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NUEVOS MÉTODOS DE EXTRACCIÓN Y FRACCIONAMIENTO DE CARBOHIDRATOS BIOACTIVOS

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NUEVOS MÉTODOS DE EXTRACCIÓN Y FRACCIONAMIENTO DE CARBOHIDRATOS BIOACTIVOS

Memoria presentada por:

LAURA RUIZ ACEITUNO

Para optar al grado de

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CERTIFICAN:

Que el presente trabajo, titulado "**NUEVOS MÉTODOS DE EXTRACCIÓN Y FRACCIONAMIENTO DE CARBOHIDRATOS BIOACTIVOS**", y que constituye la Memoria que presenta Dª. Laura Ruiz Aceituno para optar al grado de Doctor en Ciencia y Tecnología de Alimentos, ha sido realizado en el Departamento de Análisis Instrumental y Química Ambiental del Instituto de Química Orgánica General del C.S.I.C, bajo nuestra dirección.

Y para que así conste, firman el presente certificado en Madrid a 8 de julio de 2015.

Fdo. Dª. María Luz Sanz Murias **Fdo. Dª. Lourdes Ramos Rivero**

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En definitiva, esta tesis está dedicada a aquellas personas que más de cerca han vivido los momentos más prósperos y también los más difíciles en relación a la realización de ésta.

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ÍNDICE

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LISTA DE ACRÓNIMOS

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[BMIM][$MeSO_4$]: 1-butyl-3-methylimidazolium methylsulfate / 1-butil-3-metilimidazolio metil sulfato

[EMIM][DCA]: 1-Ethyl-3-methylimidazolium dicyanamide / 1-etil-3-metililimidazolio de dicianamida

[EMIM][OAc]: 1-ethyl-3-methylimidazolium acetate / 1-etil-3-metililimidazolio acetato

[HMIM][Cl]: 1-Hexyl-3-methylimidazolium chloride / 1-hexil-3-methilimidazolio de cloruro

[MMIM][Me_2PO_4]: 1,3-dimethylimidazolium dimethylphosphate / 1,3-dimetilimidazolio dimetil fosfato

ASE: Accelerated Solvent Extraction / Extracción acelerada con disolventes

BSA: Bis(trimethylsilyl)acetamide / bis(trimetilsilil)acetamida

BSTFA: N,O-Bis(trimethylsilyl)trifluoroacetamide / N,O-Bis(trimetilsilil)trifluoroacetamida

DMSO: Dimethyl sulfoxide / dimetil sulfóxido

DP: Degree of polymerization / Grado de polimerización

EI: Electron Impact / Impacto Electrónico

FOS: Fructooligosaccharides / Fructooligosacáridos

GC: Gas chromatography / Cromatografía de gases

GC-FID: Gas chromatography–flame ionization detection / Cromatografía de gases con detector de ionización de llama

GC-MS: Gas chromatography–mass spectrometry / Cromatografía de gases acoplada a espectrometría de masas

GOS: Galactoooligosaccharides / Galactooligosacáridos

HMDS: Hexamethyldisilazane / hexametildisilazano

HMF: 5-hydroxymethylfurfural / 5-hidroximetilfurfural

HPLC-MS: High Performance Liquid Chromatography–Mass Spectrometry / Cromatografía Líquida de Alta Resolución acoplada a Espectrometría de Masas

HPLC-RID: High Performance Liquid Chromatography– Refractive Index Detector/ Cromatografía Líquida de Alta Resolución acoplada a Detector de Índice de Refracción

HPLC-UV: High Performance Liquid Chromatography– Ultraviolet detection / Cromatografía Líquida de Alta Resolución acoplada a Detector de Ultravioleta

IEC: Ion Exchange Chromatography / Cromatografía de intercambio iónico

ILs: Ionic liquids / Líquidos iónicos

$\boldsymbol{I^T}$**:** Retention indices / Índices de Retención Cromatográfica

IUPAC-IUB: International Union of Pure and Applied Chemistry – International Union of Biochemistry / Unión Internacional de Química Pura y Aplicada – Unión Internacional de Bioquímica

LC: Liquid chromatography / Cromatografía de líquidos

LLE: Liquid-Liquid Extraction / Extracción Líquido-Líquido

LMWC: Low Molecular Weight Carbohydrates / Carbohidratos de Bajo Peso Molecular

LOD: Limit of Detection / Límite de Detección

LOQ: Limit of Quantification / Límite de Cuantificación

MAE: Microwave Assisted Extraction / Extracción Asistida con Microondas

MS: Mass spectrometry / Espectrometría de Masas

PCOS: Polycystic Ovary Syndrome / Síndrome de Ovario Poliquístico

PLE: Pressurized Liquid Extraction / Extracción con Líquidos Presurizados

PSE: Pressurized Solvent Extraction / Extracción con Disolventes Presurizados

RF: Response factors / Factores de respuesta

RTILs: Room temperatura Ionic Liquids / Líquidos iónicos a temperatura ambiente

SEC: Size Exclusion Chromatography / Cromatografía de Exclusión Molecular

SFE: Supercritical Fluids Extraction / Extracción con Fluidos Supercríticos

SHWE: Superheated Water Extraction / Extracción con Agua Sobrecalentada

SLE: Solid Liquid Extraction / Extracción Sólido-Líquido

TFA: Trifluoroacetic acid / ácido trifluoroacético

TMCS: Trimethylchlorosilane / trimetilclorosilano

TMS: Trimethylsilyl derivatives / trimetilsilil derivados

TMSI: Trimethylsilylimidazole / trimetilsililimidazol

TMSO: Trimethylsilyl oximes / trimetilsilil oximas

tR: Retention times / Tiempos de retención

UAE: Ultrasonic Assisted Extraction / Extracción Asistida con Ultrasonidos

RESUMEN / SUMMARY

RESUMEN

La obtención de carbohidratos bioactivos, como son los inositoles y prebióticos, presenta un gran interés para su uso como ingredientes alimentarios, por los beneficios que éstos suponen para la salud de los consumidores. Dichos compuestos están implicados en diversos procesos biológicos relacionados, en especial, con alteraciones metabólicas o con la inducción de cambios en la microbiota intestinal. El potencial empleo de estos ingredientes bioactivos por parte de la industria alimentaria y/o farmacéutica hace que la búsqueda de nuevas vías para su obtención, que sean eficaces y ventajosas frente a las ahora disponibles, cobre cada vez mayor importancia.

En línea con esta observación, el objetivo principal de esta tesis doctoral es **el desarrollo de nuevos métodos para la extracción y el fraccionamiento selectivo de carbohidratos bioactivos (inositoles y prebióticos)**.

Para alcanzar este objetivo, se han abordado dos líneas principales de investigación: (i) la evaluación del empleo de nuevos disolventes (líquidos iónicos, ILs) para el fraccionamiento selectivo de carbohidratos en función de su estructura química, y (ii) el desarrollo de nuevos métodos de extracción y enriquecimiento de carbohidratos bioactivos a partir de matrices naturales.

En primer lugar, fue necesario desarrollar un método de derivatización que permitiera el análisis de carbohidratos de bajo peso molecular (LMWC) disueltos en ILs por cromatografía de gases (GC). El método optimizado y validado resultó ser eficaz para este fin, siendo la primera vez que carbohidratos disueltos en ILs eran analizados por esta técnica. Posteriormente, y dada la escasez de datos bibliográficos sobre la solubilidad de LMWC en ILs, se llevaron a cabo estudios de solubilidad de distintos monosacáridos, polialcoholes, disacáridos y trisacáridos en ILs basados en metil-imidazolio pudiéndose relacionar dichos valores con su estructura química. Los ILs resultaron ser disolventes prometedores para el fraccionamiento selectivo de aldosas y cetosas bioactivas, tales como lactosa y lactulosa, así como de distintos polialcoholes y otros LMWC.

Previo al desarrollo de métodos de extracción, se llevaron a cabo avances en la determinación cuali- y cuantitativa de los LMWC (en particular de inositoles y prebióticos) presentes en distintas matrices alimentarias (piñones, vegetales, legumbres) por GC-MS previa derivatización a sus correspondientes trimetilsilil oximas (TMSO) determinándose compuestos no identificados hasta el momento en estas matrices.

En esta Tesis, se seleccionaron la extracción con líquidos presurizados (PLE) y la extracción asistida por microondas (MAE) con el fin de evaluar su potencial en este campo de aplicación, así como sus correspondientes ventajas e inconvenientes. Los métodos de PLE y MAE desarrollados permitieron obtener rendimientos en inositoles superiores a los conseguidos por el procedimiento convencional de extracción sólido-líquido, pero con tiempos de extracción más cortos y con un menor consumo de disolvente. Además, los métodos desarrollados consiguieron por primera vez la extracción conjunta de inositoles e inulina a partir de subproductos de la industria alimentaria.

Por último, se ha profundizado en el fraccionamiento de carbohidratos usando un procedimiento biotecnológico basado en el uso de *Saccharomyces cerevisiae* que permitió la eliminación de azúcares coexistentes en los extractos que podrían interferir con la bioactividad de los carbohidratos de interés.

Los resultados descritos en esta Tesis suponen una contribución destacable en el estudio de carbohidratos, así como en la obtención de nuevos ingredientes bioactivos, tema éste de gran actualidad y repercusión tanto científica como económica.

SUMMARY

The extraction of bioactive carbohydrates, such as inositols and prebiotics, from natural sources is of great interest for their use as food ingredients due to the benefits they represent to consumers' health. These compounds are involved in several biological processes related with metabolic disorders or inducing changes in gut microbiota. The potential use of these bioactive ingredients from the food and / or pharmaceutical industry makes that the search for new extraction alternatives, more effective and advantageous than the available ones, gains an increasing importance.

Therefore, the main objective of this PhD dissertation is the development of new methods for the extraction and selective fractionation of bioactive carbohydrates (inositol and prebiotics).

To achieve this goal, two main research lines have been addressed: (i) the evaluation of the use of new solvents (ionic liquids, ILs) for the selective fractionation of carbohydrates based on their chemical structure, and (ii) the development of new methods of extraction and enrichment of bioactive carbohydrates from natural matrices.

First of all, it was necessary to develop a derivatization method that allows the analysis of low molecular weight carbohydrates (LMWC) dissolved in ILs by gas chromatography (GC). The optimized and validated method proved to be effective for this purpose, being this the first time that carbohydrates dissolved in ILs are analyzed by this technique. Subsequently, and due to the scarce data published so far in the literature on the solubility of LMWC in ILs, solubility studies of different monosaccharides, polyols, disaccharides and trisaccharides in methyl-imidazolium based-ILs were carried out, and these values were related to their chemical structure. ILs were found to be promising solvents for the selective fractionation of aldoses and bioactive ketoses, such as lactose and lactulose, as well as several polyols and other LMWC.

Prior to the development of extraction methods, significant advances in qualitative and quantitative determination of the LMWC (particularly inositol and prebiotics) present in different food matrices (pine nuts, vegetables, legumes) were carried out by GC-MS after derivatization to their corresponding trimethylsylil oximes (TMSO). Different carbohydrates were identified for the first time in these matrices.

In this thesis, pressurized liquid extraction (PLE) and microwave assisted extraction (MAE) were selected to evaluate their efficiency in the extraction of bioactive carbohydrates, as well as their respective advantages and disadvantages. The developed PLE and MAE methods allowed to obtain greater yields of inositols than those achieved by the conventional solid-liquid extraction method, using shorter extraction times and with a lower solvent consumption. Moreover, the developed methods allowed, for the first time, the simultaneous extraction of inositol and inulin from food industry by-products.

Finally, a biotechnological process based on the use of *Saccharomyces cerevisiae* which allowed the removal of coexisting sugars in the extracts that might interfere with the bioactivity of the carbohydrates of interest has been optimized.

The results described in this thesis represent a remarkable contribution to the study of carbohydrates, as well as to the development of new ingredients and bioactive supplements, which is a subject of great interest and scientific and economic impact.

ESTRUCTURA DE LA MEMORIA

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La primera parte de esta memoria es una Introducción general (**Sección 1**) que recoge una revisión detallada de las principales características y propiedades de los carbohidratos objeto de estudio en este trabajo, en particular inositoles y carbohidratos prebióticos, así como una descripción de su contenido en distintos alimentos. También se describen y discuten las técnicas de extracción de carbohidratos bioactivos empleadas en la actualidad, prestando especial atención a la extracción sólido-liquido convencional, a la extracción mediante líquidos presurizados (PLE) y a la asistida por microondas (MAE), estas dos últimas como técnicas avanzadas, así como los principales métodos de fraccionamiento de carbohidratos, incluyendo una mención al potencial empleo de líquidos iónicos (IL) como nuevos disolventes. Por último, se revisan las técnicas instrumentales más comúnmente empleadas para el análisis de carbohidratos, prestando especial atención a la cromatografía de gases y su acoplamiento a espectrometría de masas (GC-MS). En esta sección se recogen parcialmente los artículos de revisión publicados en las revistas "Alimentación, Nutrición y Salud" (2012. Vol 19 (1), Pp 1-12) y "Trends in Analytical Chemistry" (2013. Vol 43, Pp 121 -145) y un capítulo del libro "Polysaccharides", (Ramawat, K. G.; Mérillon, J.-M., Eds. Springer International 2014; pp 1-18).

Los objetivos planteados en el trabajo aparecen detallados en la **Sección 2** (Objetivos, metodologíay plan de trabajo). Estos objetivos se han establecido en base al estado actual de los conocimientos descritos en la primera sección de la memoria y se han centrado en el desarrollo de nuevos métodos de extracción y fraccionamiento de carbohidratos bioactivos.

Los resultados más relevantes alcanzados en el marco de este estudio se presentan en forma de publicaciones científicas en las **Secciones 3 y 4** de esta memoria. Teniendo en cuenta las limitaciones de los métodos existentes en la actualidad para el fraccionamiento de carbohidratos, el desarrollo de nuevos procedimientos que supongan un avance sobre los existentes presenta un gran interés. Así, la **Sección 3** evalúa el potencial de los ILs para el fraccionamiento selectivo de carbohidratos. En primera instancia, en la **Sección 3.1,** se desarrolla un método de derivatización para el análisis por GC de carbohidratos disueltos en

ILs, ya que hasta el momento no se había descrito ningún procedimiento que permitiera la aplicación de esta técnica al análisis directo de carbohidratos disueltos en estos disolventes. Los parámetros optimizados para este primer estudio se emplearon en estudios siguientes orientados a la evaluación de la solubilidad de distintos mono-, di- y trisacáridos (**Sección 3.2)** y polialcoholes (**Sección 3.3)** en cuatro ILs. El empleo de estos ILs resultó ser también eficaz para el fraccionamiento de monosacáridos/polialcoholes; disacáridos/polialcoholes (**Sección 3.3**) y aldosas/cetosas (**Sección 3.4**), en este último caso, tanto para mezclas binarias como para un producto real de síntesis que contenía lactulosa, lactosa y monosacáridos.

Los resultados obtenidos en relación con el desarrollo de nuevos métodos de extracción y enriquecimiento de carbohidratos bioactivos a partir de matrices naturales se recogen en la **Sección 4**. Así, en primer término, se estudió la composición en carbohidratos de bajo peso molecular (LMWC), prestando especial atención a la búsqueda de inositoles, en varias matrices vegetales, como el piñón (**Sección 4.1**), diversos vegetales (incluyendo las familias Asteraceae, Amarantaceae, Brassicaceae, entre otras) (**Sección 4.2**) y legumbres (**Sección 4.3**). Los LMWC extraídos fueron derivatizados a sus TMSO y analizados por GC-M. Su identificación se basó en datos de retención cromatográfica, además de la interpretación de sus espectros de masas. Por otra parte, se ha propuesto una metodología basada en el uso de *Saccharomyces cerevisiae* para el fraccionamiento de inositoles de otros LMWC presentes en extractos de legumbres **(Sección 4.3**). Dado el gran interés que existe en la actualidad en la obtención de ingredientes naturales que aporten propiedades específicas, es preciso disponer de procesos de obtención que aseguren su extracción con un alto rendimiento. En la **Sección 4.4** se propone el empleo de PLE para la obtención de extractos ricos en inositoles a partir de piñones, evaluando y optimizando los distintos parámetros experimentales que afectan a la eficacia del proceso (disolvente, temperatura, tiempo, número de ciclos). Además, se evalúa la eliminación de carbohidratos interferentes coextraidos usando el tratamiento con *S. cerevisiae* antes desarrollado. Finalmente, en la **Sección 4.5**, se describe la optimización de sendos procedimientos de extracción basados en PLE y MAE para la obtención de inositoles e inulina a partir de hojas externas de alcachofa, como residuo agroalimentario. Se optimizan las distintas variables que afectan al proceso (tiempo, temperatura, cantidad de muestra) mediante

diseños de experimentos para la obtención simultánea, por primera vez, de estos carbohidratos bioactivos.

Por último en la **Sección 5** se expone una discusión integradora de todos los resultados obtenidos, teniendo en cuenta los diferentes aspectos investigados y en la **Sección 6** se recogen las conclusiones más destacadas de esta Tesis en relación a los objetivos inicialmente planteados.

En cumplimiento con la normativa vigente referente a la presentación de tesis con mención internacional, en esta memoria aparecen redactados el resumen y las conclusiones en dos idiomas oficiales de la Comunidad Europea, inglés y español. Únicamente se presentan en inglés los trabajos publicados en revistas internacionales, el resto del trabajo está redactado en español.

1. INTRODUCCIÓN

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1.1. Definición y clasificación de los carbohidratos

Los **carbohidratos** son unas de las moléculas orgánicas más abundantes de la Tierra, ya que están presentes en las células de todos los organismos vivos y son los componentes mayoritarios de plantas y árboles (Sanz y Martínez-Castro 2007). Se clasifican en monosacáridos, oligosacáridos y polisacáridos según su grado de polimerización.

Los **monosacáridos** son los carbohidratos más sencillos y están constituidos por una cadena de átomos de carbono a los que se unen grupos hidroxilo. Uno de los átomos de carbono tiene unido un grupo carbonilo; según éste se encuentre en posición terminal o no, reciben el nombre de aldosas o cetosas (Biermann 1989; Collins y Ferrier 1995).

En disolución, las conformaciones abiertas aparecen en equilibrio con sus correspondientes formas cíclicas, estando estas últimas más favorecidas. Este equilibrio puede verse modificado en función del disolvente, la composición y temperatura de la disolución (Biermann 1989). Las estructuras cíclicas (hemiacetales) se obtienen como resultado de la reacción del grupo carbonilo con uno de los grupos hidroxilo de la molécula. Las formas cíclicas más frecuentes son las de anillos de 5 átomos de carbono (denominadas furanosas) y de 6 átomos (piranosas). Tras la ciclación, el grupo carbonilo (carbono anomérico) permite dos orientaciones en el espacio del grupo hidroxilo unido a él, originando dos posibles anómeros, α y β. Si el grupo hidroxilo unido al carbono anomérico se encuentra libre, ese azúcar es reductor (a través del mismo pueden reaccionar con otras moléculas), mientras que si está unido a otra molécula, es no reductor (este grupo está bloqueado).

Los **oligosacáridos** están constituidos por varias unidades (de 2 a 8) de monosacáridos unidos por enlaces O-glicosídicos. Dependiendo del número de unidades, los oligosacáridos se denominan disacáridos, trisacáridos, tetrasacáridos, etc.

Los **polisacáridos** pueden definirse como polímeros constituidos por un elevado número de monosacáridos formando cadenas lineales o ramificadas, que pueden estar unidos por enlaces glicosídicos iguales o de distinto tipo. Entre los más comunes se encuentran la inulina, el almidón, la celulosa, el glucógeno y la quitina.

Dentro del término genérico "carbohidrato" se incluyen, además de los monosacáridos, oligosacáridos y polisacáridos, otras sustancias derivadas de los mismos por reducción de los grupos carbonilo (alditoles), por oxidación de uno o más grupos terminales a ácidos carboxílicos, o por sustitución de uno o más grupos hidroxilos por átomos de hidrógeno, grupos amino, grupos tioles o grupos similares heteroatómicos (Angyal 1984).

Los **alditoles** se nombran a partir de la aldosa de la cual se generaron sustituyendo el sufijo "-osa" por "-itol": por ejemplo, la reducción de la D-glucosa origina D-glucitol (también denominado, sorbitol). Estos alditoles no cíclicos son conocidos generalmente como polialcoholes (Brimacombe y Webber 1972). Entre estos compuestos, los de mayor importancia en la industria alimentaria son el xilitol (pentitol) y el manitol (hexitol) (Belitz *y col.* 2009).

Los **ciclitoles** son polialcoholes cíclicos en los que al menos tres de los carbonos poseen un grupo hidroxilo. Dentro de esta categoría se encuentran los inositoles, cuya estructura es un anillo de seis carbonos con un hidroxilo en cada uno de ellos. Existen nueve isómeros, que se diferencian entre sí por la disposición axial o ecuatorial de sus grupos hidroxilo y se nombran con los prefijos: *cis*-, *epi*-, *allo*-, *neo*-, *myo*-, *muco*-, D-*chiro*-, L–*chiro*- y *scyllo*- (**Figura 1.1**) (Angyal y Anderson 1959).

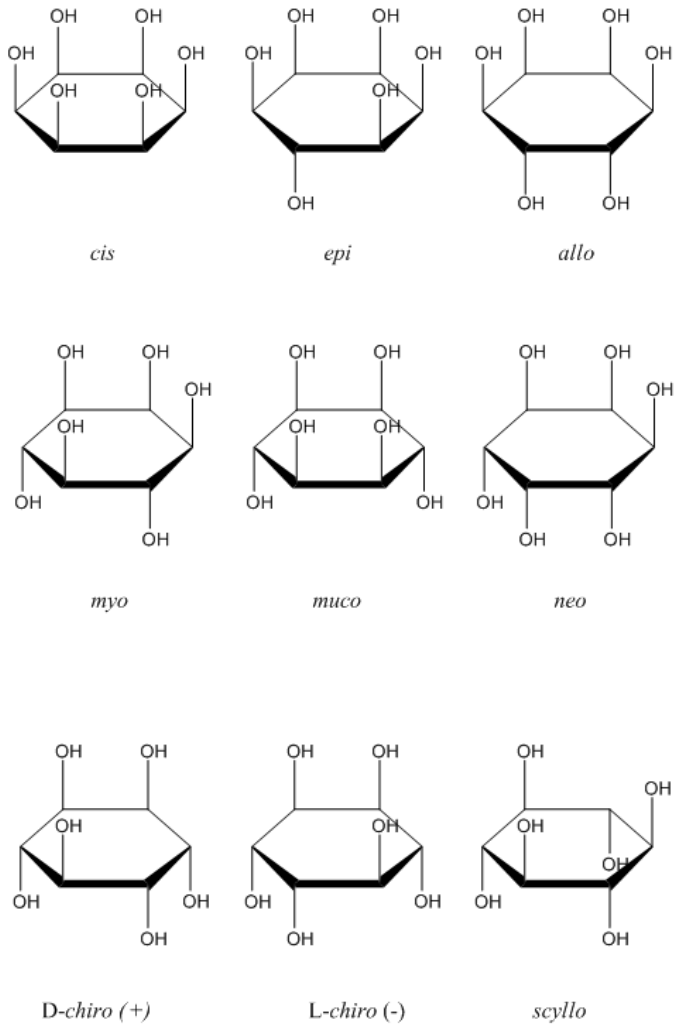

Figura 1.1. Estructura de los inositoles.

La nomenclatura de estos compuestos se recoge en las recomendaciones IUPAC-IUB 1973 para ciclitoles. Así, la configuración relativa de los grupos hidroxilos en cuanto a la posición en el anillo se describe como una fracción en la que los números situados en el numerador representan grupos hidroxilo u otros grupos diferentes al hidrógeno por encima del plano del anillo, mientras que los del denominador se refieren a aquellos que están por debajo del plano (Angyal y Anderson 1959). Como ejemplo, la forma de fracción 1,2,4,5/3,6 corresponde al *muco*-inositol (**Figura 1.1**). Más información sobre este tema, incluyendo las normas de nomenclatura para los compuestos quirales, se puede encontrar en http://www.chem.qmul.ac.uk/iupac/cyclitol.

En la naturaleza se encuentran al menos el *myo*-, *chiro*-, *scyllo*-, *neo*- y *muco*-inositol, así como algunos derivados como los metil-inositoles (con uno o dos de los hidroxilos metilados) y desoxi-inositoles (con uno o dos hidroxilos menos). En cambio, el *epi*-, *allo*- y *cis*-inositol sólo se obtienen por síntesis (Anderson 1972). Los inositoles pueden encontrarse en forma libre, o bien glicosilados, fosforilados, o formando parte de fosfolípidos.

El *myo*-inositol es el isómero más ampliamente distribuido en la naturaleza, seguido por los isómeros ópticamente activos D-(+)- y L-(-)-*chiro*-inositol. El *myo*-inositol se encuentra en los tejidos de casi todas las especies vivas: en animales mayoritariamente formando parte de fosfolípidos, y en vegetales como fosfatos (sería, por ejemplo, el caso del ácido fítico o fitato, *myo*-inositol hexafosfato, y sus correspondientes sales).

El *myo*-inositol pertenece al grupo de los 22 compuestos orgánicos (incluyendo 13 aminoácidos) que son necesarios para la supervivencia y crecimiento de las células humanas. Los humanos pueden sintetizar *myo*-inositol de forma endógena a partir de glucosa y, aunque a veces es considerado una vitamina, realmente no lo es (Angyal y Anderson 1959).

El D-(+)- y L-(-)-*chiro*-inositol están presentes en plantas superiores, sobre todo como metil-éteres [D-(+)-pinitol y L-(-)-quebrachitol]. La **Tabla 1.1** recoge los nombres y estructuras de algunos de los inositoles y derivados más comunes en la naturaleza.

Tabla 1.1. Clasificacion de inositoles más frecuentemente encontrados en la naturaleza.

	O-Metil-			**C-Metil-**	**Glicosil-**		**Glicosil-metil-**		
***Myo*-inositol** **(1,2,3,5/4,6)-ciclohexanohexol**	Bornesitol (1-*O*-metil-*myo*-inositol)	Ononitol (4-*O*-metil-*myo*-inositol)	Sequoyitol (5-*O*-metil-*myo*-inositol)	Laminitol (4-C-metil-*myo*-inositol)	Galactinol (*O*-α-D-galactopiranosil (1→3)-D-*myo*-inositol)	Isómeros galactinol	Galactosil bornesitol (latiritol)	Galactosil ononitol	Galactosil sequoyitol
					Digalactosil-*myo*-inositol (*O*-α-galactopiranosil-(1→6)-*O*-α-D-galactopiranosil-(1→3)-*myo*-inositol)			Digalactosil sequoyitol	Digalactosil ononitol
					Trigalactosil-*myo*-inositol ([*O*-α-galactopiranosil-(1→6)]$_2$-*O*-α-D-galactopiranosil-(1→3)-*myo*-inositol)				
					Tetragalactosil-*myo*-inositol				
***Muco*-inositol** **(1,2,4,5/3,6)-ciclohexanohexol)**	1-*O*-metil-*muco*-inositol	Viscumitol (1,2-di-*O*-metil-*muco*-inositol)							

Tabla 1.1. Continuación.

	O-Metil-			**C-Metil-**	**Glicosil-**		**Glicosil-metil-**		
***Chiro*- inositol** **(D) (1,2,5/3,4,6)- y (L) (1,2,4/3,5,6)-ciclohexanohexol**	Pinitol (3-*O*-metil-D-*chiro*-inositol)	Quebrachitol (2-*O*-metil-L-*chiro*-inositol)	Pinpollitol (1,4-*O*-dimetil-*chiro*-inositol)		Fagopiritol A1 (*O*-α-galactopiranosil-(1→3)D-*chiro*-inositol)	Fagopiritol B1 (*O*-α-galactopiranosil-(1→2)-D-*chiro*-inositol)	Galactopinitol A (*O*-α-galactopiranosil (1→2)-4-*O*-metil-1D-*chiro*-inositol)	Galactopinitol B (*O*-α-galactopiranosil (1→2)-3-*O*-metil-1D-*chiro*-inositol)	Galactopinitol C
					Fagopiritol A2 (*O*-α-galactopiranosil -(1→6)-*O*-α-galactopiranosil-(1→3)-D-*chiro*-inositol)	Fagopiritol B2 (*O*-α-galactopiranosil-(1→6)-*O*-α-galactopiranosil (1→2)-D-*chiro*-inositol)	Ciceritol (*O*-α-D-galactopiranosil–(1→6)-*O*-α-D-galactopiranosil (1→2)-4-*O*-metil-1D-*chiro*-inositol)		
						Fagopiritol B3 (*O*- [α-galactopiranosil-(1→6)]$_3$-(1→2)-D-*chiro*-inositol)	Trigalactopinitol A (*O*-[α-D-galactopiranosil (1→6)]$_2$-*O*-α-galactopiranosil (1→2)-4-*O*-metil-1D-*chiro*-inositol)		
***Scyllo*-inositol** **(1,3,5/2,4,6)-ciclohexanohexol**	*O*-metil-*scyllo*-inositol			Mitilitol (2-C-metil-*scyllo*-inositol)					

Respecto a los **glicosil-inositoles**, los fagopiritoles están constituidos por una o más moléculas de galactosa unidas a D-*chiro*-inositol mediante enlaces α-(1→3) para los isómeros A1, A2, A3, y α-(1→2) en el caso de los isómeros B1, B2, B3 (**Tabla 1.1**). El galactopinitol está compuesto por una molécula de pinitol y una de galactosa unidas mediante enlace α-(1→2), mientras que el ciceritol tiene dos moléculas de galactosa unidas por enlace α-(1→6) y una de ellas unida al pinitol mediante enlace α-(1→2).

1.2. Propiedades de los carbohidratos

1.2.1. Propiedades fisicoquímicas

Los carbohidratos, en general, son sustancias cristalinas, no reductoras, de alto punto de fusión y químicamente estables al calor, a ácidos y a álcalis. Son polares y muy poco o nada volátiles.

Existen varias propiedades importantes de los carbohidratos en relación con su uso como ingredientes alimentarios. Entre ellas destacan su solubilidad en disolventes polares, higroscopicidad, estabilidad térmica y poder edulcorante (Davis 1995).

La **solubilidad** de los carbohidratos en agua y disolventes polares es función de su peso molecular. Los carbohidratos de peso molecular bajo o medio (mono- y oligosacáridos) son solubles en agua, alcoholes y disolventes polares como la piridina, dimetilsulfóxido y dimetilformamida, mientras que presentan una baja solubilidad en etanol y son insolubles en disolventes poco polares como éter, cloroformo o benceno. Los polisacáridos son menos solubles, siendo algunos de ellos (como la celulosa) insolubles en agua (Collins y Ferrier 1995; Belitz *y col.* 2009). Los homopolisacáridos lineales son, en general, insolubles en agua como resultado de la configuración de la molécula. Los homosacáridos unidos mediante distintos enlaces y los heterosacáridos son más solubles que los polímeros homogéneos en agua y disolventes polares (Whistler 1973). Así, por ejemplo, la inulina es moderadamente soluble en agua (con una solubilidad del 10%, p/v, a temperatura ambiente, pero su solubilidad aumenta con la temperatura) (Bosscher 2009).

La **higroscopicidad** de los azúcares varía dependiendo, entre otros factores, de su estructura, de la mezcla de isómeros y de su pureza. En disoluciones concentradas de azúcares, como por ejemplo jarabes de glucosa, esta propiedad contribuye al mantenimiento de la humedad de algunos alimentos, como es el caso de algunos productos de panadería. Sin embargo, puede resultar adversa en el caso de polvos y granulados que contengan azúcares, ya que la preparación de disoluciones se verá dificultada debido a la formación de aglomerados (Belitz *y col.* 2009).

Los carbohidratos tienen una **estabilidad térmica** muy baja, ya que se deshidratan con facilidad y a temperaturas elevadas sufren importantes degradaciones, como las reacciones de caramelización (Claude y Ubbink 2006).

Una propiedad interesante de los azúcares es su capacidad de desviar la luz polarizada, lo que se conoce como **poder rotatorio**. Cada uno de los azúcares lo hace con un sentido e intensidad determinados, lo que ayuda a su caracterización (Belitz *y col.* 2009). Se denominan dextrógiros los azúcares que desvían el plano de polarización de la luz hacia la derecha y levógiros a los que la desvían hacia la izquierda.

Los mono- y oligosacáridos, así como los polialcoholes correspondientes, poseen, con pocas excepciones, sabor dulce. La sacarosa destaca entre los demás por su sabor especialmente agradable, incluso a altas concentraciones, y es el azúcar más usado como edulcorante. Sin embargo, existen otros azúcares como la β-D-manosa con sabor entre dulce y amarga y algunos oligosacáridos, como la gentiobiosa, que son amargos. La intensidad del sabor disminuye en los oligosacáridos en general conforme aumenta la longitud de la cadena (Belitz *y col.* 2009). Los polialcoholes destacan por su **poder edulcorante** (además de apenas aportar valor calórico y no ser cariogénicos) y también se usan como **humectantes** en el procesado de alimentos, aunque se utilizan en dosis moderadas debido a su efecto laxante (Collins y Ferrier 1995). Ciertos oligosacáridos, como la inulina, se añaden a productos alimentarios por aportar **textura, mejorar la palatabilidad y como sustitutos de grasas** (Bryan 2003).

1.2.2. Propiedades biológicas

Los carbohidratos son la principal fuente de energía para la mayoría de las células, como es el caso del almidón en plantas y el glucógeno en animales. Por otra parte, algunos carbohidratos de alto peso molecular como celulosa, pectina o xilano determinan la estructura de las plantas, y otros, como la quitina, son el principal componente del exoesqueleto de insectos y crustáceos. Además de sus funciones energéticas y estructurales, los carbohidratos están implicados en diversos procesos biológicos relacionados, en especial, con alteraciones metabólicas o inducción de cambios en la microbiota intestinal (Belitz *y col.* 2009; Moreno y Sanz 2014).

Entre los carbohidratos con mayores propiedades bioactivas para el ser humano destacan los inositoles y los oligosacáridos prebióticos.

- ***Inositoles***

Hay un gran número de propiedades biológicas atribuidas a los **inositoles y sus derivados**, la mayoría asociadas a su estructura.

El ***myo*-inositol** desempeña un papel importante en la utilización de la grasa, como promotor del crecimiento y exhibe cierta habilidad para mejorar la conductancia nerviosa en diabéticos (Holub 1986). Se ha especulado que las alteraciones en el metabolismo del *myo*-inositol juegan un papel en la patogénesis de neuropatías asociadas a la diabetes mellitus y al fallo renal crónico, tales como depresión o ansiedad (Clements y Darnell 1980; Nemets *y col.* 2001).

El *myo*-inositol es precursor de fosfatidilinositol, un componente de las membranas celulares, y de varias moléculas que actúan como segundos mensajeros, como el inositol-1,4,5-trifosfato (que modifica los niveles de calcio intracelular) y el fosfatidilinositol-3,4,5-trifosfato (que participa en la transducción de la señal). La actividad de algunos segundos mensajeros está relacionada con la activación de los receptores de serotonina. Esto podría explicar los posibles efectos beneficiosos atribuidos al *myo*-inositol en el tratamiento de la depresión y de los trastornos obsesivo compulsivos (Nemets *y col.* 2001). También se ha demostrado el valor terapéutico del *myo*-inositol en la bulimia nerviosa (Gelber *y col.* 2001), abriendo la posibilidad de su uso en nuevos tratamientos para estos pacientes.

Algunos estudios parecen indicar que la administración de *myo*-inositol disminuye la probabilidad de daño en la retina y en los pulmones de recién nacidos prematuros con síndrome de dificultad respiratoria, y la disminución en la mortalidad por fallo respiratorio (Hallman *y col.* 1992). Como consecuencia, este compuesto se emplea como suplemento en algunas fórmulas infantiles y productos clínicos nutricionales (Tagliaferri *y col.* 2000).

Finalmente, cabe mencionar que el *myo*-inositol también tiene un uso cosmético, pues mejora la oxigenación del tejido cutáneo (Rolland *y col.* 2004), por lo que existen preparaciones cosméticas para su aplicación en la piel.

El **D-*chiro*-inositol** ha demostrado ser efectivo contra la insulinorresistencia (Ostlund y Sherman 1996). Este compuesto posee una actividad semejante a la insulina al favorecer la captación de glucosa hacia el interior de las células musculares (Yap *y col.* 2009), contribuyendo así a disminuir el nivel de azúcar en sangre.

El síndrome de ovario poliquístico (PCOS) está asociado con hiperinsulinemia y con resistencia a la insulina (Nestler *y col.* 1999). Según ciertos estudios clínicos, la suplementación oral con D-*chiro*-inositol puede mejorar la ovulación, los parámetros metabólicos y la sensibilidad a la insulina en mujeres con este síndrome (Nestler *y col.* 1999).

El D-*chiro*–inositol, junto a otros inositoles (L-*chiro*, *allo*-, *cis*-, *epi*-, *muco*-, *neo*- y *scyllo*-inositol), se usan para tratar alteraciones como dislipemias, hipercolesterolemia o enfermedad cardiovascular cuando éstas pueden ser tratadas con niacina, aportando propiedades fisiológicas mejoradas con respecto a las que parece aportar el hexaniacinato de *myo*-inositol (Hendrix 2008).

Se han atribuido también diversas propiedades antioxidantes y crioprotectoras a varios **metil-inositoles**, como quebrachitol, ononitol, D-1-*O*-metil-*muco*-inositol y, en especial, pinitol (Orthen y Popp 2000), al que se le atribuyen numerosas propiedades beneficiosas. El pinitol, al igual que el *chiro*-inositol, mimetiza la acción de la insulina al estimular la captación de glucosa por parte de los tejidos sensibles a ésta (tejido muscular y adiposo) e inhibir la liberación de glucosa del hígado. Ello se debe a que ambos compuestos forman parte de la estructura de dos mediadores de insulina que posibilitan muchas de sus

acciones. El efecto hipoglicemiante del pinitol ha sido verificado en ratas con diabetes mellitus, en las que se comprobó un descenso significativo en la glucosa sanguínea (Kim *y col.* 2005). Además, se ha estudiado el efecto del pinitol en la bajada de los niveles de ácidos grasos plasmáticos y en aquellas patologías asociadas con la resistencia a la insulina (como las resultantes de la diabetes mellitus y sus complicaciones crónicas), obesidad, hiperlipidemias, aterosclerosis, hipertensión, enfermedades cardiovasculares y otras complicaciones diversas (Ostlund y Sherman 1996). En 1996, Ostlund y Sherman mostraron por primera vez que la resistencia a la insulina podía ser tratada en humanos con pinitol, el cual disminuía los niveles de insulina, glucosa y ácidos grasos en sangre cuando éstos resultan muy superiores a lo normal. Por el contrario, otros estudios observaron que al tratar con pinitol a pacientes diabéticos tipo 2 se producía una alteración en el metabolismo de la glucosa, pero no en los perfiles lipídicos (Kim *y col.* 2007).

Respecto a los **glicosil-inositoles,** los fagopiritoles son de interés para el tratamiento de pacientes con diabetes mellitus tipo 2 y con síndrome de ovario poliquístico, probablemente por la similitud estructural de estos compuestos con un mediador de insulina que es deficiente en estos pacientes. De hecho existe un tratamiento patentado basado en la administración de estos compuestos a pacientes con diabetes tipo 2 (Obendorf y Horbowicz 2004).

- ***Oligosacáridos prebióticos***

Se define como prebiótico a aquel ingrediente que produce una estimulación selectiva del crecimiento y/o actividad(es) de uno o de un limitado número de géneros/especies de microorganismos en la microbiota intestinal confiriendo beneficios para la salud del hospedador (Roberfroid *y col.* 2010). Por tanto, para que un ingrediente o alimento pueda considerarse como prebiótico debe cumplir una serie de requisitos tales como: (i) no ser hidrolizado o absorbido en el tracto gastrointestinal (GIT) superior (esófago, estómago y duodeno) y, por lo tanto, ser resistente a la acidez gástrica, a la hidrólisis por enzimas digestivas y no absorberse en el intestino delgado; (ii) ser fermentado selectivamente por

bacterias beneficiosas de la microbiota intestinal y; (iii) ser capaz de inducir efectos fisiológicos beneficiosos para la salud (Gibson *y col.* 2004; Roberfroid 2007).

A los oligosacáridos prebióticos se atribuyen un gran número de implicaciones positivas para la salud, tales como la estimulación del sistema inmune, incremento de la resistencia a la invasión de las bacterias intestinales patógenas, actividad intestinal mejorada (regulación de la motilidad intestinal, producción de ácidos grasos de cadena corta, prevención de diarrea y estreñimiento), protección contra el cáncer, efecto en la biodisponibilidad de los minerales (con implicaciones en la osteoporosis), la regulación del peso corporal y apetito (al incrementar la saciedad), o la modulación del metabolismo lipídico y de carbohidratos [con implicaciones a nivel cardiovascular y para diabéticos, por reducir el exceso de glucosa circulante y colesterol (Kaur y Gupta 2002) y mejorar la sensibilidad a la insulina (Bryan 2003; Bosscher 2009; Johnston *y col.* 2010; Saad *y col.* 2013)].

Los oligosacáridos prebióticos pueden encontrarse de forma natural en productos vegetales, leche (materna, de vaca y de cabra) o miel, pero pueden también obtenerse de forma sintética, mediante hidrólisis ácidas o procesos enzimáticos. Aunque en el mercado mundial se comercializan un gran número de carbohidratos prebióticos, como por ejemplo xylooligosacáridos (XOS), oligosacáridos de soja (SOS), rafinosa, lactosacarosa e isomaltooligosacáridos (IMO) (Rastall 2010; Lamsal 2012) etc., solamente existe evidencia científica de sus propiedades beneficiosas en humanos para fructanos tipo inulina y fructooligosacáridos (FOS), galactooligosacáridos (Baghurst y Mingos), lactulosa y oligosacáridos de leche humana (HMO) (Corzo *y col.* 2015).

- **Inulina:** Está compuesta por varias unidades de fructosa y una glucosa terminal, unidas mediante enlaces β-(2→1). Es un carbohidrato no digestible y ha formado parte de la dieta durante muchos años, al estar presente de manera natural en muchas plantas comestibles. Respecto al grado de polimerización (DP), como ejemplo, la inulina presente en la achicoria se compone de una mezcla de oligo- y polísacaridos en los que el DP varía de 2 a ~ 60 unidades con un DP medio de 12 (Eggleston y Côté 2003).

- **FOS:** Son oligosacáridos que se obtienen por hidrólisis de la inulina o mediante transfructosilación enzimática, a partir de sacarosa, utilizando fructosiltransferasas. Su fórmula general puede ser GFn indicando la presencia de varias unidades de fructosa unidas a una glucosa terminal mediante enlaces glicosídicos β-(2→1) o FFn, indicando la presencia de unidades de fructosa unidas también por enlaces β-(2→1) (Corzo *y col.* 2015). Los FOS obtenidos por síntesis enzimática a partir de sacarosa se encuentran constituidos por unidades de fructosa y se unen mediante enlaces glicosídicos β-(2→1) entre sí o a una glucosa terminal, aunque en este último caso las uniones pueden ser del tipo β-(2→6) (Gibson *y col.* 2004). El fructano más simple es el trisacárido kestosa, que existe como 3 isomeros: 1- kestosa, 6-kestosa y neo-kestosa.

- **GOS**: Son compuestos obtenidos industrialmente a partir de la lactosa del permeado de suero de quesería, mediante transglicosilación catalizada por β-galactosidasas (lactasas). Estas enzimas, en determinadas condiciones, son capaces de catalizar tanto la hidrólisis de la lactosa como la formación de un enlace β–glicosídico entre la galactosa liberada en la hidrólisis y la lactosa u otros carbohidratos presentes en el medio de reacción para dar lugar a β–GOS. Los oligosacáridos de la familia de la rafinosa [trisacárido compuesto por una molécula de galactosa unida a una de sacarosa mediante enlace α-(1→6)] son α-galactooligosacaridos (α-GOS) caracterizados por la presencia de enlaces α-(1→6) entre moléculas de galactosa y también a una molécula de sacarosa terminal (Martínez-Villaluenga y Frías 2014). Asi, moléculas sucesivas de galactosa unidas a la rafinosa originan el tetrasacárido estaquiosa, el pentasacarido verbascosa y el hexasacarido ajugosa.

- **HMO**: Se encuentran en concentraciones comprendidas entre 12-14 mg/mL, encontrándose los mayores niveles en el calostro (22-24 mg/mL). La fracción que constituye los HMO es muy compleja ya que se estima que puede estar formada por al menos 1000 componentes (Corzo *y col.* 2015).

- **Lactulosa**: es un disacárido (4-O-β-D-galactosil-D-fructosa) que se obtiene industrialmente mediante isomerización en medio básico de la lactosa presente en el permeado del suero de quesería. También puede obtenerse por síntesis enzimática utilizando lactosa y fructosa y β-galactosidasas de diferentes orígenes (Villamiel *y col.* 2014).

1.3. Presencia de carbohidratos bioactivos en alimentos

Como ya se ha indicado, los carbohidratos son componentes mayoritarios de muchos alimentos y tienen una importancia decisiva en la alimentación ya que pertenecen al grupo de nutrientes básicos implicados en la nutrición y en el metabolismo. Además de estar presentes de forma natural, pueden añadirse para mejorar las propiedades nutritivas, tecnológicas o funcionales del alimento, por ejemplo, como edulcorantes, gelificantes, espesantes, estabilizadores y precursores de compuestos con aroma o color (Belitz *y col.* 2009).

A continuación se describe el contenido de los carbohidratos en los que se centra esta memoria en distintos alimentos.

- ***Inositoles***

Los inositoles se encuentran sobre todo en los alimentos de origen vegetal, siendo su presencia en los alimentos de origen animal menos frecuente y más baja. Las principales fuentes de **inositoles** y su contenido en diferentes grupos de alimentos se muestran en la **Tabla 1.2**.

En general, el ***myo*-inositol** se encuentra en cantidades relativamente altas (2 – 22 mg/g) en muchos alimentos. En las frutas, los mayores contenidos se han detectado en algunos cítricos, kiwi y en la variedad de melón "cantalupo" (1,36 - 3,55 mg/g), mientras que las bayas suelen presentar menores concentraciones (0,13 – 1,73 mg/g) (Clements y Darnell 1980). Los frutos secos presentan también contenidos relativamente altos (superiores a 1 mg/g), y lo mismo sucede con los cereales y las legumbres. Dentro de este último grupo, destacarían por su elevado contenido las judías (1,9 mg/g). Las verduras presentan contenidos más bajos (menos de 0,5 mg/g). Entre los alimentos ricos en *myo*-inositol, es notable la alta cantidad presente en el café soluble (22 mg/g) (Ruiz-Matute *y col.* 2007). Hay que destacar también la llamada "miel de palma", un tipo de jarabe preparado en las Islas Canarias a partir de savia de la palmera *Phoenix canariensis*, con niveles en el intervalo de 2,9 a 8,4 mg/g (Ruiz-Matute *y col.* 2010); y algunas otras mieles, como las de abeto y sauce, donde alcanza niveles de 1,1 y 2,2 mg/g, respectivamente (de la

Fuente *y col.* 2007). En alimentos de origen animal, como los productos cárnicos, las cantidades son en general menores, encontrándose las concentraciones más altas en hígado. También se han detectado pequeñas cantidades en leche de vaca, huevos y pescado (0,01 – 0,19; 0,05 – 0,34 y 0,02 – 0,25 mg/g, respectivamente). Se ha observado que el contenido en *myo*-inositol en leche materna es relativamente alto (0,33 mg/mL) en comparación con las fórmulas infantiles (0,075 mg/mL) (Pereira *y col.* 1990).

El ***chiro*-inositol** está presente en zumos de cítricos, destacando en este sentido el zumo de mandarina (1,08 mg/mL) (Sanz *y col.* 2004). Su presencia ha sido también descrita en cantidades relativamente altas (3-7 mg/g) en algunas legumbres, como la soja, y en cereales como el salvado de arroz negro (Kong *y col.* 2008). También se ha detectado la presencia de *chiro*-inositol en algunas mieles, siendo inusualmente alto el nivel hallado en miel de madroño (2,0 mg/g) (de la Fuente *y col.* 2007), en vino (Carlavilla *y col.* 2006) y en frutos secos como los piñones (Kim *y col.* 2005).

El ***scyllo*-inositol** se ha encontrado en zumos de frutas cítricas, siendo el de pomelo el que presenta los contenidos más altos (0,15 mg/mL). También se ha detectado en zumo de uva (Sanz *y col.* 2004) y en otros productos derivados de la uva, como arropes o vino (Carlavilla *y col.* 2006; Ruiz-Matute *y col.* 2010). Este compuesto ha sido identificado también en algunos vegetales como zanahoria, perejil, cilantro, hinojo (Soria *y col.* 2009), así como en algunos sucedáneos del café que contienen achicoria (Ruiz-Matute *y col.* 2007).

Tabla 1.2. Contenido (mg/g, mg/mL) de inositoles en distintos tipos de alimentos.

Alimento	***myo*-INOSITOL**	***chiro*-INOSITOL**	***scyllo*-INOSITOL**	***muco*-INOSITOL**
Frutas frescas y zumos	0,01-3,55 mg/g (Clements y Darnell 1980)	Zumos cítricos: 0,06 - 1,08 mg/mL Zumo de frambuesa: trazas (Sanz *y col.* 2004)	Uva: 0,08 mg/g Zumos cítricos: trazas-0,15 mg/mL (Sanz *y col.* 2004)	
Legumbres y verduras	0,03-1,93 mg/g (Schweizer *y col.* 1978; Clements y Darnell 1980; Soria *y col.* 2009)	Soja: 4,36 – 6,75 mg/g (Kim *y col.* 2005)	Zanahoria: 1,50–5,80 mg/g [a] Perejil, cilantro e hinojo: 1,60-2,40 mg/g [a] (Soria *y col.* 2009)	
Cereales	0,03– 0,42 mg/g (Clements y Darnell 1980) Salvado de arroz negro: 7,85 – 8.52 mg/g (Kong *y col.* 2008)	Salvado de arroz negro: 3,41- 6,60 mg/g (Kong *y col.* 2008)		
Miel y jarabes	Miel: 0,10-2,20 mg/g (de la Fuente *y col.* 2007) Jarabes: 0,4 – 8,4 mg/g (Ruiz-Matute *y col.* 2010)	Miel: 0,10-2,00 mg/g (Kim *y col.* 2005; de la Fuente *y col.* 2007)	Arropes: trazas (Ruiz-Matute *y col.* 2010)	Miel: trazas-1,10 mg/g (Sanz *y col.* 2004; de la Fuente *y col.* 2007)
Frutos secos	2,78 – 0,12 mg/g (Clements y Darnell 1980)	Piñones: 1,74 mg/g (Kim *y col.* 2005)		
Café y sucedáneos	trazas-22,00 mg/g (Ruiz-Matute *y col.* 2007)	Trazas (Ruiz-Matute *y col.* 2007)	Achicoria en polvo: 0,20-0,40 mg/g (Ruiz-Matute *y col.* 2007)	
Alimentos de origen animal	Carne: 0,05 – 1,31 mg/g (Clements y Darnell 1980) Leche y productos lácteos: 0,01-0,19 mg/mL (Clements y Darnell 1980; Pereira *y col.* 1990) Leche humana: 0,33 mg/mL (Pereira *y col.* 1990) Huevos: 0,05-0,34 mg/g (Clements y Darnell 1980) Pescado: 0,02-0,25 mg/g (Clements y Darnell 1980)			
Vino	0,20-0,48 mg/mL (Carlavilla *y col.* 2006)	Trazas-0,02 mg/mL (Carlavilla *y col.* 2006)	0,01-0,07mg/mL (Carlavilla *y col.* 2006)	

[a:] Referido a peso seco.

Hasta el momento, el ***muco*-inositol** sólo ha sido detectado en concentraciones muy bajas en mieles (desde trazas hasta 1,10 mg/g) (Sanz *y col.* 2004; Tabla 1.2). Se piensa que este compuesto procede directamente del néctar o mielato recogido por las abejas y que no se altera por la acción de las enzimas que transforman el néctar en miel.

