline; MA, Malic acid; Gly, Glycerol; Glu, Glucose; TA, Tartaric acid; Bet, Betaine; Xyl, Xylose; Fru, Fructose.

* Corresponding author.

E-mail address: maria.jose.esteve@uv.es (M.J. Esteve).

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Article

Sustainable Development and Storage Stability of Orange By-Products Extract Using Natural Deep Eutectic Solvents

Clara Gómez-Urios ^{1,†} , Adriana Viñas-Ospino ^{1,†} , Pablo Puchades-Colera ¹, Daniel López-Malo ² , Ana Frígola ¹, María José Esteve ^{1,*}  and Jesús Blesa ¹ 

¹ Nutrition and Food Science, Faculty of Pharmacy, University of Valencia, Avenida Vicent Andrés Estellés, s/n, 46100 Burjassot, Spain

² Faculty of Health Science, European University of Valencia, 46100 Valencia, Spain

* Correspondence: maria.jose.esteve@uv.es

† These authors contribute equally to this work.

Abstract: The citrus industry produces large amounts of waste rich in bioactive compounds that have important effects on human health. Their extraction was performed using organic solvents, and a greener alternative to those solvents are natural deep eutectic solvents (NADES). The present study aimed to obtain and optimize extracts rich in polyphenols and flavonoids from orange peels using NADES and monitor polyphenol stability in the extracts for 30 days. The software COSMOtherm (conductor-like screening model) was used to screen fourteen NADES. The most promising solvents were lactic acid:glucose (LA:Glu) with an extraction yield of 1932 ± 7.83 mgGAE/100 gdw for TPC (total polyphenol content) and 82.7 ± 3.0 mg/100 gdw for TFC (total flavonoid content) and in the case of L-proline:malic acid (LP:MA) was 2164 ± 5.17 mgGAE/100 gdw for TPC and 97.0 ± 1.65 mg/100 gdw for TFC. The extraction process using LA:Glu and LP:MA was optimized, and the results showed that the selected variables (%NADES, solid:liquid ratio, and extraction time) had a significant influence on the extraction of TPC and TFC. Results showed that NADES improve the stability of TPC. These findings revealed that NADES are efficient for the extraction of bioactive compounds from orange by-products, and these extracts can represent an alternative for the food industry to enrich food products with natural ingredients.

Keywords: natural deep eutectic solvents; green extraction; orange peels; bioactive compounds; polyphenols; COSMOtherm



Citation: Gómez-Urios, C.; Viñas-Ospino, A.; Puchades-Colera, P.; López-Malo, D.; Frígola, A.; Esteve, M.J.; Blesa, J. Sustainable Development and Storage Stability of Orange By-Products Extract Using Natural Deep Eutectic Solvents. *Foods* **2022**, *11*, 2457. <https://doi.org/10.3390/foods11162457>

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1. Introduction

Global orange production is estimated at around 115.5 million tons, and from this amount, Spain produces over 3.3 million tons [1]. Fifty percent of the orange weight is by-products, and these are discarded, representing an important environmental problem. Orange by-products include peel (flavedo and albedo), pulp, and seeds, which are known sources of valuable compounds with biological effects on human health [2,3]. For example, orange peel extracts have been demonstrated to decrease the risk of developing cancer in mouse models [4]. These biological benefits can be attributed to the wide range of biomolecules present in orange peels as fermentable sugars, carbohydrate polymers, flavonoids, polyphenols, vitamins, carotenoids, and essential oils [5]. Especially, polyphenols have gained much interest due to their antioxidant and inflammatory effects [6,7].

