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AVANCES PARA EL ANÁLISIS DE MEZCLAS COMPLEJAS MEDIANTE TÉCNICAS CROMATOGRÁFICAS MULTIDIMENSIONALES

Advances for the analysis of complex mixtures by multidimensional techniques

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INTERNATIONAL THESIS 2018

UAM
UNIVERSIDAD AUTONOMA
DE MADRID

Universidad Autónoma de Madrid

Consejo Superior de Investigaciones Científicas

UNIVERSIDAD AUTÓNOMA DE MADRID

FACULTAD DE CIENCIAS

Departamento de Química Analítica

Avances para el análisis de mezclas complejas mediante técnicas cromatográficas multidimensionales

"Advances for the analysis of complex mixtures by multidimensional techniques"

Memoria presentada por:

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Para optar al grado de:
DOCTOR EN QUÍMICA APLICADA
(MENCIÓN INTERNACIONAL)

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CERTIFICAN:

Que el presente trabajo, titulado "**AVANCES EN EL ANÁLISIS DE MEZCLAS COMPLEJAS MEDIANTE TÉCNICAS CROMATOGRÁFICAS MULTIDIMENSIONALES**", y que constituye la Memoria que presenta D. Cipriano Carrero Carralero para optar al grado de Doctor en Ciencias Químicas, 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 23 de marzo de 2018.

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

A mis padres y abuelos…

AGRADECIMIENTOS

Hemos navegado en mares tropicales y en las más fieras tormentas, hasta que finalmente divisamos en el horizonte esta tesis. Creo que podría rellenar con los agradecimientos todas las hojas de este, mi cuaderno de bitácora. Pero me temo que tendré que ceñirme a unas pocas líneas.

Todo esto comenzó mientras trabajando entre vinos, los cantos de la investigación comenzaron a seducirme. Así que, oteando el horizonte, divisé un tentador anuncio en la antigua web del Instituto de Fermentaciones Industriales, respondí, y sin dudarlo, puse rumbo a los madriles a una entrevista con Mariluz Sanz y Javier Moreno. Días después de esa entrevista, sonó mi teléfono y era Mariluz invitándome a bordo como grumete. Muchas gracias a ambos por ese voto de confianza en ese inicio. Pero sobre todo a Mariluz, que ha sido la patrona del barco todos estos años y que, a pesar del paso por tormentas y tempestades, ha sabido aprovechar el viento dominante al máximo y llevar a este grumete hasta al avistamiento del puerto. Una profesional excelente y de una calidad humana doblemente mejor, por ello me siento realmente afortunado por haberte tenido como mentora todos estos años. Al poco apareció Lourdes con su transatlántico, así que lancé un cabo y me uní a su tripulación. Muchas gracias por la confianza puesta en mí en ese inicio y dejarme seguir la estela de tu embarcación, por tu conocimiento, bien hacer durante todos estos años y por introducirme al apasionante mundo del GCxGC. Por supuesto, gracias al Ministerio de Economía y Competitividad por mi beca predoctoral, sin lo cual nada de todo esto hubiera sido posible.

Dentro de la tripulación fui conociendo a numerosos marineros y grumetes a lo largo de los años. No juzgo lo que he aprendido en Química Analítica, eso lo dejo al juicio del tribunal de esta tesis, pero en lo que se refiere a humanidad, creo que hemos batido records. Por orden de aparición son Sonia Rodriguez, Laura Ruiz, María Francesca de Agostino, Inmaculada Calvillo, Andrea Martín, María Jesús García, Drashti Manthsucani y las incorporaciónes de última hora: Adal Mena y Ana María Zuluaga. Igualmente quiero agradecer al personal del puente de mando: Ana Cristina Soria y Ana Ruiz Matute. Gracias por vuestra amistad, sois lo mejor!. Un cariñoso recuerdo siempre voy a guardar de Jesus Sanz e Isabel Martínez por su ayuda y sabiduría, que nos han guiado cuando atravesábamos peligrosos estrechos de espectros de masas indiscernibles. Igualmente, quisiera agradecer a Gonzalo Almendros por su ayuda en el fascinante mundo de los lípidos del suelo. También a los de líquidos, los informáticos, los de ambientales del piso de arriba y la familia de los Quintanilla.

Pero este mi bote, que poco a poco iba añadiendo velas y convirtiéndose en velero, hizo alguna incursión a alta mar tras la estela de otros grandes buques como el del CIAL (2012-2013), en aquella estancia de 6 meses en la que pasé muy buenos momentos. Y mi estancia en la "University of Amsterdam" donde agradezco a Peter Schoenmakers el brindarme la posibilidad de poder unirme a su tripulación durante el 2015, al igual que a todos los chicos de Ámsterdam por su cálida bienvenida.

GRACIAS a Cipriano Carrero Fuentes y María África Carralero Fernández, mis padres, a los que se debe más que a nadie que usted pueda estar leyendo estas líneas. Por su apoyo incondicional e incansable todos estos años, que no ha flojeado en ningún momento. Han sido los propulsores desde el principio de un muy largo camino de colegio, instituto, universidad, master y doctorado. Siempre han estado ahí, a pesar de lo que ocurriera. Un ejemplo a seguir, trabajadores, totalmente volcados en la familia, atentos a que nada nos falte y que todo vaya bien. En el fondo el que el niño haya salido científico es culpa de mi madre y Parque Jurásico. Y nada que decir de las aportaciones a la ciencia que ha hecho mi padre con el huerto de Colmenar de Oreja, con todas las muestras que hemos sacado de allí para el laboratorio. He de deciros que llegó a cultivar la judía munga (*Vigna radiata*) y con unos resultados excepcionales. En fin, UN LUJO DE PADRES!. A mis abuelos Filomena Fernández Cámara, Bautista Carralero del Castillo y Cipriano Carrero Mingo, que seguro que estarán súper orgullosos ahora mismo; siempre os llevaré en el corazón. A mi abuela Carmen Fuentes Fernández, mi mejor fan y a la que quiero con locura. Y mis hermanas, por supuesto!

Y ahora sí que sí el momento Paco Martinez Soria. He de confesaros que durante este trayecto la barca vadeó un poco, y llegó hasta un archipiélago de Suecia. Allí entre la bruma aparecieron dos destellos azules rodeadas de halos dorados que me cautivaron instantáneamente. No lo dudé y salté a las frías aguas, sin importarme la distancia ni el esfuerzo para alcanzarlos. Fue una odisea psicodélica, extraña e incluso subrealista, al más alto estilo Massimo Bottura. Pero cuando volví a cubierta, me di cuenta que había encontrado el mayor tesoro que jamás me hubiera imaginado: Victoria. La puse en la proa de mi velero para siempre.

Y nada, después de esta tesis, ponemos rumbo a Italia, no sé hacia dónde nos llevará el viento, si habrá tormentas o por el contrario la mar estará en calma. Pero tan solo sé que mientras vaya acompañado de mi fiel tripulación de a bordo y esté rodeado de personas tan maravillosas como con las que he estado estos últimos años, no habrá problema.