La **Tabla 1.3** resume el contenido medio de **metil-inositoles** encontrados en ciertos alimentos. Entre los derivados de *myo*-inositol, el bornesitol está presente en el café (1,0-9,5 mg/g) (Ruiz-Matute *y col.* 2007), el ononitol en alfalfa (0,80 mg/g) (Binder y Haddon 1984; Campbell y Binder 1984) y en legumbres del género *Vigna*, como las judías carillas (Ford 1982). La soja es una planta rica en metil-*myo*-inositoles. En ella se ha detectado la presencia de bornesitol, sequoyitol y ononitol en hojas y nódulos (Schweizer *y col.* 1978; Binder y Haddon 1984; Streeter 1985) pero existen pocos datos aún respecto a su posible presencia en semillas (Chiera *y col.* 2006).

En cuanto a los metil derivados del *chiro*-inositol, el más frecuente en los alimentos es el pinitol. El nombre "pinitol" deriva de "pine" (pino) debido a que este compuesto fue aislado por primera vez de la madera de este árbol. El pinitol se ha detectado en leguminosas, siendo especialmente abundante en la algarroba (50-75 mg/g peso seco) y en la soja (6-9 mg/g peso seco) (Schweizer *y col.* 1978; Quemener y Brillouet 1983; Baumgartner *y col.* 1986; Kim *y col.* 2005). No obstante, también se encuentra en cantidades relativamente altas en otras legumbres como garbanzos, lentejas o judías (4,5-12,6 mg/g peso seco) (Schweizer *y col.* 1978; Åman 1979; Ostlund y Sherman 1996). El pinitol también se ha detectado en cantidades variables en alfalfa (Ostlund y Sherman 1996), frutos secos como el cacahuete (Binder y Haddon 1984) y en miel (Sanz *y col.* 2004).

El quebrachitol se ha detectado en jarabe de arce (Stinson *y col.* 1967; Binder y Haddon 1984), donde se considera un marcador de genuinidad, y en las bayas del espino amarillo, un arbusto del género *Hippophae* (Yang *y col.* 2011) consumido en ciertas regiones de Europa y Asia (Li *y col.* 2007).

Tabla 1.3. Presencia y contenido (en mg/g o mg/mL) de metil-inositoles en diferentes tipos de alimentos.

Compuesto	Alimento	Contenido	Referencia
Bornesitol	Café	1,00 - 9,50	Ruiz-Matute *y col.* 2007
	Achicoria en polvo	0,30	Ruiz-Matute *y col.* 2007
Ononitol	Alfalfa	0,80	Campbell y Binder 1984
	Judia adzuki	--*	Peterbauer *y col.* 2003
	Cacahuetes	--*	Binder y Haddon 1984
Pinitol	Algarroba	50,00 -75,00 [a]	Baumgartner *y col.* 1986
	Habas de soja	6,00 -9,00 [a]	Schweizer *y col.* 1978
	Lentejas	4,00 [a]	Schweizer *y col.* 1978
	Alubias	2,00 [a]	Schweizer *y col.* 1978
	Altramuz	3,00 [a]	Quemener y Brillouet 1983
	Garbanzo	4,50 – 12,60 [a]	Schweizer *y col.* 1978; Quemener y Brillouet 1983
	Alfalfa	3,80	Campbell y Binder 1984
	Miel	0,09-7,85	Sanz *y col.* 2004
	Cacahuetes	--*	Binder y Haddon 1984
Quebrachitol	Jarabe de arce	0,95-1,37	Stinson *y col.* 1967
	Bayas de espino amarillo	2,27-6,15	Kallio *y col.* 2009
Metil-*scyllo*-inositol	Fríjol mungo	3,00 -8,00 [a]	Åman 1979
Metil-*muco*-inositol	Miel	0 – 3,63	Sanz *y col.* 2004

* --: No se proporcionan datos cuantitativos; [a] :Referido a peso seco;

Por último, el metil-*scyllo*-inositol ha sido identificado en frijol pequeño o "judías Mung" (*Vigna radiata* y *Vigna mungo*) (Ford 1982) en concentraciones de 3-8 mg/g peso seco, mientras que el metil-*muco*-inositol sólo se ha detectado en algunas mieles (0-3,63 mg/g) (Sanz *y col.* 2004). Se cree que ambos compuestos deben provenir del néctar (Sanz *y col.* 2005).

Los **glicosil-inositoles** se encuentran principalmente en alimentos de origen vegetal y, en particular, en legumbres. La **Tabla 1.4** resume el contenido en estos compuestos en distintos alimentos. En cuanto a su concentración, destaca la presencia de los fagopiritoles (glicosil-*chiro*-inositoles) en trigo sarraceno (Fagopiritol A1, B1, B2 y B3), soja, altramuz, lentejas y garbanzos (Fagopiritol B1; Quemener y Brillouet 1983; Yasui 1985; Piotrowicz-Cieślak *y col.* 2003; Gomes *y col.* 2005). El galactinol se ha encontrado en remolacha (Brown y Serro 1953), alfalfa (Horbowicz *y col.* 1995) y trigo sarraceno, aunque en este último aparece en menor cantidad (0-1,6 μg/embrión) que los fagopiritoles (220,1 – 317,5 μg/embrión) (Horbowicz *y col.* 1998; Horbowicz y Obendorf 2005).

Tabla 1.4. Contenido en glicosil-inositoles (en mg/g, mg/embrión o mg/cotiledones) en diferentes tipos de alimentos.

Compuesto	**Alimento**	**Contenido**	**Referencia**
Galactinol	Alfalfa	1,27-1,69 [a]	Horbowicz *y col.* 1995
	Trigo sarraceno	Trazas	Horbowicz y Obendorf 2005
	Altramuz	0,86-3,00 [a]	Quemener y Brillouet 1983; Piotrowicz-Cieślak *y col.* 2003
	Habas	0,60 [a]	Quemener y Brillouet 1983
	Judías	0,50 [a]	Quemener y Brillouet 1983
	Lentejas	1,20 [a]	Quemener y Brillouet 1983
	Garbanzos	0,80 [a]	Quemener y Brillouet 1983
	Soja	0,10-0,40 [a]	Yasui 1985
Digalactosil *myo*-inositol	Alfalfa	0,31-0,90 [a]	Horbowicz *y col.* 1995
	Trigo sarraceno	Trazas	Horbowicz y Obendorf 2005
	Altramuz	0-0,14 [a]	Piotrowicz-Cieślak *y col.* 2003)
Galactosil ononitol	Judía roja adzuki (Vigna angularis)	--*	Yasui 1980)
	Judía carilla (Vigna sinensis o unguiculata)		
Digalactosil ononitol	Judía roja adzuki	--*	Peterbauer *y col.* 2003

Tabla 1.4. Continuación

Compuesto	**Alimento**	**Contenido**	**Referencia**
Fagopiritoles	Trigo sarraceno	A1 (0,02-0,04 [b])	Horbowicz *y col.* 1998; Horbowicz y Obendorf 2005
		A2 (0-0,01 [b])	Horbowicz *y col.* 1998; Horbowicz y Obendorf 2005
		B1 (0,16-0,26 [b])	Horbowicz *y col.* 1998; Horbowicz y Obendorf 2005
		B2 (0-0,02 [b])	Horbowicz *y col.* 1998; Horbowicz y Obendorf 2005
		B3 (Trazas)	Horbowicz *y col.* 1998; Horbowicz y Obendorf 2005
	Soja	B1 (1,05 [c])	Gomes *y col.* 2005
		B2 (0,15 [c])	Gomes *y col.* 2005)
	Altramuz	B1 (0-0,73 [a])	Quemener y Brillouet 1983; Peterbauer *y col.* 2003
		B2 (0-0,17 [a])	Quemener y Brillouet 1983; Peterbauer *y col.* 2003
	Lentejas	B1 (0,12-2,78 [a])	Quemener y Brillouet 1983
	Garbanzos	B1 (0,08 [a])	Quemener y Brillouet 1983

Tabla 1.4. Continuación

Compuesto	**Alimento**	**Contenido**	**Referencia**
Galactopinitoles	Soja	Galactopinitol A (3,00-7,00 [a])	Schweizer *y col.* 1978
		Galactopinitol B (0,50 [a])	Quemener y Brillouet 1983
		Ciceritol (0,80)	Yasui 1985
	Lentejas	Galactopinitol A (3,00 [a])	Schweizer *y col.* 1978
		Galactopinitol B (1,10 [a])	Quemener y Brillouet 1983
		Ciceritol (16,00)	Sánchez-Mata *y col.* 1998
	Garbanzos	Galactopinitol A (5,00 -8,00 [a])	Quemener y Brillouet 1983
		Galactopinitol B (0,60 [a])	Quemener y Brillouet 1983
		Ciceritol (25,00-28,00 [a])	Sánchez-Mata *y col.* 1998
		Trigalactopinitol A --*	Nicolas *y col.* 1984
	Altramuz	Galactopinitol A (0,03-1,10 [a])	Piotrowicz-Cieślak *y col.* 2003
		Galactopinitol B (0-0,21 [a])	
		Ciceritol (0-3,42[a])	
		Trigalactopinitol A (0-2,56 [a])	
	Judías	Galactopinitol A (2,78-0,12 [a])	Quemener y Brillouet 1983
		Ciceritol (trazas)	
	Alfalfa	Galactopinitol A(1,13-1,36 [a])	Horbowicz *y col.* 1995
		Galactopinitol B (0-0,23 [a])	
		Ciceritol (2,74-5,57 [a])	

* --: No se proporcionan datos cuantitativos; [a]: Referido a peso seco; [b]: Referido a peso del embrión; [c]: Referido a peso del cotiledón

Se ha descrito la presencia de algunos derivados del ononitol, como galactosil- y digalactosil-ononitol, en la judía adzuki (*Vigna angularis*) (Yasui 1980; Peterbauer *y col.* 2003).

También se ha descrito la presencia de galactopinitoles en legumbres (galactopinitol A, galactopinitol B y ciceritol; (Nicolas *y col.* 1984; Horbowicz y Obendorf 1994). Entre ellos, el ciceritol es el azúcar mayoritario en los garbanzos (25,14 – 27,86 mg/g peso seco). Este compuesto también se ha detectado en lentejas, alfalfa y soja (Quemener y Brillouet 1983; Sánchez-Mata *y col.* 1998), si bien a niveles de concentración inferiores.

En cuanto a los **desoxi-inositoles**, es de destacar el quercitol (1,3,4/2,5-ciclohexanopentol) presente en mielatos de roble (0.1–15 mg/g; Sanz *y col.* 2005) y en vino envejecido en barricas de roble (Carlavilla *y col.* 2006).

- ***Oligosacáridos prebióticos***

Respecto a los **oligosacáridos prebióticos**, se han encontrado cantidades considerables de **FOS e inulina** en la familia de las Asteraceas, como por ejemplo en la achicoria (*Cichorium intybus* L.), con un contenido de 15,2–20,5 g de inulina/100 g (Kaur y Gupta 2002; Bosscher 2009; Moser *y col.* 2014); la alcachofa de Jerusalén (*Helianthus tuberosus* L.), con un 6-20,5 g/100 g peso muestra, y en raíces de bardana (*Arctium lappa* L.) y estevia (*Stevia rebaudiana*), en las que llega a suponer un 17% del peso de la muestra. Los FOS también están presentes, aunque en menor medida, en la familia de las Asparagaceae (espárragos, con hasta 2,6 g/100g), Poaceae (trigo y arroz, con un contenido de hasta 3 g/100 g de fructanos, mayoritariamente del tipo β-(2→6)) y Amarilidaceas (1,1 a 7,1 g/100 g en cebolla, hasta 17,4 g/100 g y bulbo de ajo y hasta 10 g/100 g en puerro) (Bosscher 2009; Martínez-Villaluenga y Frías 2014).

En referencia a los **GOS**, las fuentes principales de **oligosacáridos de la familia de la rafinosa** son las legumbres, principalmente soja y altramuz, en los que puede representar hasta un 14,5%, y la remolacha donde puede llegar a suponer hasta un 22,6 % (ambos valores expresados en peso seco), encontrándose en menores cantidades en otros vegetales y en cereales (Martínez-Villaluenga y Frías 2014).

1.4. Extracción de carbohidratos bioactivos

En la actualidad, existe un gran interés en la obtención de compuestos bioactivos que puedan ser empleados como ingredientes alimentarios. Este interés aumenta si dichos compuestos provienen de matrices alimentarias y, en especial, si los procesos de obtención resultan adecuados para su escalado industrial. La extracción de carbohidratos bioactivos a partir de fuentes naturales, sobre todo de residuos agroalimentarios, es uno de los campos de investigación más activos dentro del área de Ciencia y Tecnología de Alimentos. En este tipo de estudio, la matriz o fuente rica en carbohidratos se somete a un secado y triturado para reducir el tamaño de partícula de muestra. En general, la extracción de estos carbohidratos se lleva a cabo mediante **extracción sólido-líquido** (SLE) de la matriz con disolventes orgánicos o acuosos.

La SLE de carbohidratos se suele acelerar mediante agitación mecánica (Burbano *y col.* 1995) o la aplicación de ultrasonidos (Kong *y col.* 2008). Para aumentar la eficacia del proceso, la extracción se debe repetir sucesivamente hasta el agotamiento del compuesto de interés en la matriz original. Las temperaturas aplicadas oscilan desde la ambiental hasta la ebullición de los diferentes disolventes extractantes, que suelen ser polares, principalmente agua, etanol o metanol, o mezclas de los mismos (Martínez-Villaluenga *y col.* 2004; Obendorf y Horbowicz 2004; Kim *y col.* 2005; Fuentes-Alventosa *y col.* 2009; Li *y col.* 2012). En algunos casos se emplean dichos disolventes ligeramente acidulados con HCl en concentraciones de 0,1-0,5 M (Burbano *y col.* 1995). La eliminación de grasas u otros constituyentes apolares de los alimentos se puede realizar mediante lavados con éter de petróleo, diclorometano, heptano, etc., dependiendo de si el propósito de la extracción es sólo analítico o si es para su posterior uso en la industria alimentaria. Los principales inconvenientes de esta técnica sencilla son el elevado consumo de disolventes, en general de elevada calidad, y los largos tiempos de procesado.

Para poder obtener extractos ricos en carbohidratos bioactivos a partir de muestras naturales o de interés alimentario es necesaria la aplicación de técnicas de extracción que permitan la recuperación total de los compuestos de interés de una forma más rápida, económica y eficaz. Las limitaciones de las técnicas de extracción convencional para alcanzar estos objetivos han hecho que en los últimos años se haya intensificado la

búsqueda de técnicas alternativas en las que estos procesos se aceleren mediante la aplicación de algún tipo de energía. A este grupo de técnicas de extracción pertenecen la extracción con líquidos presurizados (PLE) (Splechtna *y col.* 2001), la extracción con fluidos supercríticos (SFE) y las extracciones asistidas con microondas (MAE) o con ultrasonidos (UAE). Entre ellas, la PLE y la MAE son técnicas de extracción relativamente recientes pero que cuentan ya con una amplia aceptación en diversos campos de aplicación, incluido el análisis de alimentos. Estas han sido las técnicas elegidas en este trabajo de investigación y sus principios y fundamentos se describen a continuación.

- **Extracción con Líquidos Presurizados**

En un proceso de PLE, la muestra, empaquetada en una celda de extracción metálica, es extraída con un disolvente (orgánico o inorgánico) a temperaturas que oscilan desde la ambiental hasta los 200ºC y a presiones elevadas (en general, en el intervalo 4-20 MPa). Las elevadas temperaturas aplicadas aumentan la velocidad de difusión, la solubilidad de los analitos, y la transferencia de masa, y disminuyen la viscosidad y tensión superficial de los disolventes. Las altas presiones, además de ayudar a mantener el disolvente en estado líquido durante el proceso de extracción, favorecen la penetración del mismo en la estructura de la matriz. Estos cambios contribuyen a aumentar el contacto entre los analitos a extraer y el extractante, mejorando la eficacia del proceso de extracción, que puede así ser completado en tiempos más cortos y con un consumo de disolvente inferior a los requeridos por otras técnicas convencionales (Ramos *y col.* 2002; Herrero *y col.* 2005). En este sentido, la principal limitación de la técnica vendría determinada por la posible termolabilidad de los analitos y, en ocasiones, de la matriz.

La **Figura 1.2** muestra el esquema básico de un equipo de PLE. El sistema consta de un depósito de disolvente conectado a una bomba de alta presión, que es la encargada de bombear el disolvente extractante hacia la celda de extracción. Ésta se encuentra situada en el interior de un horno con el que se controla la temperatura de extracción. Existen también varias válvulas que permiten ajustar la presión en los distintos puntos del sistema. A la salida de la celda de extracción, se dispone un vial colector para recoger el extracto resultante una vez despresurizado. El sistema puede incluir también un dispositivo

refrigerante para reducir la temperatura del eluyente a la salida de la celda de extracción. Este elemento es especialmente importante cuando se trabaja con compuestos volátiles o a temperaturas muy elevadas. También puede incluir un circuito de nitrógeno que sirve para purgar la celda y las líneas una vez finalizada la extracción o para secar la muestra, así como un bucle previo a la celda de extracción, para el calentamiento del disolvente cuando es necesario alcanzar temperaturas de extracción elevadas (Herrero *y col.* 2005). Como elemento de seguridad, suele incluir una membrana que se rompe en caso de sobrepresión en la celda.

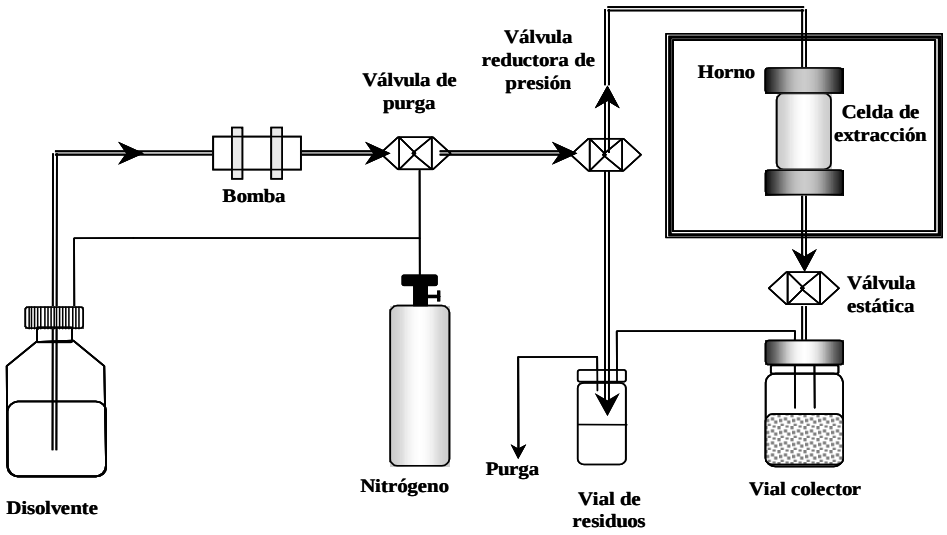

Figura 1.2. Esquema básico de un equipo de PLE (Ruiz-Matute, 2007).

En la actualidad existen varios equipos comerciales de PLE, como el ASE (Extracción acelerada con disolventes, de Dionex), y el PSE (Extracción con disolventes presurizados, de Applied Biosystems). Por otra parte, sobre la base de estos sistemas comerciales, se han realizado diferentes modificaciones correspondientes a diseños de laboratorio que incluyen, por ejemplo, depósitos y bombas específicas para disolventes de lavado, y algunos prototipos miniaturizados (Ramos *y col.* 2000; Pena-Abaurrea *y col.* 2013).

Cuando la temperatura aplicada durante la PLE es superior al punto de ebullición del disolvente e inferior a la de su punto crítico, la extracción tiene lugar en condiciones subcríticas. En esta categoría resulta especialmente interesante el caso del agua, ya da lugar a la técnica denominada extracción con agua subcrítica (Sweeley *y col.* 1963) o extracción con agua sobrecalentada (SHWE). La elevada temperatura aplicada en este caso propicia un cambio en la constante dieléctrica del agua que permite la extracción de compuestos que no podrían ser extraídos a presión atmosférica con este disolvente, es decir, apolares y/o fuertemente retenidos en la matriz.

La PLE ha sido utilizada para el análisis de diversos contaminantes (hidrocarburos aromáticos policíclicos, bifenilos policlorados, dibenzofuranos y dibenzodioxinas, pesticidas, metales, residuos de medicamentos de uso veterinario y micotoxinas) en alimentos (Carabias-Martínez *y col.* 2005; Mendiola *y col.* 2007). Se ha aplicado también a la extracción de antioxidantes en una gran variedad de matrices vegetales (Herrero *y col.* 2006; Herrero *y col.* 2012). Dentro de este último campo de aplicación tiene especial relevancia el uso de disolventes de bajo coste y considerados "seguros" desde el punto de vista alimentario, también denominados disolventes GRAS (Generally Recognized As Safe), entre los cuales se encuentran el etanol y el agua. Sin embargo, la aplicación de la PLE en el campo de los carbohidratos ha sido hasta ahora más bien escasa (Alañón *y col.* 2009) y la mayoría de los estudios se han centrado en el empleo de esta técnica para el fraccionamiento de estos compuestos (Ruiz-Matute *y col.* 2007; Ruiz-Matute *y col.* 2008). En cuanto a la extracción de carbohidratos bioactivos, hasta el momento sólo se ha descrito el uso de la PLE para la obtención de extractos enriquecidos en iminoazúcares a partir de hojas de morera (Rodríguez-Sánchez *y col.* 2013) y de inulina a partir de alcachofa de Jerusalén (Saengkanuk *y col.* 2011), pero no se ha aplicado a la extracción de inositoles u otros prebióticos.

- **Extracción Asistida por Microondas**

Las microondas son un tipo de radiación electromagnética no ionizante con una frecuencia de entre 300 MHz y 300 GHz, aunque la mayoría de equipos comerciales para uso doméstico o industrial operan a frecuencias fijas, en general de 2.45 GHz. Su uso en procesos de extracción se describió por primera vez en 1986 (Ganzler *y col.* 1986).

La aplicación de las microondas a un medio origina la migración electroforética de los iones siguiendo el campo eléctrico producido por las microondas (Nadagouda *y col.* 2011). La dirección de los iones cambia tantas veces como lo haga el signo del campo. La resistencia del medio a esta migración provoca colisiones entre las moléculas, lo que genera calor. Al mismo tiempo, las moléculas dipolares tratan de alinearse con el campo eléctrico. El consecuente proceso de alineación, cambio de dirección y realineación de los dipolos conduce a colisiones entre ellos y las moléculas circundantes, lo que genera energía y un aumento de la temperatura del medio. Estos procesos de conducción iónica y de rotación de dipolos ocurren de manera simultánea y resultan en la transformación de la energía de microondas en energía térmica (Zhang *y col.* 2011) y en un calentamiento casi inmediato de la muestra. De hecho, cuando las microondas se aplican a muestras disueltas o sólidas, la energía es absorbida de manera directa y uniforme por el medio en el que están embebidas las moléculas, dando lugar a un proceso de calentamiento más eficiente que cualquier otro proceso de calentamiento térmico convencional, en el que primero se calientan las paredes del recipiente y después el calor es trasferido a la muestra. La naturaleza del proceso de calentamiento en MAE hace posible modular la selectividad y eficacia de la extracción en función de las condiciones experimentales seleccionadas y hace que esta técnica resulte más adecuada para la extracción de compuestos termolábilies que otros procedimientos basados en el calentamiento térmico convencional del recipiente que contiene la muestra.

En general, la capacidad de las moléculas para absorber la energía de microondas aumenta al hacerlo su constante dieléctrica (ε'). Sin embargo, los disolventes con constantes dieléctricas bajas también se pueden utilizar en procesos de MAE. En este caso, las microondas son absorbidas por la matriz, lo que provoca un calentamiento interno y la rotura de la estructura celular por expansión y la liberación al medio el analito de interés (Eskilsson y Björklund 2000; Romanik *y col.* 2007). En la mayoría de las aplicaciones, se utilizan como extractantes disolventes polares o mezclas de disolventes no polares y

polares. La **Tabla 1.5** resume algunas constantes físicas relevantes para los disolventes más comunes en procesos de MAE.

Tabla 1.5. Constantes físicas para los disolventes comúnmente usados en MAE (reproducido a partir de Eskilsson y Björklund 2000)

Disolvente	Constante dielectrica[a], ε'	Momento dipolar [b]	Factor de disipacion, tan δ ($x10^{-4}$)	Punto de ebullición [c] (ºC)	Temperatura en recipiente cerrado [d] (ºC)
Acetona	20.7			56	164
Acetonitrilo	37.5			82	194
Etanol	24.3	1.96	2500	78	164
Hexano	1.89			69	-[e]
Metanol	32.6	2.87	6400	65	151
2-Propanol	19.9	1.66	6700	82	145
Agua	78.3	2.3	1570	100	
Hexano-acetona (1:1)				52	156

[a] Determinado a 20 ºC, [b] Determinado a 25 ºC, [c] Determinado a 101.4 kPa, [d] Determinado a 1207 kPa, -[e] Sin calentamiento por microondas.

Mientras que la ε' de un disolvente puede ser considerada un indicativo de su capacidad para ser polarizado en un campo eléctrico, la llamada pérdida dieléctrica (ε") es una medida de la eficacia del disolvente para disipar la energía microondas absorbida en calor. La relación entre estos dos valores es el factor de disipación ($\ln \delta = \varepsilon'' / \varepsilon'$) y determina la eficiencia del proceso de extracción.

En la actualidad, se comercializan dos tipos de sistemas para MAE: los de recipiente abierto y los de recipiente cerrado. En los primeros, la temperatura máxima de operación es el punto de ebullición del disolvente a presión atmosférica y se han utilizado sobre todo para la extracción de metales y compuestos organometálicos (Takeuchi *y col.* 2008). Los sistemas cerrados utilizan recipientes de extracción cerrados, lo que permiten trabajar a temperaturas por encima del punto de ebullición del disolvente sin evaporación significativa del mismo. Estas elevadas temperaturas de operación suelen resultar en eficacias de extracción altas en tiempos cortos y con un mínimo consumo de disolvente. En general, este tipo de sistemas permiten el tratamiento simultáneo de entre 4 y 48 muestras, si bien la temperatura y presión dentro de los recipientes se controla, en general, sólo en uno de ellos. Esto obliga a que todas muestras tratadas en un análisis sean idénticas en

cuanto a la naturaleza del disolvente(s) extractante y su proporción respecto a la cantidad de muestra sólida. Como alternativa, se comercializan también algunos equipos de MAE en los que la temperatura se controla en cada recipiente por medio de sensores infrarrojos. Estos sistemas permiten aplicar diferentes potencias y ciclos de extracción en cada vaso de extracción, por lo que son los más utilizados en la actualidad.

La configuración básica de los equipos de MAE con recipiente cerrado incluye un magnetrón (o varios), un horno donde se colocan los recipientes de extracción que contienen la muestra y el disolvente extractante seleccionado sobre un carrusel giratorio, y diferentes sensores de temperatura y presión. También puede incluir la opción de agitación magnética y distintos dispositivos electrónicos de seguridad, como un sensor de vapor de disolvente en el horno, una cámara para la recolección de posibles vapores conectada a cada recipiente de extracción, un ventilador para la eliminación de vapores del horno, aislantes extra en la cavidad del horno, o una puerta móvil para liberar la sobrepresión en la cavidad. Otros posibles elementos de seguridad serian membranas de fractura en los recipientes de extracción que se rompen a presiones superiores a 200 psi, o vasos con autocierre que se abren y cierran rápidamente para evitar sobrepresiones. Los vasos o recipientes de extracción suelen ser de materiales transparentes a las microondas, como vidrio o algunos plásticos (poliéter imida, tetrafluoromethoxyl, Teflon®, etc.). La elección entre los distintos materiales disponibles depende de los disolventes a emplear y de las temperatura y presión de trabajo.

La MAE se ha aplicado a la extracción de polisacáridos con una gran variedad de estructuras, como pectinas de frutas (Bagherian *y col.* 2011; Bélafi-Bakó *y col.* 2012; Li *y col.* 2012; Prakash Maran *y col.* 2013; Holck *y col.* 2014; Prakash Maran *y col.* 2014), celulosa de remolacha (Fishman *y col.* 2011), galactomananos y arabinogalactanos de granos de café (Passos y Coimbra 2013), xilanos de fibra de maíz (Benkő *y col.* 2007), polisacáridos sulfatados del alga *Fucus vesiculosus* (Rodriguez-Jasso *y col.* 2011) y carragenanos de algas *Hypnea musciformis* (Vázquez-Delfín *y col.* 2014). Sin embargo, no se ha aplicado hasta el momento para la extracción de carbohidratos de bajo peso molecular, ni para la extracción conjunta de estos carbohidratos y otros oligosacáridos bioactivos.

1.5. Fraccionamiento de carbohidratos

Los carbohidratos se encuentran presentes en forma de mezclas complejas en matrices naturales (plantas, alimentos,…) que incluyen compuestos en muy diversa concentración.

La extracción de carbohidratos bioactivos lleva normalmente implícita la co-extracción de otros carbohidratos que pueden interferir en sus propiedades, siendo por tanto necesaria una etapa de fraccionamiento entre ellos. Dicho fraccionamiento es necesario tanto para obtener muestras más sencillas que permitan el análisis o caracterización de carbohidratos en mezclas complejas (Sanz y Martínez-Castro 2007), como para obtener una fracción enriquecida en carbohidratos bioactivos para su uso posterior como ingredientes alimentarios. Por tanto, dependiendo de la composición de la muestra y la finalidad del extracto, puede ser necesaria la separación de carbohidratos según sus enlaces glicosídicos, las unidades monoméricas que los constituyen o su DP. Sin embargo, el fraccionamiento de los carbohidratos no es sencillo debido a la similitud de sus estructuras, la diferencia de las concentraciones a las que se encuentran en los productos naturales y la complejidad de las mezclas.

Los principales métodos de fraccionamiento aplicados a la separación de carbohidratos se basan en el uso de membranas (ultra- y nano-filtración), técnicas cromatográficas (adsorción en carbón activo, cromatografía de exclusión molecular, SEC, cromatografía de intercambio iónico, IEC), tratamientos microbiológicos, enzimáticos, y con disolventes orgánicos.

El uso de **membranas**, los tratamientos con **carbón activo** y la **SEC**, en general, se emplean para el fraccionamiento de oligosacáridos de distinto DP, no siendo válidos para la separación de carbohidratos en base a sus enlaces glicosídicos o composición monomérica. Estas técnicas permiten la obtención de altos rendimientos (aunque dependientes de la mezcla de partida) y purezas aceptables, aunque son procesos largos y laboriosos.

La **IEC**, tanto aniónica como catiónica, se ha empleado para el fraccionamiento selectivo de carbohidratos con cargas, como los iminoazúcares. También se ha empleado para el fraccionamiento de inositoles, a pesar de ser compuestos neutros. En este caso, se emplean resinas de carácter básico para separar inositoles de azucares y polialcoholes, usando agua como eluyente. Esta aproximación sugiere que la resina actúa como sustrato para una separación cromatografica y que la separación tiene lugar por afinidad más que

por intercambio iónico (Saska y Diack 1996). Los inositoles poseen una menor afinidad por la resina que los otros azúcares y pasan por la columna más rápidamente. Aunque la pureza obtenida es alta, el proceso es tedioso y los rendimientos, en general, bajos (en torno al 0,1 % en peso seco de muestra) (Asano *y col.* 2005).

Los **tratamientos microbiológicos** se usan principalmente para la eliminación selectiva de monosacáridos y disacáridos. Se han usado enzimas de origen fúngico (por ejemplo, Splechtna *y col.* (2001) usaron enzimas que reaccionaban con lactosa mucho más selectivamente que con GOS, permitiendo la eliminación de este disacárido) y también se han empleado algunas bacterias (Crittenden y Playne (2002) describieron el uso de *Zymomonas mobilis* para separar mono y disacáridos en mezclas de oligosacáridos; Li *y col.* (2008) emplearon *Kluyveromyces lactis* eficazmente en la eliminación de monosacáridos de una mezcla rica en GOS sintetizados enzimáticamente). La levadura *Saccharomyces cerevisiae* se ha usado para la eliminación de carbohidratos de bajo peso molecular en distintas preparaciones de carbohidratos (Yoon *y col.* 2003; Goulas *y col.* 2007; Li *y col.* 2008; Hernández *y col.* 2009). La mayor desventaja de las levaduras es la baja eficacia en la eliminación de algunos disacáridos (por ejemplo, lactosa o lactulosa en muestras de GOS) y la producción de etanol y trehalosa como subproductos (Hernández *y col.* 2009). Baumgartner *y col.* (1986) utilizaron *Saccharomyces bayanus* para eliminar la sacarosa, fructosa y glucosa presentes en polvo de algarroba con el fin de analizar el contenido en ciclitoles de estas muestras. Tras el tratamiento con levaduras, los carbohidratos que aún permanecían en la muestra fueron eliminados mediante cromatografía de intercambio aniónico.

Los tratamientos basados en el empleo de **disolventes orgánicos** siguen siendo muy utilizados para el fraccionamiento de carbohidratos. Dichos tratamientos se basan principalmente en las diferencias de solubilidad de estos compuestos, dando lugar a la precipitación selectiva de algunos de ellos y permitiendo así su posterior separación de la mezcla en la que están presentes. Así, por ejemplo, la tagatosa y la lactulosa, obtenidas por reacciones de isomerización o tratamientos biológicos a partir de sus correspondientes aldosas (galactosa o lactosa), presentan una mayor solubilidad que éstas en alcoholes (metanol, etanol, isopropanol y 1-propanol) (Montañés *y col.* 2007) y mezclas hidroalcoholicas (Olano 1979; Montañés *y col.* 2007). Sin embargo, la separación

utilizando estos disolventes requiere grandes volúmenes y largos tiempos de tratamiento. Con el fin de minimizar los volúmenes de disolventes usados y los tiempos requeridos en estos procedimientos, se recurre a técnicas asistidas, ya sea por microondas, líquidos presurizados o usando fluidos supercríticos.

La **SFE** se ha usado para el fraccionamiento de carbohidratos bioactivos basándose en la diferente solubilidad de estos compuestos en dióxido de carbono en estado supercrítico (SC-CO_2) y el empleo conjunto de disolventes polares (cosolventes). Esta técnica ha proporcionado buenos resultados para la separación de tagatosa y lactulosa de sus correspondientes aldosas empleando etanol como cosolvente (Montañés *y col.* 2006; Montañés *y col.* 2009). La proporción lactulosa/lactosa fue superior a la unidad empleando un 6% (p:p) de cosolvente e inferior a la unidad cuando se utilizaba un 21% del mismo. Es decir, pequeñas cantidades de cosolvente favorecieron la extracción selectiva de lactulosa en mezclas binarias de lactulosa-lactosa. Por otra parte, la proporción tagatosa/galactosa fue mayor que la unidad para todas las cantidades de cosolvente utilizadas. En este trabajo se demostró también que un incremento en la cantidad de cosolvente en la SFE aumentaba la recuperación de tagatosa manteniendo la misma selectividad

La **PLE** se ha aplicado también al fraccionamiento de distintos polisacáridos (hemicelulosa y lignina de celulosa) en fibras de lino (Kim y Mazza 2009) y paja de trigo triticale (Pronyk y Mazza 2011), o a la separación de lactosa y lactulosa en mezclas binarias (Ruiz-Matute *y col.* 2007). La combinación de PLE y adsorción en carbón activo ha sido también empleado para la eliminación de monosacáridos en mieles, con objeto de analizar carbohidratos minoritarios en estas muestras (Ruiz-Matute *y col.* 2008).

El calentamiento por **microondas** favorece la liberación de los compuestos de interés de las muestras que los contiene hacia el disolvente empleado. Hasta el momento, la técnica se ha aplicado a la separación de xilanos y celulosa (Yoshida *y col.* 2010), no habiéndose encontrado más referencias para el fraccionamiento de carbohidratos en la bibliografía especializada.

En general, la mayoría de estas técnicas de fraccionamiento han demostrado ser eficaces para la separación de carbohidratos de distinto peso molecular, pero pocas las que permiten la separación de distintos carbohidratos del mismo grado de polimerización. Además, casi todas implican el uso de disolventes orgánicos volátiles, por lo que resulta de interés la búsqueda de nuevos disolventes alternativos que permitan su reemplazo.

1.5.1. Líquidos iónicos

Los líquidos iónicos (ILs) son un grupo de disolventes no moleculares compuestos, en general, por cationes orgánicos (como imidazolio, piridinio, pirrolidino, fosfonio o cationes de amonio cuaternario), y aniones orgánicos o inorgánicos (como acetato, trifluoroacetato, tetrafluoroborato, hexafluorofosfato o bromuro). Son líquidos a temperatura ambiente (generalmente definida como inferior a 150 °C) (Raynie 2010) y, en los últimos años, han cobrado un gran interés en distintas áreas de la química fundamental y aplicada (Martín-Calero *y col.* 2011; Ma y Hong 2012).

Aunque los ILs son conocidos desde principios del siglo XIX, esta categoría de compuestos comenzó a atraer la atención científica en 1982, cuando se sintetizó el primer IL estable a temperatura ambiente con el catión 1-alquil-3-metilimidazolio (Wilkes *y col.* 1982). Sin embargo, no fue hasta 1992 cuando se sintetizó el primer IL estable en aire y agua, basado en 1-etil-3-metilimidazolio (Wilkes y Zaworotko 1992).

Las propiedades físico-químicas generales de los ILs se consideran el resultado de las propiedades combinadas de sus correspondientes cationes y aniones. En principio, este hecho se puede utilizar para modular las propiedades del IL resultante variando bien los cationes o los aniones (Huddleston *y col.* 2001). Los ILs pueden ser superácidos, básicos, hidrófilos, miscibles en agua, inmiscibles en agua e hidrófobos. En general, el anión se utiliza para controlar la miscibilidad del IL en agua, pero el catión también puede influir en la capacidad de unión del hidrógeno o la hidrofobicidad del disolvente (Huddleston *y col.* 2001).

Los ILs son considerados disolventes verdes debido a su baja volatilidad en amplios intervalos de temperatura, baja inflamabilidad y alta estabilidad térmica. Sin embargo, la toxicidad de muchos de estos disolventes no ha sido evaluada y su capacidad de distribución en el medio ambiente parece estar determinada en gran medida por su solubilidad en agua. Otras propiedades incluyen una alta conductividad eléctrica, miscibilidad en una amplia gama de disolventes orgánicos, buena capacidad de extracción de muchos materiales orgánicos, inorgánicos y organometálicos, y una alta viscosidad. Estas características explican su creciente uso en diferentes campos de investigación, incluyendo el análisis electroquímico, experimentos de catálisis y de síntesis, y procesos de extracción y preparación de muestra (Huddleston *y col.* 2001; Poole y Poole 2010; Ma y Hong 2012; Ruiz-Aceituno *y col.* 2013), en los que se usan como alternativa a los disolventes orgánicos volátiles convencionales.

En lo que se refiere al conocimiento sobre la solubilidad de carbohidratos en ILs, hasta ahora, los estudios han sido más bien escasos y se han orientado sobre todo a la solubilización (más o menos selectiva) de carbohidratos de alto peso molecular, como celulosa, almidón y quitina (Zhu *y col.* 2006; El Seoud *y col.* 2007; Zakrzewska *y col.* 2010), y su recuperación de biomasa (Zhu *y col.* 2006; El Seoud *y col.* 2007). Como ejemplo, en el estudio de Lan *y col.* (2011), se usó cloruro de 1-butil-3-metilimidazol para disolver lignocelulosa y posteriormente fraccionarla en celulosa, hemicelulosa y lignina en la muestra tratada (bagazo de caña de azúcar). Sólo se han descrito algunos estudios en los que se evalúe la solubilidad de carbohidratos de bajo peso molecular, como la glucosa, fructosa, sacarosa y lactosa en algunos ILs (Rosatella *y col.* 2009; Conceiçao *y col.* 2012). En estos estudios, la solubilidad se determina añadiendo una cantidad creciente del carbohidrato evaluado al correspondiente IL, hasta su completa disolución (Zhao *y col.* 2008; Rosatella *y col.* 2009; Conceiçao *y col.* 2012; Carneiro *y col.* 2013). Después, el analisis o medicion se lleva a cabo mediante ensayos espectrofotometricos (Liu *y col.* 2004) o por observacion de los cristales en la disolucion del IL en microscopio (Conceiçao *y col.*) y usando HPLC (Rosatella *y col.* 2009; Carneiro *y col.* 2013).

En una patente reciente, Al Nashef *y col.* (2011) proponen un método para la separación de fructosa y glucosa basado en sus diferentes solubilidades a temperatura ambiente en

dimetilfosfato de 1,3-dimetilimidazolio y etilsulfato sulfato de 1-etil-3-metilimidazolio. El primero de estos ILs tiene la capacidad de solubilizar la glucosa de 2-6 veces más que la fructosa, lo que permite enriquecer de manera notable en glucosa muestras binarias de fructosa y glucosa. Por el contrario, el etilsulfato de 1-etil-3-metilimidazolio se empleaba para enriquecer las mezclas en fructosa, ya que su solubilidad en este IL era superior a la de la glucosa (147 y 90 mg/mL, respectivamente).

1.6. Análisis de carbohidratos

En la bibliografía se han descrito distintos métodos de análisis para la determinación de carbohidratos. Estos métodos incluyen desde ensayos microbiológicos o enzimáticos (Angyal y Anderson 1959; Anderson 1972; Chaplin y Kennedy 2003) hasta métodos cromatográficos (Burbano *y col.* 1995; Tagliaferri *y col.* 2000; Sanz *y col.* 2004; Sanz *y col.* 2004) o electroforéticos (Kong *y col.* 2008). Entre ellos, las técnicas de análisis más usadas son las cromatográficas: la cromatografía de gases (GC) y la cromatografía de líquidos (LC) (Holck *y col.* 2014).

1.6.1. Análisis de carbohidratos por GC

La GC es, con diferencia, la técnica más empleada para el análisis de carbohidratos de bajo peso molecular por su alto poder de resolución, lo que asociado a una elevada sensibilidad proporcionada por los detectores a los que se acopla, permite una adecuada detección y la cuantificación de estos compuestos (Fox 2002).

El análisis de oligosacáridos de alto peso molecular y polisacáridos puede realizarse también con GC, pero debe incluir una etapa de hidrolisis total (cuyas condiciones deben ser optimizadas según el tipo de polisacárido objeto de análisis) para romper los enlaces glicosídicos y, así, los monómeros resultantes pueden ser analizados por esta técnica. La determinación estructural de los carbohidratos de alto peso molecular se puede llevar a cabo mediante la metilación de Hakomori, que incluye un proceso de metilación seguido por la hidrólisis y acetilación de los grupos hidroxilo liberados (Hakomori 1964).

Las fases estacionarias más empleadas para el análisis de carbohidratos son los polisiloxanos, con grupos metil-, fenil-, trifluoropropil- o cianoalquil-. También se usan columnas de carborano, que permiten trabajar a temperaturas más altas (Soria *y col.* 2008). La eficacia en la separación y resolución dependen de las dimensiones de la columna, siendo las más usadas las de longitud de 25-30 m con diámetro interno entre 0,25-0,32 mm y espesores de fase de 0,25 µm. La separación en GC depende, además del flujo de gas portador, de la programación de temperatura del horno y de las temperaturas del inyector y del detector (Soria *y col.* 2008), variables que deben ser cuidadosamente optimizadas.

- **Derivatización**

Los carbohidratos, debido a su alta polaridad, hidrofilidad y baja volatilidad, tienen que ser convertidos en derivados volátiles antes de poder ser analizados por GC. En este proceso, se les confiere volatilidad y estabilidad mediante reacciones basadas en la sustitución de todos los átomos de hidrógeno activos por grupos no polares. Habitualmente, los grupos hidroxilo son sililados, acetilados o trifluoroacetilados, aunque también pueden ser metilados o etilados (Dutton 1973; Knapp 1979; Biermann 1989).

Uno de los métodos de derivatización más empleados es la sililación, con la que se consigue la introducción de grupos dimetilsilil, trimetilsilil o t-butildimetilsilil en la molécula para formar los correspondientes silil etéres. Este método fue desarrollado por Sweeley y col (1963). Para llevar a cabo la derivatización, hay que disolver la muestra en disolventes no acuosos, como piridina o dimetilsulfóxido. Como reactivos sililantes se emplean hexametildisilazano (HMDS), trimetilclorosilano (TMCS), trimetilsililimidazol (TMSI), o bis(trimetilsilil)-trifluoroacetamida (BSTFA) (Sanz y Martínez-Castro 2007), o combinaciones de los mismos. La temperatura de reacción varía desde la ambiental hasta 45 ºC. La reacción de sililación ocurre de forma instantánea, aunque algunos autores proponen desde 5 (Holligan y Drew 1971) a 30 min de equilibrio (Knapp 1979). Estos derivados son bastante volátiles y estables (Sherman *y col.* 1970); sin embargo, la desventaja de este tipo de derivatización es la formación de hasta 5 formas sililadas para los azúcares reductores, lo que puede dificultar el análisis de mezclas complejas.

Una solución para este problema es la conversión del grupo carbonilo en una oxima antes de la sililacion (**Figura 1.3**), lo que reduce a 2 el número de picos cromatográficos: los isómeros *syn* (*E*)- y *anti* (*Z*)- (Molnár-Perl y Horváth 1997). Los azúcares no reductores no experimentan el proceso de oximación y dan lugar a un solo derivado. Las trimetilsilil oximas (TMSO) poseen una alta volatilidad y han sido empleadas en la determinación de la composición de carbohidratos en muchas muestras de origen alimentario, como galletas y crackers, vegetales deshidratados, frutas, etc (Villamiel 2006, Sanz *y col.* 2004). El método resulta de particular utilidad en el análisis mezclas complejas de estos compuestos, como las presentes en la miel, por la simplificación del perfil cromatográfico. La formación de oximas se realiza empleando 2,5% de cloruro de hidroxilamina en piridina y calentando la mezcla a 75 ºC durante 30 min (Sanz *y col.* 2004). Después, los carbohidratos reductores son sililados mediante los procedimientos anteriormente descritos.

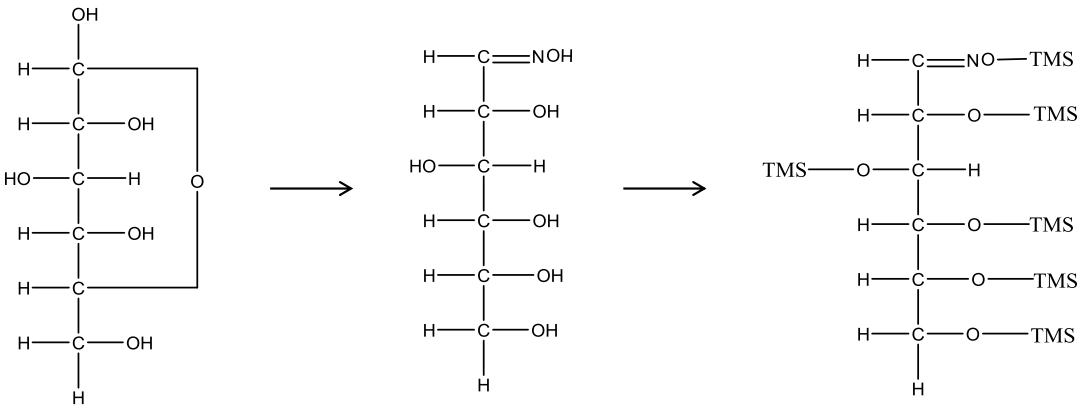

Figura 1.3. Esquema de la formación de trimetilsilil oximas.

- **Acoplamientos cromatografía de gases-espectrometría de masas**

La GC acoplada a espectrometría de masas **(GC−MS)** proporciona información estructural sobre los compuestos analizados, facilitando su caracterización. Sin embargo, en el caso de los carbohidratos, su similitud estructural hace que su caracterización por MS no sea trivial. En los acoplamientos GC–MS, los patrones de fragmentación obtenidos presentan características muy similares para distintos carbohidratos, lo que dificulta su identificación.

Los compuestos sililados poseen una ventaja adicional sobre otros derivados empleados para el análisis de carbohidratos, ya que los diferentes diastereoisómeros presentan ciertas variaciones en su espectro de masas, pudiendo asignar iones característicos a distintos carbohidratos en función de su estructura (Knapp 1979; Fox 2002).

1.6.2. Análisis de carbohidratos por LC

La LC es una técnica muy usada en el análisis, separación y aislamiento de carbohidratos, ya que la preparación de muestra previa a la inyección es mínima en la mayoría de los casos. Los modos de operación empleados para el análisis de carbohidratos incluyen la cromatografía líquida en fase normal (NPLC) e inversa (RPLC), la cromatografía de alta eficacia de intercambio aniónico (HPAEC) y catiónico (HPCEC) y la cromatografía de exclusión molecular de alta eficacia (HPSEC). En los últimos años, el modo de operación más utilizado para los análisis de carbohidratos por LC es la cromatografía de interaccion hidrofílica (HILIC).

Por otra parte, debido a la falta de grupos cromóforos, la detección de carbohidratos por LC resulta difícil. Entre las técnicas de detección más comúnmente empleadas destacan la electroquímica, de limitada estabilidad; el índice de refracción (RI), con baja sensibilidad; y la dispersión de luz (ELSD), en la que la cuantificación es problemática por la limitada reproducibilidad del factor de respuesta (Vervoort *y col.* 2008; Natalini *y col.* 2009). Como alternativa existen varios métodos de derivatización que introducen antes, durante o después de la separación cromatográfica grupos cromóforos, para la detección

por ultravioleta (UV), o fluoróforos, para la detección fluorescente (Lamari *y col.* 2003). Los reactivos más utilizados para este fin son 2-aminopiridina, 2-aminoacridona, p-aminobenceno, 2-aminonaftaleno trisulfona y 1-fenil-3-metil-5-pirazolona (Saba *y col.* 2001). La derivatización puede cambiar también propiedades de los carbohidratos, tales como la hidrofobicidad, para mejorar su resolución. Finalmente, los detectores de MS permiten el análisis de los carbohidratos sin derivatización previa, lo que constituye una ventaja en cuanto a tiempo de procesado de la muestra frente a otros métodos.

La selección de la técnica analítica más adecuada para el análisis de los carbohidratos depende de la naturaleza de la muestra, su complejidad y peso molecular esperado, entre otros factores (Sanz *y col.* 2009). En general, la resolución de mezclas complejas de carbohidratos mediante LC es menor que la conseguida con GC, y la caracterización estructural de los compuestos resulta más complicada, teniéndose que recurrir a sistemas de MS^n (Hernández-Hernández *y col.* 2012).

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2. OBJETIVOS
Y PLAN DE TRABAJO

2. OBJETIVOS, METODOLOGÍA Y PLAN DE TRABAJO

OBJETIVOS

Esta tesis doctoral tiene como punto de partida el proyecto del Plan Nacional referencia AGL2009-11909, cuyo objetivo principal era la obtención de extractos enriquecidos en carbohidratos bioactivos (inositoles, prebióticos, etc.) para su uso como ingredientes alimentarios.

En la actualidad, existe un gran interés por la obtención de ingredientes bioactivos que, al ser incorporados a alimentos, permitan que estos no solo satisfagan las necesidades nutricionales básicas, si no que proporcionen beneficios para la salud del consumidor o reduzcan el riesgo de enfermedad. Además, si estos compuestos bioactivos son de origen natural, su empleo supone un valor añadido frente a los sintéticos, ya que el consumidor busca, por lo general, alimentos constituidos por ingredientes naturales. Por tanto, su extracción mediante métodos rápidos, eficaces y respetuosos con el medio ambiente resulta de gran interés con vistas a su potencial explotación por parte de la industria alimentaria.