Polyphenol extraction has been developed in the industry using a different kind of organic solvents. However, these conventional extractions require a large amount of solvent, which causes problems such as environmental pollution and toxic effects on human health [8,9]. For this reason, the implementation of new techniques using green solvents to replace petrochemical solvents is a concern. According to one of the principles of green chemistry, reducing energy consumption by energy recovery and using innovative

Unlocking Value from Waste: a Comparative Study of Orange Peel Extraction Methods Using Choline Chloride-Based Natural Deep Eutectic Solvents

Clara Gomez-Urios, Pablo Puchades-Colera, Ana Frigola, Daniel Lopez-Malo, Maria J. Esteve,* and Jesus Blesa



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Supporting Information

ABSTRACT: Green chemistry is a fundamental aspect of the food industry in the extraction of bioactive compounds from plant byproducts, through the utilization of sustainable methodologies, such as ultrasound-assisted extraction (UAE), enhancing food safety. Conventional solvents are being replaced by green alternatives, including natural deep eutectic solvents (NADES), which are nontoxic and biodegradable. The objective of this study is to compare UAE with heating-stirring (HS) methods for the extraction of bioactive compounds from orange peels using NADES. The antioxidant capacity (AC) of the extracts will be compared by three methods [oxygen radical absorbance capacity (ORAC), DPPH, and ferric ion reducing antioxidant power (FRAP)], also the phenolic profile will be studied, and the stability of the extracted compounds will also be assessed. The AC results demonstrated that UAE yielded higher than HS with ChChl/Fruc and ChChl/LA. The FRAP revealed differences among NADES, with ChChl/LP/MA exhibiting the highest. ORAC method identified ChChl/LP/MA and ChChl/LA as the most efficient. The UAE showed higher extraction yield compared to HS, particularly for caffeic acid and hesperidin, where ChChl/Fruc and ChChl/LA were the best NADES. Concerning stability, HS yielded a more stable AC than UAE, except ChChl/Fruc. UAE demonstrated superior stability for phenolic compounds in ChChl/LP/MA and ChChl/Gly, whereas HS showed greater efficacy for ChChl/LA. In conclusion, the selection of extraction methods and solvents has an impact on the yield, stability, and AC of bioactive compounds. It is evident that the selection of NADES and extraction methods should be tailored to achieve an optimal balance between yield, AC, and long-term stability of the bioactive compounds.

KEYWORDS: natural deep eutectic solvents, bioactive compounds, green chemistry, method comparison, ultrasound-assisted extraction, orange byproducts, heating-stirring

1. INTRODUCTION

Green chemistry is now an essential aspect of the industry for generating novel food products and extracting bioactive compounds for functional food, pharmaceuticals, and cosmetics. In addition, the food industry produces a large amount of pollutant-laden plant byproducts that contain beneficial bioactive compounds.¹ Orange peel is the main waste produced in the juice industry. This peel contains a high amount of phenolic compounds, carotenoids, and ascorbic acid with high biological value.²

Extraction conditions using organic solvents, high temperatures, and long extraction times have always been used in industry.³ Recently, the search for green extraction methods has emerged, and the use of microwaves, supercritical fluids, electrical pulses, and ultrasound-assisted extraction (UAE) has increased to minimize the negative effects of conventional extraction, which helps and contributes to sustainability and food safety.⁴ The extraction of bioactive compounds has been conventionally performed by the heating-stirring extraction (HS) method used for the extraction of bioactive compounds from plant matrices^{5,6} which allows the diffusion of the target compounds to the solvents and does not imply high investment, nor high energy consumption to the food industry.

On the other hand, UAE has gained popularity in recent years. UAE has many advantages and follows the principles of green chemistry. The creation of bubbles by the cavitation phenomenon allows the breakdown of the cell wall and helps release the bioactive compounds.⁷ Among its advantages, UAE improves the physicochemical properties of food products while maintaining their safety.⁸ One problem with conventional extraction is the use of organic solvents, such as ethanol, methanol, and acetone. These solvents can be replaced by more environmentally friendly solvents, such as natural deep eutectic solvents (NADES). NADES are eutectic mixtures formed by two or more components in a specific molar ratio, they are nontoxic, inexpensive, biodegradable, and environmentally friendly, with low melting points, nonvolatile, and nonflammable.⁹ Also, the physicochemical properties (polarity, pH, and conductivity) increase their affinity for bioactive

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Choline chloride-based natural deep eutectic solvents for the extraction and stability of phenolic compounds, ascorbic acid, and antioxidant capacity from *Citrus sinensis* peel

Clara Gómez-Uríos^a, Adriana Viñas-Ospino^a, Pablo Puchades-Colera^a, Jesús Blesa^a, Daniel López-Malo^b, Ana Frígola^a, María José Esteve^{a,*}