Sé y soy consciente de que faltan muchos nombres que citar en estos agradecimientos así que, si lo estás leyendo y te das por aludido/a, tan solo quisiera darte las GRACIAS de todo corazón

Gracias

Cipriano Carrero

Resumen / Summary

RESUMEN

La cromatografía de gases (GC) es una técnica analítica ampliamente aceptada para la resolución de mezclas relativamente complejas en la que la identificación se basa en la comparación de los tiempos de retención de los analitos con los de patrones adecuados analizados en las mismas condiciones. Sin embargo, cuando la complejidad de las mezclas estudiadas aumenta, la posibilidad de coelución de los analitos de interés con otros componentes de la mezcla o de la matriz presentes en el extracto también aumenta. Es entonces cuando se hace necesario incorporar un criterio adicional de separación o identificación para evitar ambigüedades en la determinación de los componentes de la mezcla. Las técnicas multidimensionales resultantes permiten una determinación más fidedigna de los analitos al basar su identificación en al menos dos criterios independientes. En el caso de las técnicas cromatográficas, la multidimensionalidad pueden venir dada, por ejemplo, por su acoplamiento con una segunda técnica de separación cromatográfica o con una técnica de espectrometría de masas (MS) que aporte información estructural respecto al analito que eluye de la columna cromatográfica a un tiempo determinado.

En línea con esta consideración, el objetivo principal de esta tesis doctoral se centra en **el empleo de técnicas cromatográficas multidimensionales basadas en los acoplamientos GC-Q MS y GC×GC-ToF MS para la resolución de problemas de diversa índole en los campos alimentario y ambiental**.

Con este fin, se han planteado una serie de casos de estudio concretos que, bien por la complejidad de la mezcla a analizar, bien por el elevado número de analitos a determinar y la similitud de sus estructuras químicas, requieren un aumento en la resolución y/o selectividad de la determinación analítica respecto a la aportada por la correspondiente técnica cromatográfica monodimensional. Así, entre los estudios propuestos, se incluye (i) la extracción y análisis de carbohidratos y glicósidos bioactivos de muestras vegetales, (ii) la caracterización de lixiviados de envases alimentarios, y (iii) la caracterización de los lípidos y carbohidrato de bajo peso molecular de la materia orgánica del suelo.

En lo referente a la extracción y análisis de carbohidratos y glucósidos bioactivos de muestras vegetales, en primer lugar, se ha optimizado un procedimiento de extracción selectiva de carbohidratos bioactivos en *Vigna radiata* mediante MAE y los resultados se han comparado con los obtenidos mediante SLE. La metodología desarrollada ha permitido la caracterización de estos analitos mediante GC-Q MS. Asimismo, se ha llevado a cabo un estudio exhaustivo sobre los glicosil-ciclitoles, que ha permitido el establecimiento de relaciones entre la estructura química de estos compuestos y sus datos obtenidos por GC (índices de retención lineales) y MS empleando patrones, completando la hasta ahora limitada información disponible en este campo. Además, el desarrollo de un método de derivatización y análisis por GC-Q MS and GC×GC-ToF MS de glucósidos de fenilalcanoides ha permitido el análisis cuali- y cuantitativo de estos compuestos y de carbohidratos de bajo peso molecular en complementos alimenticios de raíz de *Rhodiola rosea*. El método optimizado ha permitido también la identificación de nuevos compuestos en estas muestras.

En esta Tesis, también se han optimizado las condiciones experimentales de separación mediante GC×GC y de detección mediante ToF MS de las mezclas complejas de lixiviados obtenidos al tratar envases comerciales contenedores de alimentos de polipropileno con simulantes alimentarios de distinta naturaleza. Los simulantes se seleccionaron de acuerdo a la legislación vigente y se aplicaron con el fin de emular un uso prolongados de los envases. Los resultados obtenidos en esta parte del estudio han puesto de manifiesto la capacidad de migrar desde estos envases al simulante de compuestos con muy distintas propiedades físico-químicas empleados en la formulación de estos plásticos y ha permitido determinar la presencia en los lixiviados de diferentes productos de degradación no siempre recogidos en la legislación.

Por último, se ha profundizado en la caracterización de los lípidos y los carbohidratos de bajo peso molecular presentes en matrices complejas de suelos mediante GC-MS y GC×GC-ToF MS. Con este fin, se ha procedido a realizar una evaluación cuali- y cuantitativa de la presencia de estos compuestos en extractos polares y apolares obtenidos de hoja, hojarasca y suelo de diferentes bosques mediterráneos mediante GC-Q MS. En su conjunto, los resultados obtenidos han permitido establecer relaciones en el proceso de transformación de los compuestos desde la planta hasta su incorporación al suelo. Sin embrago, la complejidad de los extractos de hojas obtenidos hizo necesario el empleo de una técnica capaz de proporcionar una mayor resolución, como es la GC×GC-ToF MS, con el fin de garantizar la determinación inequívoca de algunos de estos analitos.

Los resultados descritos en esta Tesis suponen una contribución destacable en el empleo de las técnicas cromatográficas multidimensionales basadas en los acoplamientos GC-Q MS y GC×GC-ToF MS para la resolución de problemas de diversa índole en los campos alimentario y ambiental.

SUMMARY

Gas chromatography (GC) is a widely accepted analytical technique for the analysis of complex mixtures. In GC, the analyte identification relies on the mutual agreement of the retention times of the investigated analyte and that of a suitable standard analysed under the same experimental conditions. However, as the complexity of the studied mixture increases, the possibility of coelution of the analytes with another compound or matrix component present in the extract increases, and the need to incorporate an additional identification criterion becomes mandatory. The resulting multidimensional technique should allow an unambiguous analyte determination based at least on two independent identification criteria. In the case of multidimensional chromatographic-based techniques, that second identification criterion can derive from the hyphenation with, for example, with a second chromatographic separation technique or with a mass spectrometry-based (MS) technique which provides structural information.

In line with this consideration, the main objective of this PhD thesis is **the use of multidimensional chromatographic techniques based on the GC-Q MS and GC×GC-ToF MS to provide appropriate solutions to specific problems in fields of food and environmental analysis**.

To achieve this general goal, several cases of study have been addressed which, because of the complexity of the mixture to be analyzed or the high number of analytes to be determined and the similarity of their chemical structures, required an enhanced resolution power and/or a selectivity higher than the provided by the corresponding one-dimensional chromatographic technique. Thus, the proposed studies included (i) the extraction and analysis of bioactive carbohydrates and glycosides from plant samples, (ii) the characterization of migration solutions obtained by treatment of plastic food containers with food simultants, and (iii) the characterization of the free lipids and low molecular weight carbohydrates present in the soil organic matter.