Entre dichos ingredientes se encuentran los carbohidratos bioactivos, como inositoles y prebióticos. En general, la presencia de estos compuestos, ya sea en matrices vegetales o en mezclas de síntesis, está acompañada de otros carbohidratos mayoritarios (como glucosa, fructosa, lactosa, etc.) que pueden interferir en sus propiedades bioactivas o en la evaluación de las mismas. Además, en general, estos carbohidratos interferentes poseen estructuras similares a las de los compuestos de interés. Por tanto, resulta de especial interés la puesta de punto de nuevas estrategias que permitan el fraccionamiento selectivo de los carbohidratos bioactivos.

De acuerdo con estas consideraciones, el **objetivo general** del presente trabajo es **el desarrollo de nuevos métodos para la extracción y fraccionamiento selectivo de carbohidratos bioactivos (inositoles y prebióticos).**

Para conseguir este objetivo general, se plantearon los siguientes **objetivos parciales**:

- Evaluar el potencial de los **ILs** para la **solubilización** de carbohidratos de bajo peso molecular.
- Desarrollar un **método para el análisis** de carbohidratos disueltos en ILs mediante GC.

- Evaluar las posibilidades de **fraccionamiento selectivo** de carbohidratos en función de su estructura química empleando nuevas estrategias analíticas y biotecnológicas.

- Estudiar la composición cuali- y cuantitativa de carbohidratos de bajo peso molecular en alimentos, con especial énfasis en carbohidratos bioactivos (inositoles y derivados), mediante GC−MS.

- Desarrollar **nuevas metodologías de extracción** mediante PLE y MAE para la obtención de extractos enriquecidos en inositoles y prebióticos.

METODOLOGÍA Y PLAN DE TRABAJO

Para alcanzar estos objetivos parciales, el plan de trabajo seguido (**Figura 2.1**) se ha estructurado en dos grandes bloques: (i) la evaluación de nuevos disolventes (ILs) para el fraccionamiento selectivo de carbohidratos en función de su estructura química (**Sección 3**) y (ii) el desarrollo de nuevos métodos de extracción y fraccionamiento de carbohidratos bioactivos a partir de matrices naturales (**Sección 4**). A su vez, cada uno de estos bloques está constituido por cuatro y cinco subsecciones, respectivamente, correspondientes a artículos ya publicados en revistas de alto índice de impacto o en proceso de revisión para su publicación.

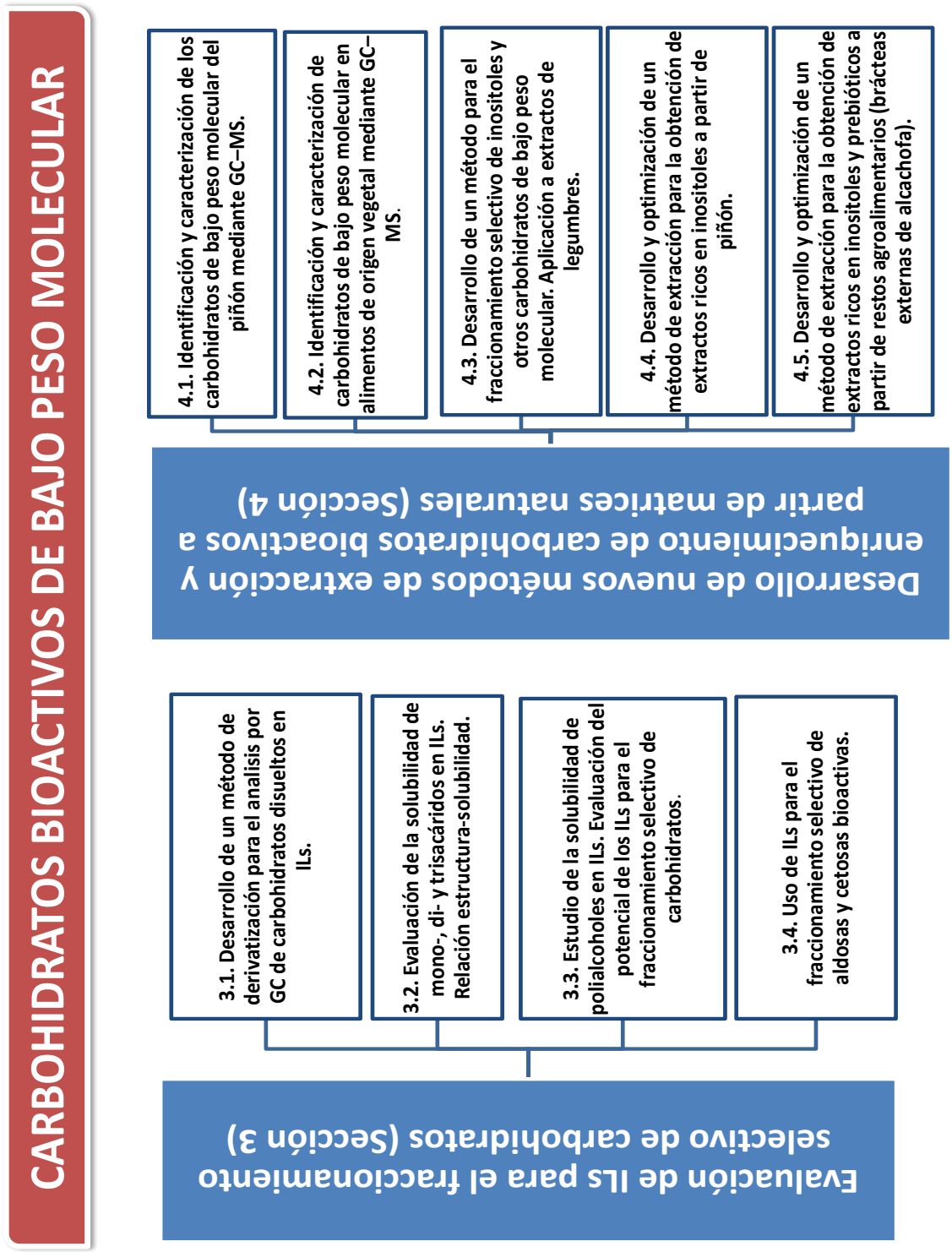

Figura 2.1. Plan de trabajo.

A continuación se detalla la metodología seguida en cada una de las secciones previamente comentadas:

- **Sección 3: Evaluación de ILs para el fraccionamiento selectivo de carbohidratos**
    - **Sección 3.1. Desarrollo de un método de derivatización para el análisis por GC de carbohidratos disueltos en ILs**
        - Evaluación y selección de los agentes derivatizantes y las condiciones de reacción óptimas.
        - Validación del método.
        - Aplicación del método desarrollado al análisis de carbohidratos presentes en muestras reales.
    - **Sección 3.2. Evaluación de la solubilidad de mono-, di- y trisacáridos en ILs. Relación estructura-solubilidad.**
        - Cálculo de solubilidad de mono-, di- y trisacáridos en distintos ILs.
        - Estudio del efecto de la temperatura en la solubilidad.
        - Influencia de la estructura química de los carbohidratos (aldosa/cetosa; grado de polimerización,…) en su solubilidad.
        - Evaluación de la degradación de carbohidratos disueltos en ILs.
    - **Sección 3.3. Estudio de la solubilidad de polialcoholes en ILs. Evaluación del potencial de los ILs para el fraccionamiento selectivo de carbohidratos.**
        - Cálculo de solubilidad de polialcoholes lineales y cíclicos (inositoles) en distintos ILs.
        - Preparación de mezclas binarias polialcohol/monosacárido y polialcohol/disacárido en ILs.
        - Evaluación de la separación selectiva de polialcoholes y otros carbohidratos presentes en las mezclas binarias.
    - **Sección 3.4. Uso de ILs para el fraccionamiento selectivo de aldosas y cetosas bioactivas.**
        - Evaluación de la solubilidad de mezclas binarias aldosa/cetosa en los ILs seleccionados.
        - Síntesis de lactulosa mediante isomerización en medio básico de la lactosa y análisis de la mezcla resultante mediante GC.
        - Evaluación de la separación selectiva de los carbohidratos presentes en la mezcla de síntesis en las condiciones optimizadas.
        - Recuperación de carbohidratos disueltos en ILs.

- **Sección 4: Desarrollo de nuevos métodos de extracción y enriquecimiento de carbohidratos bioactivos a partir de matrices naturales.**
    - **Sección 4.1: Identificación y caracterización de los carbohidratos de bajo peso molecular del piñón mediante GC–MS.**
        - Preparación de TMSO de los carbohidratos del piñón previo a su análisis por GC−MS.
        - Identificación de los carbohidratos, en especial atención a los inositoles, presentes en piñones mediante el empleo de patrones comerciales o mediante el estudio de sus datos de retención cromatográfica y elucidación estructural en base a sus espectros de masas.
        - Análisis de distintas muestras comerciales de piñones para evaluar la variabilidad de su contenido en inositoles y derivados.
    - **Sección 4.2: Identificación y caracterización de carbohidratos de bajo peso molecular en alimentos de origen vegetal mediante GC–MS.**
        - Análisis de las TMSO de los carbohidratos presentes en distintas muestras de origen vegetal por GC–MS
        - Identificación de carbohidratos de bajo peso molecular.
        - Cuantificación de dichos carbohidratos y selección de las fuentes más apropiadas para la obtención de carbohidratos bioactivos.
    - **Sección 4.3: Desarrollo de un método para el fraccionamiento selectivo de inositoles y otros carbohidratos de bajo peso molecular. Aplicación a extractos de legumbres.**
        - Puesta a punto de métodos de fraccionamiento basados en la incubación con levaduras (*Saccharomyces cerevisiae*) de extractos de legumbres.
        - Análisis de las TMSO de los carbohidratos mediante GC−MS. Selección de las condiciones óptimas del fraccionamiento.
        - Identificación y caracterización de carbohidratos de bajo peso molecular en legumbres.
    - **Sección 4.4: Desarrollo y optimización de un método de extracción para la obtención de extractos ricos en inositoles a partir de piñón.**
        - Optimización de condiciones experimentales (disolvente, temperatura, tiempo de extracción, y número de ciclos) para la SLE de inositoles a partir de piñón.

- Optimización de condiciones experimentales (temperatura, tiempo de extracción y número de ciclos) para la PLE de inositoles a partir de piñón.
- Comparación estadística de ambos métodos.
- Eliminación de carbohidratos interferentes mediante un tratamiento optimizado con levaduras.

- **Sección 4.5: Desarrollo y optimización de un método de extracción para la obtención de extractos ricos en inositoles y prebióticos a partir de restos agroalimentarios (brácteas externas de alcachofa).**
  - Optimización de condiciones experimentales (temperatura, tiempo de extracción y número de ciclos) para la PLE de inositoles e inulina a partir de brácteas de alcachofa.
  - Optimización de condiciones experimentales (temperatura, tiempo de extracción, cantidad de muestra y número de ciclos) para la MAE de inositoles e inulina a partir de brácteas de alcachofa.
  - Comparación estadística de ambas técnicas.
  - Eliminación de carbohidratos interferentes mediante un tratamiento optimizado con levaduras.

3. EVALUACIÓN DEL EMPLEO DE ILs PARA EL FRACCIONAMIENTO SELECTIVO DE CARBOHIDRATOS

3. EVALUACIÓN DEL EMPLEO DE ILs PARA EL FRACCIONAMIENTO SELECTIVO DE CARBOHIDRATOS

Como se ha descrito en la introducción general (**Sección 1**), los carbohidratos son las moléculas más abundantes de la naturaleza y se les atribuyen un gran número de propiedades bioactivas, tales como poder prebiótico o acción contra la insulinorresistencia. Estos carbohidratos bioactivos, tanto si son extraídos directamente de productos naturales como obtenidos por vía sintética, se encuentran formando mezclas complejas con otros carbohidratos, siendo en muchas ocasiones necesario su fraccionamiento para (i) obtener fracciones más sencillas que faciliten su caracterización, (ii) evaluar su actividad mediante sistemas *in vitro*, o (iii) para su empleo como ingredientes funcionales. Sin embargo, como ya se ha indicado, dicho fraccionamiento no resulta trivial, debido a la similitud de estructuras de los carbohidratos y a las diferentes abundancias de los mismos en las mezclas.

En esta sección se ha explorado el empleo de nuevos disolventes, en concreto los ILs, para el fraccionamiento de carbohidratos atendiendo a sus diversas estructuras. Para ello, en primera instancia, fue necesario desarrollar un método que permitiera la derivatización de los carbohidratos disueltos en ILs para su posterior análisis mediante GC. En este estudio, se prefirió el empleo de esta técnica cromatográfica para el análisis instrumental de los carbohidratos por ofrecer una mayor capacidad de resolución que la LC, aspecto ventajoso a la hora de abordar la separación de mezclas complejas siendo necesario optimizar un método de derivatización previo al análisis por GC. Por otra parte, la GC puede ser acoplada a diferentes detectores suficientemente sensibles como para garantizar la adecuada detección de los analitos objeto de estudio en matrices reales, mientras que la introducción de ILs en el sistema de LC suele dar problemas debido al aumento del ruido de fondo y/o contaminación de los detectores, por lo que su uso implica la necesidad de la eliminación de ILs de las muestras previo al análisis cromatográfico.

La **Sección 3.1** incluye los resultados del trabajo titulado “Development of a carbohydrate silylation method in ionic liquids for their gas chromatographic analysis” de Ruiz-Aceituno y col. publicado en *Analytica Chimica Acta 787 (2013) 87– 92*. En esta

publicación se presenta el primer método de análisis de LMWC disueltos en ILs mediante GC descrito en la bibliografía. Como ya se ha indicado, esta técnica se seleccionó por su gran poder de resolución para el análisis de mezclas complejas, aunque requiere de una derivatización previa de los carbohidratos. Dicho proceso de derivatización ha de ser total y los derivados formados estables durante al menos unas horas. Estos requisitos básicos de cualquier proceso de derivatización no habían sido hasta el momento explorados empleando ILs como disolventes, siendo necesaria la optimización y validación del método. Los parámetros estudiados fueron la temperatura y tiempo de reacción, los reactivos de sililación (naturaleza y cantidad) y las condiciones de agitación para la derivatizacion de los carbohidatos estudiados disueltos en distintos ILs de base imidazolium. El método optimizado y validado por comparación de los resultados obtenidos con los encontrados empleando disolventes orgánicos convencionales, se aplicó al análisis de carbohidratos presentes en muestras reales (zumos de frutas) y se utilizó en las Secciones 3.2, 3.3 y 3.4.

El artículo titulado: "Influence of chemical structure on the solubility of low molecular weight carbohydrates in room temperature ionic liquids" de Carrero-Carralero y col. publicado en *Industrial and Engineering Chemical Research*, 53 (2014) 813843-13850 constituye la **Sección 3.2**. Este trabajo aporta nuevos datos de solubilidad para varios LMWC (monosacáridos, disacáridos y trisacáridos) disueltos en cuatro líquidos ionicos (1-etil-3-metililimidazolio de dicianamida, [EMIM][DCA]; 1-hexil-3-methilimidazolio de cloruro, [HMIM][Cl]; 1-butil-3-metilimidazolio metil sulfato, [BMIM][$MeSO_4$]; y 1,3-dimetilimidazolio dimetil fosfato, [MMIM][Me_2PO_4]). Se ha estudiado también la relación entre dichos datos de solubilidad y la estructura química de los carbohidratos objeto de estudio, evaluándose además el efecto catalizador de los ILs para reacciones de deshidratación de carbohidratos formando 5-hidroximetilfurfural. Los resultados obtenidos dan pie a nuevas aplicaciones en el campo de la química de carbohidratos.

La **Sección 3.3**. está constituida por el artículo titulado "Solubility of sugar alcohols in ionic liquids. Selective fractionation from other low molecular weight carbohydrates" enviado para su publicación. En este estudio, se evalua por primera vez la solubilidad de distintos polialcoholes lineales e inositoles en los ILs que mejores resultados proporcionaron en el estudio recogido en la sección previa. Las diferencias de

solubilidad encontradas entre estos polioles y los azúcares previamente estudiados puso de manifiesto la potencial eficacia de determinados ILs (principalmente [EMIM][OAc] and [MMIM][Me_2PO_4]) para su fraccionamiento selectivo. Este aspecto se demostró mediante su aplicación al fraccionamiento de mezclas binarias constituidas por un poliol y un mono- o disacárido.

Por último, se evalua la capacidad de los ILs para el fraccionamiento de cetosas bioactivas de sus correspondientes aldosas. Los resultados obtenidos se resumen en el trabajo "Use of Room Temperature Ionic Liquids for the selective fractionation of bioactive ketoses from aldoses" publicado por Carrero-Carralero y col. en *Separation and Purification Technology* 149 (2015) 140–145, que constituye la **Sección 3.4** de esta memoria. Partiendo de los datos de solubilidad de aldosas y cetosas, se seleccionan distintos ILs para su fraccinamiento, aplicándose a un producto real de síntesis (lactulosa obtenida a partir de lactosa). Se han evaluado también distintos procedimientos para la recuperación de los carbohidratos disueltos en los ILs, prestando especial atención además a la posibilidad de reutilización de estos disolventes.

3.1. Development of a carbohydrate silylation method in ionic liquids for their gas chromatographic analysis

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Development of a carbohydrate silylation method in ionic liquids for their gas chromatographic analysis

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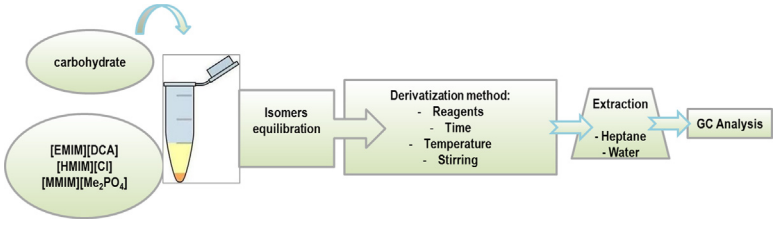
HIGHLIGHTS

- A silylation procedure of carbohydrates in ionic liquids, particularly in [EMIM][DCA], is optimized.
- Trimethylsilylimidazole was the most effective silylation reagent.
- Other silylation reagents used under ultrasonic agitation provide variable results depending on the carbohydrate nature.
- Ionic liquids can be used as alternative to non-volatile organic solvents for carbohydrate derivatization.

GRAPHICAL ABSTRACT



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ABSTRACT

This paper reports on the feasibility of silylation of low molecular weight carbohydrates dissolved in different ionic liquids (ILs) for their further analysis by gas chromatography (GC). Derivatization reagents (nature and amounts), temperature and time of reaction and stirring conditions were evaluated for different carbohydrates (i.e., glucose, mannose, fructose and lactose) dissolved in 1-ethyl-3-methylimidazolium dicyanamide [EMIM][DCA]. Evaluation of conformational isomerism of glucose dissolved in [EMIM][DCA] revealed the effect of the time of dissolution in the equilibration of α- and β-furanoses (up to 3% and 6%, respectively, after 70 h of incubation) and that 21 h sufficed to obtain results similar to those provided by the reference method involving pyridine. Once optimized, the proposed derivatization procedure provided satisfactory yields (i.e., close to 100%) using 100 μL of trimethylsilylimidazole (TMSI) at mild conditions (25 °C) for a relatively short time (1 h) for most of the investigated carbohydrates. Under these experimental conditions, linear responses (i.e., R^2 better than 0.974) were obtained in the tested range of 0.25–1 mg of the derivatized target compounds. Other reagents, such as *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% trimethylchlorosilane (TMCS), were successfully used under ultrasonic conditions for aldose monosaccharides and disaccharides derivatization, while BSTFA was useful for ketose monosaccharides. The possibility of using the proposed method for the derivatization of selected carbohydrates dissolved in different ILs and the efficiency of the method applied to the analysis of carbohydrates present in real samples (fruit juices) have also been investigated.

1. Introduction

Room temperature ionic liquids (or simply ionic liquids, ILs) are low melting point salts that exist in liquid phase at relatively low temperatures (generally speaking, below 150 °C). ILs possess unique and attractive properties such as low volatility, variable viscosity, chemical and thermal stability, and tunable solubility properties, among others [1,2]. These properties make them to be considered a promising recyclable alternative to traditional volatile organic solvents for a high number of applications [1,3–5], including carbohydrate synthesis and processing [6,7]. The low solubility of these compounds in a large majority of conventional organic

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solvents makes the use of ILs a good alternative in carbohydrate chemistry.

Gas chromatography (GC) is a powerful separation technique for the analysis of complex mixtures of low molecular weight carbohydrates (LMWC), for which shows high resolution and sensitivity. Nevertheless, for GC carbohydrate analysis a previous derivatization step is mandatory. Silylation is the most frequently used procedure for the derivatization of carbohydrates which provides them with the required volatility and stability [8]. The formation of per-trimethylsilyl (TMS) ethers is generally carried out using individual or combined silylation agents, such as trimethylsilylimidazole (TMSI), hexamethyldisilazane (HMDS) or trimethylchlorosilane (TMCS), and an aprotic solvent at different temperatures for different reaction times [9]. The good solubility of the carbohydrates and their derivatives in pyridine makes this solvent the most frequently used for sugar analysis. However, ILs could be considered a greener and more safety alternative replacement for pyridine, which is toxic and flammable.

Although not orientated to analytical purposes, silylation of cellulose dissolved in ILs has been widely studied [10,11]. As an example, HMDS has been proposed for the silylation of cellulose dissolved in different 1,3-dialkylimidazolium-based ILs, the solubility of the derivatization reagent in the IL was a key parameter determining the derivatization efficiency [12]. However, to the best of our knowledge, the effectiveness of silylation of LMWC dissolved in ILs, previous to their GC analysis, has not been evaluated before. It is important to consider that the previously reported procedures for derivatization of LMWC dissolved in common solvents are not directly valid in ILs because the efficiency of the silylation process markedly depends on solubility, viscosity and other physicochemical properties of ILs (they can act as catalyst, as retardant, or do not act). Moreover, the most common silylation reagents are usually very effective silyl-donors in pyridine, but when carbohydrates are dissolved in other solvents, these reagents could behave in a different manner: the high number of hydroxyls in the molecule, the presence of different tautomeric forms in solution and the steric hindrance must be taken into account when a different solvent is chosen [9]. There are only a limited number of references regarding the use of ILs for silylation of low molecular weight compounds containing hydroxyl groups [13]. 1-Butyl-3-methylimidazolium hexafluorophosphate has been proved to be useful as a green recyclable alternative to conventional solvents for the silylation of alcohols and phenols with t-butyldimethylchlorosilane (i.e., the derivatization yields ranged from 90 to 99% for all alcohols tested) [14].

In this study, the efficiency of a silylation method for mono-, di- and trisaccharides dissolved in ILs before their GC analysis was evaluated and applied to the analysis of carbohydrates present in real samples (fruit juices). The experimental parameters evaluated for method optimization included the time required for carbohydrate equilibration, the nature and amount of the silylation reagent used, the time and temperature of reaction and the stirring conditions.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used in this work were analytical or research grade. Cellobiose was acquired from Difco Laboratories (Detroit, MI, USA). Glucose, fructose, tagatose, galactose, mannose, xylitol, mannitol, lactose, lactulose, gentiobiose, raffinose, melezitose, phenyl-β-D-glucoside, TMSI, HMDS and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + 1% TMCS were obtained from Sigma Chemical Co. (St. Louis, USA). Heptane, TMCS and *N*,*O*-bis(trimethylsilyl)acetamide (BSA) were purchased from Merck (Darmstadt, Germany). BSTFA was acquired from ACROS organics (Geel, Belgium) and *n*-tetracosane from PolyScience Corporation (Illinois, USA).

1-Ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]), 1-hexyl-3-methylimidazolium chloride ([HMIM][Cl]), 1-butyl-3-methylimidazolium methyl sulfate ([BMIM][$MeSO_4$]) were obtained from Sigma Chemical Co. and 1,3-dimethylimidazolium dimethyl phosphate ([MMIM][Me_2PO_4]) from Alfa Aesar (Massachusetts, USA). These ILs were selected on the base of LMWC solubility data available in the literature [3,15,16]. Other criteria such as diversity of anion and cation, availability, and low cost were also considered.

2.2. Dissolution of carbohydrates in ILs

ILs were dried at 60 °C under vacuum for 3 days before use. Carbohydrates (1 mg), previously indicated in Section 2.1, were individually dissolved in 50 μL of the tested IL and the resulting mixtures were equilibrated by stirring using a Thermomixer (Eppendorf, Hamburg, Germany). Different times and temperatures (see Table 1) were assayed using glucose to optimize equilibration conditions.

2.3. Samples

Fresh fruits (strawberry, grape, orange and tangerine) were purchased at a local market in Madrid (Spain) and juices were produced from them in the laboratory, after removing skins and seeds when necessary. They were then centrifuged at 10,000 rpm for 20 min at 5 °C and the supernatant was filtered to remove any suspended solid material. Juices (0.5 mL) were diluted to 25 mL with 70% methanol, as previously done by Sanz et al. [17]. After that, 0.5 mL of the solution was evaporated under vacuum in a Speed Vac (Genevac, Ipswich, UK).

2.4. General procedure for the silylation reaction

Silylation of the assayed carbohydrates dissolved in the corresponding IL was carried out with 100 μL of different silylation reagents assayed [i.e., TMSI, TMSI:TMCS (1:1, v/v), HMDS, HMDS:TMCS (1:1, v/v), BSTFA, BSTFA + 1% TMCS and BSA] for different times (0, 30, 60 and 120 min), at different temperatures (25, 45 and 60 °C) and under different agitation conditions (i.e., ultrasonic agitation, conventional stirring and static incubation). 200 μL of ultrapure water were added to finish the reaction. The derivatized carbohydrates were then recovered by liquid–liquid extraction (LLE) with 100 μL of heptane. After optimization of the derivatization procedure, two more successive LLEs with heptane were performed to ensure quantitative recovery of the derivatized analyte. In this case, extracts were jointly collected and directly analyzed by GC. Otherwise specified, all assays were carried out in triplicate.

Carbohydrates present in fruit juices were derivatized by adding to the dried extracts (i) pyridine (100 μL) and TMSI (100 μL) as reference method [9], and (ii) [EMIM][DCA] (100 μL) and TMSI (100 μL) under optimized conditions. Reaction was stopped by adding water and the derivatized carbohydrates were recovered as indicated above for their further GC analysis.

2.5. GC analysis

GC analysis of the derivatized carbohydrates was carried out using an HP 7890A gas chromatograph equipped with a flame ionization detector (FID) from Agilent Technologies (Palo Alto, CA, USA) using nitrogen as carrier gas (flow rate, 0.7 mL min^{-1}). Derivatized glucose tautomers were analyzed using a 7890A

Table 1
Percentage of isomeric forms of TMS glucose dissolved in [EMIM][DCA] and equilibrated under different conditions and derivatized using TMSI at 25 °C for 30 min. Standard deviation (S.D.) in parenthesis ($n = 3$).

Equilibration conditions		% glucose tautomers (S.D.)			
Time (h)	Temperature (°C)	α-Pyranose	β-Pyranose	α-Furanose	β-Furanose
0.5	60	67.6 (0.3)	26.8 (0.1)	3.1 (0.2)	2.5 (0.1)
0.5	25	84 (5)	14 (6)	0.6 (0.1)	0.7 (0.3)
21	25	47.9 (0.2)	50.8 (0.3)	0.5 (0.1)	0.8 (0.1)
27	25	45.2 (0.3)	51 (1)	1.2 (0.6)	2.3 (1.0)
45	25	40.6 (0.1)	53.7 (0.1)	1.9 (0.1)	3.8 (0.2)
70	25	39.2 (0.3)	52 (3)	3.0 (1.0)	6 (2)

Table 2
Silylation yields (%) of glucose dissolved in [EMIM][DCA] using TMSI under different derivatization conditions. Standard deviation (S.D.) in parenthesis ($n = 3$).

	Yield (S.D.)					
Temperature (°C)	25				45	60
Time (min)	0*	30	60	120	60	60
Yield (S.D.)	80 (1)[a]	96 (1)[b]	103 (2)[c,A]	101 (1)[c]	105 (4)[A]	102 (2)[A]

Different letters indicate significant differences ($p < 0.05$) for each time at the same temperature (lower case letters), and for each temperature at the same time (capital letters).
* Immediately after reagent addition.

Table 3
Silylation yields (%) of carbohydrates dissolved in [EMIM][DCA] using different reagents at 60 °C for 1 h under ultrasound agitation. Standard deviation (S.D.) in parenthesis ($n = 3$).

Reagent	Glucose	Mannose	Fructose	Lactose
HMDS	–	–	–	–
HMDS + TMCS	103 (1)	100 (2)	21 (5)	13 (6)
BSTFA + TMCS	103.2 (0.3)	104 (2)	–	97 (4)
BSTFA	87 (23)	89 (2)	100 (5)	94 (3)
TMSI	96 (10)	102 (4)	107 (1)	100 (3)
TMSI + TMCS	82 (11)	89 (14)	93 (2)	96 (19)
BSA	–	–	–	–

gas chromatograph coupled to a 5975 C quadrupole mass detector (GC–MS, both from Agilent Technologies) using helium at 1 mL min^{-1} as carrier gas. In both cases, a fused silica capillary column coated with 100% dimethylpolysiloxane (30 m × 0.25 mm i.d. × 0.25 μm *df*) was used (Zebron, Phenomenex, CA, USA). The oven temperature was programmed as follows: 180 °C to 250 °C (10 °C min^{-1}) and held for 3 min, and then to 300 °C at 15 °C min^{-1} and held for 20 min. The injection port was heated to 300 °C and injections were made in the split mode, with a split ratio 1:20. FID temperature was 320 °C. The MS was operated in electron impact (EI) mode at 70 eV, scanning the 35–700 *m*/*z* range. The transfer line and ionization source were heated at 280 and 230 °C, respectively. Chromatographic peaks were measured using an HPChem acquisition system (Agilent Technologies).

2.6. Quantitative analysis

Quantitation analyses were performed in triplicate using the internal standard procedure. For this purpose, calibration curves of the derivatized carbohydrate in the 0.1–1 mg range were constructed. Response factors of glucose, *myo*-inositol, lactose and raffinose (which were used for monosaccharides, *myo*-inositol, disaccharides and trisaccharides quantitation, respectively) relative to two internal standards (*n*-tetracosane and phenyl-β-D-glucoside) were determined for quantitative analysis. The reproducibility of the method was determined on the basis of the intra-day and inter-day precision, and estimated as the relative standard deviation (RSD) of the calculated concentrations for each carbohydrate standard in five separate determinations.

2.7. Statistical analysis

Statistical analysis was performed using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Univariate analysis of variance (ANOVA) and Fisher test were used to evaluate significant differences among the several derivatization treatments, and also to evaluate the efficiency of the silylation method using real samples. The differences were considered to be significant when $p < 0.05$.

3. Results and discussion

Information available in the literature about the solubility of mono-, di- and trisaccharides in ILs is rather scarce [15]. However, [EMIM][DCA] has been described as a good solvent for different carbohydrates such as glucose and sucrose, for which solubilities above 10% wt have been reported [18]. Therefore, this IL was selected for the optimization of the proposed derivatization method. The effectiveness of the derivatization procedure was initially evaluated using glucose and TMSI as silylation reagent due to the experimentally determined miscibility of this reagent in [EMIM][DCA].

3.1. Evaluation of conformational isomerism of glucose

α- and β-pyranoses have been identified as the most abundant isomers of glucose dissolved in pyridine (45 and 53%, respectively) at room temperature; meanwhile, α- and β-furanoses only appear at low levels (0.6 and 1%, respectively) [19]. However, no data regarding conformational equilibrium of glucose dissolved in ILs can be found in the literature. Therefore, the equilibrium of the

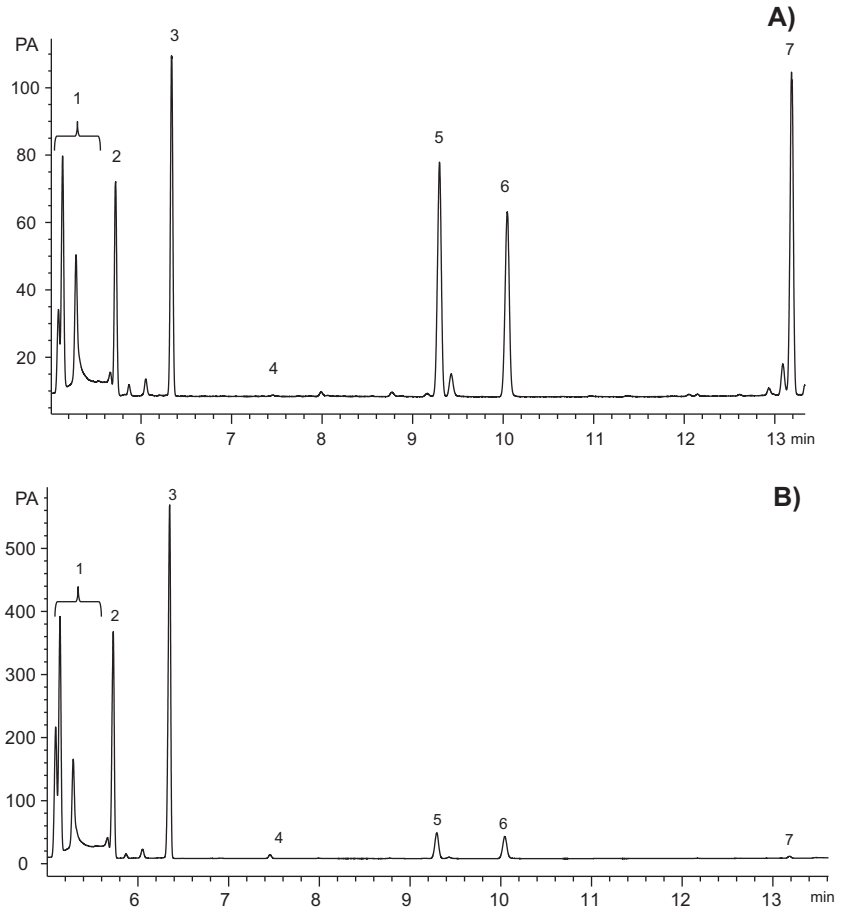

Fig. 1. Gas chromatographic profiles of strawberry (A) and grape (B) juices derivatized using [EMIM][DCA] and TMSI. Peak identification: (1) fructose, (2) α-glucopyranose, (3) β-glucopyranose, (4) *myo*-inositol, (5) phenyl-β-D-glucoside (internal standard), (6) *n*-tetracosane (internal standard), and (7) sucrose.

tautomeric forms of crystalline glucose dissolved in [EMIM][DCA] was evaluated taking into account two main experimental parameters, namely temperature and time of dissolution.

For comparative purposes, the identification of the tautomers of sugars silylated in pyridine was carried out by GC–MS according to Martínez-Castro et al. [20].

When crystalline glucose was dissolved in [EMIM][DCA] at 60 °C for 30 min (longer times were not assayed to avoid degradations) and then silylated with TMSI, α-pyranose was noticeably more abundant (68%) than the other tautomers. This percentage was even higher at 25 °C (84%; Table 1). However, when the glucose solution in [EMIM][DCA] was kept at 25 °C for 21 h before silylation, similar percentages of TMS α- and β-pyranose were obtained (48 and 51%, respectively). Times of equilibration longer than 27 h resulted in percentages of α- and β-furanoses higher than those previously found in pyridine solutions [19] and arose up to 3% and 6%, respectively, after 70 h of incubation. According to these results, for the following experiments, carbohydrates were dissolved in [EMIM][DCA] and kept at 25 °C for 21 h before derivatization.

3.2. Optimization of the silylation procedure in [EMIM][DCA]

To evaluate the effectiveness of the derivatization of glucose, two internal standards were used: phenyl-β-D-glucoside, as a derivatizable carbohydrate, and *n*-tetracosane, as a non-derivatizable standard. The solubility of phenyl-β-D-glucoside in [EMIM][DCA] was experimentally confirmed in the laboratory, while *n*-tetracosane was insoluble in this IL but fully soluble in heptane.

Reactions were developed at 25 °C for 1 h, recovery of TMS-glucose derivatives being carried out by single LLE with 100 μL of heptane. Different yields were calculated for glucose depending on the internal standard used for quantitation. Whereas the recovery calculated for glucose using phenyl-β-D-glucoside as internal standard was 99 ± 4%, its yield relative to *n*-tetracosane was 85 ± 4%. This discrepant result could be associated to either an incomplete derivatization of both glucose and phenyl-β-D-glucoside or to an incomplete extraction of these compounds into the heptane layer. The latter possibility was evaluated by successive LLEs of the derivatized mixture with heptane. It was found that quantitative recoveries of both glucose and phenyl-β-D-glucoside from the [EMIM][DCA] was only achieved after three successive LLE with 100 μL of heptane, yields being 87 ± 5%, 11 ± 4% and 2 ± 1%, respectively. Therefore, this recovery procedure was followed for further experiments and the following data are only expressed related to phenyl-β-D-glucoside.

The time and temperature of the reaction and the volume of silylation reagent were also optimized. As shown in Table 2, silylation was not immediately produced after addition of TMSI and at least 30 min were required to achieve acceptable yields of 96%. Complete derivatization was only achieved after 1 h of treatment (yield, 103%) with no significant improvement at longer reaction times (e.g., the yield after 2 h of reaction was 101%; Table 2). Therefore, 1 h was selected for following assays. Under these conditions, no significant differences existed between the different temperature assayed, 25 °C being chosen for following experiments. Different volumes (100 and 200 μL) of TMSI were then evaluated for the silylation reaction and similar results were obtained in both cases (i.e.,

Table 4
Silylation yields (%) of different low molecular weight carbohydrates dissolved in [EMIM][DCA] and submitted to the proposed optimized method. Standard deviation (S.D.) in parenthesis ($n = 3$).

Compound	Yield (S.D.)
Xylitol	100 (6)
Mannitol	101 (6)
myo-Inositol	101 (2)
Glucose	96 (10)
Mannose	102 (4)
Galactose	102 (1)
Tagatose	99 (2)
Fructose	100 (6)
Maltose	98 (2)
Lactose	100 (3)
Lactulose	101 (5)
Cellobiose	110 (4)
Raffinose	101 (7)
Maltotriose	95 (10)
Melezitose	106 (9)

$103 \pm 2\%$ for 100 μL and $99 \pm 6\%$ for 200 μL). Therefore, 100 μL of TMSI were proved to be enough to achieve a complete derivatization.

The influence of different stirring procedures on the efficiency of the derivatization process was also investigated. No significant differences were observed when the reaction was carried out under static conditions ($101 \pm 6\%$), stirring with a vortex ($101 \pm 4\%$) or under ultrasonic agitation ($101 \pm 1\%$). Therefore, the three procedures could be considered suitable for the silylation of glucose with TMSI.

Besides TMSI, other silylation reagents (i.e., HMDS, BSTFA, BSA and their combinations with TMCS) were investigated. Except TMSI, the other evaluated reagents were not miscible in [EMIM][DCA] and they formed viscous/jelly solutions or remained as a separated layer. Nevertheless, they dissolved using ultrasounds agitation. Therefore, the efficiency of all these reagents for the silylation of carbohydrates was evaluated under sonication conditions. In addition, these experiments were not only limited to glucose, but also extended to other LMWC, i.e. mannose, fructose and lactose, as representative analytes for aldoses, ketoses and disaccharides, respectively. Table 3 summarizes the results obtained in this part of the study. Whereas TMSI was effective for derivatization of all the carbohydrates analyzed, different behaviors were observed for the other investigated reagents depending on the nature of the carbohydrate. Silylation of aldose monosaccharides was also effective using HMDS + TMCS and BSTFA + TMCS, albeit these reagents failed for fructose derivatization. Silylation of this ketose was effective using BSTFA, whereas lactose was effectively silylated using BSTFA + TMCS, BSTFA, and TMSI + TMCS. However, with the latter reagent the dispersion was high as reflects its standard deviation (19%, Table 3), and derivatization could not be considered reliable. HMDS and BSA were not efficient for the silylation of the carbohydrates evaluated and different by-products were formed after reaction. The behavior of these reagents indicates that silylation of sugars is more difficult in ILs than in the usual solvents. It seems that it is not a mere problem of solubility; probably viscosity also contributes impairing the movement of molecules in the medium; or maybe also other molecular properties of ILs could act as retardant.

Table 5
Silylation yields (%) of carbohydrates using TMSI at 60 °C for 1 h under ultrasonic agitation. Standard deviation (S.D.) in parenthesis ($n = 3$).

Reagent	Glucose	Fructose	Lactose
[HMIM][Cl]	98 (6)	103 (6)	96 (11)
[BMIM][$MeSO_4$]	6 (2)	3.4 (0.1)	11 (5)
[MMIM][Me_2PO_4]	96 (6)	101 (12)	93 (5)

Regarding sample handling, TMSI was also the best choice, considering that clear solutions were obtained in all cases and that the recovery of silylated carbohydrates from the miscible solution was easier. Moreover, it could be simultaneously applied for all the carbohydrates studied.

3.3. Linear response and reproducibility of the complete analysis

Good linear relationship between carbohydrate amount and peak areas was obtained for calibration curves in the evaluated range (0.1–1 mg); correlation coefficients (R^2) ranged from 0.974 to 0.999. Intra- and inter-day reproducibilities were evaluated derivatizing 1 mg of glucose as a reference compound ($n = 5$) under the optimized conditions (i.e. 100 μL of TMSI at 25 °C for 1 h). Relative standard deviation was lower than 4%.

3.4. Application to different carbohydrates

Different carbohydrates, including mono-, di- and trisaccharides, dissolved in [EMIM][DCA] were silylated following the optimized derivatization procedure. As shown in Table 4, the proposed derivatization procedure was effective for most of the assayed carbohydrates, providing yields in the range of 96–110% with SD below 10 ($n = 3$).

These yield and repeatability values are in the range of those reported in the literature for the derivatization of carbohydrates using more toxic solvents such as pyridine [21–23].

3.5. Use of different ILs

Once optimized, the straightforward silylation procedure was finally extended to other ILs, i.e. [HMIM][Cl], [BMIM][$MeSO_4$] and [MMIM][Me_2PO_4], for glucose and fructose taken as representatives of monosaccharides (aldose and ketose) and lactose representing a disaccharide (Table 5). Considering that [HMIM][Cl] was not miscible with TMSI and has a high viscosity (7500 cP at 25 °C), derivatization had to be performed under ultrasounds agitation at 60 °C. Silylation failed in [BMIM][$MeSO_4$] (mean value of $6 \pm 2\%$, $3.4 \pm 0.1\%$, and $11 \pm 5\%$; for glucose, fructose and lactose yield, respectively) and different by-products were formed. However, carbohydrates were correctly derivatized in [HMIM][Cl] and [MMIM][Me_2PO_4] (recovery values in a range of 96–98%, and 93–101%, respectively). The formation of by-products was not observed and clean chromatograms were obtained in all cases, something which make possible the accurate determination of the carbohydrates even if a non selective detector as FID was used.

3.6. Application to real sample analysis

The optimized derivatization method was applied to four different hand-made fruit juices (grape, strawberry, orange and tangerine). Fig. 1 shows GC profile of strawberry (A) and grape (B) samples dissolved in [EMIM][DCA] and silylated using 100 μL of TMSI at 25 °C for 1 h. According to previous works [17], fructose, glucose, sucrose and *myo*-inositol were detected in all the juices. Table 6 shows the concentrations (mg mL^{-1}) of these low molecular weight carbohydrates found in the juices when using pyridine or [EMIM][DCA] as solvents in the derivatization procedure. Grape juice had the highest concentration of fructose and glucose and the lowest of sucrose, whereas tangerine juice showed the highest content of sucrose and *myo*-inositol. When both derivatization procedures were compared, no statistically significant differences were observed between determined concentrations of the four carbohydrates in all cases which confirmed the high efficiency of the optimized procedure.

Table 6
Concentrations (mg mL^{-1}) of carbohydrates dissolved in either pyridine or [EMIM][DCA] in different fruit juices and submitted to the proposed optimized method. Standard deviation (S.D.) in parenthesis (n = 3).

	Fructose		Glucose		Sucrose		*myo*-Inositol	
	Pyridine	IL	Pyridine	IL	Pyridine	IL	Pyridine	IL
Strawberry	19.2 (2.4)[a*]	19.2 (2.1)[a]	10.8 (1.8)[a]	10.4 (0.9)[a]	13.2 (0.9)[a]	9.8 (1.5)[a]	0.04 (0.02)[a]	0.05 (0.02)[a]
Grape	122.4 (11.8)[a]	128.4 (8.3)[a]	83.5 (7.2)[a]	88.0 (4.4)[a]	0.6 (0.4)[a]	0.5 (0.1)[a]	0.4 (0.2)[a]	0.6 (0.1)[a]
Orange	24.7 (3.2)[a]	28.4 (2.4)[a]	17.5 (3.4)[a]	18.8 (0.4)[a]	46.3 (1.9)[a]	48.1 (5.1)[a]	1.4 (0.04)[a]	1.5 (0.1)[a]
Tangerine	54.8 (1.8)[a]	55.1 (5.1)[a]	30.8 (1.7)[a]	31.7 (1.4)[a]	51.2 (6.2)[a]	51.5 (4.7)[a]	2.6 (0.1)[a]	2.7 (0.1)[a]

[*] Similar letters indicate not significant differences (p < 0.05) for each carbohydrate concentration in each juice.

4. Conclusions

Silylation of LMWC dissolved in selected ILs, particularly in [EMIM][DCA], has been proved to be effective under optimized conditions, so avoiding the use of toxic solvents such as pyridine. The use of TMSI under mild conditions for relatively short periods of time (1 h) resulted in good derivatization yields for most of the carbohydrates investigated. Other silylation reagents such as the mixtures of HMDS and BSTFA with TMCS can be used under ultrasonic agitation conditions but variable derivatization efficiencies have been obtained depending on the nature of the carbohydrate, especially for aldoses or ketoses. The application of this green solvent based methodology could also be extended to the analysis of other polar compounds present in real samples.

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3.2. Influence of chemical structure on the solubility of low molecular weight carbohydrates in room temperature ionic liquids

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Influence of Chemical Structure on the Solubility of Low Molecular Weight Carbohydrates in Room Temperature Ionic Liquids

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ABSTRACT: The use of room temperature ionic liquids (ILs) as green solvents is an emerging and innovative technology, which is quickly expanding in carbohydrate chemistry. However, information about solubility of low molecular weight carbohydrates (LMWC) in ILs is still scarce. This study provides new solubility data for several LMWC dissolved in methylimidazolium-based ILs. Different solubility values have been obtained for carbohydrates depending on their structure. In general, ketoses are more soluble than their corresponding aldoses, and trisaccharides are more soluble than mono- and disaccharides, although some exceptions have been observed depending on the tested IL. [BMIM][$MeSO_4$] and [HMIM][Cl] acted both as solvent and as catalyst for dehydration of ketoses (fructose and tagatose) into 5-hydroxymethylfurfural at 318 K. These findings pointed out the potential of ILs as an interesting alternative solvent for new applications in applied carbohydrate chemistry.

1. INTRODUCTION

Carbohydrates, either naturally occurring or synthesized by chemical or enzymatic reactions, are normally found as complex mixtures and are involved in a high number of industrial processes and are relevant in different application fields, including industrial chemistry, bioenergy, food, pharmacy, and medicine. Fractionation of carbohydrates is a challenging task due to this structural complexity and/or similarity. Although different techniques have been proposed for the selective fractionation of carbohydrates,[1,2] most of them involve the use of different solvents and the differences in carbohydrate solubility on them.[3]

As it is known, carbohydrates are soluble in water and poorly soluble in almost any organic solvent except for a few exceptions, such as dimethylformamide, dimethyl sulfoxide (DMSO), or pyridine. However, these solvents have many undesirable properties and are not compatible with many applications of carbohydrate-derived products.[4] Differences in monosaccharide composition, position of the carbonyl group (aldoses and ketoses), and degree of polymerization (mono-, di-, oligo-, and polysaccharides) of carbohydrates dramatically affect their solubility properties. As an example, Montañés et al.[3] reported that lactose (β-D-galactopyranosyl-(1→4)-D-glucose) was scarcely soluble in methanol at 22 °C (0.96 g/L), whereas its corresponding ketose, lactulose (β-D-galactopyranosyl-(1 → 4)-D-fructose), showed a solubility value of 19.4 g/L.

Room temperature ionic liquids (RTILs, or simply ILs) are nonmolecular ionic solvents resulting from the combination of organic cations (e.g., imidazolium, pyridinium, pyrrolidinium, or phosphonium, or quaternary ammonium) and organic or inorganic anions (e.g., acetate, trifluoroacetate, tetrafluoroborate, hexafluorophosphate or bromide). They are liquid at or near room temperature (with melting points below 100 °C).[5] Their overall physicochemical properties, such as low volatility, nonflammability, odorlessness, thermal stability, recyclability, etc., result from the combined properties of both cation and anion.[6−8]

In general, ILs are considered environmentally friendly solvents and seem to constitute a safe alternative to the use of traditional volatile organic solvents. Nowadays, a large variety of applications of ILs can be currently found in the literature, mainly as media and/or catalysts in organic synthesis,[9,10] but also for analytical determinations (derivatization reactions, extractants, chromatographic stationary phases, etc.).[11]

ILs are able to dissolve numerous nonpolar and polar compounds, including highly polar compounds such as carbohydrates.[6,12] They have been used as green solvents to improve pretreatment and fractionation procedures for lignocellulosic biomass to obtain a variety of value-added products[13,14] and different chemical reactions, including the conversion of cellulose and cellulose-derived glucose by oxidation, hydrogenation, or dehydration reactions into value-added chemicals and liquid fuels.[15]

Although many studies have reported the solubility of polysaccharides such as cellulose in different ILs, data about solubility of low molecular weight carbohydrates (LMWC) are still limited in the literature. Most studies have focused on the investigation of the solubility of glucose, fructose, and/or sucrose in some ILs.[12,16−18] Apart from these sugars, solubility of lactose, galactose, and xylose have also been reported in a few imidazolium-based ILs.[16,18,19]

Overall, the use of ILs in carbohydrate chemistry is an emerging and innovative technology, which is quickly expanding. Therefore, it is crucial to increase the knowledge

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about the solubility of LMWC in ILs in order to evaluate their potential use for the selective separation of carbohydrate mixtures and to spread its application within industrial procedures. In this work, the solubility of different monosaccharides (glucose, galactose, fructose, and tagatose), disaccharides (lactose, lactulose, and maltose), and trisaccharides (maltotriose and raffinose), in five imidazolium-based ILs are investigated at different temperatures, most of them for the first time. Differences in solubility between aldoses and ketoses, structural isomers, and carbohydrates with different degree of polymerization (DP 1−3) have also been discussed. Moreover, the effect of ILs in the degradation of these carbohydrates with temperature has been evaluated.

2. EXPERIMENTAL SECTION

2.1. Chemicals and Reagents. Analytical standards of fructose, glucose, galactose, tagatose, lactose, lactulose, raffinose, maltose, maltotriose, phenyl-β-D-glucoside, and 5-hydroxymethyl-2-furaldehyde were obtained from Sigma Chemical Co. (St. Louis, US). The investigated ILs (see Figure 1 for their chemical structure and Table 1 for their physicochemical properties), 1-hexyl-3-methylimidazolium chloride ([HMIM][Cl]), 1-butyl-3-methylimidazolium methyl sulfate ([BMIM][$MeSO_4$]), 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]), and 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]), were also obtained from Sigma Chemical Co. 1,3-Dimethylimidazolium dimethylphosphate ([MMIM][Me_2PO_4]) was from Alfa Aesar (Massachusetts). Trimethylsilylimidazole (TMSI) was obtained from Sigma Chemical Co. and heptane from Merck (Darmstadt, Germany).