^a Nutrition and Food Science, Faculty of Pharmacy, University of Valencia, Avda. Vicent Andrés Estellés, S/n, 46100, Burjassot (Valencia), Spain

^b Faculty of Health Sciences, European University of Valencia, Valencia, Spain

ARTICLE INFO

Keywords:

NADES
Polyphenol
Ascorbic acid
Orange peel
Stability

ABSTRACT

Orange peel is a great source of bioactive compounds like phenolic compounds and ascorbic acid (AA), which have high antioxidant capacity. New methodologies have called attention for their extraction employing greener solvents, as natural deep eutectic solvents (NADES). There is a lack of information about the protective role and stability of bioactive compounds in NADES. This study aims to find the most suitable NADES for the extraction of bioactive compounds from orange peel. The first step was screening eight NADES, determining its antioxidant capacity (AC), AA, total phenolic content (TPC) and total flavonoids (TFC). The extract stability was monitored during storage for twelve days, determining phenolic compounds, AA, and AC. The results showed that choline chloride:malic acid (ChCl:MA) (TPC: 1053 mgGAE/100 g. TFC: 94.7 mg/100 g. AA: 430 mg/100 g), and choline chloride:glycerol (TPC: 903 mgGAE/100 g. TFC: 169 mg/100 g. AA: 416 mg/100 g) were the most suitable NADES for the extraction of bioactive compounds. AA and phenolic compounds are more stable in ChCl:MA. The capacity of NADES to improve the stability and enhance the AC of the extracts makes these formulations suitable for their application in the food formulations avoiding the solvent elimination process.

1. Introduction

The orange is one of the most used fruits in the food industry for the production of fruit juices, also, it is a widely cultivated and marketed fruit. About two-thirds of the citrus fruits worldwide are produced in China, Brazil, India, Mexico, the USA, and Spain (Food and Agriculture Organization of the United Nations, 2017). Its composition makes the orange useable not only in the food industry, but also in the perfumery, cosmetics, and pharmaceutical industry (Alexandre, Moreira, Castro, Pintado, & Saraiva, 2018). The citrus industry generates a wide range of orange waste, including peel and seeds, representing 50% of the total weight of the fruit (Ozturk, Parkinson, & Gonzalez-Miquel, 2018). Orange peel, a by-product of the juice industry, has significant biological value (Romano et al., 2022), antioxidant capacity (AC), and health-promoting qualities (Teixeira et al., 2020). However, due to their high production, it also represents a problem for the environment.

Polyphenols and ascorbic acid are one of the most abundant bioactive compounds in orange peel, with biological properties such as

antioxidant and antibacterial (Fu et al., 2022), they also are great additives in food products (Lazar, Talmaciu, Volf, & Popa, 2016). Different phenolic compounds are found in orange peel, being hesperidin the most abundant (Anticon, Blesa, Frígola, & Esteve, 2020). The structure of the phenolic compounds present in orange peel differs depending on the group (phenolic acid and flavonoid). Their molecular structure (Fig. S1) gives the compounds different chemical characteristics, such as their biological activity (Singh, Singh, Kaur, & Singh, 2020). When phenolic acids undergo condensation reactions with glucose and quinic acid, they take on a water-insoluble form. Low molecular weight phenolic acids exhibit water-soluble qualities throughout subsequent processing as well as during human digestion as can be checked in Table S3 (Bennour, Mighri, Eljani, Zammouri, & Akrou, 2020).

To obtain these bioactive compounds, different extraction methods, like mechanical extraction and agitation, could increase the extraction power by combining with ultra-sound, microwaves, or electric pulses (Pradana, Sadewo, Haryanto, & Sudibyo, 2021). According to the principles of green chemistry, the industry is more focused in searching

* Corresponding author.

E-mail address: Maria.jose.esteve@uv.es (M.J. Esteve).