Regarding the extraction and analysis of carbohydrates and bioactive glycosides from plant samples, first of all, a new MAE-based sample preparation methodology was optimized to allow the selective extraction of bioactive carbohydrates from *Vigna radiata*, and the results were compared with those provided by the more conventional SLE. The developed method allowed the characterization of the target analytes by GC-Q MS. Then, an exhaustive study of the investigated glycosyl-cyclitols was carried out, which finally allowed the establishment of relationships between the chemical structure of these compounds and their GC (retention index) and MS data. These data represented a relevant contribution to the, up to now, scarce data reported concerning this topic. On the other hand, the investigation performed regarding the derivatization of glycosides of phenylalkanoids and their analysis by GC-Q MS and GC×GC-ToF MS have resulted in a novel method for the qualitative and quantitative determination of these analytes and low molecular weight carbohydrates in *Rhodiola rosea* root food supplements. This methodology also allowed the identification of some not previously described compounds.

In another study, the experimental conditions affecting the separation by GC×GC and the detection by ToF MS of the analytes present in the complex mixtures obtained by treatment of commercial polipropilene food containers with appropriate food simulant solutions has been optimized. Food simulants were selected according to current EU legislation and treatment conditions were those corresponding to long time use. Results obtained in this study evidenced that a relatively large number of compounds with different physicochemical properties and included in these plastic formulations were able to migrate from the container into the several assayed food simulants. In addition, a number degradation compounds not included in current legislation were also identified.

The last study included in this PhD book leaded with the characterization of the free lipids and low molecular weight carbohydrates present in complex soil matrices by GC-MS and GC×GC-ToF MS. In this case, GC-Q MS was used for the qualitative and quantitative determination of these compounds in polar and non-polar extracts obtained from leaves, litter and soil from different Mediterranean forests. Altogether, the results obtained allowed to establish a relationships between the degradation process of the analytes and the composition of the soils organic matter. However, the complexity of the leaves extracts made advisable the use of an analytical technique able to provide an enhanced separation power, such as GC×GC-ToF MS, to ensure the unambiguous determination of some of the target analytes.

The results summarized in this PhD Thesis do contribute to illustrate the potential of the multidimensional chromatographic techniques GC-Q MS and GC×GC-ToF MS to unravel the composition of complex mixtures obtained from food and environmental matrices and involved in relevant case studies in these applications fields.

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1. Introducción

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1.1 La cromatografía en la resolución de problemas analíticos

Uno de los principales objetivos de la Química Analítica es el desarrollo de métodos que permitan dar respuesta a los nuevos retos que se plantean en distintas áreas de la Ciencia tales como la ciencia y tecnología de alimentos, la medicina, la edafología, o el medio ambiente, entre otras, y con objetivos tan diversos como identificar marcadores químicos que permitan establecer la genuinidad de un alimento, detectar una posible enfermedad, identificar signos de degradación de los ecosistemas, evaluar la presencia de contaminantes en muestras ambientales o encontrar productos que eviten el deterioro de obras de arte, por mencionar algunos ejemplos.

Entre todas las posibles herramientas de separación que emplea la Química Analítica para dar respuesta a estos problemas, las técnicas cromatográficas y, en concreto la cromatografía de líquidos (LC) y la cromatografía de gases (GC), son las más empleadas por su robustez, eficacia y la posibilidad de modular su selectividad. La elección entre una u otra técnica vendrá condicionada principalmente por las características de los analitos a determinar (peso molecular, volatilidad, polaridad, etc.). Así, la LC ha sido en general empleada para el análisis de compuestos polares y semi-polares de peso molecular "relativamente" elevado. Por su parte, la GC se ha considerado tradicionalmente la técnica de elección para el análisis de compuestos volátiles. Sin embargo, el análisis de compuestos semi-volátiles o de baja volatilidad es también posible con GC, si bien en este caso requiere el empleo de reacciones de derivatización de los analitos de interés previo a su análisis instrumental.

A pesar de la gran eficacia de estas técnicas cromatográficas monodimensionales, en algunos casos no son capaces de resolver de manera completa algunas de las mezclas complejas existentes en la naturaleza. En estos casos, pueden tener lugar coeluciones entre los analitos entre sí o con otros componentes coextraídos de la matriz en la que se encuentran y que el proceso de preparación de muestra no ha sido capaz de eliminar en su totalidad. Es entonces cuando se hace necesario el empleo de técnicas cromatograficas multidimensionales. Este término hace referencia al acoplamiento de dos técnicas que proporcionen información complementaria respecto a la identidad de los analitos detectados siendo al menos una de ellas cromatográfica. Los ejemplos más comunes son los acoplamientos multidimensionales del tipo GC–GC, LC–LC, y los bidimensionales GCxGC y LCxLC, así como los de dichas técnicas en sus formatos mono- y multi-dimensional con la espectrometría de masas (MS).

En el caso de la cromatografía multidimensional clásica (por ejemplo, GC–GC, LC-LC), dos columnas de dimensiones convencionales pero con fases estacionarias distintas se acoplan en serie mediante una interfase que permite la transferencia selectiva de una o varias fracciones los denominados heart-cuts que eluyen de la primera columna hacia una segunda columna para la mejora de la separación de los analitos respecto a lo conseguido en la primera. Es una técnica muy útil cuando el análisis en una sola dimensión no permite la separación de los compuestos por presentar estructuras y/o propiedades físico-químicas muy similares o incluso idénticas, como es el caso de los compuestos quirales (Bordajandi, 2005). Sin embargo, el empleo de columnas convencionales en ambas dimensiones limita en la práctica el número de transferencias o heart-cuts que pueden ser transferidos entre ambas columnas sin comprometer la separación conseguida en la primera dimensión. Este hecho hace que, cuando en un análisis hay que realizar varias transferencia, sea necesario emplear análisis suesivos del mismo extracto, lo que puede dar lugar a un aumento considerable del tiempo de análisis. Este hecho, unido a la complejidad creciente de muchos de los extractos reales investigados, ha estimulado el interés por las técnicas cromatograficas bidimensionales, en particular LC×LC y, sobre todo, por su mayor nivel de madurez GC×GC.

A diferencia de la cromatografía muldimensional antes descrita, en las técnicas bidimensionales la separación en la segunda dimensión tiene lugar en condiciones de cromatografía rápida, de tal forma que todo el eluyente de la primera columna es transferido a la segunda dimensión y el tiempo de análisis total es similar al de un análisis minodimensional. De esta forma, la totalidad de la muestra es sometida a dos procesos de separación diferentes en un único análisis, pero siempre preservando la separación conseguida en la primera dimensión , consiguiendo así un aumento importante del poder de separación (Giddings, 1987).