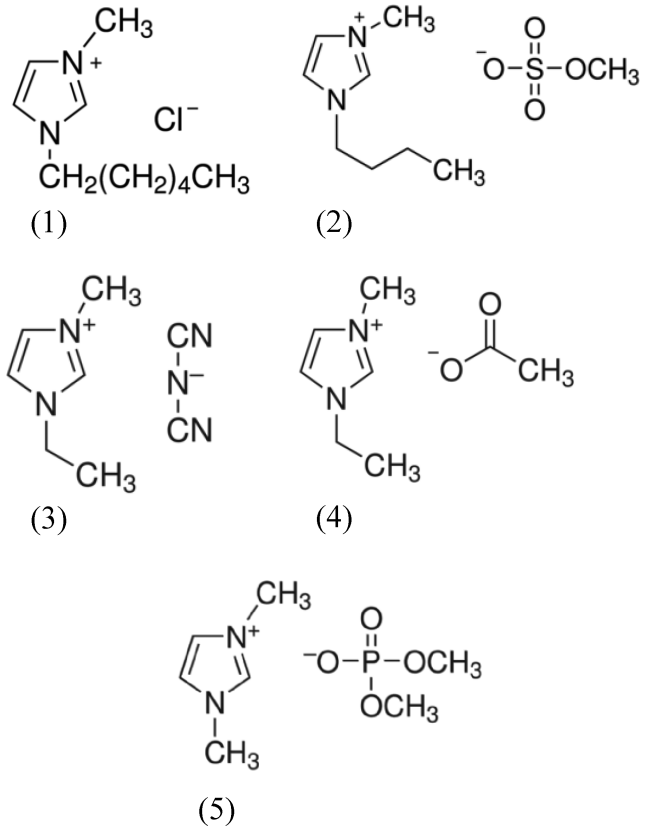

Figure 1. Structures of tested ionic liquids: (1) [HMIM][Cl], (2) [BMIM][$MeSO_4$], (3) [EMIM][DCA], (4) [EMIM][OAc], and (5) [MMIM][Me_2PO_4].

2.2. Sample Preparation. *Dissolution of Carbohydrates in IL.* For solubility studies, each carbohydrate was individually dissolved in the corresponding test IL, with a slight excess. Samples were stirred during 24 h at 1350 rpm and left to stand for another 24 h more. Solubility was evaluated at three temperatures, i.e., 299, 318, and 348 K, using a Thermomixer (Eppendorf, Hamburg, Germany). Then, an aliquot of the resulting mixture was collected from the upper layer, derivatized, and analyzed by gas chromatography (GC).

2.3. Analytical Methods. *2.3.1. Determination of the Water Content.* Water content in each IL was measured using a C20 Compact Karl Fischer Coulometer (Mettler Toledo, OH). HYDRANAL-Coulomat AG from Sigma Chemical Co. was the reagent used for volumetric titration.

2.3.2. Study of the Browning Reaction. Color development was evaluated as the absorbance of the samples at 420 nm, following the method of Meydav et al.[21] using Spectra Max Plus 384 Microplaque Reader (Molecular Devices, CA). The absorbance was measured for carbohydrates dissolved in sodium phosphate buffer (pH, 7.02; control value), as well as in [BMIM][$MeSO_4$] and [HMIM][Cl]after dilution 1:6 (v/v) in water.

IL-mediated formation of HMF was analyzed using a HPLC-UV equipment (see HPLC Analysis section for detailed description).

2.3.3. Gas Chromatography Analysis. Derivatization was carried out using 10 mg of the mixture of carbohydrate and the corresponding IL and 0.3 mg of phenyl-β-D-glucoside, which was used as internal standard. Silylation was done according to Ruiz-Aceituno et al.[11] In brief, TMSI (100 μL) was added to the sample extracts and the reaction was allowed to proceed for 1 h. Then, the reaction was stopped by water addition. Trimethylsilyl carbohydrates derivatives were then extracted onto 100 μL of heptane. Two successive extractions with heptane were done to ensure complete recovery of the derivatized carbohydrates.

An HP 7890A gas chromatograph equipped with a flame ionization detector (FID) from Agilent Technologies (Palo Alto, CA) was used for the determination of the derivatized carbohydrates. The GC separation was performed on a silica capillary column coated with 100% dimethylpolysiloxane (30 m × 0.25 mm i.d. × 0.25 m d.f.) was used (Zebron, Phenomenex, CA). The carrier gas was nitrogen at a constant flow rate of 0.677 mL/min. The GC oven temperature program started at 200 °C and increased at 2 °C/min to a final temperature of 290 °C. The inlet and detector temperatures were set at 300 °C. Samples were injected (1 μL) with a split ratio of 20:1.

Quantitation was done using the internal standard method. For this, standard solutions of the studied carbohydrates were dissolved in each IL at concentrations in the 0.25−1 mg range. Response factors of each carbohydrate were used for quantitative analysis.

2.3.4. HPLC Analysis. Chromatographic analyses of carbohydrates dissolved in [BMIM][$MeSO_4$] were performed using a HPLC-RID system (Agilent Technologies 1220 Infinity LC System-1260 RID, Boeblingen, Germany). An amino column (100-NH_2, 250 mm × 4.6 mm and 5 μm of particle size) from Kromasil (Bohus, Sweden) was used for separation. The mobile phase was a mixture of acetonitrile:water in a ratio 70:30 (v/v) and the flow rate was 1.0 mL/min. The mobile phase was degassed by an ultrasonic bath and filtered through a

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Table 1. Physicochemical Properties of the ILs Investigated

	[HMIM][Cl]	[BMIM][$MeSO_4$]	[EMIM][DCA]	[EMIM][OAc]	[MMIM][Me_2PO_4]
chemical formula	$C_{10}H_{19}ClN_2$	$C_9H_{18}N_2O_4S$	$C_8H_{11}N_5$	$C_8H_{14}N_2O_2$	$C_2H_{15}N_2O_4P$
M (g mol^{-1})	202.50	250.31	177.21	170.21	161.97
T_{fus}(K)	198[8]	253[8]	252[8]	253[8]	–[a]
viscosity at RT (mPa s)	7500[8]	180[8]	21[8]	91[8]	363[20]

[a](–) No data available.

0.45 μm membrane filter under vacuum. Data acquisition and processing were performed using Agilent ChemStation software (Agilent Technologies, Boeblingen, Germany). Quantitation analyses were carried out using the external standard method by using solutions of carbohydrate standards dissolved in the corresponding IL in the range 2–25 mg/100 mg.

HMF determination was carried out using a HPLC-UV 1200 series with G1314B VWD Detector (Agilent Technologies), using a XDB-C18 column (Zorbax, 5 μm particle size and 80 Å pore size, 150 × 4.6 mm i.d.). Methanol–water with a linear binary gradient was used as mobile phase at a flow rate of 1 mL/min, following the method proposed by Vinas et al.[22] HMF quantitation was carried out using the external standard method and a calibration curve in the 0.025–0.112 mg/mL range. Detection was performed using a variable-wavelength UV detector set at 280 nm.

2.4. Statistical Analysis. Data were evaluated using the software Statistica 7.0 (StatSoft, Inc., Tulsa, OK). Analyses of variance (ANOVA) were used to evaluate significant differences among solubility values calculated for the tested carbohydrates in the different test ILs. Differences were considered to be significant when $p < 0.05$.

3. RESULTS AND DISCUSSION

Water has been recognize as a factor with large influence on the solubility measurement on ILs.[19] As an example, it has been demonstrated that just 1 wt % of water significantly reduced the solubility of carbohydrates such as cellulose,[12,23] but also that it improved the solubility of glucose at different extent depending on the IL used.[16] Therefore, the presence of water in ILs should be carefully controlled during the experiments.

IL water content was measured before solubility assays and the results are summarized in Table 2. The water content was below 1.1 wt % in all test ILs, except for [HMIM][Cl], which had 2.0% water.

Table 2. Water Content in the Test ILs Measured by Karl-Fisher[a]

solvent	mass % of water
[HMIM][Cl]	2.041 (0.027)
[BMIM][$MeSO_4$]	0.035 (0.001)
[EMIM][DCA]	0.755 (0.001)
[EMIM][OAc]	1.099 (0.008)
[MMIM][Me_2PO_4]	0.411 (0.001)

[a]Standard deviation (s.d.) is given in parentheses (n = 3).

3.1. Solubility of Carbohydrates in ILs. To determine the solubility of the carbohydrates considered in this study (see Table 3 for their identification and physicochemical properties[24–29]), individual mixtures of each carbohydrate and each investigated IL were stirred for 24 h at the selected temperature. Preliminary experiments confirmed that this stirring time was enough to obtain reproducible and accurate results. As previously discussed by Carneiro et al.,[18] stirring time during solubility experiments is a crucial parameter to obtain accurate measurements due to the high viscosity of ILs

Table 3. Physicochemical Properties of the Investigated Carbohydrates

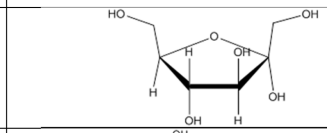
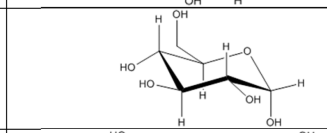
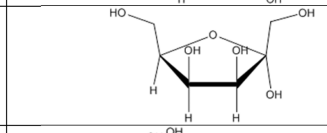
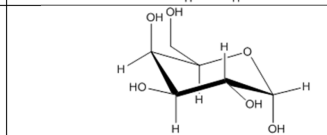
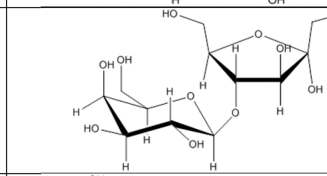
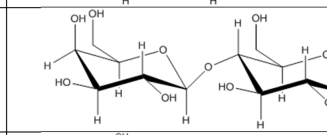
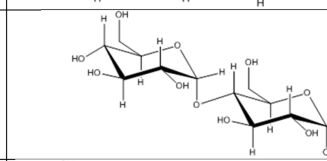
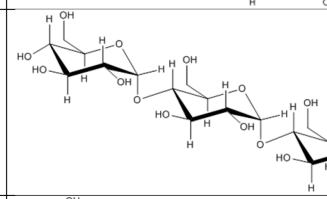
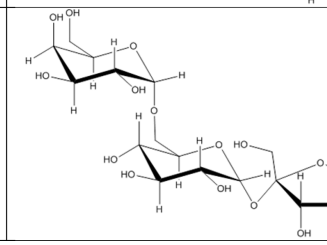
Carbohydrate	Structure	Mw (g mol^{-1})	T_{fus} (K)
Fructose [24,25]		180.16	378.1
Glucose [24,25]		180.16	423.1
Tagatose [26]		180.16	407.0
Galactose [27]		180.16	436.1
Lactulose [28]		342.30	449.0
Lactose [26]		342.30	475.8
Maltose [28]		342.30	375.0
Maltotriose [26]		504.44	132.0
Raffinose [29]		504.42	543.4

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Table 4. Solubility (%, w/w) at 299 K of the Studied Carbohydrates in the Test ILs[a]

	[HMIM][Cl]	[BMIM][$MeSO_4$]	[EMIM][DCA]	[EMIM][OAc]	[MMIM][Me_2PO4]
fructose	20.2[a] (2.4)	13.2[a] (1.9)	55.0[a] (5.5)	37.4[a] (2.5)	29.8[a,b] (3.2)
glucose	9.3[b,c] (0.2)	10.3[a,b] (0.4)	28.1[b] (0.8)	22.6[c] (0.9)	29.4[a,b] (3.8)
tagatose	29.5[d] (6.3)	8.0[a,b,c] (0.9)	38.0[d] (0.8)	27.2[b,c,d] (1.2)	31.4[a,b] (0.3)
galactose	8.1[c] (1.6)	1.1[c] (0.1)	8.5[c] (0.3)	25.7[b,c] (1.0)	39.8[c] (2.3)
lactulose	13.0[b,c] (0.9)	4.4[b,c] (0.8)	44.9[e] (2.4)	33.0[a,d,e] (2.7)	25.2[a,b] (1.9)
lactose	3.3[e] (0.4)	8.2[a,b,c] (0.1)	7.9[c] (0.2)	30.8[b,d,e] (1.1)	30.9[a,b] (2.0)
maltose	14.1[b] (1.8)	23.3[d] (1.4)	39.4[d,e] (3.9)	27.2[b,c,d] (0.4)	24.5[a] (1.5)
maltotriose	32.1[d] (6.4)	92.3[e] (12.1)	94.8[f] (13.1)	33.8[a,d] (2.3)	32.9[b,c] (2.8)
raffinose	–	–	34.4[b,d] (3.9)	196.5[f] (7.9)	188.3[d] (13.0)

[a]Standard deviation is given in parentheses (n = 3). Superscript roman letters a–f: Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level. No data is provided due to carbohydrate degradation in the symbol – of the table.

(Table 1). Noteworthy, this parameter has been omitted in most of the previously reported studies regarding carbohydrates solubility measurements, making the reproduction of their experimental conditions extremely difficult. Moreover, considering the ILs viscosities, the precipitation of the carbohydrate excess is also a key experimental parameter to obtain a representative aliquot for its subsequent analysis. In the present study, a static time of 24 h at each selected temperature was required. In the case of [HMIM][Cl], which has the highest viscosity (Table 1), centrifugation at 8000g for 5 min also helped to this step.

As indicated in the Experimental Section, the solubility of the investigated carbohydrates in ILs was determined by gas chromatography (GC) after a derivatization step. Carbohydrates dissolved in [BMIM][$MeSO_4$] were only partially derivatized,[11] so these samples were analyzed by high performance liquid chromatography with refractive index detection (HPLC-RID).

3.1.1. Solubility of Carbohydrates in ILs at 299 K. Solubility data of mono-, di-, and trisaccharides at 299 K in different ILs are shown in Table 4. Highly variable solubility values were obtained for the studied carbohydrate depending on the IL considered. The highest solubility values were found for [MMIM][Me_2PO_4] and [EMIM][DCA]. This result, in combination with the relatively low viscosity of this latter IL (Table 1), could represent an additional advantage for their potential alternative use for carbohydrates processing.

In general, the lowest solubilities were observed in [BMIM][$MeSO_4$] and [HMIM][Cl] (despite the fact that the latter had the highest water content). This fact could be justified because, among the ILs considered in this study, [BMIM][$MeSO_4$] and, specially, [HMIM][Cl] contain the cations with the longest alkyl chains, which negatively affects to the solubility of carbohydrates.[12,30] Chloride anion is a strong proton acceptor and so it plays a key role in the dissolution process. However, because of the high melting point and high viscosity of ILs containing this ion, the processing of carbohydrates with chloride-based ILs is considered to be somehow expensive and inefficient.[12]

Regarding monosaccharides, glucose was found to be highly soluble in [MMIM][Me_2PO_4] and [EMIM][DCA] (29.4 and 28.1%, respectively), followed by [EMIM][OAc] (22.6%). Results for [EMIM][DCA] agreed with those found by MacFarlane et al.,[31] who reported that ILs containing this anion dissolved glucose in concentrations above 100 g L^{-1}. As reported, the high solubility of carbohydrates in DCA-based ILs could be justified by the high capacity of this anion for hydrogen bonding with hydroxyl groups.[18] The same reasoning could be applied to [MMIM][Me_2PO_4] and [EMIM][OAc].

When the solubilities of glucose were compared with those of its C-4 epimer (galactose), some different behaviors were observed depending on the IL considered. These sugars exhibited essentially similar solubility values in [HMIM][Cl] and [EMIM][OAc] (around 8% and 24%, respectively). However, glucose was significantly more soluble than galactose in [BMIM][$MeSO_4$] and in [EMIM][DCA], but it was less soluble in [MMIM][Me_2PO_4]. This behavior has been also observed by other authors using other immidozolium-based ILs (e.g., solubility of glucose at 298.2 K of 15.5% and solubility of galactose 6.88% in [EMIM][$EtSO_4$]).[32]

The solubility of glucose was also compared with values obtained for its corresponding ketose (fructose). In general, fructose exhibited significantly higher solubility values than glucose in the evaluated ILs, except for [BMIM][$MeSO_4$] and [MMIM][Me_2PO_4], for which similar solubility values were obtained for both monosaccharides. This behavior was also observed for tagatose (ketose) and galactose (aldose) in [HMIM][Cl] and [EMIM][DCA]. Although further investigation can be recommended, our results pointed out that these ILs could be useful for the selective separation of these isomeric carbohydrates. The results reported by Rosatella et al.[16] evidenced a higher solubility of fructose as compared to glucose in four ILs ([EMIM][BF_4], [EMIM][TfO], [BMIM]-[BF_4], and [BMIM][TfO]), and a similar behavior was also observed by Carneiro et al.[32] in [EMIM][EtSO4] and by Paduszynski et al.[30] in [BMIM][DCA], which would contribute to support of this statement. Carneiro et al.[18] concluded that the higher solubility of fructose compared to that of glucose in different ILs (mainly DCA-based) was due to its different carbon skeleton, which gives fructose a more stable and energetic structure, causing its lower melting temperature (Table 3) and enthalpy, which are the key factors (specific interactions and solute melting properties) affecting solubility. This explanation could be also applied to tagatose and galactose, considering the lower melting temperature of the former. To the best of our knowledge, this is the first time that solubility data of tagatose in ILs are reported in the literature. Considering the several properties attributed to this functional monosaccharide (e.g., low-caloric, low-glycemic, noncariogenic sweetener, texturizer, stabilizer, humectant, prebiotic, etc.),[33] the potential use of ILs for the efficient fractionation of tagatose from galactose should be considered in the future.

Regarding disaccharides, both lactulose and maltose were found to be highly soluble in [EMIM][DCA], but, strikingly,

lactose showed very low solubility in this IL (7.9%), a trend similar to that observed for galactose. On the contrary, the three investigated disaccharides exhibited high solubilities on [EMIM][OAc] and [MMIM][Me_2PO_4], with values ranging from 24.5 to 33.0%. Lactose and maltose are directly found in nature. Meanwhile, lactulose is a synthetic disaccharide produced by chemical isomerization of lactose using different catalyzers, which is currently attracting high attention considering its functional properties, such as prebiotic and laxative activities or for the treatment of portal systemic encephalopathy, among others.[34] Similarly to tagatose, no previous data concerning the solubility of lactulose in ILs can be found in the literature and the data presented in this work could provide useful information for future applications in different research fields.

Individual solubility data obtained for lactulose and lactose showed a similar trend to that observed for ketose/aldose monosaccharides. Lactulose was more soluble than lactose in [HMIM][Cl] and [EMIM][DCA], while no significant differences were found regarding the solubility data of these disaccharides in [BMIM][$MeSO_4$], [MMIM][Me_2PO_4], and [EMIM][OAc] (Table 4).

The tested trisaccharides (maltotriose and raffinose) exhibited highest solubility values in the investigated ILs among the different carbohydrates considered in this study. This was especially significant for maltotriose in [EMIM][DCA] (94.8%) and raffinose in [EMIM][OAc] and [MMIM][Me_2PO_4] (196.5 and 188.3%, respectively). Moreover, significant differences were found among the solubilities calculated in the different ILs for both trisaccharides, which could be mainly attributed to their different monosaccharide composition (Table 4). It should also be noted that, when raffinose dissolved in [HMIM][Cl] and [BMIM][$MeSO_4$] was analyzed, peaks corresponding to sucrose and galactose coming from the hydrolysis of this trisaccharide were observed. Therefore, no solubility data for raffinose in these two particular ILs was provided.

In general, an increase in solubility values of carbohydrates in the tested ILs was detected as their molecular weight increased. This result is clearly observed for the solubility values of glucose, maltose, and maltotriose (carbohydrates with one, two, and three glucose units) in the different ILs, mainly in [HMIM][Cl], [BMIM][$MeSO_4$], and [EMIM][DCA] (Table 4). This behavior diverged from that observed for organic solvents, in which the solubility of carbohydrates used to decrease as the molecular weight increases (e.g., solubility in methanol).[3]

Finally, significant differences were found when solubility values of different carbohydrates in ILs with the same cation, [EMIM][OAc] and [EMIM][DCA], were compared (Table 4). This was particularly evident for galactose, lactose, maltotriose, and raffinose. This finding agreed with previous observations pointing to the interaction between the carbohydrate and the anion of an IL as predominant compared with the interaction with the cation during the dissolution process.[4] That is, the solubility of carbohydrates in ILs looks to be more dependent on the nature of the anion than on that of the IL cation.

3.1.2. Effect of Temperature in the Solubility of the Different Carbohydrates. Considering the lower solubility of carbohydrates in [HMIM][Cl] and [BMIM][$MeSO_4$], higher temperatures were assayed in order to evaluate the possible influence of this parameter on the dissolution process. Table 5 shows the solubility data obtained for the investigated carbohydrates in these two ILs at 318 K. Solubility values in [EMIM][DCA] at this temperature have also been included for comparative purposes.

Table 5. Solubility (%, w/w) at 318 K of Different Carbohydrates in Selected ILs[a]

	[HMIM][Cl]	[BMIM][$MeSO_4$]	[EMIM][DCA]
fructose	–	–	85.7[a] (4.7)
glucose	21.0[a] (6.7)	3.5[b] (0.4)	37.3[c] (0.7)
tagatose	–	–	49.8[d] (0.9)
galactose	14.6[a] (4.6)	1.0[a] (0.1)	13.4[b] (0.3)
lactulose	42.9[b] (5.9)	–	67.8[e] (5.8)
lactose	17.6[a] (3.0)	4.2[b] (0.5)	13.6[b] (0.6)
maltose	39.2[b] (2.0)	–	75.9[e] (5.2)
maltotriose	–	–	–
raffinose	–	–	167.7[f] (18.1)

[a]Standard deviation is given in parentheses (n = 3). Superscript roman letters a–f: Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level. No data is provided due to carbohydrate degradation in the symbol – of the table.

Notable differences were observed for the three compared ILs. Solubility of aldoses (except maltose in [BMIM][$MeSO_4$] and maltotriose in all ILs) increased by a factor of 2–5 in [HMIM][Cl] when the temperature was raised from 299 to 318 K, while a much more moderate increase, i.e., 1–2-fold, was observed in [EMIM][DCA]. In contrast, the solubility of glucose and lactose decreased noticeably in [BMIM][$MeSO_4$] under these new experimental conditions, whereas that of galactose remained at similar low levels despite the increase of temperature. When solubility of ketoses was evaluated in [HMIM][Cl] and [BMIM][$MeSO_4$] at 318 K, a brown color was observed (except for lactulose in [HMIM][Cl]). This browning effect became also evident when dissolving maltotriose in [HMIM][Cl] and [BMIM][$MeSO_4$], and for maltose in this latter IL, making evident the degradation of these carbohydrates during the treatment. Consequently, data concerning these experiments were not considered reliable and were not included in Table 5. Carneiro et al.[18] reported a decrease of the solubility values when this brown color appeared as the temperature increased. With the only exception of maltotriose, no degradation was observed for carbohydrates dissolved in [EMIM][DCA], so solubility data provided of these analytes at 318 K was considered reliable (Table 5).

Similarly to that observed at 299 K, raffinose was hydrolyzed at 318 K [HMIM][Cl] and [BMIM][$MeSO_4$], but no degradation was seen to occur in [EMIM][DCA], in which the increase of temperature promoted a 5-fold increase in the solubility of this carbohydrate.

At 348 K, reliable solubility data was only obtained for aldoses (lactose and galactose) in [EMIM][DCA]; i.e., browning reactions occurred in the other two ILs assayed. As somehow expected, for these two sugars, the solubility was found to increase gradually with the temperature, reaching values of 17.8% of lactose (w:w) and 20.0% of galactose (w:w) in this IL at the higher assayed temperature. Even under such drastic temperature conditions, the obtained solubility values were lower than those obtained in [EMIM][OAc] and [MMIM][Me_2PO_4] at 299 K for these two sugars.

3.1.3. Carbohydrate Degradation. To evaluate the degradation of carbohydrates in [HMIM][Cl] and [BMIM]-

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[$MeSO_4$], a control assay of each carbohydrate dissolved in sodium phosphate buffer (pH 7.02, 1 mM) was subjected to experimental conditions similar to those used in the solubility experiments (318 K, 24 h under stirring followed by 24 h of static time). The absorbance of the mixtures was recorded at 420 nm to determine the nonenzymatic degradation, i.e., browning of the solution. When sodium phosphate buffer was used as solvent, a sharp increase in the absorbance was observed for tagatose, those recorded for lactulose and fructose being also significant but less pronounced (Figure 2).

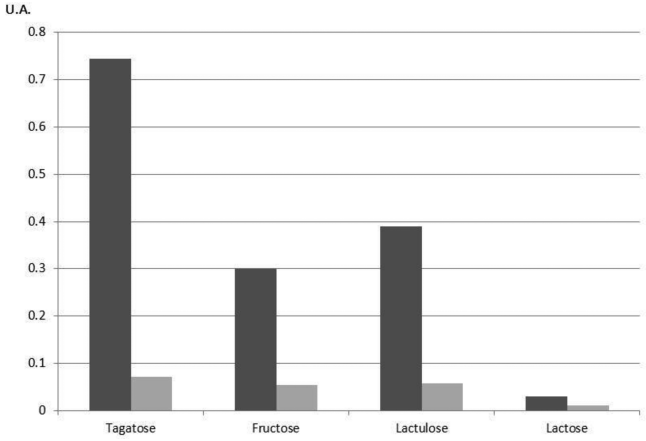

Figure 2. Absorbance (420 nm, UA) of selected carbohydrates dissolved in [BMIM][$MeSO_4$] (dilution 1:6 v/v in water) (black) and in sodium phosphate buffer (pH = 7.02, 1 mM) at 318 K (gray).

Nevertheless, it should be highlighted that, although evident, the absolute increments in the absorbance values were relatively small and in all instances values below 0.071 units of absorbance (UA) were obtained. Absorbance measurements under these experimental conditions kept close to zero for galactose, glucose, lactose, maltose, matotriose, and raffinose (data not shown).

On the contrary, a 1:6 (v/v) water dilution was mandatory before measuring the absorbance of carbohydrates dissolved in [BMIM][$MeSO_4$] at 318 K (Figure 2). As shown in Figure 2, absorbance values were higher for ketoses (between 0.30 and 0.75 units) than for aldoses (between 0.059 and 0.067 units). A similar behavior was observed in [HMIM][Cl], where tagatose also showed the most evident browning and the highest absorbance (1.30 UA) compared to the phosphate buffer solution. In this IL, fructose showed an absorbance value of 0.03 UA, nearly similar to that of the control solution (0.06 UA), whereas no browning effect was observed on solutions containing lactulose and aldoses.

Several studies have demonstrated that some ILs catalyze the degradation of carbohydrates in the presence of other cosolvents such as DMSO, methanol, supercritical dioxide, metal halides, etc. even at room temperature.[35] It has also been demonstrated that fructose dissolved in acidic ILs is more prone than glucose to be converted into dehydration products, such as 5-hydroxymethylfurfural (HMF) without any additional catalyst. This process has been reported to occur mainly at high temperatures (353−373 K), but also at lower temperatures a significant conversion takes place.[36,37] This finding could agree with our observations for ketoses, in particular for tagatose, dissolved in [BMIM][$MeSO_4$] and [HMIM][Cl] at 318 K, although browning did not became apparent in [EMIM][DCA]. To further confirm this possibility, carbohydrate mixtures in the acidic ILs, [BMIM][$MeSO_4$] and [HMIM][Cl], at 318 K were tested for HMF. The results of this part of the study are summarized in Table 6. The highest HMF

Table 6. HMF (%, w/w) Produced during Solubility Assays Carried out at 318 K[a]

	[HMIM][Cl]	[BMIM][$MeSO_4$]
fructose	9.98[a] (0.98)	3.32[b] (0.69)
tagatose	4.92[c] (0.20)	4.21[c] (1.43)
lactulose	–	3.19[b] (0.46)
maltose	–	0.004[d] (0.015)
maltotriose	0.021[d] (0.002)	0.02[d] (0.02)
raffinose	0.07[d] (0.01)	0.42[d] (0.01)

[a]Superscript roman letters a−d: Entries followed by the same letter showed no statistically significant differences for their mean values at the 95.0% confidence level. Standard deviation is given in parentheses (n = 3). No HMF production in the symbol − of the table.

concentration was produced in the solution containing fructose in [HMIM][Cl], in which a ratio as large as 10% (w/w) was found. Tagatose showed a significant degradation in both investigated ILs with HMF:carbohydrate ratios of 4.9% w/w in [HMIM][Cl] and 4.2% w/w in [BMIM][$MeSO_4$], whereas the HMF:carbohydrate ratio of lactulose was 3.2% in the latter IL. These findings agreed with those of Moreau et al.,[36] who demonstrated that the acid-catalyzed dehydration of fructose and sucrose into HMF occurred easily in the presence of [HMIM][Cl] which acted as both solvent and catalyst. On the basis of these results, a number of imidazolium-based ionic liquids, alone or in combination with other catalysts, have been used for the dehydration of fructose, glucose, and even polysaccharides, as recently reviewed by Teong et al.[38] Our results contribute to support the idea that a similar approach could be applied in the case of tagatose and lactulose.

HMF is one of the top building block chemicals obtained from biomass and can be used to synthesize a broad range of value added compounds.[35] Therefore, [HMIM][Cl] and [BMIM][$MeSO_4$] and the evaluated ketoses might be used for the production of this compound at moderate temperatures, so reducing energy consumption as compared to other previously reported methods.

4. CONCLUSION

This study provides new solubility data for different mono-, di-, and trisaccharides in selected ILs. In general, ketoses were found to be more soluble than their corresponding aldoses in the evaluated ILs, although some exceptions have been observed depending on the considered IL. [BMIM][$MeSO_4$] and [HMIM][Cl] acted both as solvent and as catalyst for dehydration of specific carbohydrates into HMF at 318 K. This fact was especially evident for fructose and tagatose, while raffinose underwent a hydrolysis reaction at 299 and 318 K in these two ILs. Although further studies should be addressed, these findings point out the potential of ILs as an interesting alternative solvent for the efficient fractionation of LMWC which opens new applications in applied carbohydrate chemistry.

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Notes

The authors declare no competing financial interest.

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3.3. Solubility of sugar alcohols in ionic liquids. Selective fractionation from other low molecular weight carbohydrates.

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Enviado para su publicación

Solubility of sugar alcohols in ionic liquids. Selective fractionation from other low molecular weight carbohydrates.

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ABSTRACT

Sugar alcohols such as xylitol, sorbitol and inositols, are added-value carbohydrates, used either by their bioactive or technological properties. Extraction of these compounds from natural sources is interesting, however, the fractionation of unavoidable coextracted low molecular weight carbohydrates (LMWC) is becoming a mandatory task.

In this article, solubility of sugar alcohols and inositols in ionic liquids (ILs) is investigated. The fractionation of these added-value carbohydrates from common sugars involving the use of ILs is also studied.

Results showed a broad range solubility values of inositols and sugar alcohols (1.7-84.7%) in the studied ILs (i.e., 1-hexyl-3-methylimidazolium chloride ([HMIM][Cl]), 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]), 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) and 1,3-Dimethylimidazolium dimethylphosphate ([MMIM][Me_2PO_4])). Highest solubility values were observed in [EMIM][OAc] and [MMIM][Me_2PO_4]. Inositols and sugar alcohols were successfully fractionated from mono- and disaccharides by precipitation after IL treatment.

These findings indicated a potential approach in forthcoming optimization of the choice of ILs for the efficient separation of LMWC having different properties for their further/possible/eventual use as ingredients in commercial preparations.

KEYWORDS

Ionic liquids, gas chromatography, inositols, polyalcohols, fractionation, carbohydrates.

1. INTRODUCTION

Polyols, polyhydroxyalcohols or sugar alcohols are those compounds obtained when the aldo- or keto- group of a sugar is reduced to the corresponding hydroxyl group (Bieleski 1982). These carbohydrates occur naturally in plants and can be divided into acyclic or linear polyols and cyclic polyols (cyclitols) such as inositols.

Acyclic sugar alcohols are carbohydrates commonly used as technological ingredients; for instance, as sweeteners, due to their non-cariogenic properties and their lower contribution to raise blood glucose compare to sucrose (Plouvier 1963, Brimacombe and Webber 1972). The most common acyclic sugar alcohols are mannitol, sorbitol and xylitol.

Inositols are considered bioactive carbohydrates used in treatment of polycystic ovary syndrome and several affections related to insulin resistance (Nestler et al. 1999, Kim et al. 2007).

These added-value carbohydrates coexist in natural products with other sugars (mainly, mono- and disaccharides) which could interfere in their bioactive or technological properties. Several techniques, such as chromatographic (Saska and Diack 1996, Hernández et al. 2009), membrane-based (Goulas et al. 2003) or microbiological (Ruiz-Aceituno et al. 2013), have been proposed for the separation of carbohydrates, however, fractionation is not straighforward considering the similarities of carbohydrate structures. Therefore, the search for fractionation techniques which allow to obtain high selectivity and efficiency, using small solvent volumnes, environmentally friendly, is of high interest for both researchers and industries (Moreno and Sanz 2014).

The use of organic solvents for carbohydrate fractionation is widespread; differences in their solubility result in the selective precipitation of specific carbohydrates, which can be easily separated from the extraction mixture (Montañés et al. 2008, Montañés et al. 2009). However, these methods usually require high volumes of organic solvents and new alternatives are proposed such as the use of ionic liquids (Ils) (Carrero-Carralero et al. 2015).

ILs are composed of organic cations, and organic or inorganic anions. They have different and tunable physicochemical properties, noting a low volatility and viscosity

and a high thermal stability (Ruiz-Aceituno et al. 2013). These properties make them to be more demanded in several applications, and are considered more environmentally friendly than volatile organic solvents.

ILs have shown to dissolve cellulose and other polysaccharides more efficiently than organic solvents (Zhu et al. 2006, El Seoud et al. 2007, Abe et al. 2010, Zakrzewska et al. 2010), and also allowed the depolymerization of these compounds to obtain monosaccharides (Hyvärinen et al. 2010). However, only few studies reporting solubility of low molecular weight carbohydrates (LMWC) in ionic liquids can be found in the literature (Liu et al. 2005, El Seoud, et al. 2007, Rosatella et al. 2009, Conceiçao et al. 2012, Carrero-Carralero et al. 2014). Regarding sugar alcohols, Conceiçao *et al* (2012) studied the solubility of xylitol and mannitol in different ILs constituted by imidazolium, pyridinium and phosphonium cations. However, to the best of our knowledge, no more data regarding these added value carbohydrates can be found in the literature.

The use of ILs for the selective fractionation of carbohydrates has been mainly applied to polysaccharides. Lan *et al.* (2011) used ILs to dissolve lignocellulose, and then cellulose could be fractionated from lignin in the treated sample. Moreover, releasing of monosaccharides from lignocellulosic wood material through IL was investigated (Hyvärinen, et al. 2010). Regarding LMWC separation, Al Nashef *et al.* (2011) proposed a method for the separation of fructose and glucose in binary mixtures based on their different solubility in 1,3-dimethylimidazolium dimethylphosphate and 1-ethyl-3-methylamidazolium ethylsulfate at room temperature. More recently, separation of bioactive ketoses from their aldoses (i.e., fructose/glucose, tagatose/galactose, lactulose/lactose) using ILs were also studied (Carrero-Carralero, et al. 2015).

In this work, solubility measurements were conducted for the first time for several polyhydroxyalcohols (acyclic sugar alcohols and inositols) dissolved in imidazolium-based ionic liquids. Additionally, this information was used to design a new fractionation procedure of binary mixtures of these polyols and other LMWC using ILs.

2. MATERIALS AND METHODS

2.1.Chemicals and reagents

All chemicals and reagents used in this work were analytical or research grade. *myo*-Inositol, *chiro*-inositol, xylitol, mannitol, phenyl–β-D-glucoside, 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]), 1-hexyl-3-methylimidazolium chloride ([HMIM][Cl]), 1-ethyl-3-methylimidazolium acetate ([EMIM][OAc]) and trimethylsilylimidazole (TMSI), were obtained from Sigma Chemical Co. (St. Louis, USA). 1,3-Dimethylimidazolium dimethyl phosphate ([MMIM][Me_2PO_4]) was from Alfa Aesar (Massachusetts, USA). Pinitol (3-O-methyl-D-*chiro*-inositol), quebrachitol (2-O-methyl-D-*chiro*-inositol) and galactinol (1-O-α-D-Galactopyranosyl-L-*myo*-inositol) were from Carbosynth (Berkshire, UK). Heptane was purchased from Merck (Darmstadt, Germany) and *n*-tetracosane from PolyScience Corporation (Illinois, USA).

2.2. Water content determination

Before solubility assays, water content of ILs was measured using a C20 Compact Karl Fischer Coulometer (Mettler Toledo; Ohio, US). HYDRANAL®-Coulomat AG from Sigma Chemical Co. was the reagent used for volumetric titration. Values ranged between 0.41of [MMIM][Me_2PO_4] and 2.04 % of [HMIM][Cl].

2.3.Dissolution of carbohydrates in ILs

2.3.1. Solubility procedure

Each carbohydrate was individually dissolved in the corresponding IL for solubility studies, using a slight excess. Samples were stirred during 24 hours at 1350 rpm and left to stand for another 24 hours more. Solubility was evaluated at two temperatures, 25 and 45 ºC, using a Thermomixer (Eppendorf, Hamburg, Germany). Then, an aliquot of the resulting mixture was collected from the upper layer, derivatized and analyzed by gas chromatography (GC). Solubility assays were made in triplicate.

2.3.2. Dissolution of binary mixtures of low molecular weight carbohydrates in ILs

Four binary mixtures of different low molecular weight carbohydrates were studied: mixture 1 (fructose : *myo*-inositol), 2 (pinitol : maltose), 3 (mannitol : glucose) and 4 (mannitol : maltose).

These mixtures (50%, w/w, of each carbohydrate) were dissolved in 100 mg of the specified IL with slight excess (a 10% above the corresponding limit of solubility).

Samples were stirred at 1350 rpm during 24 h at 25 ºC and left to stand for another 24 h at this temperature, using a Thermomixer (Eppendorf, Germany). Then, an aliquot of the upper liquid layer of the solution mixture was extracted and analyzed by GC, as indicated in section 2.4 and 2.5.

2.4. General procedure for the silylation reaction

Silylation of the tested carbohydrates dissolved in the corresponding IL was carried out as described elsewhere (Ruiz-Aceituno et al. 2013), directly in the ILs and avoiding the use of pyridine. In brief, 100 µL of TMSI was added to each upper layer collected and kept under ultrasonic agitation during 1 hour. Afterwards, 200 µL of ultrapure water were added to finish the reaction. The derivatized carbohydrates were then recovered by liquid-liquid extraction (LLE) with 100 µL of heptane that contained *n*-tetracosane. Two more successive extractions with heptane were performed to ensure quantitative recovery of the derivatized analytes. Extracts were jointly collected and directly analysed by GC.

2.5. GC analysis

Analysis of the derivatized carbohydrates was carried out using an HP 7890A gas chromatograph equipped with a flame ionization detector (FID) from Agilent Technologies (Palo Alto, CA, USA) using nitrogen as carrier gas (flow rate, 0.7 mL min^{-1}). A fused silica capillary column coated with 100% dimethylpolysiloxane (30 m x 0.25 mm i.d. x 0.25 µm *df*) was used (Zebron, Phenomenex, CA, USA). The oven temperature was programmed as follows: 180 ºC to 250 ºC (10 ºC min^{-1}) and held for 3 min, and then to 300 ºC at 15 ºC min^{-1} and held for 20 min. The injection port was

heated to 300 ºC and injections were made in the split mode, with a split ratio 1:20. FID temperature was 320 ºC. Chromatographic peaks were measured using a HPChem acquisition system (Agilent Technologies).

Quantitative analyses were performed in triplicate using the internal standard procedure. For this purpose, calibration curves of the derivatised carbohydrate in the 0.1 – 1 mg range were constructed. Response factors of these compounds relative to two internal standards (*n*-tetracosane and phenyl-β-D-glucoside) were determined for quantitative analysis.

2.6. Statistical analysis

Statistical analysis was performed using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Analysis of variance (ANOVA) was used to evaluate differences in solubility values.

3. RESULTS AND DISCUSSION

3.1. Solubility of sugar alcohols in ILs

Solubility data of sugar alcohols at 25 ºC in the different ILs are shown in Table 1. In general, the highest solubility values were found for [EMIM][OAc], followed by [MMIM][Me_2PO_4]. Previous studies have described acetate as an efficient anion to dissolve carbohydrates (Zhao et al. 2008, Carneiro et al. 2013). In contrast, low values were obtained in [HMIM][Cl] and [EMIM][DCA]. It is worth noting the differences found in this last IL between these results and those previously found for mono- and disaccharides (Carrero-Carralero, Ruiz-Aceituno et al. 2014). Whereas sugar alcohols were slightly soluble in [EMIM][DCA], high solubility values were found for monosaccharides such as fructose and glucose.

Moreover, high differences were also found in solubility values of the sugar alcohols in ILs with the same cation ([EMIM][OAc] and [EMIM][DCA]). Previous studies have pointed out that the solubility of carbohydrates in ILs is more dependent on the nature of the anion than on that of the IL cation (Liu, et al. 2005, Carrero-Carralero, et al. 2014).

Regarding inositols, *myo*-inositol showed lower solubility than *chiro*-inositol in all the ILs under study. Pinitol and quebrachitol, both derived from *chiro*-inositol, showed a different behavior depending on the IL. These methyl-inositols were 3.7 and 2.7 times less soluble than *chiro*-inositol in [HMIM][Cl] and 3.5 and 2.4 times more soluble than the inositol in [EMIM[[OAc]. Solubility values of *chiro*-inositol were in between those found for both methyl-inositols in [EMIM][DCA] and [MMIM][Me_2PO_4].

Galactinol (a glycosyl-*myo*-inositol) showed higher solubility values than inositols and methyl-inositols in all the studied ILs, except for [MMIM][Me_2PO_4], for which no significant differences with *chiro*-inositol and pinitol were detected. Carrero-Carralero et al. (2014) also observed an increase in solubility values of carbohydrates dissolved in these ILs with the increase of their molecular weight (e.g. disaccharides were more soluble than monosaccharides).

Considering acyclic sugar alcohols, xylitol was 5.3 times more soluble than mannitol in [EMIM][DCA]. This result agreed with those found by Conceicao *et al*. (2012) who found that xylitol was more soluble than mannitol in [BMIM][SCN], [BMIM][HSO_4], [EMIM][$MeOEtOEtSO_4$], [BMIM][$C(CN)_3$] and [(i-Bu)$_3$MeP][TsO]). However, in our study, mannitol was more soluble than xylitol in [HMIM][Cl] and [EMIM][OAc], whereas no statistical significant differences where observed in solubility values of these compounds in [MMIM][Me_2PO_4].

The effect of the temperature in the solubility of these sugar alcohols was also evaluated in [HMIM][Cl] and [EMIM][DCA] (Table 2), for which the target carbohydrates presented low values at 25ºC. As expected, solubility values at 45 ºC were found to be higher than those at 25 ºC. In the case of galactinol, it was hydrolyzed when mixed with [HMIM][Cl] at this temperature. This fact was confirmed by the presence of their monomers (*myo*-inositol and galactose) in the resulting GC profile. Hydrolysis of other carbohydrates in this IL was also observed in previous studies, such as raffinose and maltotriose (Carrero-Carralero et al. 2014).

3.2. Fractionation of sugar alcohols from mono- and disaccharides in binary mixtures

Binary mixtures of mannitol and *myo*-inositol, as representative of acyclic sugar alcohols and inositols, respectively, and mono- and disaccharides were prepared to

evaluate the efficiency of IL for their selective fractionation based on precipitation of one of the sugars.

The fact that *myo*-inositol has a low solubility is considered in section 3.1., and is used in this part of the manuscript to aboard the separation of LMWC mixtures using ILs.

[EMIM][OAc], [MMIM][Me_2PO_4] and [EMIM][DCA] were found to be the best candidates for testing fractionation of binary carbohydrates mixtures. Sugars studied presented high solubility values in [EMIM][OAc] and [MMIM][Me_2PO_4], and [EMIM][DCA] presented high differences in individual solubilities with other carbohydrates having similar chemical structure, according to results from Carrero-Carralero et al (2014).

Considering solubility data obtained, 25 ºC were used for the fractionation assays, due to the high differences observed. Comparing the solubility data obtained in this study for sugar alcohols and inositols, with solubility values of sugars (mono- to trisaccharides) in [EMIM][DCA] and [HMIM][Cl] published previously by our research group (Carrero-Carralero et al. 2014), a lower solubility of inositols is noticed.

As differences in solubility in ILs of different compounds have been observed, equimolar binary mixtures were submitted to a fractionation study. Figure 1 shows the content (as %) of the carbohydrates solubilized in the specific IL for the equimolar binary mixtures after treatment with each IL. The following mixtures were studied: i) monosaccharide:inositol, ii) disaccharide:inositol, iii) monosaccharide:sugar alcohol, iv) disaccharide:sugar alcohol. These carbohydrates were chosen not only due to their different solubility values but also because they represent different chemical structures.

In the mixture of a monosaccharide and a inositol (*myo*-inositol:fructose), namely mixture 1, it can be observed a noticeable enrichment of fructose in [EMIM][DCA], which was nearly totally dissolved (97.1%) whereas only 2.2% of *myo*-inositol remained in the treated mixture. Binary mixture number 2 was submitted to treatment using the same IL, achieving a solubilization of 91.6% of the disaccharide maltose, although pinitol was partially soluble (40%).

Mannitol was totally dissolved in [EMIM][OAc] in mixture 3, whereas only 32.8% of glucose remained in the treated sample/mixture. [MMIM][Me2PO4] was able to solubilize almost all part of mannitol (95.4%) and 63.7% of maltose.

The analysis of fractionation using ILs demonstrates the different behaviour between inositols and sugar alcohols: selective precipitation of inositols occurred in ILs

in these carbohydrates mixtures, while the latter compounds may be solubilized in the ILs tested for these mixtures. Due to this different observed behaviour, precipitate was considered for recovery of inositols, and solubility for recovery of sugar-alcohols (Figure 2).

These solvents could be a choice for separation of polyols from common sugars coextracted or present in a matrix, nevertheless further investigations are required.

4. CONCLUSIONS

This works provides new data about solubility of carbohydrates in ILs (specifically, inositols), contributing to expand the knowledge on this field.

Results of this study suggest that some ILs can be utilized in separation of carbohydrates dissolved in ILs (such as [EMIM][DCA], whose use was useful in fractionating binary mixtures of carbohydrates when solubilized in it).

Those achievements are starting findings for the development of new strategies for further use in fractionation. As ILs have tunable properties, an advantage from them could be taken to develop carbohydrate processing applications.

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FIGURE CAPTIONS

Figure 1. Content (%) of sugars and inositols (binary mixtures) after treatment at 25 ºC in selected ILs. SD are shown as error bars (n=3).

Figure 2. Chemical structures of tested polyalcohols.

TABLES

Table 1. Solubility values (%, w/w) of polyols at 25 ºC. Experimentally determined SD are shown in parenthesis (n=3).

	[HMIM][CL]	**[EMIM][DCA]**	**[EMIM][OAC]**	**[MMIM][Me_2PO_4]**
***myo*-Inositol**	7.3 (1.1) [a,b*]	1.7 (0.1) [a]	17.1 (1.6) [a]	41.9 (4.9) [a]
***chiro*-Inositol**	11.4 (0.2) [c,d]	10.8 (0.1) [b]	19.4 (0.9) [b]	56.6 (4.3) [b]
Pinitol	3.1 (0.1) [e]	11.8 (1.0) [c]	67.6 (6.0) [c]	63.9 (3.9) [c]
Quebrachitol	4.2 (0.5) [e,b]	9.8 (0.3) [d]	46.4 (3.7) [d]	18.1 (0.7) [d]
Xylitol	8.5 (0.9) [a,c]	21.6 (0.3) [e]	58.6 (0.2) [e]	48.2 (0.2) [e]
Mannitol	11.9 (1.0) [d]	4.1 (0.3) [f]	84.7 (0.3) [f]	49.4 (1.0) [e]
Galactinol	57.1 (4.3) [f]	18.2 (0.7) [g]	82.9 (3.2) [g]	59.3 (4.5) [b,c]

*Different letters indicate significant differences.

Table 2. Solubility values (%, w/w) of polyols at 45 ºC in selected ILs. Experimentally determined SD are shown in parenthesis (n=3).

	[HMIM][CL]	**[EMIM][DCA]**
***myo*-Inositol**	24.9 (1.4) [a*]	2.9 (0.1) [a]
***chiro*-Inositol**	21.9 (2.3) [a]	14.5 (2.2) [b]
Pinitol	29.3 (2.0) [b]	19.2 (0.8) [c]
Quebrachitol	25.4 (0.5) [a,b]	15.5 (0.8) [b]
Xylitol	61.4 (5.6) [c]	38.4 (3.7) [d]
Mannitol	37.4 (0.7) [d]	5.9 (0.1) [e]
Galactinol	**	28.5 (0.6) [f]

*Different letters indicate significant differences.

**hydrolyzed sample

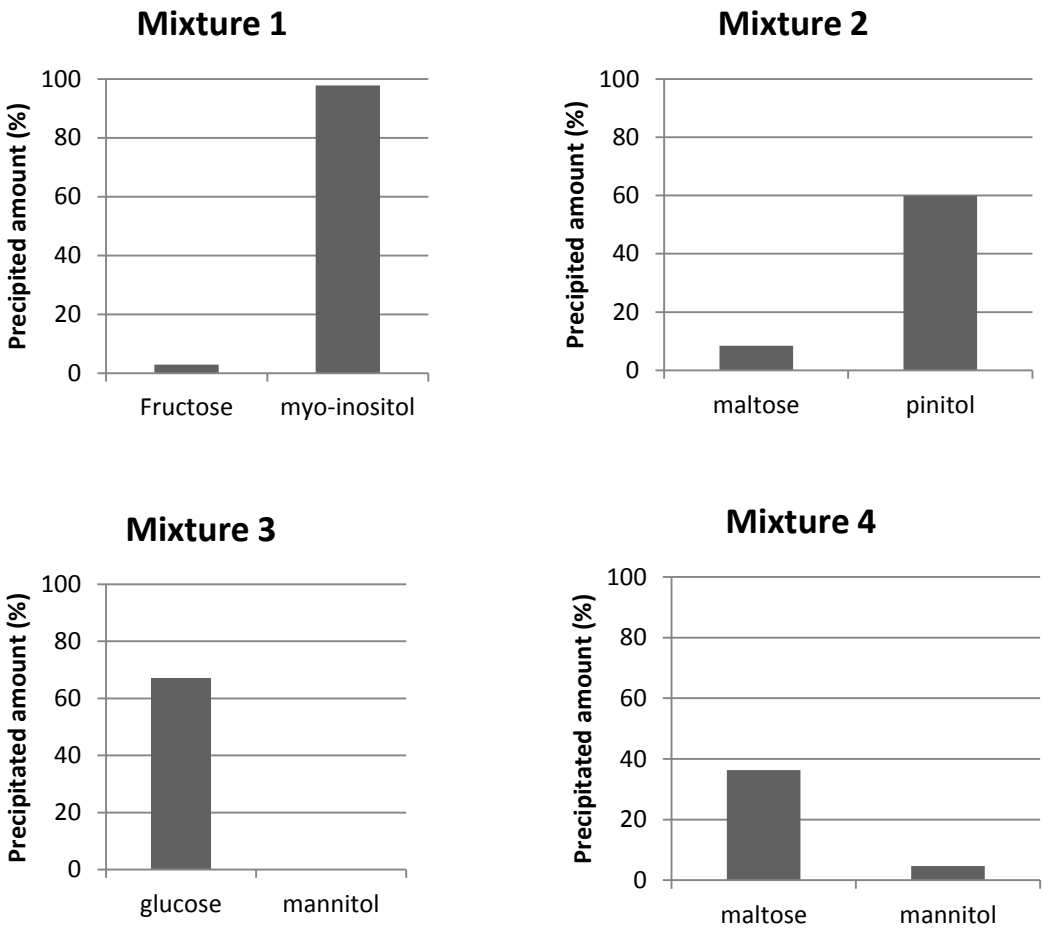

Mixture 1
Precipited amount (%)
100
80
60
40
20
0
Fructose
myo-inositol
Mixture 2
Precipited amount (%)
100
80
60
40
20
0
maltose
pinitol
Mixture 3
Precipitated amount (%)
100
80
60
40
20
0
glucose
mannitol
Mixture 4
Precipitated amount (%)
100
80
60
40
20
0
maltose
mannitol

Figure 1.