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Anexo XIII. Conferences

Autores: Gómez-Urios, Clara; Viñas-Ospino, Adriana; López-Malo, Daniel; Frígola, Ana; Blesa, Jesús; Esteve, Maria J

Título: Valorization of food waste: comparison of the polyphenol profile, extracted by ultrasound using natural deep eutectic solvents

Tipo de participación: Poster

Congreso: 10th International Symposium on Recent Advances in Food Analysis-RAFA 2022

Ciudad: Praga

País:: REPÚBLICA CHECA

Año: 2022

Autores: Gomez-Urios, Clara; Puchades-Cólera, Pablo; Esteve, Maria J.; Frigola, Ana; Lopez-Malo, Daniel; Blesa, Jesús

Título: Sugar and polyalcohol-based NADES: Stability of Ascorbic acid during storage at 25°C and 4°C.

Tipo de participación: Poster

Congreso: 5th International Symposium on Green Chemistry

Ciudad:

País:: FRANCIA

Año: 2022

Autores: Puchades-Cólera, Pablo; Gomez-Urios, Clara; Esteve, Maria J.; Frigola, Ana; Lopez-Malo, Daniel; Blesa, Jesús

Título: Natural deep eutectic solvents to the extraction of bioactive compounds from orange peel and antioxidant capacity of extracts. Replacing organic conventional solvents?

Tipo de participación: Poster

Congreso: 5th International Symposium on Green Chemistry

Ciudad:

País:: FRANCIA

Año: 2022

Autores: Gomez-Urios, Clara; Viñas-Ospino, Adriana; Puchades-Cólera, Pablo; Esteve, Maria J.; Frigola, Ana; Blesa, Jesús; Lopez-Malo, Daniel

Título: Extracción de ácido ascórbico en residuos de la industria citrícola con solventes naturales (NADES)

Tipo de participación: Poster

Congreso: Nutrición Práctica XXVI Jornadas Internacionales. XV Congreso internacional de SEDCA

Publicación: Nutrición Clínica y Dietética Hospitalaria, Vol. 42 Núm. supl. 1 (2022)

Ciudad: Madrid

País:: ESPAÑA

Año: 2022

Autores: Puchades-Cólera, Pablo; Gomez-Urios, Clara; Viñas-Ospino, Adriana; Frigola, Ana; Blesa, Jesús; Lopez-Malo, Daniel; Esteve, Maria J.

Título: Evaluación de la capacidad antioxidante (DPPH y ORAC) de NADES y su impacto sobre la estabilidad del ácido ascórbico

Tipo de participación: Poster

Congreso: Nutrición Práctica XXVI Jornadas Internacionales. XV Congreso internacional de SEDCA

Publicación: Nutrición Clínica y Dietética Hospitalaria, Vol. 42 Núm. supl. 1 (2022)

Ciudad: Madrid **País::** ESPAÑA **Año:** 2022

Autores: Viñas-Ospino, Adriana; Gomez-Urios, Clara; Puchades-Cólera, Pablo; Esteve, Maria J.; Frigola, Ana; Lopez-Malo, Daniel; Blesa, Jesús

Título: Natural Deep Eutectic Solvents for the green alternative carotenoid extraction from orange peels.

Tipo de participación: Poster

Congreso: Nutrición Práctica XXVI Jornadas Internacionales. XV Congreso internacional de SEDCA

Publicación: Nutrición Clínica y Dietética Hospitalaria, Vol. 42 Núm. supl. 1 (2022)

Ciudad: Madrid **País::** ESPAÑA **Año:** 2022

Autores: Gómez-Urios, Clara; Mbuy, Inés; López-Malo, Daniel; Frígola, Ana; Esteve, Maria J, Blesa, Jesús

Título: Reusing food waste: Ascorbic acid extraction from orange peel using ultrasound assisted extraction and natural deep eutectic solvents.

Tipo de participación: Poster

Congreso: The 3rd international electronic conference on Foods: Food, Microbiome, and Health - A Celebration of the 10th Anniversary of Foods' Impact on our Wellbeing

Ciudad: On-line **País::** PAÍSES BAJOS **Año:** 2022

Autores: Puchades-Colera, Pablo; Gómez-Urios, Clara; Esteve, Maria J.; Frigola, Ana; Blesa, Jesús; Lopez-Malo, Daniel

Título: Determination of hesperidin and evaluation of total antioxidant capacity on orange peel extracts obtained by natural deep eutectic solvents (nades)

Tipo de participación: Poster

Congreso: VI Congreso Internacional ACOFESAL: Calidad y Seguridad Alimentaria

Ciudad: **País::** ESPAÑA **Año:** 2022

Autores: Gómez-Urios, C.; Viñas-Ospino, A.; Penadés-Soler, A.; Frígola, A.; López-Malo, D.; Esteve, MJ ; Blesa, J.