Otro de los problemas que presentan las técnicas cromatográficas monodimensionales (y en ocasiones también las multidimensionales a pesar de su mayor capacidad de resolución) es que, en ciertos casos, la simple comparación de los tiempos de retención de los analitos con los de sus correspondientes patrones analizados en las mismas condiciones resulta insuficiente para la garantizar la identificación inequívoca de los compuestos de interés por existir la posibilidad de coelución con otros compuestos de estructura similar en la mezcla problema. Este hecho se ve agravado en aquellos casos en los que no se dispone de patrones para todos y cada uno de los analitos de interés o cuando estos se encuentran presentes en la naturaleza como mezclas complejas de isómeros. En estos casos, los acoplamientos de las técnicas cromatográficas antes indicadas con la espectrometría de masas (MS) supone una posible solución al problema al

aportar la MS información estructural referente al analito, lo que contribuye a facilitar su identificación/caracterización de forma más fiable. Sin embargo, la caracterización de los compuestos en base a su espectro de masas no es siempre sencilla y requiere un estudio detallado de los fragmentos característicos de dichos espectros.

En esta tesis nos centraremos en el desarrollo de métodos de separación de compuestos orgánicos semi-volátiles y de volatilidad baja mediante GC y GC×GC, así como mediante los acoplamientos de estas técnicas con la MS, para la resolución de problemas relativos a diversas áreas de conocimiento, en particular relacionadas con la ciencia y tecnología de alimentos, la fitoquímica, y la edafología.

1.2. Metodologías analíticas para el análisis de compuestos semi-volátiles y de baja volatilidad mediante cromatografía de gases

Al margen de las etapas de muestreo y pre-tratamiento de la muestra (esta última entendida aquí como cualquier operación encaminada a definir y obtener una sub-muestra representativa, seca y homogénea para su análisis), cuya descripción excede el objetivo de esta Introducción, el análisis de muestras complejas como las abordadas en este trabajo implica necesariamente un tratamiento de la muestra previo a su análisis instrumental. El objetivo básico de un proceso de preparación de muestra es extraer el analito de la matriz en la que se encuentra atrapado para, una vez purificado y concentrado, ser sometido a su proceso de separación y determinación inequívoca mediante una técnica instrumental, en este caso cromatográfica, preseleccionada. La necesidad de una mayor o menor exhaustividad en las etapas de extracción y purificación, y de una mayor o menor demanda de capacidad de separación cromatográfica y sensibilidad en la detección vendrán determinadas por el objetivo del análisis, la complejidad de la matriz y del extracto a analizar y el nivel de concentración de los analitos en la muestra de partida.

Las siguientes secciones discuten algunos aspectos de interés en relación con las metodologías analíticas desarrolladas en esta tesis.

1.2.1. Preparación de muestra

La preparación de muestra es actualmente uno de los principales puntos de interés en el desarrollo del método analítico. Es necesario el desarrollo de nuevos métodos de preparación de muestra que, manteniendo los estándares de calidad y eficacia de los procedimientos convencionales, sean más rápidos, respetuosos con el medio ambiente y de menor coste que los disponibles en la actualidad.

La preparación de muestra para el análisis de compuestos semivolátiles y de baja volatilidad en muestras complejas implica siempre una primera etapa de extracción de los analitos de interés de la matriz de que forman parte. A continuación, y debido a la escasa selectividad de muchos de estos procesos de extracción, suele ser necesario llevar a cabo una purificación de los extractos obtenidos, con el fin de eliminar todos aquellos potenciales interferentes que puedan haber sido coextraído junto con los analitos de interés en la etapa anterior y que puedan interferir en su posterior determinación instrumental. Finalmente, en el caso de los compuestos poco volátiles o termolábiles, es necesario proceder a su derivatización previa al análisis mediante GC.

1.2.1.1. Extracción

El objetivo de esta etapa es recuperar los analitos de la matriz de forma completa y con las menores interferencias posibles. En la bibliografía, se describen una variedad de técnicas para la extracción de compuestos de volatibilidad baja o media en función de la naturaleza de los analitos y de la matriz. A continuación, se describen de manera sucinta las empleadas en el marco de este estudio.

La **extracción líquido-líquido (LLE)** se basa en la transferencia del analito de una muestra líquida (normalmente acuosa) hacia un disolvente inmiscible (en general, orgánico). Este proceso es sencillo y no requiere el uso de instrumentación especial. Además, su rendimiento puede mejorarse de manera sencilla con la aplicación de extracciones sucesivas o mediante el uso de sales, agentes acomplejantes o derivatizantes.

El reparto de un compuesto orgánico dado entre fases acuosa y orgánica inmiscibles depende de su coeficiente de distribución (K). K es esencialmente la relación de las concentraciones del soluto entre dos disolventes diferentes una vez que el sistema alcanza el equilibrio. En concreto,

en la LLE, K es la relación de la solubilidad del soluto disuelto en la fase orgánica respecto de la solubilidad del mismo analito disuelto en la capa acuosa (Chiou, 1977):

$$K = \frac{\text{Solubilidad en el disolvente orgánico (g/100mL)}}{\text{Solubilidad en el dicolvente acuoso (g/100mL)}}$$

Así, los compuestos solubles en agua permanecerán en la fase acuosa, mientras que las moléculas más apolares pasarán a la fase orgánica. Mediante el uso de disolventes adecuados, un analito puede fraccionarse fácilmente y extraerse prácticamente en su totalidad desde una fase hacia otra de distinta polaridad. Dado que el coeficiente de distribución es una relación, a menos que K presente un valor elevado, no es esperable que todo el analito sea transferido a la fase orgánica en un único proceso de extracción. Por este motivo, es común llevar a cabo una serie de extracciones sucesivas en las mismas condiciones hasta el agotamiento del compuesto de interés en la fase acuosa de partida.

A pesar de su sencillez, la LLE es por lo general una técnica lenta, muy manipulativa, con el consecuente riesgo de pérdida y/o contaminación de los extractos y cuya robustez puede verse afectada por la formación de emulsiones. Además, suele involucrar volúmenes de disolvente relativamente elevados, por lo que los extractos obtenidos suelen estar bastante diluidos, lo que hace imprescindible su concentración previa al análisis instrumental.

La **extracción sólido-líquido (SLE)** es una de las técnicas más comúnmente utilizadas para la extracción de compuestos insertos en matrices sólidas y semisólidas. Su fundamento es similar al de la LLE. En esta técnica, la muestra se mezcla con un disolvente extractante preseleccionado y la mezcla se agita durante un periodo de tiempo determinado, de tal manera que los compuestos pasen al disolvente en función de su afinidad al mismo. Una vez concluido el proceso de extracción, se procede a la separación del sólido de la fase líquida mediante filtración, decantación o centrifugación. (Nielsen, 2010).

Se pueden diferenciar tres etapas en el proceso de extracción sólido-líquido:

1. Una primera etapa en la que el disolvente penetra en la estructura de la matriz, lo cual se suele favorecer mediante la agitación de la mezcla, por ejemplo, por vía magnética o utilizando un baño de ultrasonidos.
2. En segundo lugar, se produce la difusión del compuesto de interés través de los poros del sólido hacia la superficie externa de la partícula de la matriz.
3. En la tercera etapa, se produce la transferencia del soluto desde la matriz al disolvente líquido que la rodea por solubilización del analito en este último.