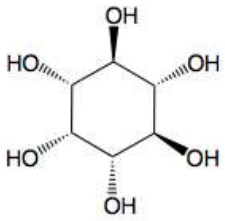
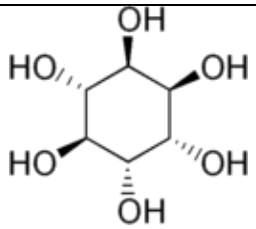
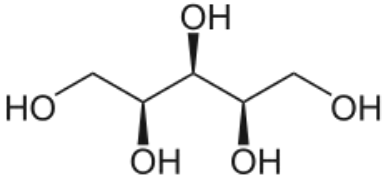
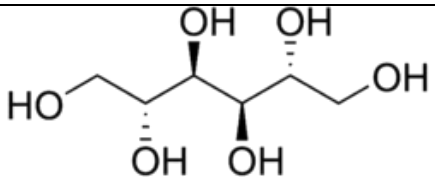
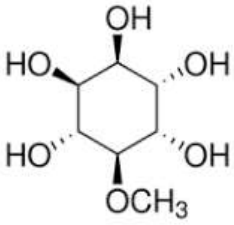
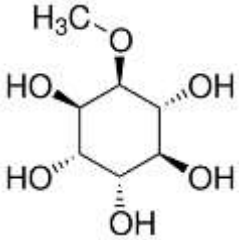
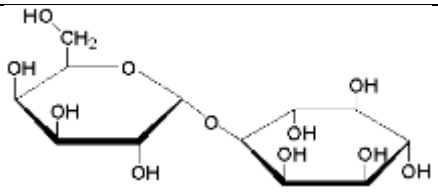
Carbohydrate	Structure	Mw (g mol^{-1})	Melting point (^{o}C)
myo-inositol		180.16	225
D-*chiro*-inositol		180.16	230
Xylitol		152.15	94
Mannitol		182.17	168
Pinitol		194.18	179-185
Quebrachitol		194.18	190-198
Galactinol		342.29	221

Figure 2.

3.4. Use of room temperature ionic liquids for the selective fractionation of bioactive ketoses from aldoses

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Use of room temperature ionic liquids for the selective fractionation of bioactive ketoses from aldoses

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ABSTRACT

This work deals with the effective fractionation of bioactive ketoses, i.e. lactulose and tagatose, from their corresponding aldoses, lactose and galactose, in equimolar binary mixtures driven by room temperature ionic liquids, i.e. 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]) and 1-butyl-3-methylimidazolium methyl sulfate ([BMIM][$MeSO_4$]), respectively. Under assayed conditions, tagatose was found to be 6-fold more soluble on [BMIM][$MeSO_4$] than galactose; meanwhile lactulose was 3 times more soluble than lactose on [EMIM][DCA]. As an application example in a more complex sample, a lactose isomerization mixture containing in addition lactulose and monosaccharides was enriched in this ketose by using [EMIM][DCA]. Carbohydrates were then successfully recovered from the ionic liquid following an activated charcoal-based treatment. Overall, lactulose content was enriched from a 24% in the initial isomerization reaction mixture to a 62% in the purified sample. These experimental results demonstrated the potential of ionic liquids as green alternative solvents for the selective fractionation of bioactive ketoses from their corresponding aldoses in food and beverage production.

1. Introduction

Fractionation of food carbohydrates is considered a challenging task due to the complexity of the mixtures and the structural similarity among them. Most of the available procedures are suitable for the fractionation of carbohydrate mixtures with different degree of polymerization [1]. However, the fractionation of carbohydrates having the same molecular weight but different monomeric composition, glycosidic linkages and/or carbonyl group position (e.g., aldoses and ketoses) is particularly difficult.

Ketoses, such as tagatose or lactulose, are considered bioactive carbohydrates with potential pharmaceutical and/or food applications due to their functional properties, which include prebiotic activity among others [2,3]. Both carbohydrates can be obtained by alkaline isomerization or by enzymatic treatment from their corresponding non bioactive aldoses, i.e. galactose or lactose, respectively. However, subsequent isolation of these carbohydrates from the synthesis mixtures remains as a difficult task. Montañés et al. [4] studied the individual solubility of three aldoses (glucose, galactose and lactose) and their respective ketoses (fructose, tagatose and lactulose) in different alcohols (methanol, ethanol, 1-propanol and 2-propanol) at several temperatures (295, 303 and 313 K). In general, ketoses were found to be more soluble than aldoses in these solvents. These authors also applied thermodynamic models to predict the solubility of sugars to further select the best solvent to fractionate these ketoses from mixtures with other carbohydrates. Despite the usefulness of these methods, they usually require large volumes of organic solvents, which are in sharp contrast to the increasing demand for more cost-effective and green analytical methodologies involving small solvent volumes.

During the last years, environmental friendly techniques based on supercritical fluid (SFE) and pressurized liquid (PLE) extraction have been evaluated for the selective fractionation of food carbohydrates. As an example, Montañes et al. [5,6] efficiently separated tagatose or lactulose from binary mixtures with different aldoses using supercritical carbon dioxide with different co-solvents (ethanol/water mixtures, isopropanol, methanol, etc.) to increase the carbohydrate solubility. Under the experimental conditions proposed, purities above 90% of ketoses and recoveries higher than 75% were obtained. PLE has also been employed with successful results for the fractionation of lactulose from lactose with a purity of 97% and a yield of 64% [7].

Room temperature ionic liquids (RTILs or simply ILs) are solvents constituted by organic cations (imidazolium, piridinium, pirrolidinium, phosphonium, etc) and different organic and inorganic

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anions (acetate, trifluoroacetate, tetrafluoroborate, bromide, etc). These solvents show melting points below 373 K, are considered environmentally friendly, and have many extra advantageous features, including low volatility and viscosity, tuned selectivity, capacity to dissolve compounds of different nature and recycling feasibility [8]. In consequence, ILs could be considered a good and safe alternative to the use of traditional organic volatile solvents in carbohydrate chemistry [9]. However, the solubility of carbohydrates of low molecular weight in different ILs has only been evaluated in few studies [10–14]. Al-Nashef et al. [15] patented a method to separate fructose from glucose in binary mixtures based on their different solubility in 1,3-dimethylimidazolium dimethylphosphate and 1-ethyl-3-methylamidazolium ethylsulfate at room temperature. Recently, the individual solubilities of lactulose, lactose, tagatose and galactose, among others, in different ILs (i.e., 1-ethyl-3-methylimidazolium dicyanamide, 1-hexyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium methyl sulfate) have been determined [16]. In general, ketoses were found to be more soluble in ILs than aldoses, a finding that pointed out the potential of ILs as alternative solvents for the efficient fractionation of low molecular weight carbohydrates. The main objective of this work is to evaluate the feasibility of three ILs, 1-hexyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium methyl sulfate and 1-ethyl-3-methylimidazolium dicyanamide, for the selective separation of ketoses with potential pharmaceutical and/or food applications such as lactulose, fructose and tagatose from their corresponding aldoses (i.e., lactose, glucose and galactose) in binary mixtures. The proposed methodology has been applied for the fractionation of lactulose from lactose isomerization reaction mixtures and the final recovery of this ketose from IL was also evaluated.

2. Materials and methods

2.1. Chemicals and reagents

Analytical standards of fructose, glucose, galactose, tagatose, lactose, lactulose, phenyl-β-D-glucoside and activated charcoal (Darco G60, 100 mesh) were obtained from Sigma–Aldrich (St. Louis, USA), and tetracosane from Polyscience Corp (Illinois, USA). The three assayed ionic liquids, [HMIM][Cl], [BMIM][$MeSO_4$], [EMIM][DCA], dichloromethane and trimethylsilylimidazole (TMSI) were also purchased from Sigma–Aldrich. *n*-Heptane was from Merck (Darmstadt, Germany), acetone from Carlo Erba Reagents (Val de Reuil, France), and ethyl acetate, absolute ethanol, methanol and isopropanol extra pure from Scharlab (Sentmenat, Spain).

2.2. Dissolution of ketose:aldose mixtures in the test ILs

For solubility studies, binary mixtures of fructose:glucose, tagatose:galactose and lactulose:lactose (50%, w/w, of each carbohydrate) were dissolved in 100 mg of the test IL with slight excess (a 10% above the corresponding limit of solubility). Samples were stirred at 12,100 *g* using a Thermomixer (Eppendorf, Germany) during 24 h at 299 K and left to stand for another 24 h at this temperature. Then, an aliquot of the solution mixture was extracted from the upper liquid layer and analyzed by gas chromatography with flame ionization detector (GC-FID) and/or high performance liquid chromatography with refractive index detector (LC-RID) as indicated in Section 2.5.

2.3. Synthesis of lactulose and subsequent fractionation with ILs

Isomerization of lactose was carried out following the method of Montilla et al. [17]. In brief, 2 mL of a 250 mg/mL solution of lactose were added to 8 mL of potassium phosphate buffer 0.05 M, pH 6.6. Pulverized egg shell was added to this solution (final concentration, 30 mg/mL) to act as catalyst for lactose isomerization. The mixture was heated at 398 K in a bath of glycerol under continuous stirring and reflux for 150 min. Reaction was stopped by immersion in an ice bath. Finally, egg shell was removed by filtration through a 0.4 μm paper filter (Millipore) and the sample was freeze-dried.

[EMIM][DCA] at 299 K was used for the fractionation of lactulose from the isomerization mixture. For this, 600 mg of [EMIM][DCA] was mixed with 320 mg of the freeze-dried isomerization mixture following the method described in Section 2.2 for the dissolution of binary mixtures of ketoses and aldoses. Aliquots of supernatant were analyzed by GC-FID according to Section 2.5.

2.4. Extraction of lactulose from IL

Different methods were evaluated and optimized for the extraction of lactulose from IL.

2.4.1. Effect of cooling

Binary mixtures of lactose:lactulose dissolved in [EMIM][DCA] were kept at temperatures of 277, 253 and 193 K, respectively, up to one week. Aliquots of the corresponding supernatants were taken at different times and subjected to analysis for the evaluation of the precipitation of carbohydrates.

2.4.2. Solvent treatment

Miscibility of [EMIM][DCA] on ethyl acetate, ethanol, isopropanol, and hexane was firstly evaluated.

Binary mixtures of lactose:lactulose dissolved in [EMIM][DCA] were vigorously stirred at 298 K for 15 min with the immiscible solvents, i.e. either ethyl acetate or hexane in a solvent:IL ratio of 10:1 (w/w), and then left to stand during 3 min. Thereafter, aliquots of 100 μL of the organic layer were taken for further analyses.

The antisolvent method was also evaluated following the method described by Hassan et al. [11]. Briefly, solubility of binary mixtures of lactose:lactulose was evaluated in ethanol and isopropanol, which were miscible solvents with [EMIM][DCA], by using a solvent:IL ratio of 10:1 (w/w). Mixtures were homogenized at 313 K by stirring for 1 h and centrifuged at 12,100 *g* for 5 min. Finally, the supernatant was recovered and dried before analysis as indicated in Section 2.5.1.

2.4.3. Active charcoal treatment

Binary mixtures of lactose:lactulose dissolved in [EMIM][DCA] were treated with activated charcoal as indicated by Hernandez et al. [18] but varying the solvent composition. In the optimized experiment, 165 mg of the carbohydrates mixtures dissolved in [EMIM][DCA] were treated with 655 mg of activated charcoal mixed with 3 mL of water (Fig. 1). The slurry was stirred for 1 h to allow the adsorption of carbohydrates on the carbon surface. Then, the mixture was filtered through a Whatman No. 1 paper (Whatman International Ltd., Maidstone, UK) under negative pressure and the filtrate (IL + water) was removed. Activated charcoal was washed with 2 mL of water by stirring the slurry for 1 h to assure the complete IL removal and then filtered as indicated above. Desorption of carbohydrates from the activated charcoal was done by washing the sorbent with 12 mL of ethanol:water (50:50, v/v) under agitation for 1 h. Phase separation was done by filtration as previously indicated. One mL of the filtrate was finally evaporated under vacuum at 40 °C and analyzed as indicated in Section 2.5.1. This procedure was also applied to the isomerization mixture dissolved in [EMIM][DCA].

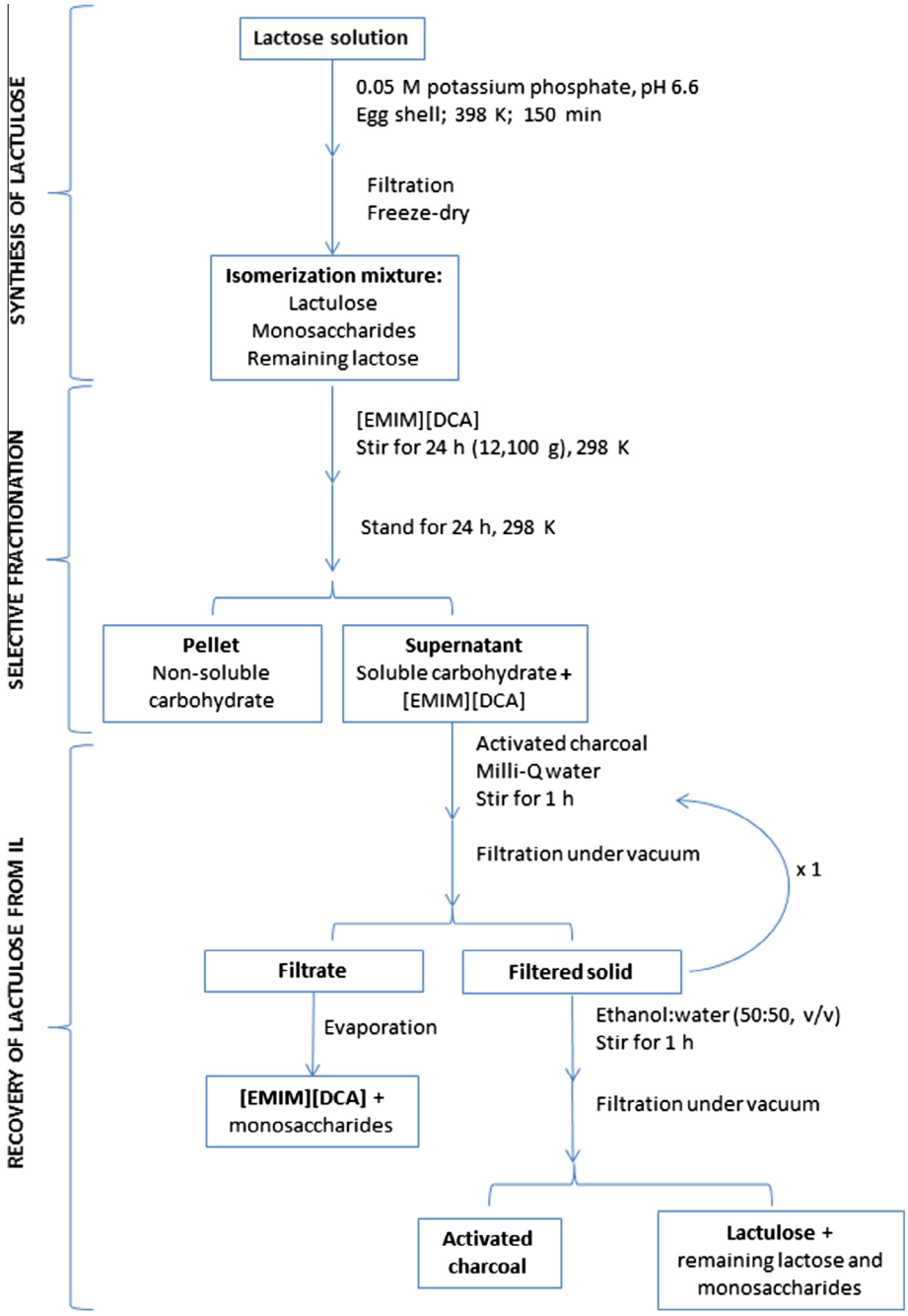

Fig. 1. Scheme of the general process of synthesis, fractionation, and recovery of lactulose obtained from lactose. Pulverized egg shell was used as catalyst for lactose isomerization, [EMIM][DCA] as fractionation agent and active charcoal for the recovery of lactulose from IL.

2.5. Analytical methods

2.5.1. GC-FID analyses

The GC system was an HP 7890A equipped with a FID from Agilent Technologies (Palo Alto, CA, USA). The GC separation was performed on a silica capillary coated column DB-17 with 50% phenyl and 50% polysiloxane (30 m × 0.25 mm i.d. × 0.25 μm *df*; Agilent Technologies). Nitrogen was used as carrier gas at a constant flow of 0.677 mL/min. The GC oven temperature program started at 200 °C and increased at 2 °C/min up to 290 °C. The inlet and detector temperatures were set at 300 °C. Samples were injected (1 μL) with a split ratio of 20:1.

In all cases, 10 mL of a solution containing phenyl-β-D-glycoside (internal standard) at a concentration level of 1 mg/mL in *n*-heptane were added to aliquots of 10 mg of the mixtures of carbohydrate and IL. Analytes derivatization to trimethylsilyl (TMS) ethers was done according to Ruiz-Aceituno et al. [19]. In brief, 100 μL of trimethylsilylimidazole (TMSI) were added to the samples and stirred at room temperature for 1 h. The reaction was stopped by adding 200 μL of water. Trimethylsilyl carbohydrates were extracted by liquid–liquid extraction (LLE) with *n*-heptane. The extraction was repeated twice to ensure total carbohydrate recovery.

Quantitation was carried out using the internal standard method. For this, solutions of carbohydrate standards dissolved

in ILs in the 0.25–1 mg range were prepared. Calculated response factors for each carbohydrate relative to two internal standards (*n*-tetracosane and phenyl-β-D-glucoside) were used for quantitative analysis. All GC analyses were carried out, at least, in triplicate.

2.5.2. LC-RID analyses

LC analyses were performed using an Agilent Technologies 1220 Infinity LC System-1260 (Boeblingen, Germany) equipped with a RID. LC separation was carried out on an amino column (100-NH_2, 250 mm × 4.6 mm, 5 μm particle size) from Kromasil (Bohus, Sweden) using isocratic elution with acetonitrile:water at 70:30 (v/v) as the mobile phase and at flow rate of 1.0 mL/min for 20 min.

Samples involved in the solubility studies were dissolved in a 1:1 (v/v) acetonitrile:water solution to yield a concentration of 10 mg/mL and 50 μL were injected in the LC system. Acquisition and processing were performed using the Agilent ChemStation software (Agilent Technologies). All LC analyses were carried out, at least, in triplicate.

Quantitation analyses were carried out using the external standard method. For this, solutions of 2–27 mg carbohydrate standards in 100 mg of ILs were diluted with acetonitrile:water (1:1, v/v) to 10 mL.

2.6. Statistical analysis

Data treatment was done using the software Statistica 7.0 (Stat Soft Inc., Tulsa, OK, USA). Differences were considered to be significant when $p < 0.05$; analyses of variance (ANOVA) using the Fisher test were used to evaluate significant differences.

3. Results and discussion

3.1. Fractionation of ketoses from aldoses in equimolar binary mixtures by ILs

ILs used in this work were chosen based on preliminary results published by Carrero-Carralero et al. [16], who determined the solubility of single aldoses and ketoses in selected ILs at different temperatures. Consequently, 1-hexyl-3-methylimidazolium chloride ([HMIM][Cl]), 1-butyl-3-methylimidazolium methyl sulfate ([BMIM][$MeSO_4$]) and 1-ethyl-3-methylimidazolium dicyanamide ([EMIM][DCA]) were selected for the fractionation of equimolar binary mixtures of fructose:glucose, tagatose:galactose and lactulose:lactose, respectively, at 299 K. These ILs showed the highest differences between individual solubility values calculated for a given ketose and its corresponding aldose which, in principle, could lead to effective fractionation in binary mixtures. According to reported solubility values, tagatose was 7 times more soluble than galactose in [BMIM][$MeSO_4$], lactulose was 4-fold more soluble than lactose in [EMIM][DCA], and fructose was 2-fold more soluble than glucose in [HMIM][Cl]. The temperature was set at 299 K to avoid ketose dehydration into 5-hydroxymethylfurfural [16].

Table 1 shows the solubility data (%, w/w) obtained for ketoses and aldoses in the equimolar binary mixtures in the corresponding studied IL. Solubility values of the investigated carbohydrates in [HMIM][Cl] and [EMIM][DCA] were determined by GC-FID after a derivatization step. Carbohydrates dissolved in [BMIM][$MeSO_4$] were only partially derivatized with the proposed methodology [19]. Therefore, these samples were analyzed by LC-RID.

Remarkably, tagatose was 6-fold more soluble than galactose in [BMIM][$MeSO_4$], whereas lactulose was 3 times more soluble than lactose in [EMIM][DCA] (Table 1). These results agreed with the individual solubility data previously reported by Carrero-Carralero et al. [16] and point out the feasability of using these ILs for their efficient fractionation. However, solubility of fructose in [HMIM][Cl] in the presence of glucose (9.4%) was meaningfully lower than that previously described for individual samples (20.2%) [16]. This fact could be attributed to the high viscosity of [HMIM][Cl], determined as 7500 cp [20], which could impair the solubilization of fructose [21].

Table 1
Solubility values, as % (w/w) of the carbohydrate in the mixture, of aldoses and ketoses in binary mixtures (1:1, w/w) on selected ILs at 299 K. Experimentally determined standard deviations (SD) are shown in parenthesis ($n = 3$).

[EMIM][DCA][a]		[HMIM][Cl][a]		[BMIM][$MeSO_4$][b]	
Lactose	9.7 (1.7)	Glucose	9.0 (0.4)	Galactose	1.2 (0.2)
Lactulose	28.0 (1.3)	Fructose	9.4 (0.2)	Tagatose	7.1 (0.3)

[a] Solubility values determined by GC-FID analysis.
[b] Solubility values determined by LC-RID analysis.

3.2. Fractionation of lactulose from isomerization reaction mixtures

To evaluate the efficiency of ILs in the fractionation of a carbohydrate real mixture, the isomerization of lactose in basic media catalyzed by egg shell was carried out. Fig. 2A displays the GC-FID chromatogram of this reaction mixture. This process had a yield of 24% in lactulose, and the rest of carbohydrate composition consisted of 48% lactose and 28% monosaccharides (galactose and glucose). The yield of lactulose was in accordance with the values reported by Montilla et al. [17].

Considering the data obtained from the study of solubility of the standard binary mixture of lactulose and lactose (Table 1), [EMIM][DCA] was used for the fractionation of lactulose from the isomerization reaction mixture at 299 K. As it can be observed in Fig. 2B, the use of [EMIM][DCA] resulted in a noticeable enrichment of lactulose which became the predominant carbohydrate. Fig. 3 shows the percentages of lactose, lactulose and monosaccharides before and after the fractionation using [EMIM][DCA] (grey

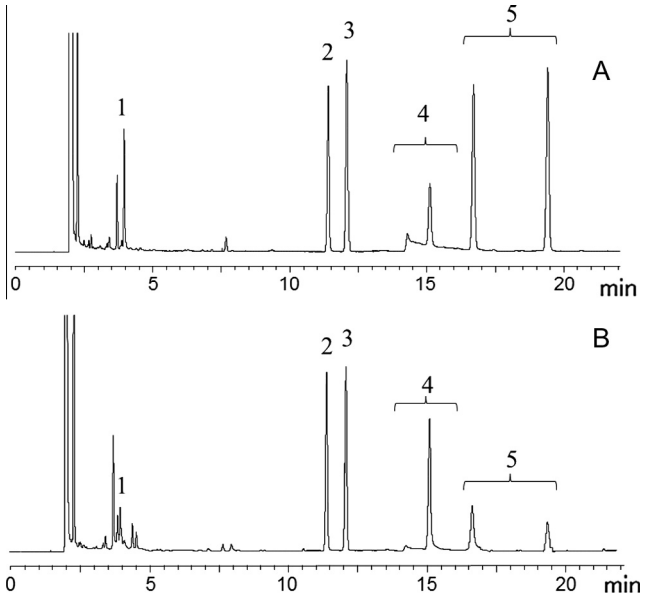

Fig. 2. GC-FID profiles of the reaction mixture derived from the alkaline isomerization with egg shell of lactose to lactulose before (A) and after (B) fractionation with [EMIM][DCA]. Labelled peaks are as follows: (1) Monosaccharides, (2) tetracosane (non-derivatized internal standard), (3) phenyl-β-D-glucoside (derivatized internal standard), (4) lactulose and (5) lactose. Both chromatograms use the same abundance scale.

and white bars, respectively). Lactulose percentage in the isomerization mixture increased more than 2-fold as compared to values obtained before treatment. On the contrary, lactose and monosaccharides percentages decreased notably after this treatment. Thus, the carbohydrate content of the isomerization reaction mixture after the fractionation with [EMIM][DCA] was 58% lactulose, 31% lactose and 11% monosaccharides. Regarding extraction yields, it can be mentioned that lactulose was totally dissolved in the IL whereas only 28% of lactose and 19% of monosaccharides remained in the treated mixture. These results demonstrated that treatment of the isomerization reaction mixture with [EMIM][DCA] resulted in a notable enrichment of lactulose.

3.3. Recovery of lactulose from [EMIM][DCA]

Different procedures were evaluated to isolate lactulose from the corresponding IL, i.e. [EMIM][DCA]: (i) effect of cooling, (ii) solvent or antisolvent treatment, and (iii) adsorption on activated charcoal. Likewise, these treatments could simultaneously contribute to lactulose enrichment and the recovery of the IL for subsequent recycling.

As it was previously observed by Carrero-Carralero et al. [16], solubility of lactose and lactulose in [EMIM][DCA] decreased as the temperature does. Therefore, sample cooling could in principle lead to a higher precipitation of carbohydrates, so allowing their effective fractionation from the IL. Al-Nashef et al. [15], also proposed a cooling procedure to separate glucose and fructose from 1-ethyl-3-methylimidazolium ethylsulfate or 1,3-dimethyl-imidazolium dimethylphosphate. In the present study, binary mixtures of lactose and lactulose dissolved in [EMIM][DCA] were kept at 279, 253 and 193 K up to one week. None of these treatments were effective for lactose and lactulose separation from IL by precipitation. Best separation was obtained at 193 K and resulted only in 4.3% of lactulose and 2.1% of lactose precipitation.

Regarding the use of solvents, two different approaches were followed: LLE using solvents immiscible with [EMIM][DCA], and the antisolvent method, which involves the use of a solvent miscible with the selected IL, but in which lactose and lactulose were not at all or only partially soluble. Ethyl acetate and *n*-hexane were assayed as solvents for the former approach. Solubility of lactose and lactulose in *n*-hexane was almost negligible, whereas 47% of lactulose and 50% of lactose were dissolved in ethyl acetate and so recovered from [EMIM][DCA]. These results allowed a low recovery of lactulose, making difficult the potential recyclability of the IL for further usages.

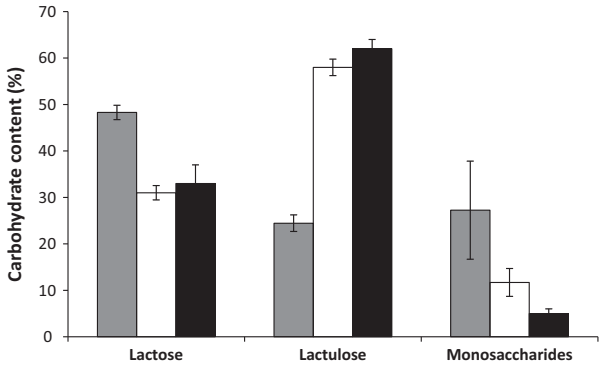

Fig. 3. Content (%) of lactose, lactulose and monosaccharides in the alkaline isomerization mixture before (grey bars) and after treatment with [EMIM][DCA] (white bars), and after removing the IL by treatment with activated charcoal (black bars). SD are shown as error bars ($n = 3$).

Ethanol and isopropanol, solvents miscible with [EMIM][DCA], were evaluated as antisolvents to separate the binary mixtures of lactose and lactulose from this IL. Isopropanol allowed the highest removal of lactulose (66%) and lactose (96%) from [EMIM][DCA]; meanwhile, ethanol was not able to recover lactulose while 88% of lactose was extracted. According to these results, we conclude that the use of ethanol and isopropanol as antisolvents were not useful for the recovery of lactulose from IL mixtures since lactose was notably enriched in relation to lactulose. However, these results would indicate that these solvents could be of great interest in carbohydrate chemistry, mainly for lactose extraction. Previously, ethanol has been suggested as a good antisolvent to recover glucose from mixtures with 1-ethyl-3-methylimidazolium thiocyanate [11] and with ILs based on 1-methyl-3-alkylimidazolium as cation and chloride, bromide, acetate, and hydrogen sulfate as anions [22].

Finally, the effect of activated charcoal on the separation of binary mixtures of lactose and lactulose in [EMIM][DCA] was evaluated and latter being applied to the treatment of the isomerization reaction mixture. Different ethanol:water ratios, i.e. 5:95, 1:99 and 0:100, (v/v) were assayed according to Hernandez et al. [18] to allow the maximum adsorption of carbohydrates in the charcoal and the IL removal. Treatments were carried out twice to assess the total IL removal. Ethanol:water 5:95 (v/v) resulted in a complete desorption of carbohydrates from the sorbent, whereas 33% lactose and 44% lactulose were removed with ethanol:water 1:99 (v/v). Best results were obtained using water as eluent, resulting only in a 10% removal of lactose and lactulose.

Recovery of disaccharides from charcoal using ethanol:water 50:50 (v/v) was remarkably high (89% and 90% for lactose and lactulose, respectively), whereas only 24% of monosaccharides remained in the eluate. Absence of detectable IL in these extracts was confirmed by pre-concentration of the extract to dryness and subsequent analysis. IL was recovered by evaporation of the filtrates derived from the two first washes in the lactulose recovery process with activated charcoal (Fig. 1). In consequence, IL could be recycled for further uses in lactulose fractionation. This is an important aspect to be considered for the balance of the cost of the ILs and, consequently, for the viability of the whole process. Considering these results, carbohydrate mixtures were enriched in lactose and, mainly, lactulose, which accounted for 33% and 62% of total carbohydrates in the mixture (Fig. 3, black bars).

To sum up, the overall process (including IL treatment and the activated charcoal step) allowed the recovery of 90% lactulose, 25% lactose and 4.6% monosaccharides from the original isomerization mixture, whereas purity of this mixture was 62% lactulose, 33% lactose and 5% monosaccharides.

4. Conclusions

ILs are a promising alternative to conventional organic volatile solvents for the selective fractionation of aldoses and ketoses. The results reported in the present study are the first evidence of the usefulness of [EMIM][DCA] for the enrichment of lactulose in its product of synthesis by isomerization of lactose in basic media. Recovery of carbohydrates from ILs was also successfully achieved using an activated charcoal treatment. This last step could also facilitate the potential recycling of ILs favoring, thus, the development of a cost-effective process. The reported results demonstrate that this procedure was more effective than the antisolvent or the cooling method for the fractionation of ketoses from aldoses.

As a whole, the proposed methodology represents a novel, environmental friendly and valuable alternative to conventional organic solvent-based procedures in use for carbohydrates

fractionation. Their positive features, such as simplicity, straightforward nature and efficiency, make to consider it an interesting methodology with potential for scaling up processes.

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4. DESARROLLO DE NUEVOS MÉTODOS DE EXTRACCIÓN Y ENRIQUECIMIENTO DE CARBOHIDRATOS BIOACTIVOS A PARTIR DE MATRICES NATURALES

4. DESARROLLO DE NUEVOS MÉTODOS DE EXTRACCIÓN Y ENRIQUECIMIENTO DE CARBOHIDRATOS BIOACTIVOS A PARTIR DE MATRICES NATURALES

Como se ha comentado en la **Sección 1**, la obtención de carbohidratos bioactivos, tales como prebióticos o inositoles, puede llevarse a cabo en algunos casos mediante reacciones de síntesis. Sin embargo, su extracción a partir de matrices naturales, sobre todo de subproductos agroalimentarios, presenta un gran interés para la industria alimentaria. El desarrollo de métodos de extracción de carbohidratos bioactivos rápidos, eficaces y fácilmente escalables es imprescindible para obtener rendimientos óptimos desde el punto de vista económico. En esta Tesis, se seleccionaron la PLE y la MAE con el fin de evaluar su potencial y sus correspondientes ventajas e inconvenientes en este campo concreto, dados los escasos antecedentes bibliográficos existentes sobre este tema.

Previo a la optimización de métodos de extracción de carbohidratos bioactivos, es necesario tener un conocimiento de la composición de dichos carbohidratos, así como de otros potencialmente interferentes con su actividad, con el fin de asegurar la posterior eliminación de estos últimos. En el caso de los inositoles, la mayoría de los estudios previos se centran en el análisis de *myo*-inositol en diversas matrices (Clements and Darnell 1980), pero existen pocas referencias bibliográficas que aporten datos detallados sobre la presencia y contenido de otros inositoles y sus derivados (Sanz *et al.* 2004; Sanz *et al.* 2005; Ruiz-Matute *et al.* 2007). Por tanto, los objetivos de esta sección fueron (i) el estudio exhaustivo de la composición en carbohidratos de bajo peso molecular (LMWC), con especial énfasis en los inositoles, en diversas matrices alimentarias de origen vegetal; (ii) la evaluación de métodos de fraccionamiento para la eliminación de carbohidratos interferentes en extractos vegetales; y (iii) la optimización de métodos de PLE y MAE para la obtención de carbohidratos bioactivos.

Para alcanzar estos objetivos, en primer lugar, se llevaron a cabo una serie de estudios preliminares orientados a determinar el contenido en inositoles de distintas muestras de origen vegetal, con el fin de seleccionar las muestras objeto de estudio. Se eligieron muestras vegetales poco estudiadas hasta el momento o de relevancia por su consumo, tales como frutos (piñón, naranja, fresa, uva), legumbres (cacahuete, soja,

garbanzo, lenteja) y verduras (achicoria, endivia, escarola, alcachofa, rábano, calabacín y cebolla), cuyos resultados se presentan a continuación.

En esta parte del trabajo, las muestras trituradas y homogeneizadas se sometieron a SLE empleando agua como extractante durante 2 h a 50 ºC. Los extractos, una vez filtrados, fueron analizados por GC−MS, previa conversión a sus TMSO (Sanz *et al.*, 2004).

Es sabido que los inositoles poseen un espectro característico con iones *m/z* 305 y 318, de forma que los compuestos que mostraron dicho espectro fueron cuantificados de manera conjunta. La **Figura 4.1** muestra los resultados correspondientes al contenido en inositoles de estos extractos. Como se puede observar, el piñón fue el que mostró los contenidos más elevados (4,02 mg/g), seguido por la soja (3,58 mg/g). Por su parte, la escarola, endivia, fresa y el rábano mostraron los menores contenidos (0,09; 0,07; 0,05 y 0,04 mg/g o mg/L, respectivamente).

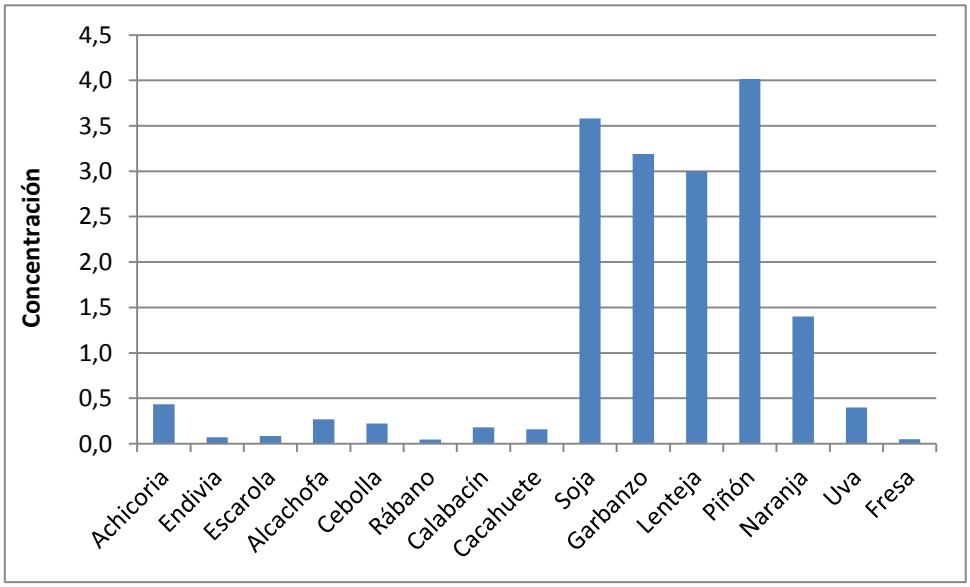

Figura 4.1. Contenido en inositoles (mg/g o mg/mL) de distintas muestras vegetales.

Considerando los resultados obtenidos en estos estudios previos, su elevada producción en España y la escasa información bibliográfica existente sobre su composición en carbohidratos, se llevó a cabo un estudio exhaustivo del contenido en LMWC del piñón por GC−MS. Los resultados obtenidos se recogen en la **Sección 4.1,** que constituye el trabajo titulado “Low molecular weight carbohydrates in pine nuts

from *Pinus pinea L*." de Ruiz-Aceituno y col., publicado en *Journal of Agricultural and Food Chemistry*, 60 (2012), 4957−4959. En este trabajo, se identificaron algunos azúcares y varios inositoles y sus derivados que no habían sido descritos hasta ese momento en este tipo de matriz.

Otras matrices de interés en cuanto al contenido en inositoles son verduras como la achicoria y la alcachofa. En la **Sección 4.2** se incluye un estudio exhaustivo de la composición cuali- y cuantitativa en LMWC de estos vegetales, además de en otros de las familias Asteraceae, Amarantaceae, Amarylidaceae, Brassicaceae, Dioscoreaceae y Solanaceae mediante GC−MS. Los resultados más relevantes de este trabajo de investigación se resumieron en el artículo titulado "Determination of free inositols and other low molecular weight carbohydrates in vegetables" de Hernandez-Hernandez y col., publicado en *Journal of Agricultural and Food Chemistry* 59 (2011), 2451–2455. Este estudio permitió la identificación de distintos azúcares e inositoles, además de otros polialcoholes y glicósidos, algunos de ellos carbohidratos con reconocidas propiedades beneficiosas para la salud.

Considerando que las legumbres también mostraron ser fuentes ricas en inositoles, a continuación se procedió a determinar la composición en LMWC por GC−MS en garbanzo, lenteja, almorta, algarroba, soja y carilla. Los resultados de este trabajo, que constituye la **Sección 4.3** de esta tesis, se recogen en el artículo titulado "Optimisation of a biotechnological procedure for selective fractionation of bioactive inositols in edible legume extracts" de Ruiz-Aceituno y col., publicado en *Journal of the Science of Food and Agriculture* 93 (2013) 2797-2803. Cabe destacar la identificación de nuevos LMWC, como por ejemplo bornesitol y latiritol en almorta. Además, en esta parte del estudio, se optimizó un método de fraccionamiento limpio, seguro y de fácil escalado, para la eliminación selectiva de LMWC interferentes con las propiedades de los inositoles (como glucosa o fructosa) de los extractos enriquecidos basado en el uso de *Saccharomyces cerevisiae.*

Una vez determinada la composición en inositoles de las muestras de interés y seleccionado el método para el fraccionamiento selectivo de LMWC que permitía la recuperación de los inositoles, se procedió a la optimización de un método de extracción basado en PLE para su recuperación a partir de muestras vegetales. La **Sección 4.4,** correspondiente al trabajo de Ruiz-Aceituno y col., titulado "Optimization of

pressurized liquid extraction of inositols from pine nuts (*Pinus pinea* L.)" publicado en *Food Chemistry* 153 (2014) 450–456, resume los resultados correspondientes a la optimización, por primera vez, de un método de PLE para la obtención de extractos enriquecidos en inositoles a partir de piñones. Empleando la regresión múltiple por pasos, se evaluó la influencia de la temperatura, el tiempo y los ciclos de extracción en el rendimiento y composición del extracto. Los resultados obtenidos se compararon con los proporcionados por la SLE convencional (una vez optimizada también cantidad de muestra, volumen de extractante, tiempo de agitación y temperatura del tratamiento) y se valoraron y discutieron las ventajas y desventajas de ambos procedimientos. El método de fraccionamiento desarrollado previamente y basado en el empleo de *S. cerevisiae* (**Sección 4.3)** se aplicó para la eliminación de otros carbohidratos interferentes co-extraidos de los extractos PLE.

La **Sección 4.5** resume los resultados más relevantes obtenidos al poner a punto sendos métodos basados en MAE y PLE para la extracción simultánea, por primera vez, de inositoles e inulina a partir de un subproducto de la industria agroalimentaria, en concreto las brácteas externas de la alcachofa. En este estudio, se optimizaron los diferentes parámetros experimentales que afectaban a la extracción de estos carbohidratos a partir de la matriz investigada, aplicando diseños de experimentos del tipo Box-Behnken y factorial 3^2. La determinación de los carbohidratos de interés en los extractos obtenidos se llevó a cabo mediante GC−FID (previa derivatización a sus correspondientes derivados TMSO) antes y después de ser sometidos a un tratamiento enzimático con inulinasa. Una vez determinadas las condiciones óptimas de extracción, los extractos así obtenidos se sometieron al tratamiento con levaduras descrito en la **Sección 4.3**. Los resultados de este estudio constituyen la base del artículo enviado para su publicación a la revista *Food Chemistry* por Ruiz-Aceituno y col. titulado "Extraction of bioactive carbohydrates from artichoke (*Cynara scolymus* L) external bracts using microwave assisted extraction and pressurized solvent extraction".

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4.1. Low molecular weight carbohydrates in pine nuts from *Pinus pinea* L.

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Low Molecular Weight Carbohydrates in Pine Nuts from *Pinus pinea* L.

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ABSTRACT: Low molecular weight carbohydrates in pine nuts from *Pinus pinea* L. (n = 7) have been studied by gas chromatography−mass spectrometry as their trimethylsilyl oximes. Besides previously reported components, such as glucose, fructose, sucrose, and raffinose, several soluble carbohydrates have been identified for the first time in this product, including saccharides (galactose, maltose, and planteose) and cyclitols (pinitol, galactinol, galactopinitol A1, fagopyritol B1, and other glycosyl-inositols). Most abundant cyclitols were *chiro*-inositol, fagopyritol B1, and pinitol, with concentrations ranging from 126.7 to 222.1 mg (100 g)$^{-1}$, 94.2 to 177.1 mg (100 g)$^{-1}$, and 51.2 to 282.8 mg (100 g)$^{-1}$, respectively.

KEYWORDS: *soluble carbohydrates, cyclitols, fagopyritol, pine nut, raffinose, bioactive carbohydrates*

INTRODUCTION

The edible seeds of *Pinus pinea* L. (pine nuts, pine kernels) are traditionally consumed in the countries of the Mediterranean basin, Spain being the main producer (about a 45% of world production). Besides their organoleptic properties, pine nuts present a high nutritional value.

Several compositional studies about pine nuts have been carried out,[1,2] including determination of phenolic acids,[3] phytic acid,[4] minerals,[5] lipids,[6] and antioxidants.[7] These studies have shown that pine kernels possess several interesting nutritional properties.

On the contrary, soluble carbohydrates of pine nuts have been scarcely studied. Although ionic chromatography allowed the identification and determination of glucose, fructose, sucrose, raffinose, and stachyose,[8] these carbohydrates are usually determined as "total soluble sugars".[1,2] Moreover, scarce evidence about the presence of cyclitols in pine nuts has been reported. Cyclitols, such as *chiro*-inositol and pinitol (D-3-*O*-methyl-*chiro*-inositol), are considered bioactive carbohydrates, because they have been shown to exert an acute and sustained antihyperglycemic effect in a diabetic mouse model, where the effect may be an insulin-like effect on glucose transport that is independent of insulin.[9] To the best of our knowledge, only Kim et al.[10] have reported the content of *chiro*-inositol in a sample of pine nuts (without indication of the botanical species); in that work, all the potential derivatives of *chiro*-inositol present in the sample were converted into free *chiro*-inositol and determined by HPLC.

In this work the analysis of free soluble sugars in pine kernels has been carried out, paying special attention to the determination of bioactive cyclitols, most of them identified for the first time in this product.

MATERIALS AND METHODS

Standards. Galactose, glucose, glucose 6-phosphate, fructose, sucrose, maltose, raffinose, planteose, pinitol, phenyl β-D-glucoside, *myo*-inositol, *chiro*-inositol, and galactinol were acquired from Sigma Chemical Co. (St. Louis, MO). Planteose was kindly given by Dr. G. L. Côté (USDA, Peoria, USA).

Samples. Pine nuts from pines in Tres Cantos (Madrid) were manually collected by Dr. R. Morales (Real Jardín Botánico, CSIC, Madrid, Spain). Nuts-in-shell were removed from the cones and cracked; kernels were separated from external shell and inner seed coat.

Six commercial samples of peeled pine nuts, five of them of Spanish origin and one imported from Italy, and samples of buckwheat (*Fagopyrum esculentum*), soybeans (*Glycine maxima*), and chickpeas (*Cicer arietinum*) were purchased at local markets. Samples were stored properly in dry conditions, protected from direct sunlight until their analysis. All samples were analyzed before their shelf life date.

Extraction. Pine nuts (0.5 g) were crushed in a mortar and extracted with 5 mL of milli-Q water at controlled temperature (60 °C) during 2 h (first cycle) in crystal vessels. The solid residue was removed by centrifugation at 4400g at 10 °C, and the process was repeated twice (second and third cycles) under the same conditions to achieve an exhaustive extraction. A clear solution was obtained from each cycle, which was then independently derivatized.

GC−MS analysis. Trimethylsilyl oximes (TMSO) were prepared as previously described.[11] In brief, 0.5 mL of phenyl β-D-glucoside (1 mg mL^{-1}) was added to 1 mL of extract, and the solution was evaporated under vacuum. Then, samples were treated with 350 μL of 2.5% hydroxylamine chloride in pyridine (30 min at 75 °C), and 350 μL of hexamethyldisilazane plus 35 μL of trifluoroacetic acid (45 °C for 30 min). After centrifugation, 1 μL of supernatant was taken for injection.

Gas chromatography−mass spectrometry (GC−MS) analyses were carried out on a 7890 gas chromatograph coupled to a 5975 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using helium as the carrier gas. A 30 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica column coated with TRB-1 (cross-linked methyl silicone) from Teknokroma (Barcelona, Spain) was used. The oven temperature was held at 200 °C for 15 min, then programmed to 270 at 15 °C min^{-1}, then programmed to 290 at 1 °C min^{-1}, and finally programmed to 300 °C at 15 °C min^{-1}. The final temperature was held for 30 min. The injector was at 300 °C, and injections were made in split mode with a split ratio of 1:20. The mass spectrometer was operated in electronic impact (EI) mode at 70 eV, scanning the 50−

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650 m/z range. The interface and source temperature were 280 and 230 °C, respectively. Acquisition was done using HP-ChemStation software (Hewlett-Packard, Palo Alto, CA, USA).

When commercial standards were not available, identification of low molecular weight carbohydrates (LMWC) has been carried out using linear retention indices (I^T), relative abundances of characteristic m/z fragments, and bibliographic data. I^T of each TMSO carbohydrate (i) was calculated as:

$$I^T = 100\left[\frac{t_{Ri} - t_{Rz}}{t_{R(z+1)} - t_{Rz}} + z\right]$$

where t_R was the total retention time measured at programmed temperature, z the number of carbon atoms of the n-alkane eluted before the target peak, and $(z + 1)$ the number of carbon atoms of the n-alkane eluted after the target peak, according to van den Dool and Kratz.[12] n-Alkanes from C_8 to C_{40} were considered for this study. Retention data and mass spectra for glycosyl cyclitols whose standards were not commercially available were obtained from those present in buckwheat, soybeans, and chickpeas.

Response factors (RF) relative to the internal standard were calculated over the expected concentration range. Due to the lack of commercial standards, the RF of galactinol was used for all glycosyl cyclitols. All analyses were carried out in duplicate. Precision was calculated from the results obtained for a standard mixture (glucose, fructose, *myo*-inositol, and sucrose), and a pine nut sample which was extracted, derivatized, and analyzed by quintuplicate. In all cases, relative standard deviations were lower than 10%.

RESULTS AND DISCUSSION

Figure 1 shows the GC profile of a pine nut sample. Several components with mass spectra characteristic of carbohydrates

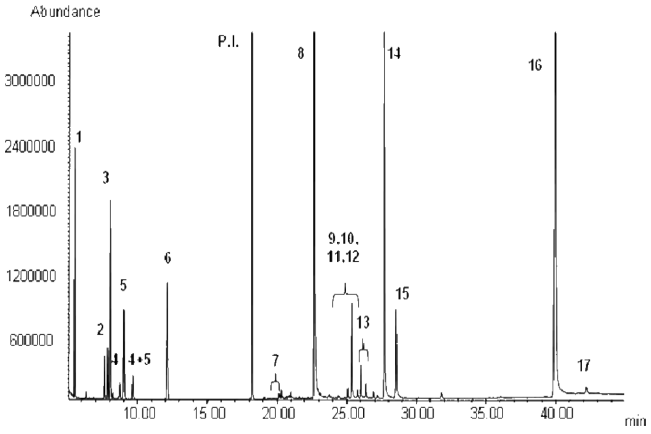

Figure 1. GC profile of low molecular weight carbohydrates in a pine nut sample as their TMSO: 1, pinitol; 2, fructose; 3, *chiro*-inositol; 4, galactose; 5, glucose; 6, *myo*-inositol; 7, glucose 6-phosphate; 8, sucrose; 9, 11, 12, unidentified glycosyl-inositols; 10, galactopinitol A1; 13, maltose; 14, fagopyritol B1; 15, galactinol; 16, raffinose; 17, planteose; PI, phenyl β-D-glucoside (internal standard).

were detected, including 3 monosaccharides, 2 disaccharides, 2 trisaccharides, 3 free inositols, 6 glycosyl-inositols, and a sugar phosphate. Identification was carried out by matching retention time and mass spectral data with those of available standards. Besides the four saccharides (namely, glucose, fructose, sucrose, and raffinose) previously reported,[8] galactose, maltose, and planteose were also identified in the studied samples. Maltotriose was found as traces in several samples.

To assess an exhaustive recovery of LMWC three different cycles of extraction were carried out. Percentages of extraction ranged 85−100% for the first cycle; 0−15% for the second cycle, and 0−1.5% for the third cycle. Table 1 shows the minimum, maximum, and average values of LMWC, expressed as mg (100 g)$^{-1}$, of the analyzed pine nut extracted within three cycles. Quantitative differences in LMWC composition found in these samples could not be attributed to a specific cause. Moreover, no real differences in the composition were observed between commercial and collected pine nut samples. I^T values have also been summarized in Table 1.

Table 1. Retention Indices (I^T) and Concentration (mg (100 g)$^{-1}$) of Low Molecular Weight Carbohydrates (as TMSO) in the Analyzed Samples of Pine Nuts (n = 7)

peak	compound	I^T	range	av[a]	SD[b]
1	pinitol	1896	51.2−282.8	146.9	92.2
2	fructose	2013, 2021	42.4−162.7	112.6	40.8
3	*chiro*-inositol	2029	126.7−222.1	176.3	39.5
4	galactose	2053, 2079	8.9−42.1	24.3	10.3
5	glucose	2061, 2079	54.9−172.7	112.9	37.1
6	*myo*-inositol	2139	45.8−89.0	61.5	15.2
7	glucose-6-phosphate	2471, 2493	traces[c]	traces	
8	sucrose	2736	1242.1−2146.0	1587.2	348.8
9	glycosyl-inositol	2882	traces−5.5	2.6	2.2
10	galactopinitol A1	2904	traces−2.1	0.9	0.7
11	glycosyl-inositol	2935	1.6−5.9	3.2	1.5
12	glycosyl-inositol	2954	19.3−39.4	27.6	6.9
13	maltose	2985, 2990	1.6−72.3	147.5	30.9
14	fagopyritol B1	3072	94.2−177.1	24.1	17.4
15	galactinol	3100	4.1−49.3	26.0	28.3
16	raffinose	3380	397.0−1067.7	815.1	216.0
17	planteose	3420	5.9−16.8	11.7	3.4

[a]av = average. [b]SD = standard deviation. [c]Limit of detection: 0.14 mg (100 g)$^{-1}$. Limit of quantitation: 0.46 mg (100 g)$^{-1}$.