Título: Natural deep eutectic solvents as main solvent for the extraction of total polyphenols of orange peel.

Tipo de participación: Comunicación

Congreso: 2nd International Electronic Conference on Foods - 'Future Foods and Food Technologies for a Sustainable World'

Ciudad: **País::** SUIZA **Año:** 2021

Observaciones: Formato virtual. Comunicación mediante Proceeding. Organizado por Sciforum plataforma de MDPI

Autores: Viñas-Ospino A.; Gómez-Urios, C.; Penadés-Soler, A.; Frígola A.; Esteve M.; López-Malo D.; Blesa J.

Título: Green extraction using deep eutectic solvents of flavonoids from orange peels.

Tipo de participación: Comunicación

Congreso: 2nd International Electronic Conference on Foods - 'Future Foods and Food Technologies for a Sustainable World'

Ciudad: **País::** SUIZA **Año:** 2021

Observaciones: Formato virtual. Comunicación mediante Proceeding. Organizado por Sciforum plataforma de MDPI

Autores: PUCHADES-COLERA Pablo; GÓMEZ-URIOS Clara; VIÑAS-OSPINO Adriana; LÓPEZ-MALO Daniel; FRÍGOLA Ana; BLESA Jesús; ESTEVE María José

Título: Evaluation of the antioxidant capacity on natural deep eutectic solvents containing organic acids, NADES

Tipo de participación: Comunicación

Congreso: FORTHEM Food Science Lab

Ciudad: **País::** ITALIA **Año:** 2022

Autores: Clara Gomez-Urios; Adriana Viñas-Ospino; Ana Frigola; Maria Jose Esteve; Daniel Lopez-Malo; Jesus Blesa

Título: Study of the skin and gastrointestinal membrane permeability of ascorbic acid in fourteen different nades

Tipo de participación: Poster

Congreso: DES Conference 2023

Ciudad: Lisboa **País::** PORTUGAL **Año:** 2023

Autores: Clara Gómez-Urios; Leila Benayada; Maria José Esteve; Jesus Blesa; Ana Frigola; Daniel Lopez-Malo

Título: Recover polyphenols from nades extracts of orange peel by three resins solid phase extraction

Tipo de participación: Poster

Congreso: EFFOST 2023

Ciudad: Valencia **País::** ESPAÑA **Año:** 2023

Autores: Sara Margherita Borgonovi; Clara Gomez-Urios; Maria Jose Esteve; Jesus Blesa; Stefania Iametti; Mattia Di Nunzio

Título: Protein profiling and modulation of digestive proteolytic enzymes by nades extracts of citrus by-product

Tipo de participación: Poster

Congreso: EFFOST 2023

Ciudad: Valencia **País::** ESPAÑA **Año:** 2023

Autores: GÓMEZ-URIOS Clara; VIÑAS-OSPINO Adriana; PUCHADES-COLERA Pablo; LÓPEZ-MALO Daniel; FRÍGOLA Ana; BLESA Jesús; ESTEVE María José

Título: Green extraction of ascorbic acid from orange peel using natural deep eutectic solvents

Tipo de participación: Comunicación

Congreso: FORTHEM Food Science Lab

Ciudad: **País::** ITALIA **Año:** 2022

Autores: C. Gómez-Urios; S.M. Borgonovi; S. Buratti; S. Benedetti; M.J. Esteve; J. Blesa; M. Di Nunzio

Título: Polyphenolic content and sensory profile assessed by e-nose and e-tongue of an orange juice fortified with polyphenol-enriched extracts using Natural Deep Eutectic Solvents

Tipo de participación: Poster

Congreso: 7th International Conference on Foodomics

Entidad organizadora: Universidad de Bolonia

Ciudad: Cesena **País::** ITALIA **Año:** 2024