Este proceso depende de diferentes variables que afectan a la velocidad y rendimiento de la extracción. Los principales factores que influyen en la SLE son:

(A) **Tamaño de partícula**: Lo ideal es que el material sólido presente un tamaño de partícula pequeño, con una distribución uniforme que favorezca una extracción homogénea. Las partículas pequeñas presentan una mayor relación superficie/volumen, favoreciendo la existencia de poros y reduciendo el tiempo requerido para la difusión a través de esos poros internos hacia la superficie que está en contacto con el extractante. Así, por ejemplo, para la preparación de material vegetal, el protocolo más habitual suele ser en primer lugar desecar la muestra y posteriormente triturarla, para favorecer la rotura de las paredes celulares que limitarían la difusión de los compuestos.

(B) **Disolvente:** El disolvente ideal debería permitir la extracción selectiva de los analitos de interés respecto del resto de los compuestos de la matriz. Una baja viscosidad del disolvente facilitará la difusión de los analitos a través de los poros de la matriz y, así, el proceso de extracción. Sin embargo, la viscosidad aumenta a medida que aumenta la concentración de soluto en el disolvente y, en consecuencia, la velocidad de extracción disminuye a lo largo del tiempo de extracción.

(C) **Temperatura:** La solubilidad y difusión del soluto en el disolvente aumentan con la temperatura, al igual que se reduce la viscosidad mejorando la circulación del fluido extractante.

(D) **Agitación**: Este factor aumenta la velocidad de transferencia de masa desde la superficie de la partícula hacia el disolvente, favorece la interacción con la superficie disponible de la partícula y evita la sedimentación de sólidos finos.

La SLE es una técnica bastante sencilla que puede favorecerse al realizar ciclos sucesivos de extracción o al aumentar la temperatura del medio. Sin embargo, también se caracteriza por una reducida selectividad, el empleo grandes volúmenes de disolvente, los largos tiempos de extracción y una elevada manipulación de los extractos.

La SLE es una técnica de extracción convencional bastante eficaz que cuenta con una amplia aceptación. De hecho, muchos de los métodos oficiales de análisis que involucran muestras sólidas y semi-sólidas siguen empleando SLE como técnica de referencia. Sin embargo, como se ha comentado, presenta diversas limitaciones. Con el fin de superar algunos de sus

inconvenientes, se han desarrollado técnicas de extracción acelerada con disolventes o fluidos, tales como la extracción con líquidos a presión (PLE), con fluidos supercríticas (SFE), o basadas en el uso de ultrasonidoso microondas. En esta tesis nos centraremos en el uso de las extracciones convenciones previamente descritas, LLE y SLE, y en la extracción asistida por microondas (MAE).

- **Extracción asistida por microondas**

La extracción asistida por microondas es una técnica adecuada para la extracción de compuestos de volatilidad media y/o baja. Durante la extracción tienen lugar diferentes interacciones fisicoquímicas: fuerzas de dispersión, difusión intersticial, fuerzas motrices e interacciones químicas que en gran parte están relacionadas con las propiedades del disolvente.

El calentamiento se produce debido a la orientación de las moléculas polares y a su rotación bipolar inducida por las microondas. Estos movimientos generan rozamiento entre las moléculas del disolvente extractante y de la matriz, y éste un calentamiento. A diferencia de la extracción convencional, en la que el calor se transfiere desde el disolvente al interior de la muestra, en la MAE el calor se disipa volumétricamente dentro del disolvente. En términos generales, los disolventes más polares absorben la energía de manera intensa debido a que presentan un momento dipolar permanente que es afectado por las microondas, mientras que en los disolventes no polares no se produce esa disipación de la energía en forma de calor.

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, 2009). 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 (ver Figura 1.2.1.), 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 la temperatura y presión de trabajo.

Figura 1.2.1. Equipo comercial de MAE con carrusel giratorio

1.2.1.2. Purificación

A pesar del empleo de disolventes lo más selectivos posibles durante la extracción, los extractos obtenidos a partir de matrices complejas suelen contener además de los analitos de interés, otros componentes interferentes procedentes de la matriz. Ello hace necesario que la mayor parte de los casos sea necesario llevar a cabo un tratamiento de purificación de los extractos obtenidos antes de proceder a su análisis instrumental (salvo, claro está, que se emplee una técnica muy selectiva para la separación y detección de los compuestos de interés). El objetivo de esta etapa del proceso de preparación de muestra es eliminar, o reducir en su concentración, aquellos componentes de la matriz coextraídos o potencialmente interferentes de los compuestos de interés durante su análisis instrumental o, en el caso de extractos alimentarios, en su actividad biológica. Estos procesos de purificación y/o fraccionamiento pueden involucrar uno o más tratamientos analíticos, en función de su selectividad y eficacia. En general, muchos de los protocolos de purificación en uso suelen implicar un primer tratamiento, o purificación, cuyo fin es de la eliminación de los compuestos presentes en el extracto de naturaleza o con propiedades físico-químicas distintas a las de los analitos de interés, seguido en un segundo tratamiento, o fraccionamiento, orientado a eliminar potenciales interferentes presentes en este extracto ya purificado, pero con estructura y/o físico-químicas muy similares a las de los compuestos investigados. La necesidad de este último paso de fraccionamiento depende en gran medida de la capacidad de separación de la técnica cromatográfica elegida y la selectividad/especificidad del detector empleado, sobre todo el en caso del análisis de compuestos a niveles residuales.

Las diferentes técnicas de purificación pueden clasificarse según presenten una naturaleza destructiva o no destructiva. La ventaja de las **técnicas no destructivas** es que respetan la naturaleza de los compuestos del extracto. Dentro de este grupo se incluyen técnicas como la diálisis, que es aconsejable cuando se tienen que eliminar grandes cantidades de analitos interferentes. Sin embrago, tiene la desventaja de involucrar grandes cantidades de disolventes orgánicos de elevada pureza y elevados tiempos de extracción. Como alternativa, la cromatografía de exclusión por tamaño (SEC), separa las moléculas en base a su tamaño mediante su elución a través de un gel. Las pequeñas moléculas se difunden en los poros del gel y su flujo a través de la columna se retarda en función de su tamaño, mientras que las moléculas grandes no entran en los poros y eluyen rápidamente. En consecuencia, las moléculas eluyen en orden de peso molecular decreciente (MW). Las principales desventajas de esta técnica son que suele involucrar volúmenes relativamente grandes de disolvente, lo que resulta en extractos diluidos, y que, dependiendo de la mezcla investigada, en muchos casos no es posible un

aislamiento completo de los analitos de interés debido a procesos de coelución parcial con otros compuestos presentes en el extracto.

Por su parte, el fraccionamiento entre los analitos de interés y otros compuestos que aún no se hubieran eliminado en las fases anteriores, suele llevarse a cabo mediante cromatografía con absorbentes diferentes como el carbón activo, Alúmina, Florisil® y ciertas fases estacionarias de cromatografía líquida de alta eficacia (como las de base de pirenil) que permiten la separación selectiva de los analitos de interés.