Sucrose was the main sugar in all examined samples, as reported by Ruggeri et al.,[8] with amounts in the 1.2−2.1 g (100 g)$^{-1}$ range. The second most abundant sugar was raffinose, which averaged 815 mg (100 g)$^{-1}$. Monosaccharides varied in a broad concentration range, but glucose and fructose always showed similar concentrations, around 100 mg (100 g)$^{-1}$. Galactose, maltose, and planteose appeared at lower amounts.

Traces of several sugar phosphates were found in all samples, but only glucose 6-phosphate could be positively identified.

Chromatograms also displayed several cyclitols and derivatives: the sum of free inositols (pinitol, *chiro*-inositol, and *myo*-inositol) averaged 385 mg (100 g)$^{-1}$, *chiro*-inositol being, in general, the most abundant of them (127−222 mg (100 g)$^{-1}$). Up to six glycosylated cyclitols were detected. Among them, the most abundant peak was identified as fagopyritol B1 by comparison of its I^T (3072) and mass spectrum with that present in an extract of buckwheat (*Fagopyrum esculentum*).[13] The abundance ratios of m/z 305/318 and 318/319 fragments were very similar to those described by Obendorf et al.[14] as characteristics for this compound. Fagopyritol B1 ranged from 94 to 177 mg (100 g)$^{-1}$ in pine nut samples. However, fagopyritol A1 was not detected. Galactinol was identified by matching with the standard. A small peak with I^T of 2904 showed the characteristic spectrum of a galactopinitol. Its retention index and mass spectrum were identical to those of galactopinitol A1 present in soybeans and in chickpeas, as described by Schweizer et al.[15]

Three compounds marked as 9, 11, and 12 in Figure 1 showed mass spectra compatible with glycosyl-inositols, probably galactosides. They could not be identified since mass spectra of glycosyl-inositols are rather similar and, besides the lack of standards, the number of published GC and MS data for this type of compounds is very limited. Peaks 9 and 11 appeared in almost all samples at low levels (around 3 mg (100 g)$^{-1}$ each), whereas peak 12 was slightly more abundant (19–39 mg (100 g)$^{-1}$).

From the presented results, it can be concluded that pine kernels from *P. pinea* have a high content of bioactive carbohydrates. The amount of total *chiro*-inositol (sum of free compound and derivatives) found in the examined samples (as deduced from Table 1) is clearly higher than that reported by Kim et al.[10](1.74 g kg^{-1}). Moreover, the concentration of fagopyritol B1 in pine nuts is similar to that found in several buckwheat flours.[13] Inositols have been shown to have favorable properties in human health[10,16,17] and to present substantial beneficial effects for the treatment of certain diseases. *chiro*-Inositol, pinitol, and glycosyl-inositols appear to exert an insulin-like effect,[9] showing positive activity for type II diabetics and patients with polycystic ovary syndrome.[15] Moreover, α-galactoside sugars, such as raffinose, are currently considered as prebiotics, i.e. able to modify the composition of the colon microflora.[18]

In conclusion, several LMWC have been found in pine nuts from *P. pinea* for the first time. Noticeable amounts of *chiro*-inositol and several glycosyl cyclitols (especially *chiro*-inositol derivatives) with relevant bioactive properties have been detected. These results point out that probably pine kernels should be considered a healthy food component, not only for their composition in phenolic compounds and unsaturated lipids but also for their low molecular weight carbohydrate content.

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Notes

The authors declare no competing financial interest.

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4.2. Determination of free inositols and other low molecular weight carbohydrates in vegetables

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Determination of Free Inositols and Other Low Molecular Weight Carbohydrates in Vegetables

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ABSTRACT: Different low molecular weight carbohydrates including saccharides, polyalcohols, sugar acids, and glycosides have been identified and quantified in different edible vegetables from Asteraceae, Amarantaceae, Amarylidaceae, Brassicaceae, Dioscoreaceae, and Solanaceae families by gas chromatography−mass spectrometry. Apart from glucose, fructose, and sucrose, other saccharides such as sedoheptulose in chicory, spinach, cabbage, purple yam, eggplant, radish, and oak leaf lettuce, rutinose in eggplant skin, and a glycosyl-inositol in spinach have been identified. *chiro*-Inositol was found in all vegetables of the Asteraceae family (3.1−32.6 mg 100 g^{-1}), whereas *scyllo*-inositol was detected in those of purple yam, eggplant, artichoke, chicory, escarole, and endive (traces−23.2 mg 100 g^{-1}). α-Galactosides, kestose, glucaric acid, and glycosyl-glycerols were also identified and quantified in some of the analyzed vegetables. Considering the bioactivity of most of these compounds, mainly chicory leaves, artichokes, lettuces, and purple yam could constitute beneficial sources for human health.

KEYWORDS: Vegetables, low molecular weight carbohydrates, *chiro*-inositol, *scyllo*-inositol, *myo*-inositol

INTRODUCTION

Vegetables have been considered healthy foods because of their vitamin and fiber content, as well as a source of other bioactive substances such as antioxidants (e.g., spinach,[1] chicory,[2] artichokes,[3] cabbage,[4] and lettuces [5]). Nevertheless, the carbohydrate fraction also deserves a detailed study.

Carbohydrates in vegetables mainly consist of cellulose and other polysaccharides, with important roles as structural materials and energy reserves. Free low molecular weight carbohydrates (LMWC) are biologically important constituents of vegetables and include saccharides, mainly fructose, glucose, and sucrose. Minor compounds in this fraction are oligosaccharides such as raffinose or kestose, as well as cyclitols, alditols, and acid sugars, many of them with positive properties.

Besides their role in plant metabolism, inositols have been shown to have favorable consequences in human health[6−8] and to present substantial beneficial effects for the treatment of certain diseases.[9] They have been proposed for treating conditions associated with insulin resistance, which can result in disorders such as diabetes mellitus, obesity, atherosclerosis, hypertension, etc.[10] Special attention has been paid to their effect on the treatment of polycystic ovary syndrome.[6] The administration of inositol to premature infants with respiratory distress syndrome who received parenteral nutrition during the first week of life is also associated with increased survival and a decrease incidence of retinopathy of prematurity.[11]

The best known and ubiquitous member of this family is *myo*-inositol;[12] other interesting and less extended inositols are *chiro*-inositol, which has been detected in soybeans,[13] citrus fruits,[14] and black rice,[15] and *scyllo*-inositol, which has been found in fruits[14,16] and also in vegetables from *Apiaceae* family.[17] The beneficial properties of these carbohydrates and their derivatives have promoted the study of their concentration in human diet. Alditols have also shown many physical properties similar to sugars but with lower caloric content, noncariogenicity, low glycemic index, and low insulin response.[18,19]

Data about saccharides in edible vegetables have been focused on common sugars (glucose, fructose, and sucrose) and a few polyalcohols (mannitol, sorbitol, and *myo*-inositol),[20−23] whereas the presence of other LMWC with possible functional properties has not been considered.

Special attention has been paid to fructans (FOS) considering their prebiotic properties.[24] Some α-galactosides such as raffinose, stachyose, and galactosyl-cyclitols have been considered as non-nutritional carbohydrates,[25] but at present, they are considered as prebiotics.[21] These carbohydrates are characteristic compounds of legumes, although some of them appear in other vegetal sources such as beet root,[26] potato,[27] and safflower.[28]

Gas chromatography coupled to mass spectrometry (GC-MS) is a powerful technique for the separation, structural elucidation, and quantification of volatile compounds, including carbohydrates previously submitted to a derivatization process.

In the present work, GC-MS has been used for the determination of LMWC in different vegetables from the market, considering the beneficial properties of these compounds and the influence of their content in the diet. Besides the more common soluble saccharides, other carbohydrates including inositols, alditols, sugar acids, heptuloses, and glycosides have been identified and quantified.

MATERIALS AND METHODS

Standards. Fructose, galactinol, galactose, glucose, *chiro*-inositol, *myo*-inositol, *scyllo*-inositol, isomaltose, kestose, maltose, mannitol, mannose, phenyl-β-D-glucopyranoside, raffinose, rutinose, and sucrose were acquired from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO), and mannoheptulose was obtained from Biosynth (Staad, Switzerland).

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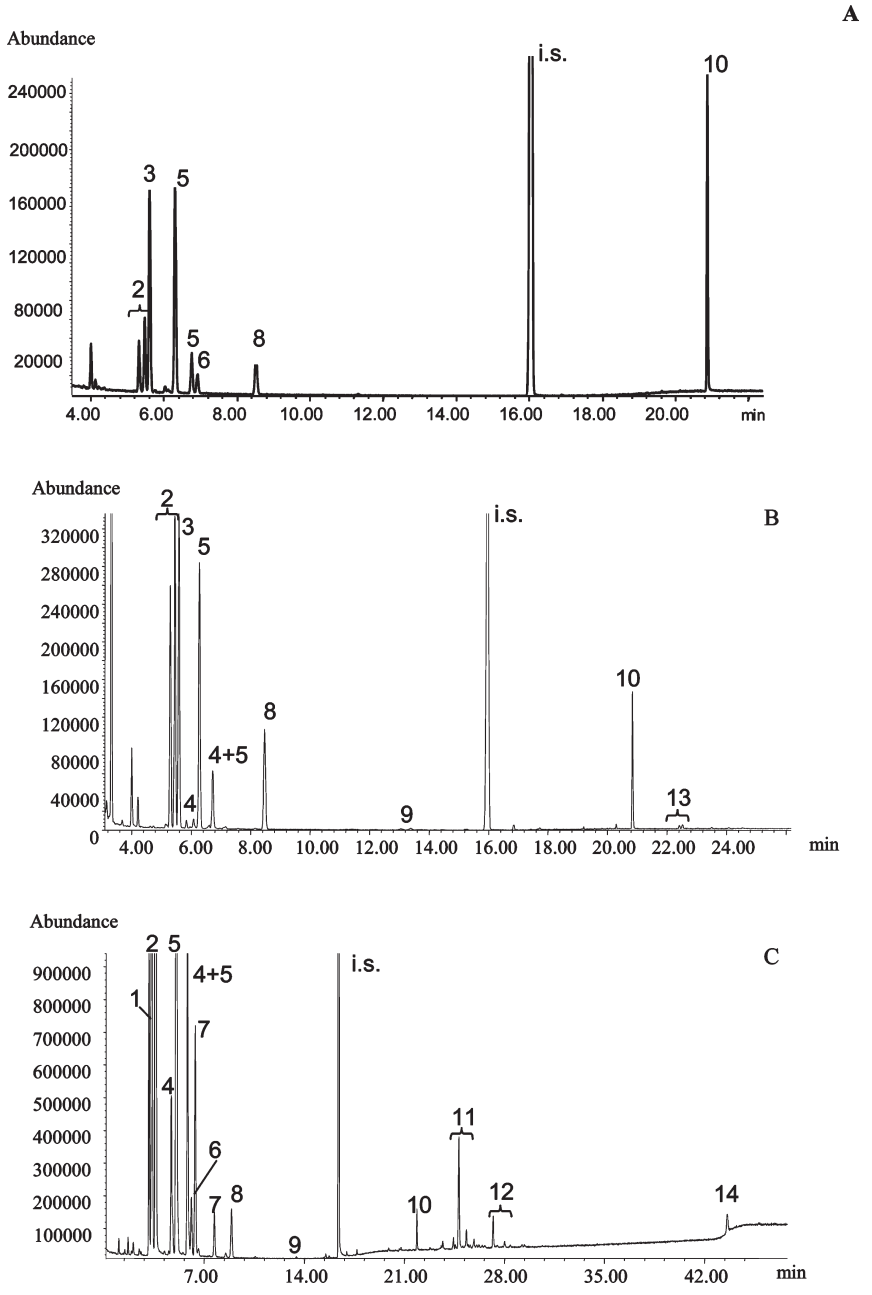

Figure 1. GC-MS profiles of TMSO of low molecular weight carbohydrates of (A) artichoke, (B) oak leaf lettuce, and (C) purple yam. Peaks: 1, mannitol; 2, fructose; 3, *chiro*-inositol; 4, galactose; 5, glucose; 6, *scyllo*-inositol; 7, glucaric acid; 8, *myo*-inositol; 9, sedoheptulose; i.s., phenyl-β-glucoside; 10, sucrose; 11, maltose; 12, isomaltotriose; 13, other disaccharides; and 14, kestose.

Samples. Good quality fresh vegetables (artichoke, chicory leaves, endive, escarole, spinach, beet, cabbage, radish, eggplant, purple yam, and lettuces) were purchased at local markets in Madrid (Spain). As the sugar content in vegetables depends on several factors including cultivar, season, and agricultural and postharvest treatments, seven different cultivars of lettuces were purchased in different seasons. The extraction was carried out using edible parts of the samples. Two or three units of each vegetable were chopped and mixed. Five grams of them was immediately extracted with 25 mL of ultrapure water +0.1% acetic acid at 60 °C for 1 h, using constant agitation. The different extracts obtained were filtered with Whatman #1 filter paper and kept at −20 °C until analysis. Edible skins from eggplant and radish were also used for analysis. All extractions were carried out in duplicate.

GC-MS Analysis. One milliliter of vegetable extract was mixed with 0.5 mL of phenyl-β-D-glucoside (1 mg mL^{-1}) and evaporated under vacuum. Dried samples were treated with 350 μL of 2.5% hydroxylamine hydrochloride in pyridine (30 min at 75 °C), and 350 μL of hexamethyldisilazane plus 35 μL of trifluoroacetic acid (45 °C for 30 min).[29,30]

Derivatized samples were centrifuged, and 1 μL of supernatant was injected into the injection port of a Hewlett-Packard 7890 gas chromatograph coupled to a 5975 quadrupole mass detector (both from Agilent, Palo Alto, CA), using helium as carrier gas (average linear velocity ~20 cm s^{-1}). A 30 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica column coated with TRB-1 (cross-linked methyl silicone) from Teknokroma (Barcelona, Spain) was used. The oven temperature was held at 200 °C for 20 min, then programmed to 270 °C at a heating rate of 15 °C min^{-1}, then programmed to 290 at 1 °C min^{-1}, and finally

Table 1. Saccharide Concentrations (mg/100g of Product) in Analyzed Vegetables[a]

family	genus	common name	fructose	galactose	glucose	sedoheptulose	sucrose	other disaccharides	raffinose	kestose
Asteraceae	*Cichorium*	chicory leaves	345.5 (48.9)	6.5 (1.5)	229.5 (25.8)	1.0 (0.2)	125.8 (51.5)			
		endive	687.1 (3.9)		829.8 (4.0)	tr[b]	32.5 (0.1)			
		escarole	358.6 (8.2)		239.3 (4.8)		31.2 (0.9)			
	Cynara	artichoke	8.9 (1.0)		15.7 (1.7)	tr	21.2 (2.7)			
	Lactuca	Batavian lettuce	294.9 (29.3)	3.3 (0.1)	194.2 (12.9)	tr	28.1 (1.9)			
		iceberg lettuce	485.6 (7.6)	1.4 (0.8)	389.8 (6.5)		23.8 (4.1)			
		oak leaf lettuce	38.4 (1.1)	1.3 (0.1)	25.3 (0.6)	0.8 (0.0)	8.7 (0.2)	0.7 (0.0)		
		Lollo Rosso lettuce	6.4 (0.3)	0.9 (0.1)	3.4 (0.2)		1.0 (0.1)	3.6 (0.2)		
		Romaine lettuce	216.1 (2.0)	tr	144.3 (1.5)		29.5 (0.6)			
		Cresta lettuce	361.3 (6.0)	2.0 (0.5)	255.6 (2.4)		38.8 (0.9)			
Amarantaceae	*Spinacia*	spinach	47.5 (6.4)	2.2 (0.1)	74.8 (10.7)	0.4 (0.3)	54.4 (14.7)	0.5[c] (0.1)		
	Beta	beet root	140.0 (58.42)	10.5 (0.5)	220.7 (75.5)	tr	10697.5 (1010.9)	30.5[d] (9.2)	37.7 (8.6)	16.9 (2.6)
Amarylidaceae	*Allium*	onion	1760.1 (434.2)	12.7 (0.9)	1538.9 (352.7)		220.6 (7.6)	2.2 (0.2)		
Brassicaceae	*Raphanus*	radish[e]	797.6 (80.9)	4.3 (0.5)	799.9 (108.3)	2.1 (0.4)	46.4 (12.6)			
	Brassica	cabbage	614.6 (4.2)	61.1 (0.5)	693.7 (19.9)	1.6 (0.0)	251.6 (50.1)		1.3 (0.1)	
Dioscoreaceae	*Dioscorea*	purple yam	2622.1 (170.7)	181.7 (3.5)	913.8 (47.4)	tr	1.6 (0.1)	145.8[f] (30.2)		19.0 (1.0)
Solanaceae	*Solanum*	eggplant	827.0 (96.8)	2.6 (0.1)	965.9 (115.7)		147 (11.93)	*g*		

[a] Standard deviations are in parentheses. [b] tr, traces. [c] Isomer of galactinol. [d] Galactinol and an isomer of sucrose (about 13 and 23 mg 100 g^{-1}, respectively). [e] Radish also contained 0.5 mg/100 g mannose. [f] Mainly maltose and isomaltose. [g] Free rutinose was detected in skin (1.9 mg 100 g^{-1}).

programmed to 300 °C at 15 °C min^{-1} and held for 40 min.[15] The injector temperature was kept at 300 °C, and injections were made in split mode with a split ratio of 1:20. The mass spectrometer was operated in electronic impact (EI) mode at 70 eV, scanning the 35−700 m/z range. The interface and source temperature were 280 and 230 °C, respectively. Acquisition was done using a HPChem Station software (Hewlett-Packard, Palo Alto, CA).

RESULTS AND DISCUSSION

The GC-MS method allowed the analysis of soluble carbohydrates (free saccharides, cyclitols, alditols, sugar acids, and glycosides) along with small amounts of free amino acids, phenolic acids, and other low molecular weight substances. Figure 1 shows the chromatographic profile of artichoke, oak leaf lettuce, and purple yam. The identity of peaks was assigned by GC-MS and confirmed by comparison of retention time and mass spectra with those of standard substances, when available. Otherwise identities were given as tentative.

Quantitative results (average values expressed as mg 100 g^{-1} and standard deviations) are shown in Tables 1 and 2. The detection (LOD) and quantitation (LOQ) limits of the method were calculated for each compound according to Foley and Dorsey.[31] Mean values of 0.13 and 0.4 mg 100 g^{-1} were obtained for LOD and LOQ, respectively.

Saccharides. Average values (mg 100 g^{-1} of product) and standard deviations of saccharides found in the vegetables are shown in Table 1. Predominant sugars in most vegetable in this study were fructose and glucose, these values being higher in onion (1.8 and 1.5 g 100 g^{-1}, respectively) and purple yam (2.6 and 0.9 g 100 g^{-1}, respectively). Sucrose appeared in all samples, ranging from 1 mg 100 g^{-1} in Lollo Rosso lettuce to 11 g 100 g^{-1} in beet root. Galactose occurred as a minor component in most samples, the highest values being found in cabbage and purple yam (61 and 181 mg 100 g^{-1}, respectively), whereas small amounts of mannose were found only in radish (0.5 mg 100 g^{-1}).

A carbohydrate with a mass spectrum compatible with a heptulose was also detected in chicory, spinach, cabbage, radish, and oak leaf lettuce at low levels (less than 2.1 mg 100 g^{-1}) and at trace levels in a few more. This saccharide was identified as sedoheptulose by comparison with a hot water extract of *Sedum spectabile* leaves. Heptuloses are common in Crassulaceae and have been detected in 26 plant families[32] and tropical fruits.[33] Soria et al.[17] have recently reported the presence of sedoheptulose in carrots ranging from 1.5 to 5.8 mg 100 g^{-1} of product, values slightly higher than those found in the vegetables analyzed in the present work. Besides sedoheptulose, other heptulose (probably manno-heptulose on the basis of its mass spectrum and retention time) appeared in spinach (0.8 mg 100 g^{-1}).

It is worth noting the presence of free rutinose (6-*O*-L-rhamnopyranosyl-D-glucose) in eggplant skin. This disaccharide is commonly present in different vegetal sources as a rutinoside. However, it is not frequently found in free form.

Apart from the small amounts of sucrose found in purple yam, some disaccharides, mainly maltose and isomaltose, were detected. The presence of maltose in yam has been previously reported.[34] The only trisaccharide detected in this plant was kestose.

A small peak eluting close to sucrose with a mass spectrum very similar to this disaccharide and differing only in the slightly higher relative abundance of the ion at m/z 271 was also detected in beet root. Taking into account its retention time and mass spectrum, it could correspond to a diastereomer of sucrose. However, it could not be confirmed considering that GC and MS data about diastereomers of sucrose are very scarce.[35] Galactinol, raffinose, and kestose, which have been reported in this tuber,[26,36] were also found in the present work.

Cyclitols. Cyclitol concentrations of analyzed vegetables are shown in Table 2. As expected, *myo*-inositol (which is the most abundant inositol in nature, occurring in both vegetal and animal kingdoms) was observed in all analyzed samples, ranging from 0.5 mg 100 g^{-1} in Lollo Rosso to 24.6 mg 100 g^{-1} in purple yam.

chiro-Inositol was found in all vegetables of *Cynara*, *Cichorium*, and *Lactuca* genus (chicory, endive, escarole, artichoke, and lettuces), all belonging to Asteraceae family. This cyclitol is a secondary messenger in insulin signal transduction, and different

Table 2. Polyalcohol and Inositol Concentrations (mg/100 g of Product) in Analyzed Vegetables[a]

family	genus	common name	mannitol[b]	*chiro*-inositol	*scyllo*-inositol	*myo*-inositol
Asteracea	*Cichorium*	chicory leaves	0.8 (0.5)	19.9 (2.3)	5.3 (0.1)	18.2 (0.4)
		endive	1.8 (0.4)	3.1 (0.2)	0.9 (0.1)	3.0 (0.0)
		escarole		4.4 (0.3)	tr[c]	4.1 (0.2)
	Cynara	artichoke		21.6 (1.7)	2.1 (0.3)	3.0 (0.2)
	Lactuca	Batavian lettuce		8.2 (1.1)		10.9 (0.9)
		iceberg lettuce		8.3 (0.2)		8.4 (1.1)
		oak leaf lettuce		32.6 (1.0)		8.0 (0.3)
		Lollo Rosso lettuce		9.0 (0.3)		0.5 (0.0)
		Romaine lettuce		3.5 (0.1)		4.1 (0.2)
		Cresta lettuce		13.2 (0.3)		18.4 (0.3)
Amarantacea	*Spinacia*	spinach	0.4 (0.5)			1.2 (0.1)
	Beta	beet root				1.7 (0.2)
Amarylidacea	*Allium*	onion				22.2 (2.1)
Brassicacea	*Raphanus*	radish	1.9 (1.7)			4.4 (0.9)
	Brassica	cabbage	3.2 (0.5)			18.1 (1.2)
Dioscoreacea	*Dioscorea*	purple yam	141.1 (7.2)		28.3 (0.1)	24.6 (1.6)
Solanacea	*Solanum*	eggplant			1.6 (0.0)	21.5 (0.2)

[a] Standard deviations are in parentheses ($n = 2$). [b] Mannitol was overlapped with small amounts of an unknown product with m/z fragments 157, 219, 244, and 375. [c] tr, traces.

studies have shown that it can help to treat women with polycystic ovarian syndrome by improving insulin sensitivity.[6,37] Previous studies have reported that *chiro*-inositol is frequently found in the Asteraceae family,[38] but to the best of our knowledge, its presence in the vegetables studied here has not been previously reported. Concentrations of *chiro*-inositol ranged from 3 mg 100 g^{-1} in endive to 33 mg 100 g^{-1} in oak leaf lettuce. It is also worth pointing out the relatively high values observed in artichoke where it was the most abundant LMWC detected along with sucrose. Its content in vegetables is lower than in citrus juices,[14] where it varied from 7 mg 100 mL^{-1} in lemon juice to 108 mg 100 mL^{-1} in mandarin orange juice.

Therapeutic properties related to cognitive deficit in AD pathologies have been attributed to *scyllo*-inositol.[39] Small amounts of *scyllo*-inositol were found in chicory, endive, escarole, artichoke, purple yam, and eggplant, varying from traces in escarole to 28.3 mg 100 g^{-1} in purple yam. This figure was lower than that reported by Soria et al.[17] in carrot, parsley, coriander, and fennel (2 mg g^{-1}). Quantitative differences in cyclitol concentrations among diverse cultivars of lettuces examined can be attributed to factors such as cultivar, season, and agricultural and postharvest treatments.

Alditols. Alditol concentrations of vegetables studied are also shown in Table 2. These polyalcohols play various physiological roles in vegetables.[18,19] Small amounts of mannitol appeared in chicory leaves, endive, spinach, radish, cabbage, and eggplant; however, its chromatographic peak was overlapped with an unknown product with fragments at m/z 157, 219, 244, and 375 in its mass spectrum. The highest level of mannitol (141.1 mg 100 g^{-1}) was found in purple yam, where the interference was not detected.

Other Compounds. Glucaric acid also appeared in a small concentration in cabbage and spinach, as previously reported;[41,42] this sugar acid was also detected in purple yam in this work at notably higher levels (about 36 mg 100 g^{-1} of product). This acid has been shown to promote some beneficial effects on health.[40,41]

Small amounts of glycosyl glycerols were detected in cabbage (1.0 mg 100 g^{-1}), purple yam (2.5 mg 100 g^{-1}), eggplant (0.17 mg 100 g^{-1}), and spinach (0.22 mg 100 g^{-1}). Minute amounts (not quantified) of sugar phosphates were found in chicory and cabbage.

Final Remarks. The exhaustive analysis of LMWC in natural sources is a difficult task, due to the high number of isomers present and the scarce availability of standards. However, the chosen GC-MS method has afforded the simultaneous quantification of saccharides, polyalcohols, acid sugars, and glycosides in different vegetables. It has also allowed the identification for the first time of several remarkable and/or bioactive compounds such as *chiro*-inositol, *scyllo*-inositol, sedoheptulose, free rutinose, α-galactosides, kestose, glucaric acid, and glycosyl-glycerols in the vegetables studied.

A recent review by Fardet[42] offered new perspectives about the health-protective effects of whole grain cereals, considering the high number of bioactive compounds present. Similar hypotheses can be set out about the vegetables studied here. They contain different bioactive LMWC, besides fiber, vitamins, antioxidants, and prebiotics.

Considering the therapeutic potential of cyclitols (treatment of conditions associated with insulin resistance, polycystic ovary syndrome, respiratory distress syndrome, AD-like pathologies, etc.), some of the vegetables such as chicory leaves, artichokes, and lettuces should be included in special diets to increase their consumption. Moreover, purple yam has shown to be a valuable source of bioactive carbohydrates. Removal of nonbioactive mono- and disaccharides (glucose, fructose, and sucrose) from these vegetable sources to obtain special ingredients for diabetics could be a new route for future investigations.

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4.3. Optimisation of a biotechnological procedure for selective fractionation of bioactive inositols in edible legume extracts.

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Optimisation of a biotechnological procedure for selective fractionation of bioactive inositols in edible legume extracts

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Abstract

BACKGROUND: Currently, disorders such as diabetes mellitus, obesity or atherosclerosis are recognised as major global health problems. The use of inositols for treating these illnesses has attracted considerable attention and their extraction from natural sources presents added value as they are considered bioactive ingredients in the food industry. Legumes are natural and rich sources of inositols; however, the co-existence of other low molecular weight carbohydrates (LMWCs) in their extracts, which interfere in their bioactivity, might constitute an important drawback, thereby making their removal essential.

RESULTS: LMWCs, including inositols, methyl-inositols and glycosyl-inositols of different legume extracts, were determined by GC-MS; the presence of bornesitol (2.35 mg g^{-1}) and lathyritol (0.27 mg g^{-1}) were reported for the first time in grass peas. The use of *Saccharomyces cerevisiae* for the selective removal of interfering carbohydrates was optimised. Incubation time (3–40 h) was highly dependent on the composition of the legume considered; inositol contents were generally stable along the treatment.

CONCLUSION: Removal of interfering LMWCs from inositol-enriched extracts was successfully achieved using a clean and easily scalable fractionation methodology. This biotechnological procedure not only represents high interest for the production of bioactive food ingredients but for applications in other research areas.

Keywords: legume; fractionation; *Saccharomyces cerevisiae*; inositols; methyl-inositol; glycosyl-inositols

INTRODUCTION

Inositols are cyclic polyalcohols with molecular formula $C_6H_{12}O_6$. There are nine inositol stereoisomers: *myo*-inositol (*cis*-1,2,3,5-*trans*-4,6-cyclohexanehexol) is the most common and abundant in nature. Other natural isomers, present in lower proportions, are *scyllo*-inositol (*cis*-1,3,5-*trans*-2,4,6-cyclohexanehexol), *muco*-inositol (*cis*-1,2,4,5-*trans*-3,6-*trans*-cyclohexanehexol), *chiro*-inositol (*cis*-1,2,4-*trans*-3,5,6-cyclohexanehexol) and *neo*-inositol (*cis*-1,2,3-*trans*-4,5,6-cyclohexanehexol).

Several bioactive properties, mainly associated with insulin resistance, have been attributed to inositols and their derivatives (methyl-inositols and galactosyl-inositols).[1] These naturally occurring carbohydrates have been proposed for treating disorders such as diabetes mellitus, obesity, atherosclerosis, hypertension and polycystic ovary syndrome.[2–4] Moreover, inositols do not show gastroenteric or hepatic side effects such as those of other conventional oral hypoglycaemics.[5]

Inositols have also been described for the treatment of premature infants with respiratory distress syndrome[6] and are widely used in cosmetic applications for improving the oxygenation of cutaneous tissues.[7] Regarding their technological functionality, inositols have been described for enhancing or accelerating swelling of thickeners in aqueous solutions of pharmaceutical, cosmetic and food compositions.[8]

The wide interest in obtaining extracts enriched in bioactive inositols for their further use in the elaboration of functional foods has promoted the search for natural food-grade sources of these compounds. Free inositols (*myo*- and *chiro*-) and methyl-inositols (pinitol derived from *chiro*-inositol; ononitol and sequoyitol from *myo*-inositol) have been detected in edible legume seeds.[4,9,10] Regarding legume galactosyl-inositols, there are four main families: those derived from *myo*-inositol (galactinol isomers), pinitol (galactopinitols), ononitol (galactosyl-ononitol) and *chiro*-inositol (fagopyritols).[11,12]

The presence in legumes of other low molecular weight carbohydrates (LMWCs) (e.g. mono- and disaccharides such as glucose, fructose or sucrose) might constitute an important drawback, as these latter compounds may (1) interfere in the bioactivity of legume extracts intended as functional foods for

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diabetic individuals, and (2) increase calorie content of legume extracts.

Ion exchange resins[13] and activated charcoal[14,15] have been commonly used to enrich extracts in inositols. Nevertheless, these treatments are tedious and poorly efficient in terms of recovery of inositols. Therefore, the development of novel fractionation methodologies that allow enrichment of inositols in these extracts and which are compatible with food industry requirements are of great interest.

Yeasts have been commonly used for the purification and removal of mono- and disaccharide by-products in carbohydrate preparations.[16,17] They have also been applied to remove major sugars present in complex mixtures thereby allowing the analysis and identification of minor carbohydrates[18,19] or the *in vitro* evaluation of their bioactivity.[20,21] Among yeast strains, *Saccharomyces cerevisiae*, considered as a safe organism,[22] has been extensively used in the field of food processing.

Therefore, the aim of this work was to optimise and evaluate a biotechnological procedure based on the use of *S. cerevisiae* for the selective removal of interfering LMWCs present in different legume extracts (black-eyed peas, buckwheat, carob pods, chickpeas, grass peas, lentils and soy beans). Furthermore, LMWC composition (including inositols and their derivatives) of these legume extracts was also studied in detail.

MATERIALS AND METHODS

Standards

Analytical standards of fructose, *chiro*-inositol, pinitol (3-*O*-methyl-D-*chiro*-inositol), glucose, galactose, *myo*-inositol, sucrose (α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside), galactinol (*O*-α-galactopyranosyl-(1 → 1)-L-*myo*-inositol), trehalose (α-D-glucopyranosyl-(1 → 1)-α-D-glucopyranoside), maltose (α-D-glucopyranosyl-(1 → 4)-D-glucopyranose), melibiose (α-D-galactopyranosyl-(1 → 6)-D-glucopyranose), melezitose (α-D-glucopyranosyl-(1 → 3)-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside), raffinose (α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside), stachyose (α-D-galactopyranosyl-(1 → 6)-α-D-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside), verbascose (α-D-galactopyranosyl-(1 → 6)-α-D-α-D-galactopyranosyl-(1 → 6)-galactopyranosyl-(1 → 6)-α-D-glucopyranosyl-(1 → 2)-β-D-fructofuranoside) and phenyl-β-D-glucoside were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Samples

Seeds of black-eyed peas (*Vigna unguiculata*), buckwheat (*Fagopyrum esculentum*), carob pods (*Ceratonia siliqua*), chickpeas (*Cicer arietinum*), grass peas (*Lathyrus sativus*), lentils (*Lens culinaris*) and soy beans (*Glycine max*) were obtained from a local market in Madrid (Spain). Samples were ground using a domestic mill (Moulinex, Barcelona, Spain) and sieved through a 500 μm (35 mesh) sieve before extraction.

Carbohydrate extraction

An exhaustive extraction of LMWCs from edible legumes (1 g) was achieved by combining three consecutive extracts, each obtained with 10 mL of hot Milli-Q water (60°C) for 2 h under constant stirring. Combined extracts were filtrated through Whatman No. 4 paper and kept at −20°C until analysis.

Yeast treatment

Yeasts (*S. cerevisiae*) (0.25 g) from two different sources, *Saccharomyces cerevisiae* type II (Sigma Chemical Co.) and bakery yeast (Maizena; Unilever, Barcelona, Spain), were activated with 1.5 mL of Milli-Q water at the selected incubation temperature (30 or 37°C) for 30 min under stirring (160 rpm).

Different mixtures of carbohydrates (5 g total weight/mixture) were used to evaluate yeast behaviour: (1) glucose + fructose + sucrose + maltose + raffinose + melezitose; (2) melibiose + sucrose; (3) *myo*-inositol; (4) *myo*-inositol + sucrose; and (5) glucose + fructose. Mixtures were incubated with activated yeasts in 25 mL of Milli-Q water at the relevant temperature and aliquots were taken at 0 (corresponding to the beginning of the experiment), 5, 24, 52 and 76 h of treatment. Samples were centrifuged at 4400 × *g* (MiniSpin; Eppendorf, Madrid, Spain) for 10 min and filtered through Whatman No. 4 paper to remove yeasts.

Aqueous legume extracts (10 mL) were incubated with 0.125 g of *S. cerevisiae* type II (Sigma Chemical Co.) at 37°C under stirring. Aliquots were taken before yeast addition (control) and after 0, 3, 5, 8, 17, 24 and 40 h of treatment. Samples were then treated as indicated above. All assays were carried out in triplicate.

Chromatographic analysis: derivatisation procedure

One millilitre of legume extract was mixed with 0.5 mL of a 70% ethanolic solution of an internal standard (phenyl-β-D-glucoside, 1 mg mL^{-1}) and evaporated under vacuum (38-40°C) prior to carbohydrate derivatisation. Trimethylsilyl oximes were prepared according to Sanz *et al.*[20] In brief, oximes were obtained by addition of 350 μL of a solution 2.5% hydroxylamine chloride in pyridine after 30 min at 75°C. They were then silylated with hexamethyldisilazane (350 μL) and trifluoroacetic acid (35 μL) at 45°C for 30 min. After reaction, samples were centrifuged at 4400 × *g* for 10 min, and the supernatant was subjected to gas chromatography–mass spectometry (GC-MS) analysis.

Gas chromatography–mass spectrometry analysis

GC-MS analysis of derivatised samples was carried out using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (both from Agilent Technologies, Palo Alto, CA, USA). Analyses were carried out on a HT5 (5% phenyl polycarborane–siloxane) capillary column (25 m × 0.22 mm i.d. × 0.1 μm film thickness; SGE, Ringwood, Australia), using helium at approx. 1 mL min^{-1} as carrier gas. The oven temperature was programmed as follows: 180°C (10 min) at 5°C min^{-1} to 200°C (15 min), then at 15°C min^{-1} to 270°C, at 1°C min^{-1} to 290°C, at 15°C min^{-1} to 300°C (15 min) and finally at 15°C min^{-1} to 360°C (15 min). Injections (1 μL) were carried out in split mode (1:20) at 300°C. The mass spectrometer was operated in electron impact mode at 70 eV, scanning the 35–700 *m/z* range. The transfer line and ionisation source were heated at 280 and 230°C, respectively. Acquisition was done using HP ChemStation software (Agilent Technologies).

Qualitative analysis was based on the evaluation of experimental linear retention indices (I^T) and mass spectra. *n*-Alkanes from C8 to C40 were considered for I^T calculation. Identities were confirmed, when possible, by using available standards. Bornesitol (D-1-*O*-methyl-*myo*-inositol) and lathyritol (α-D-galactopyranosyl-(1 → 3)-1-*O*-methyl-*myo*-inositol) were identified by comparison with those present in sweet pea (*Lathyrus odoratus* L.) extracts.[23] When standards were not available, identities were given as tentative.

For quantification, solutions of LMWC standards over the expected concentration range in legume extracts were prepared to calculate the response factor (RF) relative to phenyl-β-D-glucoside. RFs of pinitol, galactinol, maltotriose and stachyose were respectively used for bornesitol (1-*O*-methyl-*myo*-inositol) and ononitol (4-*O*-methyl-*myo*-inositol), galactosyl- and galactosyl-methyl-inositols, di-galactosyl- and di-galactosyl-methyl-inositols and tri-galactosyl-methyl-inositols, for which standards were not commercially available. For co-eluting compounds, relative abundances of characteristic *m/z* ions were considered.

Statistical analysis

Statistical analysis was performed using Statistica 7.0 (StatSoft Inc., Tulsa, OK, USA). Univariate analysis of variance and the Fisher test were used to evaluate significant differences among treatments. The differences were considered to be significant when $P < 0.05$.

RESULTS AND DISCUSSION

Low molecular weight carbohydrate composition of legume extracts

Inositols and derivatives

Table 1 shows the inositol, methyl-inositol and glycosyl-inositol content of the different legume extracts under study. *myo*-Inositol was found in all the legume extracts analysed, at concentrations ranging from 0.24 mg g^{-1} for black-eyed peas to 1.22 mg g^{-1} for chickpeas; comparable values were reported by Clements and Darnell[10] for lentils (0.45 mg g^{-1}), black-eyed peas (0.39 mg g^{-1}) and soy beans (0.88 mg g^{-1}). *chiro*-Inositol was present at lower concentrations (from 0.02 mg g^{-1} in chickpeas to 0.59 mg g^{-1} in buckwheat) and was not detected in black-eyed peas and grass peas.

As previously reported by other authors, pinitol ($I^T = 1742$) was detected in soy beans,[9] chickpeas,[9] lentils[12,24] and carob extracts;[18] its highest concentration (112.7 mg g^{-1} of sample) was found in this latter legume. A methyl-inositol, identified as ononitol according to Yasui *et al.*,[11] and with the same I^T of the first isomer of the trimethylsilyl oxime of glucose ($I^T = 1896$), was detected in black-eyed peas (Fig. 1A, solid line, peak 5). A typical mass fragmentogram (*m/z* 260) was used for quantitative analysis of this methyl-inositol (2.03 mg g^{-1}). An unknown chromatographic peak with similar mass spectra to pinitol and ononitol but different I^T value (1952) was detected in grass peas and lentils at concentrations of 2.35 and 0.08 mg g^{-1} sample, respectively. This peak was tentatively assigned to bornesitol by comparison of its experimental retention index and mass spectral data with those of the methyl-inositol found in a hot water extract of sweet pea (*Lathyrus odoratus* L.) seeds, where bornesitol had previously been identified.[23] To the best of our knowledge, this is the first time that this methyl-inositol has been found in these legumes.

Regarding glycosyl-inositols, fagopyritol B1 was found at relatively high concentrations in buckwheat (2.38 mg g^{-1}) and at lower levels in soy beans (0.10 mg g^{-1}). Small concentrations of fagopyritol A1 (0.08 mg g^{-1}) and traces of fagopyritol A2 and B2 were only detected in buckwheat. Previous studies have described the presence of fagopyritols (*O*-α-galactopyranosyl-D-*chiro*-inositols) in buckwheat.[25] Although Hoch *et al.*[26] reported high contents of fagopyritols in lentils, these compounds were not detected in the lentil extracts analysed. Galactinol was found at relatively high concentrations in chickpeas and black-eyed peas

Table 1. Linear retention indices (I^T) and content (mg g^{-1} sample) of inositols and inositol derivatives present in legume extracts

	Inositols		Methyl-inositols			Galactosyl-inositols			Di-galactosyl-inositols	Galactosyl-methyl-inositols			Di-galactosyl-methyl-inositols		Tri-galactosyl-methyl-inositols
Legume/I^T	*chiro*-1874	*myo*-2048	Pin 1742	Born 1952	Ono[a] 1896	Galac 2875	FP A1 2743	FP B1 2803	Un 2567-3012	Un 3737	Gal-ono 2814	Gal-pin A 2631	Gal-pin B 2721	Lath 2762	Cicerit 3492	Un 3518	Un 4201
Black-eyed pea	—	0.24 (0.01)[b]	—	—	2.03 (0.02)	0.23 (0.01)	—	—	—	—	0.31 (0.01)	—	—	—	—	—	—
Buckwheat[c]	0.59 (0.03)	0.78 (0.01)	—	—	—	0.02 (0.00)	0.08 (0.01)	2.38 (0.07)	0.39 (0.03)	—	—	—	—	—	—	—	—
Carob	0.57 (0.06)	0.91 (0.05)	112.71 (5.33)	—	—	—	—	—	0.18 (0.06)	—	—	—	—	—	—	—	—
Chickpea	0.02 (0.01)	1.22 (0.02)	1.95 (0.04)	tr	—	0.49 (0.03)	—	—	—	0.61 (0.22)	—	2.79 (0.09)	0.24 (0.01)	—	24.31 (0.60)	2.36 (0.07)	1.89 (0.32)
Grass pea	—	0.89 (0.06)	—	2.35 (0.10)	—	0.04 (0.04)	—	—	—	—	—	—	—	0.27 (0.01)	—	—	—
Lentil	0.09 (0.02)	0.94 (0.03)	1.97 (0.01)	0.08 (0.00)	—	0.08 (0.00)	—	—	—	0.08 (0.01)	—	0.63 (0.01)	0.07 (0.00)	—	6.08 (0.02)	1.07 (0.04)	1.06 (0.24)
Soy bean	0.11 (0.00)	0.47 (0.01)	3.48 (0.22)	—	—	—	—	0.10 (0.04)	—	—	—	0.80 (0.06)	0.66 (0.05)	—	—	—	—

[a] Quantification based on the mass fragmentogram (*m/z* 260).
[b] Average for $n = 3$ replicates. The standard deviation is in brackets.
[c] Traces of fagopyritol A2 and B2 were found in buckwheat.
Pin, pinitol; Born, bornesitol; Ono, ononitol; Galac, galactinol; FP, fagopyritol; Lath, lathyritol; Cicerit, ciceritol; Gal, galactosyl; Un, unidentified; tr, traces.

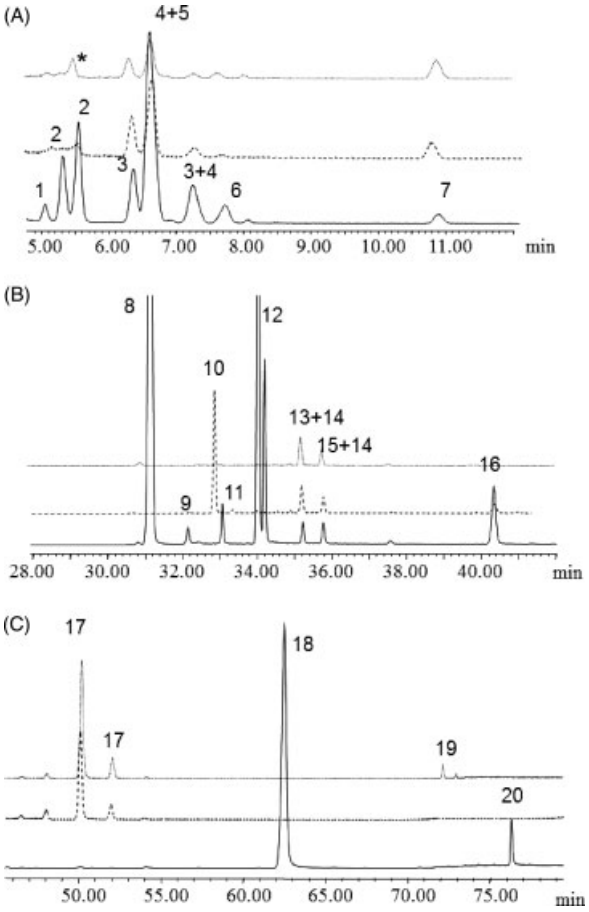

Figure 1. Chromatographic profile of black-eyed peas extract before (control; solid line) and after 3h (dashed line) and 24 h (dotted line) of yeast treatment. Peak identification: (1) unidentified monosaccharide, (2) fructose, (3) galactose, (4) glucose, (5) ononitol, (6) glucuronic acid, (7) *myo*-inositol, (8) sucrose, (9) unidentified disaccharide, (10) trehalose, (11) unidentified disaccharide, (12) maltose, (13) galactosyl-ononitol, (14) melibiose, (15) galactinol, (16) raffinose, (17) galactopyranosyl-(1 → 6)-α-galactopyranosyl-(1 → 6)-α-glucopyranoside, (18) stachyose, (19) galactopyranosyl-(1 → 6)-α-galactopyranosyl-(1 → 6)-α-galactopyranosyl-(1 → 6)-α-glucopyranoside, (20) verbascose, *interference.

(0.49 and 0.23 mg g^{-1}, respectively), and was also detected in lentils, grass peas and buckwheat at lower levels (0.08, 0.04 and 0.02 mg g^{-1}, respectively). Other unidentified galactosyl-inositols and di-galactosyl-inositols were detected in buckwheat (0.39 mg g^{-1}) and carob (0.18 mg g^{-1}), and in chickpeas (0.61 mg g^{-1}) and lentils (0.08 mg g^{-1}), respectively.

As previously reported by other studies,[9,12,24,26] galactopinitol A and B ($I^T = 2631$ and 2721) were found in chickpeas (3.03 mg g^{-1}), soy beans (1.46 mg g^{-1}) and lentils (0.70 mg g^{-1}) (Table 1), while galactosyl-ononitol ($I^T = 2814$) was only detected in black-eyed peas (0.31mg g^{-1}, Fig. 1B, solid line, peak 13).[11] Similarly to bornesitol, lathyritol ($I^T = 2762$) was detected for the first time in grass peas (0.27 mg g^{-1}), and tentatively identified by comparison with a hot water extract of sweet pea seeds where its presence had previously been reported.[23] It is also worth noting that this is the first time that lathyritol has been found in grass peas.

Remarkable concentrations of di-galactosyl-methyl-inositols, mainly ciceritol (galactopyranosil-(1 → 6)-galactopyranosil-(2 → 4)-*O*-methyl-*chiro*-inositol), and tri-galactosyl-methyl-inositols were also detected in chickpeas and lentils.

Saccharides

Total carbohydrate content (from mono- to pentasaccharides) of legume extracts ranged from 11.8 mg g^{-1} of buckwheat to 358.3 mg g^{-1} of carob. Fructose (0.33–20.51 mg g^{-1}), galactose (0.09–8.09 mg g^{-1}), glucose (0.60–23.21 mg g^{-1}) and sucrose (9.23–270.80 mg g^{-1}) were detected in all samples, except for galactose in carob (Table 2). Maltose was also present in lentils, soy beans, grass peas and black-eyed peas (0.33, 0.51, 0.71 and 11.72 mg g^{-1}, respectively), whereas other unidentified disaccharides ($I^T = 2564–2772$) were detected in carob and black-eyed peas. Different pentosyl-aldoses ($I^T = 2633$, 2672, 2680, 2715) were also found in carob.

It is widely known that oligosaccharides of the raffinose family are ubiquitous in legume seeds. Raffinose (0.20–3.28 mg g^{-1}), stachyose (14.49–50.23 mg g^{-1}) and verbascose (0.42–17.31 mg g^{-1}) were quantified in all legumes with the exception of carob and buckwheat; the highest concentrations were found in chickpeas, black-eyed peas and grass peas for raffinose, stachyose and verbascose, respectively.

Optimisation of the yeast treatment

In order to optimise a procedure for the selective removal of LMWCs that might interfere with the bioactive properties of inositols in legume extracts, the behaviour of different standards incubated with *S. cerevisiae* (Maizena) was evaluated. Figure 2 shows the evolution of a mixture of mono- (glucose and fructose), di- (sucrose and maltose) and trisaccharides (raffinose and melezitose) with incubation time (0–76 h) at 30°C (mixture 1). Raffinose and sucrose were totally removed during the yeast treatment and their relative concentrations reduced to around 0.1% just after 5 h of incubation. These carbohydrates were hydrolysed into glucose and fructose (in the case of sucrose) and into fructose and melibiose (in the case of raffinose) by the action of the extracellular invertase (β-fructosidase) present in yeast. Therefore, glucose and fructose concentrations increased at the beginning of the treatment, and they were subsequently metabolised by yeast until they disappeared after 52 and 76 h, respectively. This behaviour has previously been observed by other authors.[27] Glucose decreased more quickly than fructose, probably because both sugars share the same membrane transport components and yeast has a higher affinity for glucose than for fructose.[28] Another reason for this behaviour could be that glucose can repress the expression of specific fructose transports.[29] The melibiose produced could not be fermented by yeast and its concentration remained constant during the process. This behaviour was also confirmed by incubating melibiose and sucrose (mixture 2) with yeast: whereas sucrose immediately disappeared, melibiose remained intact throughout the entire incubation period. Regarding maltose, only a slight decrease was observed over the entire process. This behaviour was also observed by Mwesigye and Barford[30] for a mixture of sucrose and maltose and is probably due to the presence of other highly fermentable sugars in the medium, such as glucose or sucrose, which produce a catabolite repression. Melezitose concentration was constant during the incubation; invertases and α-glucosidases were not able to hydrolyse the linkages between glucose and fructose units in this non-reducing trisaccharide. Similar results were found by Yoon *et al.*[16] for this sugar.

myo-Inositol was also subjected to yeast treatment (mixture 3). No changes in the concentration of this polyalcohol were detected during the incubation time. A similar behaviour was observed when a mixture of *myo*-inositol and sucrose (mixture 4) was incubated with *S. cerevisiae*.