1.2.1.3. Derivatización

Es condición indispensable en GC que los analitos sean volátiles y térmicamente estables bajo las condiciones de análisis empleadas. Muchas muestras pueden ser sometidas a un análisis por GC directamente. Sin embargo puede ser necesario un tratamiento de derivatización de los analitos estudiados previo a la inyección de la muestra en el cromatógrafo cuando:

1. Los compuestos son demasiado volátiles para una adecuada separación en la columna cromatográfica y presentan unos tiempos de elución muy similares entre sí. El objetivo en este caso es aumentar su tamaño, disminuir su volatibilidad, y favorecer su separación del pico cromatográfico del disolvente.
2. Reducir la absorción en las paredes de la columna que pudiera ocurrir cuando se trabaja con compuestos muy polares que pudieran adherirse en exceso, lo que provocaría un indeseable ensanchamiento de la banda cromatográfica. En estos casos, se suelen sustituir los grupos polares del tipo de OH, SH o NH para evitar la formación de puentes de H entre las moléculas.
3. En el caso de analitos termolábiles, la derivatización puede aportar un aumento de la estabilidad, mejorando con ello la respuesta cromatográfica y la simetría del pico cromatográfico.

En el caso de moléculas de alto peso molecular que no sea posible volatilizar en el inyector, se suele proceder a la hidrólisis o pirólisis de la molécula, ya que aunque se pierda la estructura inicial de la misma, se puede obtener bastante información de los subcompuestos originados.

La derivatización de los analitos también puede resultar ventajosa desde el punto de vista de la detección mediante MS, ya que puede contribuir a reducir el grado de descomposición térmica

y catalítica de los compuestos y/o sus fragmentos, favoreciendo la obtención de espectros de masas más sencillos

Hay diferentes estrategias para la derivatización de los analitos, siendo los más comunes los que se basan en el reemplazo de grupos OH, SH o NH por cadenas de alquilos, acilo o sililo. También se emplean con mucha frecuencia las reacciones de condensación, que se basan principalmente en la unión de dos moléculas mediante la pérdida de una molécula de agua. Entre los compuestos susceptibles de ser derivatizados para su análisis por cromatografía de gases se encuentran los carbohidratos, los ácidos grados o los aminoácidos

1.2.2. Análisis Instrumental

1.2.2.1. Cromatografía de gases monodimensional

En la GC, la muestra se volatiliza al ser introducida en el inyector del equipo, lo que permite a los analitos ser arrastrados por un gas inerte que no interacciona con las moléculas del analito, ya que la interacción se produce únicamente entre el analito y la fase estacionaria de la columna cromatográfica. Los analitos separados en la columna cromatográfica son registrados por un detector situado al final de la columna.

Los inyectores más comúnmente empleados son los split/splitless. Se trata de un inyector de diseño sencillo, consistente en un cuerpo central que puede ser calentado y en el que se inserta una cámara de vaporización de vidrio (el linner), que es donde, de manera manual o automática, se introduce la muestra (en general, 1-2 µL del extracto purificado y concentrado disuelto en un disolvente orgánico volátil) con la ayuda de una jeringa cromatográfica. La muestra, al ser introducida en el linner (que suele estar a una temperatura de 250-300 °C), se vaporiza de manera inmediata y se mezcla con el gas portador para su transferencia a la columna cromatográfica de manera completa (modo splitless) o parcial (modo spilt). La elección entre un modo u otro de inyección dependerá fundamentalmente del nivel de concentración de los analitos en el extracto analizado, siendo el modo splitless el elegido en aquellos estudios orientados a la determinación de compuestos a nivel de trazas.

La columna capilar consiste en un tubo hueco de sílice fundido de paredes flexibles que varía en longitud entre 5 a 100 m, aunque ciertas columnas especiales pueden sobrepasar los 100 m. Los diámetros interiores de la columna suelen oscilar entre 0,1-0,18 mm (Microbore), 0,2-0,32 mm (capilar normal) y 0,53 mm (Megabore). Las columnas más empleadas son las capilares

normales, utilizándose las Microbore en análisis más rápidos de GC. Dentro de las columnas capilares, las fases líquidas que recubren su interior se caracterizan por contener fluidos de alta viscosidad o polímeros de textura gomosa en los que los analitos pueden disolverse. Así, a igualdad de punto de ebullición, los compuestos menos solubles en esta fase líquida eluirán antes, produciéndose la separación. El recubrimiento líquido está unido químicamente a las paredes de vidrio de las columnas capilares y reticulado internamente para dar espesores de fase que varían entre 0,1 y 5 µm. Las películas más gruesas retienen los compuestos más tiempo en la fase estacionaria, por lo que los analitos tendrán una interacción más prolongada en el tiempo y, por tanto, una mayor resistencia a eluir de la columna. Para separar compuestos de alto peso molecular normalmente se usan películas más finas (0,25 µm), con el fin de que los analitos permanezcan en la fase estacionaria menos tiempo (Ewing, 1997). Las columnas capilares líquidas están limitadas respecto a su temperatura de uso. Así, la temperatura más baja de uso está condicionada con el punto de fusión o ablandamiento de la fase estacionaria, mientras que el límite superior lo está por la degradación térmica o evaporación de la misma, detectable por la aparición de compuestos de degradación en el cromatograma y el incremento gradual del ruido de fondo (sangrado).

Las columnas capilares líquidas a base de polisiloxano (columnas de silicona) se encuentran entre las más usadas en la actualidad debido a ser químicamente inertes, presentar numerosas ventajas en su comportamiento térmico y carácter inerte, lo que las hace muy versátiles en sus usos. Además, presentan una buena solubilidad para diferentes solutos y baja tensión superficial (Rotzsche, 1991). Sin embargo, estas columnas no permiten trabajar a temperaturas superiores a los 360 °C. Por otra parte, presentan la posibilidad de introducir sustituyentes en la cadena de siloxano con diferentes estructuras y polaridades.

El desarrollo de nuevos materiales como el carborano ha permitido ampliar el intervalo de trabajo en GC hasta temperaturas entorno a los 430 °C (Novotny, 1972). También se han sintetizado una variedad de fases estacionarias con selectividades mejoradas,como la HT-8 [8% fenil (equiv.) policarborano-siloxano] con polaridad intermedia y diseñada para la retener específicamente a los compuestos más planos.

- **Selección de la fase estacionaria**

Un aspecto fundamental en el desarrollo del método instrumental de GC es la selección de la fase estacionaria idónea para el análisis a los compuestos de interés. Para su selección hay que tener en cuenta:

- La **selectividad**: Considerada como la capacidad de la fase estacionaria para diferenciar dos moléculas de soluto en base a sus propiedades químicas o físicas. La selectividad depende por tanto de las interacciones físico-químicas que puedan darse entre las moléculas de soluto y la fase estacionaria, consiguiéndose la separación entre dos analitos cuando dichas interacciones sean diferentes (Abraham, 1999). Dependiendo de la naturaleza de las fases líquidas de GC, existen tres tipos de interacciones principales: dispersión, interacción dipolar y enlaces de hidrógeno.