Table 2. Linear retention indices (I^T) and content (mg g^{-1} sample) of mono-, di- and oligosaccharides present in legume extracts

	Monosaccharides			Disaccharides				Oligosaccharides		
	Fructose	Galactose	Glucose	Sucrose	Maltose	Pentosyl-aldoses	Unidentified	Raffinose	Stachyose	Verbascose
Legume/I^T	1846; 1858	1888; 1920	1896; 1920	2517	2697; 2715	2633; 2672; 2680; 2715	2564–2772	3158	3980	4301
Black-eyed pea	1.64 (0.02)[a]	1.52 (0.09)	5.18 (0.13)	17.67 (0.17)	11.72 (0.30)	—	0.04 (0.00); 0.05 (0.01)	2.37 (0.08)	50.23 (1.23)	5.97 (0.35)
Buckwheat	0.33 (0.01)	0.09 (0.00)	2.18 (0.22)	9.23 (0.52)	—	—	—	—	—	—
Carob	20.51 (1.98)	—	23.21 (1.72)	270.80 (5.64)	—	43.24 (3.45)	0.51 (0.01)	—	—	—
Chickpea	0.35 (0.03)	2.99 (0.01)	0.60 (0.04)	39.03 (1.09)	—	—	—	3.28 (0.09)	20.97 (1.27)	0.57 (0.07)
Grass pea	3.25 (0.40)	4.50 (0.30)	5.22 (0.65)	12.73 (0.36)	0.71 (0.09)	—	—	0.20 (0.04)	14.49 (2.05)	17.31 (2.82)
Lentil	1.11 (0.02)	8.09 (0.21)	1.53 (0.02)	16.33 (0.15)	0.33 (0.00)	—	—	0.22 (0.00)	15.66 (0.86)	9.24 (1.23)
Soy bean	4.78 (0.09)	2.41 (0.35)	6.13 (0.17)	45.82 (1.27)	0.51 (0.05)	—	—	0.70 (0.04)	23.08 (0.65)	0.42 (0.05)

[a] Average for $n = 3$ replicates and standard deviation in brackets.

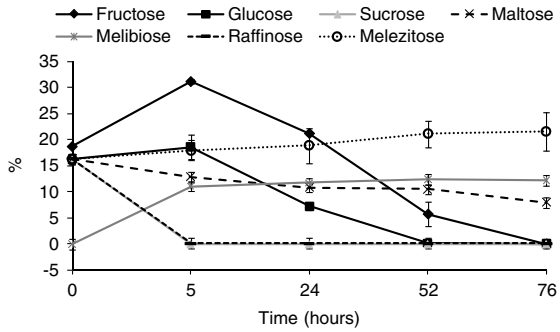

Figure 2. Behaviour of glucose, fructose, sucrose, maltose, raffinose and melezitose during incubation with *Saccharomyces cerevisiae* at 30°C for 0–76 h.

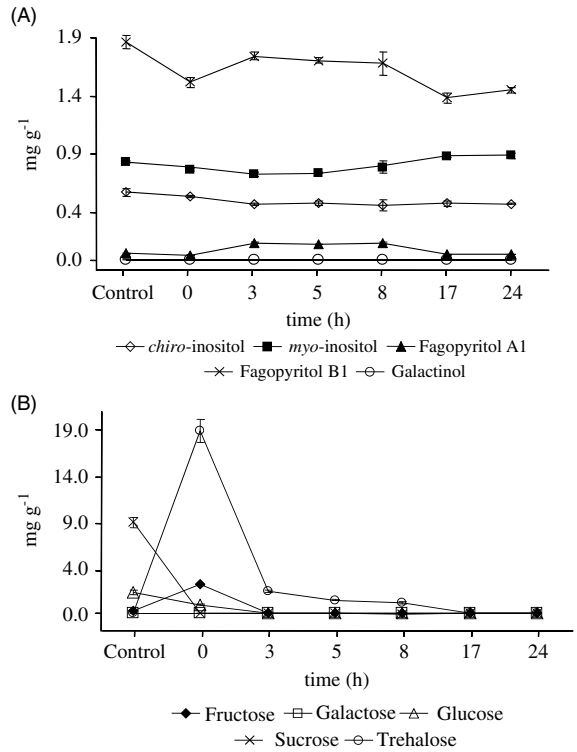

Figure 3. Evolution of the content of inositols (A) and other low molecular weigh carbohydrates (B) in buckwheat extract during yeast treatment at 37°C.

In order to evaluate if the fermentation process could be speeded up by increasing the incubation temperature, fermentation of a mixture of glucose and fructose standards (mixture 5) with *S. cerevisiae* was carried out at 37°C and results were compared with those obtained at 30°C. Whereas 76 h were necessary for glucose to disappear at 30°C, this result was achieved in only 24 h at 37°C. Regarding fructose, 25% of this carbohydrate remained after 76 h of incubation at 30°C, whereas it completely disappeared after 52 h at 37°C. Therefore, 37°C was selected for further incubations.

The effect of two different strains of yeast, *S. cerevisiae* type II (Sigma Chemical Co.) and bakery yeast (Maizena), was also evaluated on fermentation of glucose + fructose. Similar results were obtained in both cases, *S. cerevisiae* type II (Sigma Chemical Co.) was considered for further studies because of its higher feasibility for acquisition.

Table 3. Low molecular weight carbohydrate composition (mg g^{-1} sample) of legume extracts before (Control) and after the optimal time of fermentation with *Saccharomyces cerevisiae*

Legume	Optimal incubation time (h)	Free inositols	Methyl-inositols	Galactosyl-inositols	Monosaccharides	Disaccharides[a]	Oligosaccharides
Black-eyed pea	Control	0.24 (0.01)[b]	2.03 (0.02)	0.54 (0.02)	8.34 (0.25)	29.47 (0.11)	58.56 (1.67)
	24	0.28 (0.01)	1.22 (0.04)	0.57 (0.06)	0.68 (0.01)	—	25.19 (1.77)
Buckwheat	Control	1.37 (0.03)	—	3.07 (0.02)	2.60 (0.21)	9.31 (0.51)	—
	17	1.31 (0.02)	—	2.06 (0.08)	—	0.01 (0.02)	—
Carob	Control	1.47 (0.13)	112.71 (5.33)	0.21 (0.01)	46 (3.83)	316.84 (20.65)	0.51 (0.01)
	3	1.54 (0.33)	134.05 (3.16)	7.59 (1.73)	—	43.24 (3.45)	—
Chickpea	Control	1.24 (0.01)	1.95 (0.04)	31.40 (0.66)	3.94 (0.01)	39.03 (1.09)	24.83 (0.86)
	17	1.39 (0.02)	2.10 (0.15)	27.96 (2.83)	—	—	5.08 (1.62)
Grass pea	Control	0.89 (0.06)	2.35 (0.10)	0.32 (0.05)	11.57 (2.42)	13.19 (0.77)	32.03 (4.85)
	24	1.01 (0.06)	2.48 (0.08)	0.25 (0.00)	—	—	7.29 (0.53)
Lentil	Control	1.03 (0.01)	2.05 (0.01)	8.35 (0.12)	10.73 (0.21)	16.66 (0.15)	25.12 (2.08)
	24	1.08 (0.01)	2.02 (0.11)	8.10 (0.30)	0.07 (0.12)	0.25 (0.06)	3.20 (0.24)
Soy bean	Control	0.58 (0.01)	3.48 (0.22)	1.56 (0.14)	13.32 (0.52)	46.34 (1.23)	24.20 (0.65)
	40	0.76 (0.01)	3.63 (0.27)	1.30 (0.04)	—	1.34 (0.11)	12.70 (1.35)

[a] Quantification of melibiose based on mass fragmentogram (*m/z* 422).
[b] Average for $n = 3$ replicates and standard deviation in brackets.

Incubation of legume extracts with *Saccharomyces cerevisiae*

Once the behaviour of standards during incubation with yeast was determined, evaluation of the optimal time to remove interfering LMWCs while preserving bioactive inositols was carried out in the different legume extracts under study. As an example, Fig. 3 shows the evolution of carbohydrates of buckwheat during the yeast treatment. As expected, concentration of *chiro*- and *myo*-inositol (Fig. 3A) remained constant during the incubation. No significant variations were detected for fagopyritol A1 and galactinol either, whereas a slight decrease was detected for fagopyritol B1. Regarding other LMWCs (Fig. 3B), as previously observed with standards, sucrose was completely hydrolysed by yeast into glucose and fructose at the beginning of the treatment; the first monosaccharide was rapidly fermented whereas an increase in fructose content was observed, the content of this monosaccharide decreased after 3 h of incubation. Curiously, galactose, which was present at the lowest concentration in buckwheat extract (0.09 mg g^{-1}), was slowly fermented. Complete removal was detected after 17 h of treatment. This behaviour was similar to that observed for maltose in the mixture of standards and could be due to the repression effect, which ensures that the preferred sugars (glucose, sucrose, etc.) are metabolised before the consumption of alternative carbohydrates, such as maltose and galactose.[29] Other authors[21] have concluded that the level of ethanol produced during the fermentation of other carbohydrates, present at higher amounts in the sample, could inhibit the assimilation of galactose by the yeast cells. From the beginning of the treatment, trehalose was synthesised by *S. cerevisiae* as previously reported by Jules *et al.*[31] This carbohydrate was further used as a carbon source for growth and later disappeared at 17 h of treatment.

Figure 1 shows the gas chromatographic profiles obtained for the black-eyed pea extract before (control) and after 3 and 24 h of yeast treatment. As previously indicated, raffinose was quickly hydrolysed to melibiose ($I^T = 2811$; 2877, peak 14); both isomers of this disaccharide coeluted with galactosyl-ononitol ($I^T = 2814$, peak 13) and galactinol ($I^T = 2875$, peak 15), respectively. Similarly, stachyose (peak 18) and verbascose (peak 20) were rapidly hydrolysed giving rise to a tri- ($I^T = 3595$; 3663, peak 17) and a tetrasaccharide ($I^T = 4221$, peak 19), respectively. These compounds were identified as galactopyranosyl-(1 → 6)-α-galactopyranosyl-(1 → 6)-α-glucopyranoside and galactopyranosyl-(1 → 6)-α-galactopyranosyl-(1 → 6)-α-galactopyranosyl-(1 → 6)-α-glucopyranoside, taking into account the relative abundance in their mass spectra of an ion at *m/z* 422, previously described as characteristic of 1–6 linkages.[32] These oligosaccharides were not fermented during the 40 h of incubation, as α-galactosidases are not present in yeast.[16] However, for our purposes, these resulting galacto-oligosaccharides are not considered interfering compounds, as these enzymes are either not present in the upper part of the human gastrointestinal tract. Therefore, these galacto-oligosaccharides would probably reach the distal parts of the colon, where they could be selectively fermented by bacteria, conferring beneficial properties to the host as previously reported by other authors.[33] After 3 h of treatment, relatively high amounts of trehalose (produced by *S. cerevisiae*, peak 10) and galactose (peak 3) were still remaining. These carbohydrates were noticeably reduced, in the case of galactose, or totally removed, in the case of trehalose, after 24 h of incubation.

Required *S. cerevisiae* incubation time for the selective removal of LMWC in each legume extract under optimised conditions and the carbohydrate concentrations obtained after fractionation are shown in Table 3. Time of incubation was considered optimal when only 5% or less of interfering carbohydrates remained in the extracts. This time ranged from 3 h for carob extract to 40 h for soy bean extract. In general, optimal times were more dependent on the nature of the mixture of carbohydrates present in the extracts than on their concentrations. As an example, carob extracts contained the highest amount of total carbohydrates (Table 2) and 3 h of incubation were enough to substantially reduce monosaccharide and sucrose contents. However, the remaining amount of disaccharides (43.24 mg g^{-1}) remained constant during the 40 h evaluated (data not shown). The reason for this behaviour was that sucrose was the main disaccharide of carob, which was immediately hydrolysed at the beginning of the treatment; however, the remaining amount of disaccharides corresponding to pentosyl-aldoses (see Table 2) could not be metabolised by yeast. To the best of our knowledge, no information about fermentation of pentosyl-aldoses has been reported; however,

regarding pentoses, Yoon *et al*.[16] observed that xylose is not fermentable at all by yeast, whereas 33% of ribose is fermented.

Regarding soy bean extract, the long incubation time required (40 h) was due to the co-existence of small amounts of galactose with higher concentrations of preferred sugars (fructose, glucose and sucrose), which are metabolised before the consumption of alternative carbohydrates.

As previously proved with standards, free inositols were generally stable throughout the incubation time; an increase in free inositol concentration was only observed in soybeans and grass peas, probably as a result of the partial hydrolysis of corresponding galactosyl-inositols. In general, slight variations in methyl-inositol and galactosyl-inositol contents were also observed; these differences could probably be attributed to its own hydrolysis or to the breakage of carbohydrates with higher degree of polymerization taking place to different extents depending on the inositol derivative considered.

CONCLUSION

In view of that stated above, treatment with *S. cerevisiae* seems to be an effective fractionation methodology that allows the required removal of interfering carbohydrates while preserving the total inositol content of edible legume extracts. The low sample manipulation required, together with the fact of being a clean and easily scalable technique, makes this fractionation procedure an interesting, generally applicable alternative for the production of other bioactive food ingredients.

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4.4. Optimization of pressurized liquid extraction of inositols from pine nuts (*Pinus pinea* L.)

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Analytical Methods

Optimization of pressurized liquid extraction of inositols from pine nuts (*Pinus pinea* L.)

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ABSTRACT

Pressurized liquid extraction (PLE) has been used for the first time to extract bioactive inositols from pine nuts. The influence of extraction time, temperature and cycles of extraction in the yield and composition of the extract was studied. A quadratic lineal model using multiple linear regression in the stepwise mode was used to evaluate possible trends in the process. Under optimised PLE conditions (50 °C, 18 min, 3 cycles of 1.5 mL water each one) at 10 MPa, a noticeable reduction in extraction time and solvent volume, compared with solid–liquid extraction (SLE; room temperature, 2 h, 2 cycles of 5 mL water each one) was achieved; 5.7 mg/g inositols were extracted by PLE, whereas yields of only 3.7 mg/g were obtained by SLE. Subsequent incubation of PLE extracts with *Saccharomyces cerevisiae* (37 °C, 5 h) allowed the removal of other co-extracted low molecular weight carbohydrates which may interfere in the bioactivity of inositols.

1. Introduction

Inositols or 1,2,3,4,5,6-hexahydroxycyclohexanes are members of the cyclic family of carbohydrates. *myo*-Inositol is the most abundant in food and it is present in relatively high amounts in all vegetable sources and at lower concentration in meat products, followed by *chiro*-inositol which has been found in citrus fruits (Sanz, Villamiel, & Martinez-Castro, 2004), soy molasses (Saska & Diack, 1996), honey (de la Fuente, Sanz, Martínez-Castro, Sanz, & Ruiz-Matute, 2007) and some vegetables (Hernandez-Hernandez, Ruiz-Aceituno, Sanz, & Martinez-Castro, 2011). Methyl-inositols and glycosyl-inositols are also present, mainly in legumes (Peterbauer, Brereton, & Richter, 2003; Schweizer, Horman, & Würsch, 1978).

Numerous bioactive properties have been attributed to inositols and their derivatives. *myo*-Inositol is a growth factor for microorganism, a lipotropic agent for animals and it is also part of phosphatidilinositol, a frequent phospholipid in foods (Angyal & Anderson, 1959). It has been speculated that *myo*-inositol metabolism disorders have some influence in diabetic neurophaties and chronic renal failure (Clements & Darnell, 1980). D-*chiro*-Inositol and its methylated form pinitol (3-*O*-methyl-1,2,4 *cis*-3,5,6-*trans*-hexahydroxycyclohexanol) present a role in insulin action and could be used for treatments connected with diabetes mellitus, obesity, atherosclerosis, etc. (Macias Camero & Sanjuan Merino, 2004).

Chemical synthesis has been used for the production of inositols, although this process is still expensive. Consequently, different approaches have been followed for the extraction of these compounds from natural sources (Macias Camero & Sanjuan Merino, 2004). However, the development of new procedures more economical and less time consuming is of great interest.

Pressurized liquid extraction (PLE) uses the combination of both pressure and temperature to efficiently extract different compounds from vegetable matrices, generally providing a significant reduction in extraction times and solvent volumes as compared with conventional (non-solvent-enhanced) extraction procedures. PLE has been extensively used for the extraction of different contaminants such as persistent organic compounds from food (Carabias-Martínez, Rodríguez-Gonzalo, Revilla-Ruiz, & Hernández-Méndez, 2005; Mendiola, Herrero, Cifuentes, & Ibañez, 2007) and from environmental samples (Nieto, Borrull, Marce, & Pocurull, 2008; Ramos, Vreuls, & Brinkman, 2000). Regarding carbohydrates, PLE applications are very scarce in the literature. Alañón, Ruiz-Matute, Martínez-Castro, Díaz-Maroto, and Pérez-Coello (2009) used methanol at 90 °C and 1500 psi for the extraction of monosaccharides and polyalcohols from woods used in wine aging, whereas Rodríguez-Sánchez, Ruiz-Aceituno, Sanz, and Soria (2013) developed a PLE method for the extraction of iminosugars from mulberry leaves. A selective separation of lactulose from lactose using 70:30 (v/v) ethanol:water at 40 °C and 1500 psi has been proposed by Ruiz-Matute et al. (2007) whereas the combined use of PLE with an in-cell packed adsorbent bed of activated charcoal also allowed the separation of monosaccharides and

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oligosaccharides of honey (Ruiz-Matute, Ramos, Martinez-Castro, & Sanz, 2008).

Separation of inositols from sugars such as sucrose, which may interfere in the bioactivity of the extracts related to carbohydrate metabolism, has been conventionally carried out using strong anionic exchange resins of the hydroxide (OH) form and strong cationic resins (Macias Camero & Sanjuan Merino, 2004; Saska & Diack, 1996). In some cases, the process is carried out using a simulated moving bed chromatographic system (Saska & Diack, 1996). However, decomposition of the sugars in the alkaline medium can occur, and carbohydrates can be strongly adsorbed in the resin, resulting its regeneration difficult and expensive in terms of eluent and time consumption (Macias Camero & Sanjuan Merino, 2004). *Saccharomyces cerevisiae* has been used to remove monosaccharides from mixtures of carbohydrates (Baumgartner, Genner-Ritzmann, Haas, Amado, & Neukom, 1986; Ruiz-Matute, Soria, Martínez-Castro, & Sanz, 2007) and recently this method has been suggested to be effective for the production of inositol extracts free from interfering sugars from legumes (Ruiz-Aceituno, Rodríguez-Sánchez, Ruiz-Matute, Ramos, Soria, & Sanz, 2013).

Pine kernels or pine nuts are edible seeds of *Pinus pinea* L. rich in lipids and antioxidants, and are traditionally consumed in the countries of the Mediterranean basin. Noticeable amounts of *chiro*-inositol, pinitol and several glycosyl cyclitols (galactinol, galactopinitol A1, fagopyritol B1 and other glycosyl-inositols) have been recently reported in this food source (Ruiz-Aceituno, Ramos, Martinez-Castro, & Sanz, 2012).

In this work, pine kernels have been selected as a model of a complex matrix rich in inositols. The use of PLE has been evaluated for the extraction of these bioactive compounds and compared with conventional SLE. Yeast treatment was also applied for the selective separation of these compounds from other low molecular weight carbohydrates (LMWC) which could interfere in their bioactive properties.

2. Materials and methods

2.1. Samples and standards

Two commercial samples of peeled pine kernels (P1 and P2) were purchased at local markets (Madrid, Spain). Samples were stored under dry conditions and protected from direct light until their analysis. All samples were analyzed before their respective shelf life date.

Carbohydrate standards (pinitol, *chiro*-inositol, *myo*-inositol, galactinol, fructose, galactose, glucose, sucrose, maltose, phenyl-β-D-glucoside, trehalose, melibiose, planteose, raffinose) were obtained from Sigma Chemical Co. (St. Louis, USA). Sea sand was acquired from Panreac (Barcelona, Spain).

2.2. Extraction

2.2.1. Solid–liquid extraction

Extraction of inositols from 0.5 g of pine nuts (P1) previously ground in a mortar was carried out at room temperature for 2 h using 5 mL of solvent under stirring conditions. Water, methanol and ethanol (both from Sigma), the effect of temperature (60 °C) and ultrasonic agitation (Nahita ultrasonic bath (Auxilab, Navarra, Spain)) in the extraction efficiency were evaluated. The solid residue was removed by filtration through Whatman No. 4 filter paper (Whatman Scientific, Maidstone, England). Three successive extractions from the same sample were carried out. 1 mL of the resulting extracts was individually treated either with heptane (1 mL), methanol (2.25 mL) or methanol (2.25 mL) followed by activated charcoal (0.5 g) treatment to remove other interfering compounds. The optimized experimental conditions were then applied to sample P2 and extracts were kept at −20 °C until analysis. Otherwise specified, experiments were carried out in triplicate.

2.2.2. Pressurized liquid extraction (PLE)

All assays were carried out in a home-made miniaturized PLE system (Ramos, Díez, Cámara, & Ramos, 2006). The instrument consisted of an oven equipped with temperature control, in which a stainless steel extraction cell of 99 mm × 4.4 mm i.d. (total volume, 1.5 mL) was placed. This extraction cell was coupled to an isocratic pump (Hewlett–Packard 1050 series, Palo Alto, USA), which was used to deliver and pressurize the solvent via a six-port Rheodyne valve (model 7000, Rheodyne L.P., Rohnert Park, CA, USA). Another valve of the same characteristics was connected to the outlet end of the extraction cell. The extraction cell was sealed with 5 μm stainless steel frits (Supelco, Bellefonte, USA) at its lower and upper ends to avoid the access of suspended particles to the stainless-steel tubing, valves and ultimately to the extraction vial.

Pine nuts (P2) were ground in a mortar and 0.5 g were mixed with sea sand (Panreac, Barcelona, Spain) in a 1:2 ratio to avoid sample compactation.

Experimental parameters affecting the efficiency of PLE of carbohydrates were evaluated, namely extraction time, temperature and number of extraction cycles. The extraction pressure was set at 10 MPa and was separately considered during method development (Ramos, Kristenson, & Brinkman, 2002). In all cases, the solvent was completely flushed out of the cell after the static period selected for each cycle, and the extract was collected into the vial. Once water was considered as extraction solvent, static PLE times of 5, 10 and 20 min were evaluated, while extraction temperatures of 50, 100 and 150 °C were selected for optimisation. Unless otherwise specified, experiments were carried out in triplicate.

2.3. Yeast treatment

Yeast treatment was carried out as described by Ruiz-Aceituno, et al. (2013). Pine nut extract (4 mL) was treated with 1% (w/v) *S. cerevisiae* (Sigma Chemical Co.) at 37 °C under stirring for 18 h. All assays were done in triplicate. Aliquots were taken just after yeast addition (time 0) and at 3, 5, 8, 11, 15 and 18 h of treatment. Aliquots were centrifuged at 4400*g* at 10 °C for 10 min and filtered through Whatman No. 4 filters to remove yeast, and kept at −20 °C until analysis. A sample not submitted to yeast treatment was included in this study as control.

2.4. GC–MS analyses

Trimethylsilyl oximes (TMSO) were prepared as previously described (Sanz et al., 2004). In brief, 0.5 mL of phenyl-β-D-glucoside (1 mg/mL) was added to 1 mL of extract and the solution was evaporated under vacuum. Then, samples were treated with 350 μL of 2.5% hydroxylamine chloride in pyridine (30 min at 75 °C), and 350 μL of hexamethyldisilazane plus 35 μL of trifluoroacetic acid (45 °C for 30 min). After centrifugation, 1 μL of supernatant was taken for injection.

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out on a 7890 gas chromatograph coupled to a 5975 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using helium as the carrier gas. A 30 m × 0.25 mm i.d. × 0.25 μm film thickness fused silica column coated with TRB-1 (crosslinked methyl silicone) from Teknokroma (Barcelona, Spain) was used. The oven temperature was held at 200 °C for 15 min, then programmed to 270 °C at 15 °C min^{-1}, then programmed to 290 °C at 1 °C min^{-1} and finally raised to 300 °C at 15 °C min^{-1}. The final temperature was held for 30 min. The injector was heated at 300 °C and injections were made in the split mode with a split ratio

of 1:20. The mass spectrometer was operated in the electron impact (EI) mode at 70 eV, scanning the 50–650 *m/z* range. The interface and source temperature were heated at 280 and 230 °C, respectively. Acquisition was done using HP-ChemStation software (Hewlett–Packard, Palo Alto, CA, USA).

Response factors (RF) relative to the internal standard were calculated over the expected concentration range. Due to the lack of commercial standards, the RF of galactinol was used for all glycosyl cyclitols. Quantitative data were expressed in both mg/g of pine kernels and mg/g of dry extract. The first term (mg/g of pine kernel) indicates the amount of target compounds per gram of sample, whereas the second term refers to the purity of the extract in these compounds (concentration of target carbohydrates relative to that of all the co-extracted interfering compounds in the extract). Optimal conditions were chosen as a trade-off between high extraction yield of inositols (data in mg/g pine kernels) and the high enrichment on these carbohydrates caused by the lower co-extraction of interfering compounds (data in mg/g dry extract). Content of dry matter in extracts was gravimetrically measured at 110 °C up to constant weight. Precision was calculated from the results obtained for a pine nut sample which was extracted, derivatised and analyzed by quintuplicate.

2.5. Statistical analysis

Dependence of PLE efficiency for inositols and other sugars present in pine kernels with temperature and time of extraction was studied using the following quadratic linear model:

$$Y_i = \beta_0 + \beta_1 \times \text{Temp} + \beta_2 \times \text{time} + \beta_{1,1} \times \text{Temp}^2 + \beta_{2,2} \times \text{time}^2 + \beta_{1,2} \times \text{Temp} \times \text{time} \quad (1)$$

where β_0 is the intercept, β_1 and β_2 the coefficients of main effects, $\beta_{1,1}$ and $\beta_{2,2}$ the coefficients of quadratic effects and $\beta_{1,2}$ the interaction coefficient. Total relative amounts of extracted inositols, of extracted sugars and of the ratio between extracted inositols and extracted sugars amounts were the variables used as response (Y_i). Extraction temperatures (Temp) and extraction times (time) are indicated in Section 2.2.2.

The parameters were estimated through multiple linear regression (MLR) in the stepwise mode using the data analysis software program Statistica v.7.1 (StatSoft, Inc. 2005; www.statsoft.com).

Model quality and statistical significance of the coefficients were evaluated, and response surfaces and contour plots diagrams were drawn to show graphically the effect of temperature and time in the extraction of inositols and other sugars.

Cluster analysis was also carried out by using the Statistica package (Cluster analysis, Ward's method). Variables (extracted relative amounts of the quantified compounds) were grouped according to their similarity, measured as 1−Pearson *r*.

3. Results and discussion

3.1. Extraction

3.1.1. Solid–liquid extraction

The most appropriate solvent for the extraction of inositols from pine kernels (P1) by solid–liquid extraction (SLE) was firstly evaluated. Methanol, ethanol and water (5 mL) were assayed within an extraction cycle of 2 h under stirring conditions at room temperature (25 °C). As previously indicated by Ruiz-Aceituno et al. (2012), pinitol, *chiro*-inositol, *myo*-inositol, galactinol, galactopinitol A1, fagopyritol B1 and other glycosyl-inositols were detected in pine nuts. Sucrose and raffinose were the most abundant sugars, although other mono- (glucose, fructose and galactose), di- (maltose) and trisaccharides (planteose) were also present.

Total extracted content of these compounds is summarised in Table 1. To simplify data, glycosyl-inositols, mono-, di- and trisaccharides have been grouped. Ethanol was the less effective solvent for the extraction of all the LMWC whereas higher yields (mg/g pine nuts) were obtained using methanol and water. Although higher amounts of inositols were present in the water extracts, higher amounts of disaccharides were extracted with methanol. Regarding concentrations in the dry extract (mg/g dry extract), higher values were obtained with methanol as extraction solvent for *chiro*-inositol and glycosyl-inositols, but also for di- and trisaccharides. Yields of these interfering carbohydrates were lower in the water-extraction assay; therefore, this solvent was chosen for the rest of experiments.

The effect of temperature (60 °C) and ultrasonic agitation was also evaluated for the extraction of the target compounds using water as extraction solvent (Table 1). No notable differences in the concentration of total inositols (free and glycosylated inositols) relative to room temperature water were observed in these treatments. Nevertheless, higher concentrations of other LMWC (22.3 mg/g pine nut) were obtained when using ultrasonic agitation, mainly due to the higher extraction of disaccharides. As determined by data expressed by dry weight of extract, concentrations of sugars were similar for room temperature and hot water and slightly higher for water under ultrasonic agitation. Therefore, both room temperature and hot water accomplishes a low extraction of total sugars and an acceptable content of total inositols, following experiments were done using water at 60 °C.

Evaluation of the most appropriate number of successive cycles for SLE of inositols from pine nuts was also performed. The percentages of inositols found in the third SLE cycle ranged between 0.5% and 1.3%. Since these values may be considered negligible compared with those obtained in the first (85–90%) and second cycle (10–14%), only the two first cycles were performed in subsequent experiments for which both extracts were pooled before further analysis.

Once the extraction conditions were optimized, different enrichment procedures (use of methanol, heptane and methanol followed by activated charcoal treatment) were evaluated (See Supplemental material). As expected, concentration of total inositols and other low molecular weight sugars (mg/g pine nuts) was not affected by these treatments, however, some differences were observed regarding the enrichment of these extracts (mg/g dry extract). Whereas heptane did not improve the results, the use of methanol gave higher concentrations of inositols probably due to the precipitation of some co-extracted compounds. Nevertheless, concentration (mg/g dry extract) of other LMWC also increased. On the other hand, similar results of both inositols and other LMWC were obtained in those treatments with methanol and with and without the use of activated charcoal, indicating the null effect of this substance. Therefore, considering that optimal conditions were chosen as a trade-off between high extraction yields of inositols and low contents of other LMWC, none of these treatments noticeably improved the results and were not considered for following experiments.

3.1.2. Pressurised liquid extraction

For optimization of experimental conditions for PLE of inositols and other LMWC from pine kernels (sample P2) water was selected as the most appropriate solvent taking into account the above mentioned results. The effect of time (5, 10 and 20 min) and temperature (50, 100 and 150 °C) on the extraction of inositols and other LMWC was studied in order to evaluate possible different trends in the process.

As it can be seen in Table 2, a higher content of inositols and their derivatives (mg/g of pine nut) was extracted at lower temperatures; the highest values were achieved at 50 °C using extraction

Table 1
Inositols and other LMWC content (mg/g sample and mg/g dry extract) in pine nuts extracted with different solvents or conditions.

Solvent	Pinitol	*chiro*-Inositol	*myo*-Inositol	Glycosyl-inositols	Monosacc.	Disacc.	Trisacc.	Total inositols	Total sugars
Content (mg/g sample)									
Methanol	0.53 (0.02)[a]	0.78 (0.03)	0.20 (0.01)	1.9 (0.1)	n.d.	21.0 (0.9)	6.5 (0.5)	3.41 (0.05)	27.5 (1.2)
Ethanol	0.09 (0.01)	0.25 (0.01)	0.050 (0.004)	0.54 (0.01)	0.02 (0.01)	11.9 (0.6)	1.94 (0.02)	0.92 (0.01)	13.8 (0.4)
Water	1.0 (0.1)	1.1 (0.1)	0.63 (0.03)	1.9 (0.2)	0.5 (0.1)	11.9 (0.1)	7.9 (1.4)	4.7 (0.2)	20.4 (1.2)
Water (60° C)	1.3 (0.1)	1.3 (0.1)	0.676 (0.001)	1.8 (0.1)	1.4 (0.1)	11.5 (0.5)	8.8 (1.2)	5.13 (0.03)	21.8 (2.2)
Water (US)[b]	0.71 (0.02)	1.19 (0.02)	0.54 (0.02)	1.12 (0.03)	1.0 (0.1)	18.9 (0.1)	2.37 (0.03)	3.6 (0.1)	22.3 (0.2)
Content (mg/g dry extract)									
Methanol	11.2 (0.3)	16.3 (0.6)	4.1 (0.1)	39.9 (2.6)	n.d.	440 (18.1)	188.8 (17.9)	71.5 (1.0)	629.5 (25.5)
Ethanol	0.40 (0.03)	1.2 (0.1)	0.25 (0.02)	2.70 (0.04)	0.09 (0.05)	59.3 (3.2)	13.4 (0.1)	4.61 (0.04)	72.7 (2.2)
Water	11.8 (1.7)	13.6 (2.2)	6.8 (0.4)	19.0 (0.4)	6.2 (1.5)	127.4 (13.2)	74.3 (15.3)	51.2 (3.6)	207.8 (23.2)
Water (60 °C)	11.8 (0.9)	11.4 (0.6)	5.6 (0.6)	16.3 (0.6)	12.6 (0.5)	102.1 (5.0)	85.6 (15.2)	45.1 (0.6)	203.2 (20.8)
Water (US)	8.7 (0.2)	14.7 (0.2)	6.6 (0.2)	13.8 (0.4)	11.9 (0.9)	232.5 (1.2)	50.3 (0.7)	43.8 (0.1)	294.7 (0.3)

n.d.: not detected.
[a] Standard deviations in parenthesis ($n = 3$).
[b] US: Ultrasound agitation.

Table 2
Inositols and other LMWC content (mg/g sample and mg/g dry extract) in pine nuts extracted using different PLE conditions (10 MPa).

Temperature (°C)	Time (min)	Pinitol	*chiro*-Inositol	*myo*-Inositol	Glycosyl-inositols	Monosacc.	Disacc.	Trisacc.	Total inositols	Total sugars
Content (mg/g sample)										
150	5	0.6 (0.1)[a]	1.18 (0.04)	0.4 (0.1)	1.4 (0.3)	0.15 (0.05)	11.2 (4.3)	6.7 (1.3)	3.6 (0.3)	18.1 (3.0)
	10	0.9 (0.1)	1.1 (0.1)	0.44 (0.01)	1.5 (0.1)	0.199 (0.002)	10.9 (0.9)	7.7 (0.5)	3.9 (0.1)	18.8 (0.3)
	20	0.8 (0.2)	1.1 (0.2)	0.4 (0.1)	1.4 (0.3)	0.23 (0.02)	11.0 (0.6)	9.3 (2.1)	3.8 (0.7)	20.6 (1.5)
100	5	0.9 (0.2)	1.1 (0.1)	0.5 (0.1)	1.2 (0.1)	0.52 (0.01)	11.4 (1.3)	8.6 (0.5)	3.7 (0.5)	20.5 (0.8)
	10	0.8 (0.1)	1.2 (0.2)	0.5 (0.1)	1.4 (0.2)	0.5 (0.1)	13.1 (1.3)	8.5 (1.3)	3.8 (0.5)	22.2 (2.6)
	20	1.2 (0.2)	1.56 (0.02)	0.61 (0.03)	1.7 (0.1)	0.73 (0.04)	13.6 (0.2)	12.7 (0.5)	5.1 (0.1)	27.0 (0.3)
50	5	0.90 (0.04)	1.04 (0.03)	0.49 (0.03)	1.3 (0.2)	0.4 (0.1)	11.2 (1.4)	8.5 (0.8)	3.8 (0.2)	20.1 (0.5)
	10	1.1 (0.1)	1.47 (0.01)	0.65 (0.01)	1.5 (0.1)	0.79 (0.02)	12.4 (1.3)	9.7 (1.7)	4.8 (0.2)	22.9 (0.4)
	20	1.2 (0.1)	1.5 (0.3)	0.7 (0.1)	1.6 (0.2)	0.8 (0.1)	14.6 (0.8)	10.2 (0.1)	5.0 (0.6)	25.6 (0.7)
Content (mg/g dry extract)										
150	5	13.6 (3.1)	17.4 (2.1)	5.8 (1.0)	19.4 (1.7)	3.6 (1.3)	196.9 (14.9)	135.2 (16.1)	56.1 (5.7)	345.4 (15.7)
	10	12.8 (2.7)	16.5 (4.4)	6.6 (1.4)	22.4 (4.3)	3.3 (1.2)	171.1 (41.0)	135.0 (19.1)	58.3 (12.4)	309.4 (56.8)
	20	11.4 (6.0)	14.6 (4.4)	5.5 (2.1)	18.9 (6.1)	3.1 (1.2)	170.5 (54.2)	121.1 (38.3)	50.4 (18.5)	310.1 (67.1)
100	5	14.9 (2.8)	20.6 (2.7)	8.7 (1.0)	23.6 (4.4)	8.3 (1.7)	196.7 (12.3)	161.0 (23.4)	67.8 (9.3)	366.0 (22.9)
	10	16.5 (6.3)	20.7 (3.9)	8.3 (1.7)	26.6 (8.2)	7.4 (2.2)	209.5 (6.2)	164.2 (51.3)	60.6 (3.4)	353.0 (17.5)
	20	14.7 (1.7)	19.4 (0.8)	8.1 (0.4)	23.6 (1.6)	9.9 (1.3)	175.4 (11.0)	156.5 (5.4)	65.9 (2.4)	341.9 (8.7)
50	5	18.6 (1.8)	22.3 (3.3)	10.3 (1.2)	26.5 (3.8)	8.7 (1.8)	218.8 (16.0)	172.4 (18.9)	77.7 (8.3)	394.0 (9.3)
	10	19.7 (0.9)	25.5 (0.9)	11.3 (0.2)	26.1 (0.5)	13.5 (0.5)	215.4 (29.0)	168.2 (25.1)	82.5 (0.6)	397.1 (4.4)
	20	20.5 (0.2)	24.6 (2.3)	11.7 (1.4)	28.9 (2.8)	11.4 (1.5)	253.4 (3.6)	182.8 (17.1)	85.6 (3.0)	457.7 (11.7)

[a] Standard deviations in parenthesis ($n = 3$).

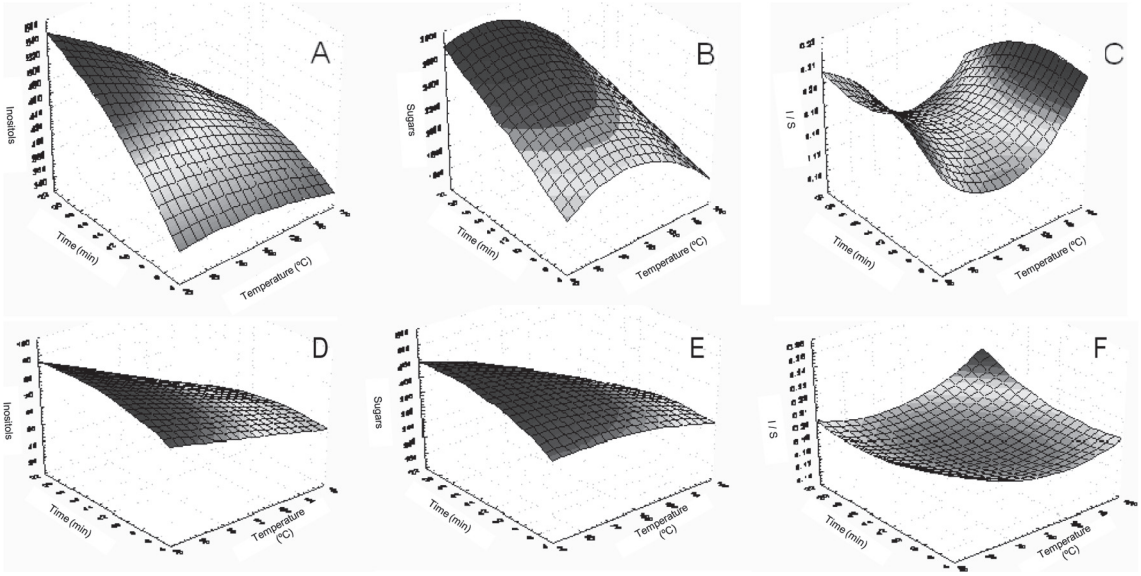
cycles of 10 and 20 min and at 100 °C with a PLE of 20 min. The lower amount of these polar compounds extracted at 150 °C could be caused by water polarity changes at subcritical conditions (Mendiola et al., 2007). Considering the dry extract yields of inositols, no influence of the extraction time was detected and the highest values were achieved at 50 °C.

From the values shown in Table 2, a study of the effect of time and temperature on the extraction of inositols from a pine nut sample relative to other LMWC was carried out in order to evaluate possible trends in the process. Extracted values were fitted to a quadratic model (Eq. (1)) using extraction times and temperatures as single factors, and extracted amounts of total sugars, of total inositols, and of the relationship between these two values as responses. Estimation of the significance of factors in the model was based on correlation coefficients, while stepwise multiple linear regression (MLR) was used to fit the model and to optimize the operation conditions.

When extracted amount values for inositols, measured as mg/g of pine nut, were used as response *Y*i, and the five model variables were individually considered (See Supplemental material), similar negative correlation coefficients (r) were found for Temp and Temp^2, while positive values of r were obtained for time and time^2. All these values were significant at the 95% level, while the correlation with the interaction Temp × time was not significant. Using as response mg of other LMWC/g of pine nut, time, time^2 (positive values), and Temp and Temp^2 (negative values) were also significant at a 95% level, when individually considered. Poorer results were found when the inositols/sugars ratio was used as response; no individual variable was significant at the 95% level.

Fig. 1(A–C) presents the results of fitting the experimental data (mg/g of pine nut) to the model of Eq. (1). The calculated coefficients allowed to plot the surface response for inositols (1 A), sugars (1 B), and the inositol/sugar ratio (I/S) (1 C). Best fit was found for sugar, where the multiple correlation coefficient (r) was 0.911. While r was also high (0.782) for inositols, the inositols/sugars ratio had the lowest fit quality ($r = 0.541$).

Extraction concentrations relative to dry weight of pine nut extract (mg/g of extract) of inositols, sugars and their ratios were fitted in a similar way to the quadratic model of Eq. (1) and correlation between individual variables in the model and concentration of inositols, sugars and their ratio were calculated (See Supplemental material). As for total content (mg/g of sample), Temp and Temp^2 showed for both total inositols and sugars a negative ef-



(A) Inositols = 276.5498+0.7086 x Temp+20.6324 x time-0.0033 x Temp^2-0.0674 x Temp x time -0.3149 x time^2; r=0.782
(B) Sugars = 1158.46+16.6592 x Temp+58.7014 x time-0.0905 x Temp^2-0.1999 x Temp x time -0.271 x time^2; r=0.911
(C) I/S = 0.2123-0.0011 x Temp+0.0046 x time+6.3619E-6 x Temp^2-1.5915E-5 x Temp x time -0.0001 x time^2; r=0.541
(D) Inositols = 75.5158-0.1564 x Temp+2.865 x time -3.2659E-5 x Temp^2-0.0093 x Temp x time -0.077 x time^2; r=0.774
(E) Sugars = 266.1994+1.1624 x Temp+20.8622 x time -0.0074 x Temp^2-0.0786 x Temp x time -0.5095 x time^2; r=0.733
(F) I/S = 0.2708-0.0012 x Temp-0.0048 x time +4.5424E-6 x Temp^2+1.6276E-5 x Temp x time +0.0001 x time^2; r=0.395

Fig. 1. Surface responses and results of the fit to quadratic linear model of inositols (A), sugars (B) and inositols/sugars ratio (C) for mg/g of pine nuts and inositols (D), sugars (E) and inositols/sugars ratio (F) for mg/g dry weight of pine nut extracts.

fect on response, significant at the 99% level. But in this case, the interaction variable Temp × time had also a negative contribution, while the effects of time and time^2 were not significant. No correlation was significant when using as variable the ratio between inositols and sugars.

Eq. (1) was also used to fit the dry weight of extract response. The model coefficients are listed in Fig. 1(D–F), where the response surfaces for inositols (1 D), sugars (1 E) and inositols/sugars ratio (1 F) are also plotted. Fit is similar for inositols (r = 0.775) and sugars (r = 0.734), but very poor (r = 0.395) for the inositols/sugars ratio.

From the surface response plots and equations in Fig. 1A and 1B, we can conclude that, for extracted amounts relative to pine nuts weight, the extraction of inositols was higher at lower temperatures, while total extracted amount of other sugars was higher at medium temperatures. Longer extraction times increased the extracted amount of both types of compounds.

If the response is expressed as mg compounds/g dry extract, maximum concentrations of inositols and sugars were obtained in both cases at low temperatures and medium times, although the effect of these parameters was not as marked as when response is given as extracted yield relative to total pine nut weight. The reason

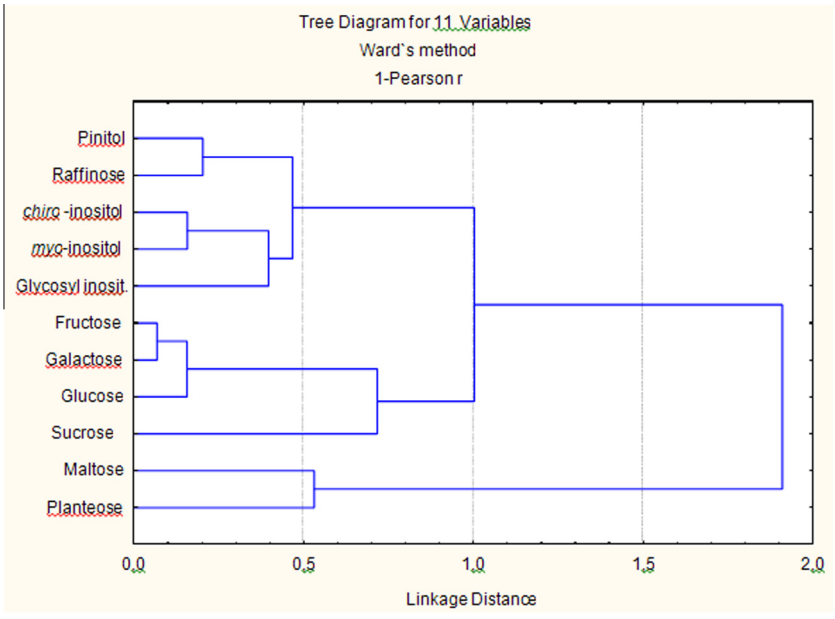


Fig. 2. Cluster analysis from the correlation coefficients of individual component concentrations (mg/g pine nut), using Ward's method for linkage.

Table 3
Comparison between the two extraction processes (sample P2).

	Content (mg/g sample)								
	Pinitol	*chiro*-Inositol	*myo*-Inositol	Glycosyl-inositols	Monosacc.	Disacc.	Trisacc.	Total inositols	Total sugars
Conventional 2 h 2 cycles	1.1 (0.1)	0.98 (0.01)	0.5 (0.1)	1.1 (0.2)	1.21 (0.04)	6.19 (0.03)	5.4 (1.3)	3.7 (0.4)	12.8 (1.4)
PLE 50 °C 18 min 3 cycles 10 MPa	1.5 (0.4)	1.6 (0.5)	0.7 (0.1)	1.7 (0.1)	0.8 (0.1)	16.2 (1.4)	12.1 (0.7)	5.7 (1.2)	28.5 (2.0)
	Content (mg/g dry extract)								
Conventional 2 h 2 cycles	17.2 (0.5)	14.4 (2.2)	7.4 (0.7)	15.6 (0.4)	19.3 (1.6)	95.5 (3.8)	75.8 (6.5)	54.7 (0.5)	190.5 (4.4)
PLE 50 °C 18 min 3 cycles 10 MPa	25.1 (1.1)	31.9 (1.1)	15.1 (0.2)	32.1 (0.6)	20.5 (1.0)	273.5 (36.7)	210.7 (31.5)	104.2 (0.8)	504.7 (6.3)

[a]Standard deviations in parenthesis ($n = 3$).

for the different behaviour is the presence in the extract of other components that are better extracted at higher temperatures.

Differences in the yields obtained for individual components in the sample were also evaluated. Correlation coefficients (r) between pairs of the 11 quantified carbohydrates were calculated: the value $1-r$ was selected as a measure of their similar extraction behaviour. These values were submitted to Cluster Analysis (Ward method), which allowed to distinguish three different groups formed by: (i) inositols and raffinose (ii) fructose, glucose, galactose, and sucrose and (iii) maltose and planteose (Fig. 2). Similar results were obtained using values relatives to total pine nut amount or to extracted amount.

Since in this study the optimization must consider both the amount of extracted inositols from pine nuts and its concentration in the extract, it is necessary to reach a compromise between these values. In view of the results, the most favourable conditions using water at 10 MPa as extractant resulted to be: low temperature (50 °C) and intermediate times (18 min), as this solvent under these conditions would extract significant quantities of inositols minimizing the presence of other undesirable compounds in the extract. Furthermore, the observed curvature of the representations in the surface response plot indicated that longer extraction times reduced the analytes yields.

The inositol yield obtained in four successive PLE extraction cycles was also determined. The amount extracted in the first cycle ranged between 73% and 80%, whereas 15–17%, 4–8% and 2–3% were achieved in the second, third and fourth cycle, respectively. The fourth cycle presented a percentage that was considered to be negligible; thereby, only three PLE cycles were considered necessary for subsequent experiments.

Finally, reproducibility of the proposed method was evaluated by extracting a sample in quintuplicate under optimized conditions. RSD percent values were below 10% for all studied compounds.

3.1.3. Comparative study of both extraction methods

Table 3 summarizes the results obtained under optimized conditions for the extraction of inositols and other carbohydrates (sample P2) for both methods, i.e., conventional SLE and PLE. The concentration of inositols extracted by PLE (5.7 mg/g) was higher than that found using conventional SLE (3.7 mg/g). However, while the extracted contents of monosaccharides were notably lower by PLE (0.8 mg/g by PLE and 1.2 mg/g by SLE), highest concentrations of di- and trisaccharides were obtained for PLE (16.2 and 12.1 mg/g by PLE and 6.2 and 5.4 mg/g by SLE).

It should be noted that PLE yielded these higher concentrations of inositols by using the same amount of pine nut sample (0.5 g), but a lower solvent volume (10 mL in SLE, and 4.5 mL in PLE) and shorter extraction times (2 cycles of 2 h in SLE, and 3 cycles of 18 min in PLE).

3.2. Selective removal of sugars

A pine nut PLE extract was submitted to a yeast treatment (*S. cerevisiae* at 37 °C) which has been previously proposed for the removal of interfering carbohydrates from bioactive iminosugars in mulberry (Rodríguez-Sánchez et al., 2013) and inositols in legumes (Ruiz-Aceituno et al., 2013). However, the time of incubation should be optimised for each matrix, depending on its concentration of carbohydrates. Therefore, incubation at different times was evaluated in the present study. In general, inositols content did not change notably with the incubation time (Fig. 3A). As shown in Fig. 3B, both sucrose and raffinose were immediately hydrolysed into glucose and fructose, and fructose and melibiose, respectively. An increase of these monosaccharides and disaccharide in the medium was consequently observed. Moreover, trehalose was also detected from the beginning of the treatment. This disaccharide markedly decreased after 3 h of digestion and almost disappeared after 5 h of yeast treatment. This behaviour can be a consequence of yeast metabolism (Jules, Guillou, François, & Parrou, 2004) and it has also been observed in previous studies (Rodríguez-Sánchez et al., 2013; Ruiz-Aceituno et al., 2013). An unknown trisaccharide (with a typical 1→6 linkage, as characterised by its mass spectra with relatively high intensity of *m/z* 422

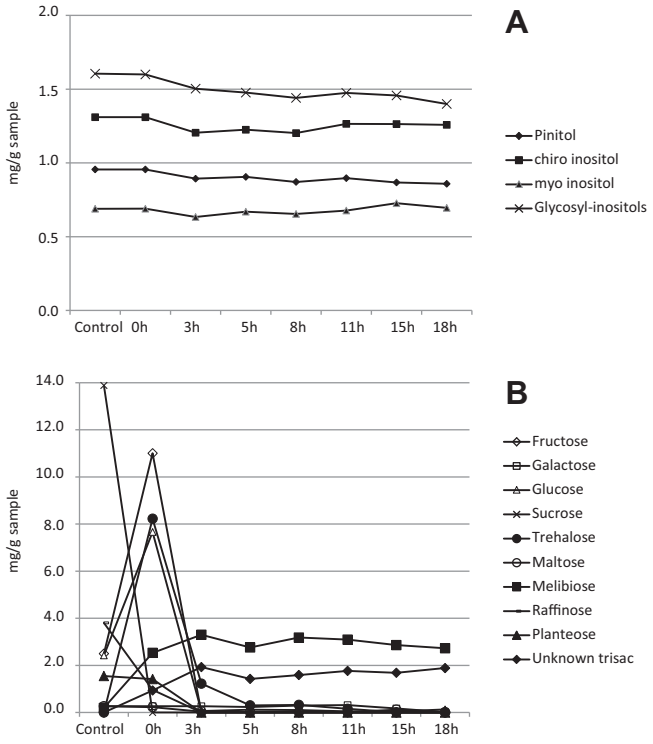


Fig. 3. Evolution of the content of inositols (A) and other LMWC (B) in pine nut extract during yeast treatment at 37° C.

ion (Hernández-Hernández, Montañés, Clemente, Moreno, & Sanz, 2011)) was found in the samples submitted to yeast treatment. This compound was probably produced by the hydrolysis of a higher molecular weight oligosaccharide (likely stachyose) that could be present in the extract.