- La **dispersión** es el tipo de interacción dominante para las fases estacionarias de polisiloxano. Se trata de fuerzas de interacción muy débiles y, por tanto, en estas fases, el factor principal que rige la elución de los analitos es la volatilidad, de manera que cuanto más volátil es el compuesto, antes eluye de la columna. Las i**nteracciones dipolares** y por **enlaces de hidrógeno** contribuyen, en caso de ser posibles, a aumentar la retención de los analitos en la columna y, por tanto, pueden favorecer separaciones específicas de los compuestos con la fase estacionaria que, en estos casos, serán de tipo polisiloxano sustituido con con cianopropilo y trifluoropropilo (para el segundo de los casos).

- La **polaridad de la fase estacionaria:** La polaridad de la fase estacionaria stá determinada por la polaridad de los grupos sustituyentes y sus cantidades relativas en la fase líquida de la columna. Si bien la polaridad no está directamente relacionada con la selectividad, este parámetro puede tener un efecto pronunciado en la retención del compuesto y, por tanto, en su potencial separación de otros compuestos presentes en el extracto: a igualdad de volatilidad, se retendrá más en una columna aquel que presente una polaridad más similar a la de fase estacionaria. Para comparar la polaridad de una fase estacionaria de GC con otra se emplean las constantes de McReynolds (McReynolds, 1970). Estas constantes permiten caracterizar el tipo de interacción predominante en cada fase en base a los índices de retención de cinco compuestos seleccionados con diferente estructura, para

cada uno de los que predomina un tipo de interacción molecular con la fase estacionaria distinto. La constante de McReynolds mide el nivel de interacción entre cada uno de estos compuestos y la fase. La diferencia entre esa interacción (medida como índice de retención) para un compuesto dado y la medida en escualeno (que es la fase no polar que se usa como referencia para el cálculo), se expresa como un índice. La magnitud de ese índice determina el nivel de polaridad de la fase en una escala normalizada, permitiendo así la comparación entre unas y otras

En general, para extractos complejos cuyo contenido no es fácil anticipar debido a la gran variedad de compuestos que pueden estar presentes en la matriz de partida, suele ser frecuente el empleo de fases estacionarias apolares, al menos como una primera aproximación. Las relativamente elevadas temperaturas a las permiten trabajar estas fases (360 °C) resultan de especial interés en este tipo de análisis para asegurar la completa elución de los analitos menos volátiles dentro de un tiempo de análisis razonable. Entre las más comunes, caben mencionar las fases 100% metil polisiloxano o 5% fenil dimetil polisiloxano.

- **Optimización de las condiciones de análisis en cromatografía de gases**

Una vez elegida la fase estacionaria y sus dimensiones, es imprescindible optimizar los distintos parámetros instrumentales que afectan a la separación en la columna cromatográfica, como el volumen de muestra inyectada, el flujo del gas portador y el modo y temperaturas de trabajo. Respecto a este último aspecto, dependiendo de la naturaleza de los analitos de interés, se pueden elegir entre llevar a cabo la separación en condiciones isotérmas o en condiciones de temperatura programada. En el primero de los casos, la temperatura del horno cromatográfico se mantiene constante durante el proceso de separación de los analitos. Esta forma de trabajo se emplea para el análisis de extractos poco complejos y que contengan compuestos relativamente similares y que no experimenten una excesiva retención en la fase, ya que la anchura de pico aumenta de manera considerable al hacerlo el tiempo de análisis. Sin embargo, la complejidad de la mayoría de los extractos de origen alimentario o ambiental hace aconsejable emplear **programas de temperatura** que permiten modificar la temperatura a lo largo del análisis, contribuyendo a la mejora de la resolución, la forma de pico a lo largo del cromatograma y la reducción del tiempo de análisis. El programa de temperaturas a aplicar debe ser cuidadosamente optimizado para cada tipo de análisis a fin de conseguir para la mejor resolución posible de los analitos objeto de estudio, tanto entre sí como de otros componentes de la matriz que pudieran permanecer en el extracto.

1.2.2.2. Cromatografía de gases completa en dos dimensiones

Como se ha comentado previamente, en GCxGC la totalidad de los compuestos que eluyen de la primera dimensión (o columna) son transferidos para su separación adicional en una segunda dimensión (o columna) gobernada por un mecanismo de separación diferente (**Figura 2**). Por tanto, y con el fin de conservar el principio de que cualquier separación conseguida en la primera dimensión debe ser mantenida en la segunda (Giddings, 1987), en principio, la separación de cada fracción transferida a la segunda dimensión ha de ser completada antes de introducir la siguiente fracción. Para poder conseguir esto, la segunda dimensión tiene que operar en condiciones de cromatografía de gases rápida. Es decir que, en GCxGC, mientras que en la primera dimensión se suele emplear una columna con dimensiones convencionales (15-30 m x 0,25 um, 0,25 um de espesor de fase), la segunda, en general, suele ser mucho más corta (1-2 m), estrecha (0,10-0,25 um) y con menor espesor de fase (0,10 um). En estas condiciones, sobre cada pico cromatográfico que eluye de la primera dimensión es posible realizar 3-4 cortes que son trasnferidos de manera sucesiva a la segunda dimensión para su separación adicional (Figura 2). A continuación, el software permite la reconstrucción de los picos eluidos de ambas columnas, asignándoles sus correspondientes tiempos de retención en ambas dimensiones y proporcionando una representación bi- (o tri-)dimensinal del cromatograma, el contour-plot, en la que los puntos de igual intensidad aparecerán unidos por una misma curvas de nivel.