Except galactose, which presence was observed until 15 h, monosaccharides and disaccharides were completely removed after 5 h of treatment. Therefore, this time was selected as the optimum for the production of inositol extracts free of most important interfering carbohydrates in pine nut extracts.

4. Conclusion

This is the first time that PLE has been used for the extraction of inositols from pine nuts. PLE has proved to be a good alternative to conventional SLE for the production of enriched inositols extracts. Additional advantages of PLE (compared with the conventional approach) are the possibility of using automated equipment requiring less manipulation of the sample and the feasibility for straightforward scaling up to industrial scale. The subsequent use of a biotechnological process (yeast treatment) allowed the removal of large quantities of co-extracted sugars which may interfere in the bioactivity of inositols. All together, the developed procedure could be considered as a valuable alternative methodology to more time-consuming analytical procedures for the enrichment of food ingredients with industrial interest. The proposed method could be applied to other types of matrices and by-products.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.12.079.

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4.5. Extraction of bioactive carbohydrates from artichoke (*Cynara scolymus* L) external bracts using microwave assisted extraction and pressurized solvent extraction.

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Extraction of bioactive carbohydrates from artichoke (*Cynara scolymus* L) external bracts using microwave assisted extraction and pressurized solvent extraction.

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Abstract

Microwave assisted extraction (MAE) and pressurized liquid extraction (PLE) methods have been optimized by means of a Box-Behnken and 3^2 composite experimental designs, respectively, for the effective extraction of bioactive carbohydrates (inositols and inulin) from artichoke (*Cynara scolymus* L.) external bracts. MAE at 60 ºC for 3 min of 0.3 g of sample allowed the extraction of higher inositol concentration than PLE at 75 ºC for 26.7 min (11.6 mg/g dry sample *vs*. 7.6 mg/g dry sample). On the contrary, under these conditions, higher inulin concentrations were extracted with the latter technique (185.4 mg/g *vs.* 96.4 mg/g dry sample), considering two successive extraction cycles for both techniques. Once optimized, both methodologies can be considered appropriate for the simultaneous extraction of these bioactive carbohydrates from this particular industrial by-product.

Keywords: inositols, inulin, PLE, MAE, artichoke external bracts.

1. Introduction

Artichoke (*Cynara cardunculus* var. *scolymus L.*) is extensively cultivated in the Mediterranean region, constituting the 85% of world production. From this vegetable, only the inner leaves (bracts) and heart are considered edible parts, whereas external bracts, leaves and stems are non-food industrial by-products (Christaki, Bonos, & Florou-Paneria, 2012). These by-products represent about 60% of the artichokes (Llorach, Espín, Tomás-Barberán, & Ferreres, 2002) and have been used for the extraction of bioactive components such as flavonoids and phenolic compounds (Ceccarelli, Curadi, Picciarelli, Martelloni, Sbrana, & Giovannetti, 2010; Lattanzio, Kroon, Linsalata, & Cardinali, 2009) or essential oils (Shimoda, Ninomiya, Nishida, Yoshino, Morikawa, Matsuda, et al., 2003) with application in food or pharmaceutical industry. These bioactives make artichoke to be considered as a functional food, exhibiting hepatoprotective and antioxidative activities, as well as the ability to inhibit cholesterol biosynthesis and low density lipoprotein oxidation (Fallah Huseini, Kianbakht, & Heshmat, 2012), due to its high content in polyphenolic compounds (Llorach, *et al.*, 2002; Sánchez-Rabaneda, Jáuregui, Lamuela-Raventós, Bastida, Viladomat, & Codina, 2003). Artichoke is also known to have a relatively high content in inulin (10.0-30.7% based on dry weight) (Lattanzio, *et al.*, 2009; G. Leroy, Mabeau, Baty-Julien, & Grongnet, 2011; Ruiz-Cano, Pérez-Llamas, Frutos, Arnao, Espinosa, López-Jiménez, et al., 2014; Van Loo, Coussement, De Leenheer, Hoebregs, & Smits, 1995), a polysaccharide composed of fructose unit chains of various lengths (linked by β-(2→1)-D-fructosyl-fructose bonds), terminated by a glucose unit linked by a α-(1→2) bond. Inulin is a reserve carbohydrate with recognized prebiotic properties and used as a technological ingredient (Gibson & Roberfroid, 1995; Gaëlle Leroy, Grongnet, Mabeau,

Corre, & Baty-Julien, 2010; López-Molina, Navarro-Martínez, Rojas-Melgarejo, Hiner, Chazarra, & Rodríguez-López, 2005)(Lattanzio, *et al.*, 2009; Ronkart, Blecker, Fourmanoir, Fougnies, Deroanne, Van Herck, et al., 2007).

Up to now, the presence of inulin in artichoke has usually been evaluated on edible parts, i.e. head or capitula. Only a couple of studies have been reported on the presence of this component in artichoke by-products (or agro-industrial wastes), such as external bracts or fractions resulting from industrial processes (López-Molina, *et al.*, 2005; Ruiz-Cano, *et al.*, 2014).

Apart from inulin, limited attention has been paid to the composition of other carbohydrates in artichoke. Few studies report the total carbohydrate content on edible parts of artichoke (Dosi, Daniele, Guida, Ferrara, Severino, & Di Maro, 2013) whereas, to the best of our knowledge, individual carbohydrate composition present in this vegetable has been only studied in a recent manuscript by (Hernández-Hernández, Ruiz-Aceituno, Sanz, & Martínez-Castro, 2011). In this latter work, low molecular weight carbohydrate (LMWC) composition of artichoke internal bracts was determined, emphasizing the content of bioactive compounds such as inositols (*chiro*-, *scyllo*- and *myo*-inositol) at levels of 2 mg/g dry weight.

Inositols (1,2,3,4,5,6-hexahydroxycyclohexanes) are cyclic polyalcohols with molecular formula $C_6H_{12}O_6$. These compounds are mainly found in vegetables, their presence in animal or meat sources being limited (Clements RS Jr & B., 1980; Ruiz Aceituno, Ramos Rivero, & Sanz Murias, 2012). Several bioactive properties have been attributed to inositols and their derivatives, mainly associated with insulin resistance, and have been used for treating disorders such as polycystic ovary syndrome, diabetes mellitus

and/or obesity (Nestler, Jakubowicz, Reamer, Gunn, & Allan, 1999; Ostlund & Sherman, 1998).

The use of advanced extraction techniques such as pressurized liquid extraction (PLE) or microwave assisted extraction (MAE) to obtain extracts rich in bioactives from industrial by-products is gaining great attention in recent years (Álvarez-Casas, García-Jares, Llompart, & Lores, 2014; Ballard, Mallikarjunan, Zhou, & O'Keefe, 2010; Machado, Pasquel-Reátegui, Barbero, & Martínez, 2015; Paes, Dotta, Barbero, & Martínez, 2014; Pap, Beszédes, Pongrácz, Myllykoski, Gábor, Gyimes, et al., 2013; Pérez-Serradilla & Luque de Castro, 2011). These techniques generally provide high yields with a significant reduction of extraction times and solvent volumes as compared with conventional (i.e., non-solvent enhanced) extraction procedures. Applications reporting on the PLE of carbohydrates are still scarce in the literature. Several studies have reported on the feasibility of PLE for the extraction of inulin from Jerusalem artichoke (*Helianthus tuberosus* L.) tubers (Saengkanuk, Nuchadomrong, Jogloy, Patanothai, & Srijaranai, 2011), of inositols from pine kernels (Ruiz-Aceituno, Rodríguez-Sánchez, Sanz, Sanz, & Ramos, 2014) and of iminosugars from mulberry leaves (Rodríguez-Sánchez, Ruiz-Aceituno, Sanz, & Soria, 2013). The concurrent extraction of monosaccharides and polysaccharides from *Cordyceps* mushroom was also carried out by using PLE (Guan, Yang, & Li, 2010). MAE has been applied to the extraction of polysaccharides (pectins, galactomannans, arabinogalactans, xylans and sulfated polysaccharides, among others) (Bélafi-Bakó, Cserjési, Beszédes, Csanádi, & Hodúr, 2012; Benkő, Andersson, Szengyel, Gáspár, Réczey, & Stålbrand, 2007; Passos & Coimbra, 2013). However, to the best of our knowledge, this technique has not been applied to the extraction of inulin in any type of

vegetable and no application to the extraction of LMWC, and more specifically to inositols, can be found in the literature.

The aim of this study is to evaluate the possibility of the simultaneous extraction of bioactive carbohydrates (inulin and inositols) from artichoke (*Cynara scolymus*) industrial by-product (i.e, external bracts) by PLE and MAE. Removal of other coextracted LMWC has also been evaluated.

2. Materials and methods

2.1. Samples and reagents

Fresh artichokes (var. "*Blanca de Tudela*") were acquired at local markets (Madrid, Spain), and the external bracts were taken and freeze-dried. Then, they were ground to fine particles using a domestic mill (Moulinex, Barcelona, Spain) and sieved (500 μm mesh). The sample was stored in a dry and hermetically closed recipient protected from light until analysis at ambient temperature.

Analytical standards of fructose, *chiro*-inositol, glucose, galactose, *myo*-inositol, sucrose and phenyl-β-D-glucoside were obtained from Sigma Chemical Co. (St. Louis, USA). Sea sand washed, thick grain, was acquired to Panreac (Barcelona, Spain).

Inulinase Novozym 960 from *A. niger* was kindly provided from Novozymes (Bagsvaerd, Denmark) with 250-400 units/g activity.

2.2. Carbohydrates extraction

2.2.1. Microwave assisted extraction (MAE)

MAE was carried out in a MARS 6 (CEM, NC, USA) system. Microwave power was set at 900 W.

In a typical experiment, dried external bracts (0.1 – 0.3 g) and 10 mL of ultra-pure water were placed in 100 mL Green Chem vessels (CEM) and submitted to MAE at different temperature and/or times, following experimental design conditions. The effect of three independent factors (temperature (T, ºC), time (t, min) and sample amount (s, g)) on carbohydrate extraction from artichoke external bracts was studied using a Box–Behnken design. A total of 15 experiments were carried out in randomized order, according to design (Table 1). The 3-level design included a subset of the runs in the full three-level factorial and 3 center points per block to estimate the experimental error. Experimental ranges for factors evaluated were: Temperature (T) = 50–120 ºC, time (t) = 3–30 min, and sample amount (s) = 0.1 – 0.5 g.

The quadratic model proposed was:

$$R = \beta_0 + \beta_1 T + \beta_2 t + \beta_3 s + \beta_{1,1} T^2 + \beta_{2,2} t^2 + \beta_{3,3} s^2 + \beta_{1,2} Tt + \beta_{1,3} Ts + \beta_{2,3} ts + \varepsilon \quad \text{(Eq1)}$$

where β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,i}$ the quadratic coefficients for i^{th} factors, $\beta_{i,j}$ the coefficients for the interaction of factors i and j and ε is the error.

Three response (R) variables were individually considered in the optimization of the MAE method: R1, total inositols amount/g of dry sample; R2, inulin amount/g of dry sample; and R3, total sugar amount/g of dry sample. The experimental conditions that independently maximized R1 and R2 and minimized R3 were obtained from the fitted models. Under optimized conditions, the number of cycles (first, second and third cycle, namely as C1, C2 and C3, respectively) were also evaluated. Obtained extracts were immediately cooled down on ice, centrifuged (4400 *g,* 10 min, 4 ºC) and keep in a freezer (-18 ºC) until analysis.

2.2.2. Pressurized liquid extraction (PLE)

Extracts were obtained using a PSE ONE system from Applied separations (PA, USA). Dried artichoke bracts (0.3 g) were introduced inside the stainless steel extraction cell (11 mL) between sand layers. Milli-Q water was used as extraction solvent and a single static extraction cycle was done in all instances. Extractions were carried out under a pressure of 100 bar, and a purge of nitrogen gas of 2 min was done at the end of the extraction cycle. The effect of two independent factors (T and t) on carbohydrate extraction from artichoke external bracts was studied using a 3^2 full factorial design with 4 replicates at the center point. Experimental ranges for factors evaluated were: T = 50–120 ºC and time = 3–30 min. The quadratic model proposed was:

$$R = \beta_0 + \beta_1 T + \beta_2 t + \beta_{1,1} T^2 + \beta_{2,2} t^2 + \beta_{1,2} Tt + \varepsilon \quad \text{(Eq. 2)}$$

where β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,i}$ the quadratic coefficients for i^{th} factors, $\beta_{i,j}$ the coefficients for the interaction of factors i and j and ε is the error.

For comparative purposes, the same response variables as in MAE, namely R1, R2 and R3 (total inositols amount/g of dry sample, inulin amount/g of dry sample and total sugars amount/g of dry sample, respectively) were considered. In total, 12 experiments were performed according to design (Table 2).

2.3. Enzymatic hydrolysis of inulin

The inulin concentration was calculated taking into account fructose, glucose and sucrose content of artichoke bracts extracts before after hydrolysis with inulinase, as previously carried out by other authors (Jaime, Martín-Cabrejas, Mollá, López-Andréu, & Esteban, 2001; Prosky & Hoebregs, 1999; Schütz, Muks, Carle, & Schieber, 2006).

MAE and PLE artichoke bract extracts (0.4 mL) were evaporated re-dissolved in 1 mL acetate buffer (pH 4.6). A volume of 50 µL of inulinase was added and the mixtures were incubated at 55 ºC while shaking at 730 *g* in a Thermomixer (Eppendorf, Hamburg, Germany). Temperature and pH conditions were chosen as the optimal for the enzyme according to the manufacturer´s recommendations. Subsamples were taken at 0, 8, 24, 48, 72 and 96 h and the enzymatic reaction was stopped by immersing the sample for 90 s on boiling water. Then, samples were cooling on ice and stored in a freezer until analysis by GC as indicated in section 2.5.

2.4. Carbohydrate fractionation

MAE extracts corresponding to C1 and C2 were mixed and incubated with 1% (w/v) yeasts (*Saccharomyces cerevisiae*, type II, Sigma Chemical Co.) at 37 ºC under stirring (90 *g*). Aliquots were taken at 0 h (corresponding to the beginning of the experiment) and after 3, 8 and 24 h of treatment. All assays were carried out in triplicate. Samples were centrifuged at 4400*g* (MiniSpin; Eppendorf, Madrid, Spain) for 10 min to remove yeasts. Samples were then treated as indicated in section 2.5.

2.5. Instrumental section

Concentration of sugars in artichoke bract extracts (before and after enzymatic treatment) was determined by GC-FID.

2.5.1. Derivatization procedure

1 mL of artichoke bracts extract was mixed with 0.5 mL of a 70% ethanolic solution of the internal standard (phenyl-β-D-glucoside, 1 mg/mL) and evaporated (38-40 ºC) prior to carbohydrate derivatization. Trimethylsilyl (TMS) oxime derivatives were prepared according to Sanz *et al*. (2005)(Sanz, Gonzalez, de Lorenzo, Sanz, & Martínez-Castro, 2005). In brief, oximes were obtained by addition of 350 μL of a solution 2.5 % hydroxylamine chloride in pyridine after 30 min at 75 °C. They were then silylated with hexamethyldisilazane (350 μL) and trifluoroacetic acid (35 μL) at 45 °C for 30 min. After reaction, samples were centrifuged at 4400 *g* for 10 min, and the supernatant was subjected to GC analysis. This derivatization procedure gives rise to a single chromatographic peak

for non-reducing sugars (corresponding to their TMS ethers) and two peaks for reducing sugars (corresponding to their *syn* (*E*) and *anti* (*Z*) oxime isomers).

2.5.2. GC analysis

GC analysis of derivatized samples was carried out using a 7890A gas chromatograph equipped with FID from Agilent Technologies (Palo Alto, CA, USA). Chromatographic analyses were carried out on a Zebron ZB-1MS methylsilicone capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Phenomenex, CA, USA), using nitrogen at 1 mL/min as carrier gas.

The oven temperature was programmed as follows: 200 ºC (14.5 min), then at 20 ºC/min to 220 ºC (3 min), and finally at 25 °C/min to 270 °C (20 min). Injections (1 µL) were carried out in split mode (1:20) at 300 °C. FID temperature was 300 ºC. Data acquisition was done using HP ChemStation software (Agilent Technologies).

Carbohydrates were identified by using available standards and previous data from literature (Hernández-Hernández, *et al.*, 2011). Quantitative data were directly obtained from peak areas. Standard solutions of LMWC were prepared using calibration curves in the 0.1 - 1 mg/mL range (5 calibration points) to calculate the response factor relative to phenyl-β-D-glucoside (n=3).

2.6. Statistical analysis

The experimental designs and the parameters of the models (Eq.1 and Eq. 2) were estimated by multiple linear regression (MLR) using StatGraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA).

3. Results and discussion

3.1. Qualitative carbohydrates composition of artichoke external bracts.

Prior to the optimization of PLE and MAE experimental parameters, characterization of the composition of artichoke external bract extracts was required. For this purpose, both a PLE and a MAE extracts (80 ºC and 17 min, and/or 85 ºC and 16.5 min, respectively) were analyzed by GC, after a previous derivatization step as indicated in section 2.5.1. Similar chromatographic profiles of carbohydrates were obtained for both extraction procedures. Figure 1A shows the GC profiles of the extracted carbohydrates from artichoke bracts using MAE. Glucose, fructose and sucrose were detected in these extracts. Regarding inositols, *chiro*-inositol was the most abundant, followed by *myo*- and *scyllo*-inositol. These results are consistent with those previously reported by Hernandez-Hernandez et al. (2011)(Hernández-Hernández, *et al.*, 2011) in artichoke internal bracts.

Previous to the analysis of inulin extracted by MAE and PLE, the optimization of the enzymatic treatment was mandatory to achieve its complete hydrolysis into glucose and fructose. Extracts were treated with inulinase, as previously reported in other works (Gaëlle Leroy, *et al.*, 2010; Ronkart, *et al.*, 2007), at 55 ºC taking sample at different times up to 96 h. Fructose, glucose and sucrose were analyzed by GC before and after inulin hydrolysis. After 48 h treatment, inulin was totally hydrolyzed into fructose and glucose and stable concentrations of these monosaccharides were obtained at following hydrolysis times; therefore this time was chosen for further analyses. Figure 1B shows the chromatographic profile of LMWC of artichoke bracts extract using MAE after inulinase treatment. As consequence of the hydrolysis, a noticeable increase of fructose (peak 2) was detected

whereas glucose only increased slightly. It is worth to mention that an abundant peak (peak S) eluting at 13.5 min and corresponding to sorbitol was detected in these samples. As described by Novozymes in their product data sheet, inulinase is supplied as a solution in 20% glycerol and 20% sorbitol. This polyalcohol coeluted with the first isomer of fructose (peak 1), hampering its direct quantitation. Therefore, quantitation of fructose in extracts treated with inulinase was done considering peak 2 and the abundance ratio of the isomeric forms (peak 1 and peak 2) of the commercial standard.

3.2. Optimization of the extraction conditions

The efficiency of two techniques (MAE and PLE) for the extraction of bioactive carbohydrates (inositols and inulin) from artichoke external bracts was compared.

3.2.1. MAE

The solvent nature, the extraction temperature and time, and the solid/liquid ratio have commonly been identified as the most relevant parameters affecting the MAE efficiency (Soria, Ruiz-Aceituno, Ramos, & Sanz, 2014). Considering previous studies regarding conventional extraction of carbohydrates (López-Molina, *et al.*, 2005; Ruiz-Aceituno, Ramos, Martinez-Castro, & Sanz, 2012; Saengkanuk, *et al.*, 2011), water was selected as solvent to achieve the highest yields. The influence of three independent variables (extraction time, temperature and sample amount) on MAE of carbohydrates from artichoke bracts was studied using a Box-Behnken design. R1 (total inositols amount) and R2 (inulin amount) were considered as response variables to be maximized, and R3 (total sugar amount) as variable to minimize, taking into account that these co-extracted

carbohydrates (glucose, fructose and sucrose) may interfere in the bioactivity related to carbohydrate metabolism of artichoke extracts.

Concentrations (mg/g dry weight) of total inositols (*chiro*-, *scyllo*- and *myo*-inositol), total sugars (glucose, fructose and sucrose) and inulin in artichoke bract extracts obtained by MAE at the different conditions evaluated by the experimental design are shown in Table 1. Inositol concentrations varied between 6.7 and 9.3 mg/g dry sample, total sugars between 50.4 and 66.7 mg/g dry sample and inulin concentrations ranged from 69.8 to 114.6 mg/g dry sample.

Response surface methodology was used to calculate the coefficients of the quadratic models proposed and to estimate the statistical significance of the regression coefficients. Regarding R1 model, the most significant coefficients ($P < 0.05$) were t and T, whereas T and s^2 were the most significant ($P < 0.05$) for R2. Figure 2 shows the surface plots for R1 and R2 of the combined effect of parameters and the model equations after excluding non-significant ($P > 0.05$) terms in the model. These quadratic models appropriately described the variability of both R1 and R2 (R^2=83% and 80%, respectively). The highest inositol yields were obtained at low temperatures and short extraction times (optimal conditions: 50 ºC, 3 min and 0.1 g of dry sample) (Figure 2A), whereas the highest inulin yields were achieved when using 120 ºC, 3 min and 0.3 g dry sample (Figure 2B). Regarding R3, experimental values could not be adjusted to an appropriate model (R^2=32%), which probably could be explained by the low variability of total sugar concentrations at the different conditions assayed. Therefore, this variable could not be minimized under the experimental conditions in this study and was not further considered.

A multiple response analysis was then performed to maximize the extraction of the bioactive carbohydrates (R1 and R2). The optimal extraction parameters were 60 ºC, 3 min and 0.26 g dry sample. Temperature was the factor showing the main differences regarding optimal conditions for R1 and R2. The increase of extraction temperatures usually improves recoveries of carbohydrates (Passos, *et al.*, 2013); however, high temperatures frequently result in an increase of undesirable coextracted materials. Under the optimal conditions cited above, 9 mg of inositols and 92 mg of inulin per gram of dried artichoke bracts were extracted. These inulin concentrations are in the range of those found in dry artichoke bracts by other authors (G. Leroy, *et al.*, 2011; Ruiz-Cano, *et al.*, 2014).

After selection of the most appropriate conditions, 3 successive extraction cycles were carried out for the exhaustive extraction of inositols and inulin from the matrix. Percentage of inositols (8.6 %) and inulin (3.0 %) found in the third cycle were very small. Since this value may be considered negligible compared with those obtained in the first (70.8% and 92.6%, respectively) and second cycle (20.6% and 4.4, respectively), only these two cycles were considered for further experiments. Therefore, 11.3 mg of inositols/g and 96.7 mg inulin/g dry samples were obtained under optimized MAE process.

3.2.2. PLE

The experimental results obtained after PLE of inositols, sugars and inulin from artichoke bracts using a 3^2 full factorial design are shown in Table 2. Variables considered in this evaluation were temperature and time. A fixed sample amount (0.3 g) was selected for all experiments considering that solid/liquid ratio in PLE changes according to the sample amount.

Figure 3 presents the results of fitting the experimental data to the model of Eq. 2 for inositols (R1, Figure 3A) and for inulin (R2, Figure 3B). Regarding R1, the most significant parameter ($P < 0.05$) was t, whereas both t and T^2 were the most significant ($P < 0.05$) for R2. These adjusted models explained the 68% and 79% of the variability of R1 and R2, respectively. Under these conditions, optimal inositol yields were achieved at 40ºC and 30 min, whereas the highest inulin yields were extracted at 75ºC and 26.7 min. Regarding R3 (total sugar extraction), the most significant parameter was T followed by t; the optimal conditions to minimize this response were 40ºC and 3 min. However, the proposed model ($R3= 48.5769 + 0.0570833T + 0.193827t$) only justified the 48% of its variability. Consequently, this parameter was not considered for further studies.

A multiple response analysis was carried out to maximize R1 and R2, alike to MAE. Optimal conditions for inositols and inulin extraction were 75 ºC and 26.7 min, whereas the extraction yield of these bioactive carbohydrates was 6.2 mg inositols/g dry sample and 141.0 mg inulin/g dry sample. At these optimal conditions, three PLE cycles were carried out. Regarding the carbohydrates extracted in each cycle (79, 19 and 3 % for inositols, and 73, 21 and 6 % for inulin, corresponding to first, second and third cycle, respectively), the third one was discarded considering the negligible amount that represented. Consequently, 7.6 mg inositols/g and 185.4 mg inulin/g dry sample were obtained using optimized conditions with this technique.

3.2.3. Comparison of extraction techniques

Under optimized conditions, MAE allowed the extraction of higher yields of inositols than PLE (11.6 mg/g *vs*. 7.6 mg/g dry sample). On the contrary, the highest yields of inulin were obtained using PLE (185.4 mg/g *vs.* 96.4 mg/g dry sample). In both cases, intermediate extraction temperatures (60-75 ºC) were used. However, extraction times were shorter with MAE (3 min *vs.* 26.7 min). As a concluding remark, the choice of the method will depend on the desired issue at a specific moment/point.

The comparison of our results with those reported in literature regarding to artichoke content in inulin (G. Leroy, *et al.*, 2011; Ruiz-Cano, *et al.*, 2014) leads to a extraction of this carbohydrate within the range of the previously reported data. About inositols, higher concentrations are extracted by these enhanced extraction techniques as compared to those concentrations previously described by Hernandez-hernandez 2011 (about 2 mg/g dry sample of total inositols *vs.* 11.3 mg/g or 7.6 mg/g dry sample for MAE and PLE, respectively). However, more studies should be done regarding variability of these carbohydrates depending on the maturity, origin, etc. of the artichoke.

3.3. Fractionation assays using yeast

Considering the unavoidable coextraction of LMWC which could interfere in the bioactive properties of inositols and inulin, and the unfeasibility of minimizing their extraction either by MAE or PLE, a fractionation procedure using *Saccharomyces cerevisiae* was applied to cycles 1 + 2 of MAE artichoke bracts extracts for their selective

removal (Rodríguez-Sánchez, *et al.*, 2013; Ruiz-Aceituno, Rodriguez-Sanchez, Ruiz-Matute, Ramos, Soria, & Sanz, 2013), as an example for the enrichment in compounds of interest.

After 3h of treatment, 40% of sugars remained in the extracts, but after 8h of treatment all of them were totally removed, except trehalose (produced as consequence of yeast metabolism) which remained at trace level. Concentration of inositols and inulin maintained unaltered during this fractionation process. Consequently, these experimental conditions were selected as optimal.

4. Conclusions

Overall, both MAE and PLE are useful tools for the effective extraction of bioactive carbohydrates (inositols and inulin) from artichoke external bracts, as an agro-industrial by-product. To the best of our knowledge, this is the first time that the simultaneous extraction of these carbohydrates is optimized by these techniques. In particular, MAE was less time-consuming than PLE. Although co-extraction of other interfering sugars could not be avoided in these extraction procedures, yeast treatment demonstrated to be effective for their subsequent removal, allowing the enrichment of extracts on these bioactive compounds. These extracts could represent an economic source of these bioactives with a potential positive combined activity to be used in food or pharmaceutical products.

Acknowledgments

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Figure Captions

Figure 1. GC-FID profile of low molecular weight carbohydrates (as TMSO derivatives) extracted from artichoke external bracts using MAE before (a) and after inulinase treatment (b). Peak identification: 1, 2: fructose; 3: *chiro*-inositol; 4, 5: glucose; 6: *scyllo*-inositol; 7: *myo*-inositol; 8: phenyl-β-D-glucoside (internal standard); 9: sucrose; 10: glycosyl-glycerol: U: unknown compounds; S: Sorbitol, present in the commercial inulinase.

Figure 2. MAE response surface plots for the extraction of inositols (A) and inulin (B).

Figure 3. PLE response surface plots for the extraction of inositols (A) and inulin (B).

TABLES

Table 1. Box–Behnken experimental design and obtained concentrations (mg/g dry weight of bracts) of the MAE of carbohydrates.

T (ºC)	t (min)	Sample amount (g)	Inositols	Sugars	Inulin
50	3	0.3	9.3 (0.2)*	66.3 (0.2)	84.5 (8.7)
50	16.5	0.1	8.7 (0.5)	66.3 (0.9)	77.7 (6.0)
50	16.5	0.5	8.2 (0.4)	57.0 (4.4)	79.4 (13.4)
50	30	0.3	8.2 (0.4)	57.3 (3.0)	69.8 (0.6)
85	3	0.1	8.8 (1.5)	64.5 (9.3)	85.9 (11.0)
85	3	0.5	7.5 (0.4)	56.7 (3.1)	80.8 (8.2)
85	16.5	0.3	6.9 (1.0)	53.6 (2.4)	89.8 (6.0)
85	16.5	0.3	7.2 (0.4)	50.4 (9.7)	98.3 (7.3)
85	16.5	0.3	8.0 (1.0)	66.7 (4.9)	96.3 (13.9)
85	30	0.1	6.8 (0.6)	50.6 (2.3)	78.3 (7.5)
85	30	0.5	7.6 (0.3)	59.1 (0.2)	78.4 (10.2)
120	3	0.3	7.5 (1.6)	58.6 (8.4)	114.6 (2.8)
120	16.5	0.1	7.6 (0.7)	56.9 (6.9)	86.0 (4.9)
120	16.5	0.5	7.6 (1.1)	56.0 (6.6)	83.0 (19.7)
120	30	0.3	6.7 (0.7)	56.9 (5.7)	87.6 (10.8)

* Standard deviations are in parentheses

Table 2. Central 3^2 composite design for optimization of carbohydrate concentration (mg/g dry weight of bracts) extraction using PLE.

T (ºC)	t (min)	Inositols	Sugars	Inulin
40	3	5.4 (0.1)*	49.6 (0.7)	67.7 (2.6)
40	17	6.1 (0.3)	55.5 (1.2)	104.8 (20.6)
40	30	6.3 (0.4)	58.4 (2.7)	123.0 (30.8)
80	3	5.3 (0.2)	54.3 (0.3)	77.6 (6.9)
80	17	6.1 (0.7)	59.5 (6.4)	137.2 (18.9)
80	17	5.8 (0.0)	54.4 (4.1)	140.3 (15.2)
80	17	6.1 (0.2)	53.9 (1.3)	142.4 (4.6)
80	17	6.0 (0.1)	54.8 (0.4)	140.1 (19.0)
80	30	6.7 (0.0)	58.5 (7.3)	130.3 (6.8)
120	3	5.7 (0.2)	55.0 (1.3)	97.1 (5.2)
120	17	6.1 (0.2)	64.5 (0.3)	85.3 (5.2)
120	30	5.4 (0.1)	57.7 (0.2)	117.9 (11.9)

* Standard deviations are in parentheses

FIGURE GRAPHICS

Figure 1

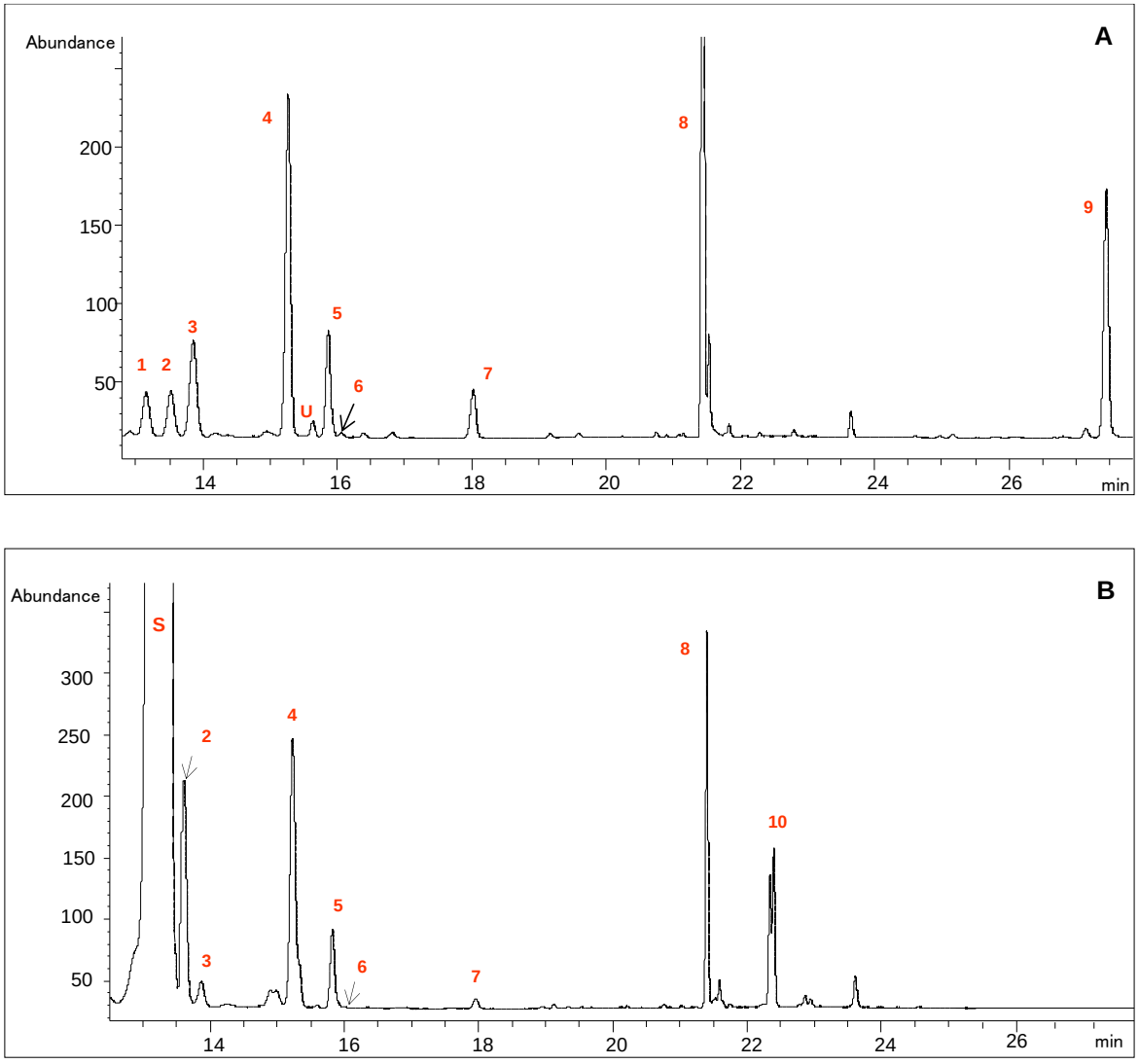
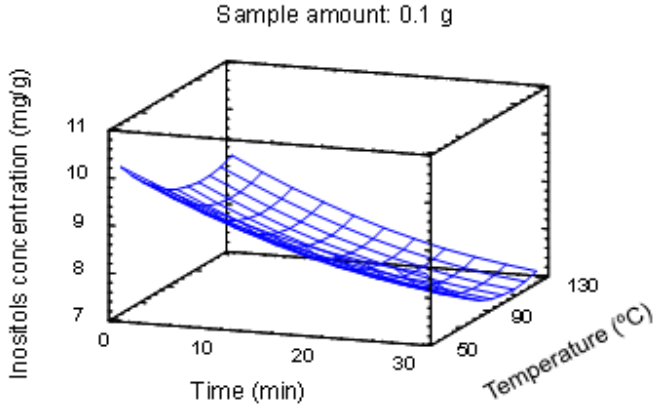


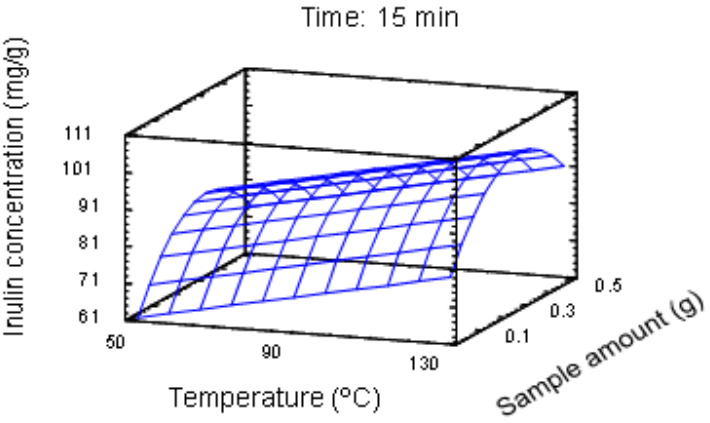
Figure 2

A



$y = 13.152 - 0.078 * T - 0.093 * t - 3.208 * s + 0.0003 * T^2 + 0.194 * t * s$

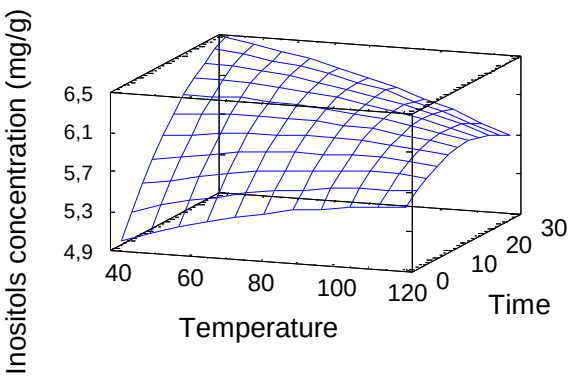
B



$y = 57.9705 + 0.213571 * T - 0.478704 * t + 155.545 * s - 259.241 * s^2$

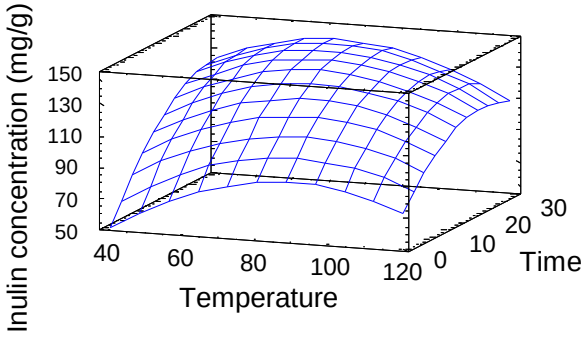
Figure 3

A



$y = 4.42716 + 0.01666*T + 0.10535*t - 0.0000625*T^2 - 0.000555*T*t - 0.001097*t^2$

B



$y = -33.1059 + 2.65729*T + 5.55453*t - 0.01483*T^2 - 0.011597*T*t - 0.08141*t^2$

5. DISCUSIÓN INTEGRADORA

5. DISCUSIÓN INTEGRADORA

Como se indica en la **Sección 2** de esta Memoria, en los trabajos aquí presentados se ha pretendido ahondar en el desarrollo de nuevas estrategias para la extracción y fraccionamiento selectivo de carbohidratos bioactivos (inositoles y prebióticos) con vistas a su posible empleo como ingredientes alimentarios.

Para conseguir este objetivo, los estudios se han centrado en:

(i) El estudio de la composición en inositoles y otros LMWC en diversos productos agrícolas (frutos como el piñón, verduras como la alcachofa, lechuga, achicoria, etc., y legumbres como el garbanzo, la almorta, etc.).

(ii) El desarrollo de métodos de fraccionamiento selectivo de carbohidratos (evaluación del empleo de ILs y de *S. cerevisiae*).

(iii) La exploración de nuevas alternativas de extracción para la obtención de extractos enriquecidos en carbohidratos bioactivos (MAE y PLE).

En cuanto a la **caracterización** de LMWC de las muestras seleccionadas (**Secciones 4.1, 4.2 y 4.3**), la GC−MS ha demostrado ser una herramienta útil para el análisis cuali- y cuantitativo de estos productos, ya que combina la buena resolución de la GC, con la sensibilidad e información estructural proporcionada por la MS. Como única desventaja se podría mencionar que para su análisis con esta técnica se requiere una etapa previa de derivatización que confiera a los carbohidratos la necesaria volatilidad y estabilidad térmica. Además, dada la complejidad de los extractos vegetales y la formación de distinto número de derivados en función del tipo de carbohidrato, es importante seleccionar aquellos derivados que contribuyan a simplificar la separación cromatográfica de dichas mezclas. Estos requisitos fueron exitosamente conseguidos mediante la formación de oximas para los azúcares reductores seguido de la requerida sililación con HMDS y TFA de todos los azúcares presentes en los extractos vegetales estudiados. Los métodos de análisis propuestos permitieron la identificación y cuantificación, por primera vez, de algunos LMWC, principalmente inositoles con descritas propiedades bioactivas, en piñón, verduras y legumbres, así como la confirmación de otros ya descritos con anterioridad en la bibliografía.

Para el desarrollo de **métodos de fraccionamiento** (**Secciones 3.3, 3.4 y 4.3**), se exploraron dos estrategias diferentes: el empleo de ILs como disolventes alternativos y más verdes que los orgánicos convencionales por su baja volatilidad y un tratamiento con levaduras (*S. cerevisiae*) como estrategia de interés biotecnológico. Previo al estudio del potencial uso de ILs para el fraccionamiento selectivo de carbohidratos fue necesario **optimizar un método que permitiera su posterior análisis con GC** (**Sección 3.1**). Los pocos estudios previos existentes en la bibliografía sobre el análisis de LMWC disueltos en ILs se basaban en técnicas colorimétricas o en el empleo de HPLC−RID. Dado que estas metodologías no permiten la caracterización estructural de los carbohidratos disueltos en ILs, requisito necesario en el análisis de muestras reales, se contempló la opción del empleo de técnicas cromatográficas acopladas a MS. En un principio, los LMWC disueltos en ILs se analizaron directamente por HPLC-MS. Sin embargo, la eliminación del IL del MS tras una inyección resultaba lenta, lo que elevaba el ruido de fondo, con el consecuente aumento de los LOD y dificultando estudios posteriores. Por tanto, la GC-MS fue la técnica de elección, si bien para su aplicación era requisito imprescindible la derivatización previa de los carbohidratos en el IL. Considerando la alta viscosidad de los ILs, esta etapa no resulta trivial y requirió la optimización y validación del procedimiento para evitar derivatizaciones parciales y asegurar la formación de derivados estables. Los resultados obtenidos para un gran número de patrones demostraron que el empleo de 100 µL TMSI como reactivo de derivatización a 25 ºC durante 1 h permitían obtener rendimientos próximos al 100% para buena parte de los compuestos estudiados. Los buenos resultados obtenidos al aplicar este método al análisis de muestras reales y la satisfactoria comparación de los resultados con los obtenidos al emplear piridina como disolvente demostraron la validez del mismo.

Una vez seleccionado y optimizado el método de análisis instrumental, se procedió a evaluar la **solubilidad de distintos carbohidratos estudiados en ILs** (**Secciones 3.2 y 3.3**). Estos datos no se encontraban hasta el momento disponibles en la bibliografía y abren nuevas vías de aplicación de los ILs en el campo de la Química de Carbohidratos, siendo los más prometedores [EMIM][DCA], [EMIM][OAc] y [MMIM][Me_2PO_4]. Un ejemplo de ello fue la aplicación exitosa de [EMIM][DCA] para el fraccionamiento selectivo del producto de síntesis de lactulosa, conocido prebiótico (**Sección 3.4**). Otro aspecto a considerar en relación con este tipo de análisis es la

recuperación de los carbohidratos disueltos en el IL. De los tres procedimientos evaluados, efecto de las bajas temperaturas, miscibilidad en distintos disolventes y tratamiento con carbón activo, fue este ultimo el que proporcionó las mayores recuperaciones de carbohidratos.

Por otro lado, también se evaluó la utilidad de la **levadura *S. cerevisiae*** para la separación selectiva de inositoles y otros LMWC (**Sección 4.3**). Se eligieron como sustrato para el estudio las legumbres por su alto contenido en inositoles, comprobándose la eficacia del procedimiento una vez optimizadas las condiciones de incubación con la levadura para cada extracto.

Los dos métodos de fraccionamiento evaluados poseen ventajas e inconvenientes. El tratamiento con IL, al tratarse de un método novedoso e incipiente, requiere de más estudios antes de proceder a su aplicación generalizada a muestras reales, entre las que se deben incluir la evaluación de posibles interferencias y efectos competitivos de otros componentes de la matriz. Una de sus ventajas es la posibilidad de extender el campo de aplicación al fraccionamiento de carbohidratos con muy diversas características estructurales (aldosas/cetosas; mono-/disacáridos; enlaces glicosídicos…). Los estudios llevados a cabo en este trabajo con 5 ILs sientan las bases de aplicaciones futuras que debería incluir la evaluación de nuevos ILs con distintos aniones y cationes (“ILs a la carta”), contribuyendo a abrir potenciales nuevas vías de exploración. Sin embargo, el empleo de ILs está condicionado en la actualidad por sus elevados costes y la comercialización de un número aún muy limitado de ILs. Además, el estudio de la toxicidad de ILs es aún una tarea pendiente en general y, en particular, en sus aplicaciones en el campo de la alimentación. Por estos motivos, en las **Secciones 4.4 y 4.5** se seleccionó el método de fraccionamiento mediante levaduras que garantiza la seguridad del producto final, ya que los sub-productos formados durante el tratamiento (glicerol, etanol, trehalosa) no son tóxicos y/o pueden ser eliminados con facilidad de las muestras.

En cuanto a los métodos desarrollados para la obtención de extractos enriquecidos en carbohidratos bioactivos (inositoles e inulina; **Secciones 4.4 y 4.5**) a partir de fuentes naturales, tanto la PLE como la MAE mostraron ventajas frente a la extracción convencional en cuanto a su mayor rapidez y menor manipulación de muestra proporcionando rendimientos de extracción similares. Comparando una con

otra, la MAE proporcionó altos rendimientos de inositoles en tiempos muy cortos (3 min) a temperaturas relativamente bajas, mientras que la PLE precisa de tiempos más largos (26.7 min), pero favorece la extracción de polisacáridos como la inulina.

Además, se ha conseguido un importante avance en la caracterización de LMWC con propiedades bioactivas en matrices alimentarias, lo que puede contribuir a la obtención y desarrollo de nuevos ingredientes alimentarios multifuncionales, siendo éste un reto de la Ciencia y Tecnología de Alimentos.

6. CONCLUSIONES / CONCLUSIONS

6. CONCLUSIONES

1. El método de derivatización de carbohidratos de bajo peso molecular disueltos en líquidos iónicos, puesto a punto y descrito por primera vez resultó ser eficaz para su posterior análisis mediante GC. Los resultados obtenidos empleando [EMIM][DCA] como disolvente y trimetilsililimidazol como agente derivatizante fueron comparables a los proporcionados por procedimientos de derivatización convencionales, incluso para el análisis de los carbohidratos presentes en zumos naturales de frutas.
2. Se han aportado datos referentes a la solubilidad de carbohidratos de bajo peso molecular (mono-, di- y trisacáridos) en distintos ILs, datos hasta ahora no disponibles en la bibliografía, y se han establecido las relaciones entre los mismos y la estructura química de los analitos investigados.
3. El empleo de ILs demostró ser eficaz para el fraccionamiento de mezclas binarias de mono- y disáridos con polialcoholes.
4. La mayor solubilidad observada, en general, para las cetosas bioactivas en relación a sus correspondientes aldosas en los ILs investigados se ha aprovechado para proponer un novedoso procedimiento de fraccionamiento entre lactosa/lactulosa y galactosa/tagatosa cuya aplicabilidad práctica se ha demostrado mediante el estudio del enriquecimiento de lactulosa a partir de su producto de síntesis por isomerización de lactosa.
5. Se ha llevado al cabo un estudio exhaustivo para caracterizar la presencia de LMWC en vegetales de distinta naturaleza, hasta ahora en muchos casos incompleta, y que ha permitiendo identificar algunos de ellos por primera vez en estas muestras, como es el caso del *chiro*-inositol, *scyllo*-inositol, sedoheptulosa, rutinosa, kestosa, ácido glucárico en vegetales; galactosa, maltosa, planteosa, pinitol, galactinol, galactopinitol A1, fagopiritol B1 en piñones; y bornesitol y latiritol en almorta. Entre todos los datos aportados, destacan los referentes a la presencia de inositoles y derivados en las diferentes matrices, por sus relevantes propiedades bioactivas.
6. Se ha propuesto un nuevo método para la PLE de inositoles a partir de piñones, que, una vez optimizado, ha proporcionado un rendimiento del 54% de inositoles, superior al conseguido por el procedimiento convencional de

extracción sólido-líquido, pero con tiempos de extracción (18 min *vs* 2 h) y gasto de disolvente (1.5 mL *vs* 5 mL) significativamente menores.

7. Se han desarrollado métodos de PLE y MAE que han permitido, por primera vez, la extracción conjunta de inositoles e inulina a partir de las brácteas externas de alcachofa, como subproducto de la industria alimentaria. Mientras que la PLE permitía obtener una mayor cantidad de inositoles, la MAE resultó ser más eficaz para la extracción de inulina.
8. El tratamiento con *Saccharomyces cerevisiae* de los extractos vegetales obtenidos aplicando las metodologías de extracción optimizadas ha permitido la eliminación selectiva de LMWC interferentes en la bioactividad de los inositoles (mono- y disacáridos). Por sus características, sencillez y facilidad de escalado, este método se considera particularmente atractivo para su potencial aplicación industrial.
9. Los estudios recogidos en esta Memoria han permitido adquirir nuevos conocimientos relacionados con la caracterización, fraccionamiento y extracción de carbohidratos bioactivos de matrices vegetales de interés alimentario.

CONCLUSIONS

1. The derivatization method of low molecular weight carbohydrates dissolved in ionic liquids implemented for the first time was found to be effective for further analysis by GC. The best results were obtained using [EMIM] [DCA] as solvent and trimethylsilylimidazole as derivatizing agent, being comparable to those obtained with conventional derivatization procedures, even to the analysis of carbohydrates present in natural fruit juices.
2. Data not available so far in the literature regarding the solubility of low molecular weight carbohydrates (mono-, di- and trisaccharides) in various ionic liquids are provided, establishing the relationship between them and the chemical structure of investigated analytes.
3. The use of ILs proved to be effective for the fractionation of binary mixtures of mono- and disaccharides from polyalcohols.
4. The higher solubility observed in general for bioactive ketoses in relation to their corresponding aldoses in the investigated ionic liquids has been used to propose a novel method of fractionation between lactose / lactulose and galactose / tagatose, whose practical applicability has been demonstrated by study of lactulose enrichment from its synthesis product by isomerization of lactose.
5. The characterization of the presence of low molecular weight carbohydrates in vegetables of different nature has been carried out, being until now incomplete, and has led to the identification of some of them for the first time in these samples, such as *chiro*-inositol, *scyllo*-inositol, sedoheptulose, rutinose, kestose, glucaric acid (vegetables), galactose, maltose, planteosa, pinitol, galactinol, galactopinitol A1, fagopiritol B1 (pine nuts), bornesitol and lathyritol (vetch). Among all of them, data concerning the presence of inositol and derivatives in different matrices is highlighted for their relevant bioactive properties.
6. A new method for PLE of inositol from pine nuts has been proposed. Once optimized, the PLE has provided a yield of 54% inositol, higher than that obtained by the conventional solid-liquid extraction method, but with extraction times (18 min *vs* 2 h) and solvent expenditure (1.5 mL *vs* 5 mL) significantly lower.

7. The methods developed by PLE and MAE have allowed for the first time the simultaneous extraction of inositol and inulin from artichoke external bracts, as a food industry byproduct. PLE allowed obtaining a larger amount of inositol to be obtained, while MAE was the most effective for extracting inulin.
8. The treatment of plant extracts using *Saccharomyces cerevisiae* has allowed the selective removal of the interfering low molecular weight carbohydrates (mono- and disaccharides) in the bioactivity of inositol. Due to its characteristics, this treatment is simple and easily scalable, which makes it particularly attractive for potential industrial application.
9. The studies included in this report have generated new knowledge related to the characterization, fractionation and extraction of bioactive carbohydrates of vegetable matrices of food interest.

7. LISTA DE PUBLICACIONES

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