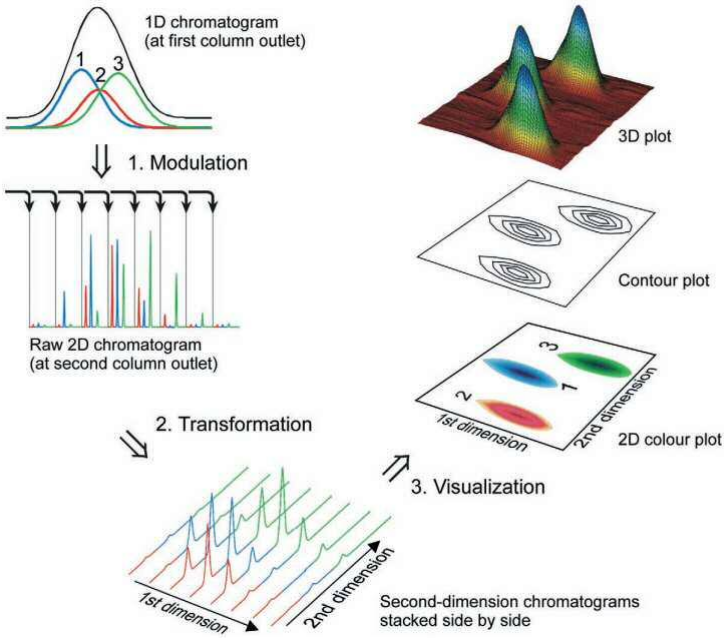

Figura 1.2.2: Descripción gráfica del proceso de separación en GCxGC, así como de la obtención del correspondiente *contour-plot* y la visualización tridimensional del cromatograma (Dallüge, 2003)

La combinación de columnas más empleada, casi con independencia del campo de aplicación emplea una columna no polar en la primera dimensión y otra polar en la segunda. Es la denominada combinación normal. De esta manera, mientras que los analitos se separan en la primera dimensión en función de sus diferentes volatilidades, la separación rápida, prácticamente isoterma, de la segunda dimensión neutraliza cualquier contribución de la volatilidad, por lo que la separación se deberá principalmente a interacciones específicas de los analitos con la fase estacionaria. En otras palabras, los dos procesos de separación cromatográfica serían pseudo-independientes y la separación pseudo-ortogonal. En estas condiciones, los cromatogramas muestran a menudo estructuras ordenadas en las que analitos pertenecientes a la misma familia pero con distinto peso molecular aparecen como bandas alineadas y paralelas (el conocido efecto tejado o "*roof tile*", **Figura 1.2.3**), de gran utilidad para identificación tentativa de compuestos estructuralmente relacionados.

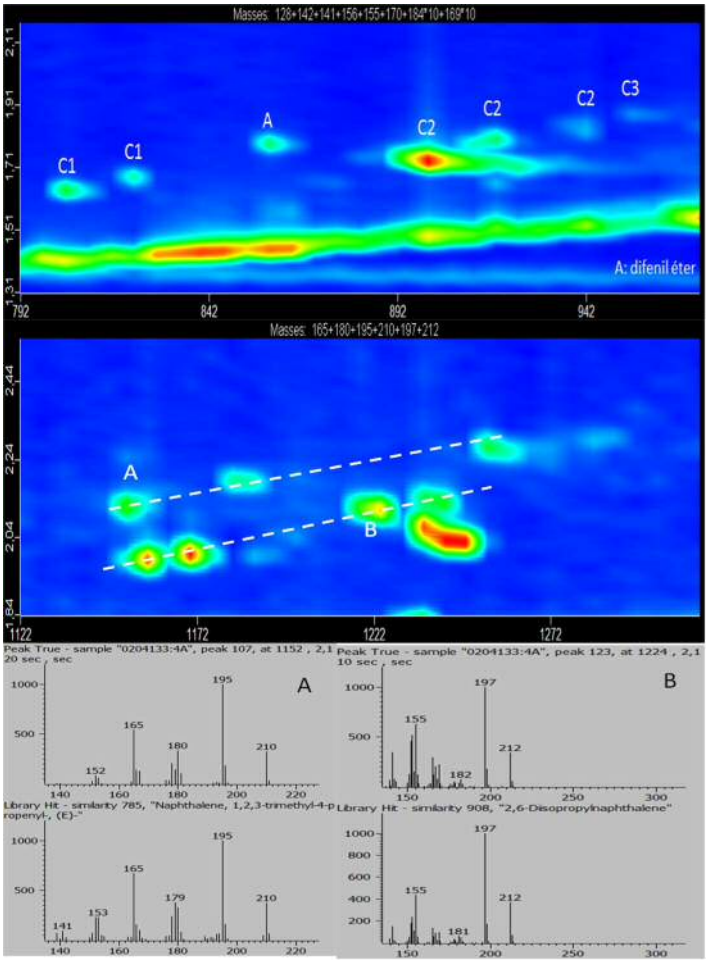

Figura 1.2.3. Perfil cromatográfico en "roof tile" obtenido por GCxGC de naftalenos C1- a C3- (cromatograma superior) y naftalenos C6 (cromatograma inferior) separados de los isómeros de trimetil-propenil (A) y diisopropilo (B), con sus correspondientes espectros de masas.

La combinación de columnas opuesta, es decir, polar-apolar suele denominarse combinación reversa.

Las dos columnas pueden estar en el mismo horno, es decir, en el horno del GC; o en distinto horno. En este caso, la segunda dimensión se introduce en un horno secundario de reducidas dimensiones que se instala dentro del horno principal y su programa de temperaturas será similar al de éste, pudiéndose aplicar un desfase (off-set) de temperaturas entre ambos o no.

El elemento central en esta técnica es el modulador. El modulador permite retener y enfocar los analitos que eluyen de la primera dimensión para su posterior reinyección total en forma de banda estrecha en la segunda dimensión. Existen distintos tipos de moduladores, siendo los más comunes los de válvulas y los térmicos, cada uno de ellos con sus respectivas ventajas e inconvenientes. Entre los últimos, los más empleados son los criogénicos, en los que se emplea un gas criogénico (por lo general, CO_2 o N_2) para el atrapamiento de los analitos que eluyen de la primera dimensión, y calor para su posterior volatilización y reinyección en la segunda. Existen distintos tipos de moduladores criogénicos. En este estudio se ha empleado un modulador de N_2 criogénico de cuatro jets en dos etapas, cuyo funcionamiento se muestra de manera esquemática en la **Figura 1.2.4**. Este modulador actúa sobre la parte inicial de la segunda dimensión y consta de dos 2 jets (o propulsores) para el enfoque, de manera alternada, de la muestra en dos puntos distintos de la columna mediante la aplicación de sendas corrientes de N2 criogénico. La liberación de los analitos atrapados en estos puntos de la columna se produce también de manera alternada, mediante la aplicación en el mismo punto, y durante un periodo de tiempo preseleccionado, de una corriente de aire caliente a una temperatura similar o superior a la del horno principal. Las condiciones de operación del modulador, es decir, la duración del periodo de modulación (P_M), de los pulsos de aire caliente y N_2 criogénico y la temperatura del aire caliente, deben ser cuidadosamente optimizadas ya que de su adecuada operación depende la eficiencia de los procesos de atrapamiento, reinyección y separación cromatográfica de los analitos en la segunda dimensión y, por tanto, del proceso de GCxGC.

Un aspecto de interés, en especial en análisis de residuos, relacionado con el proceso de modulación es que, el estrechamiento de los picos cromatográficos asociado al proceso de enfoque, suele resultar en una mejora en la sensibilidad, ya que la misma cantidad de analito alcanza el detector como una banda más estrecha de lo que lo haría de no haberse efectuado dicho enfoque. Esa mejora depende del compuesto en cuestión, pero suele oscilar, al menos, entre 10 y 25 veces respecto a la cromatografía monodimensional convencional. Esto permite

la utilización en GCxGC, incluso para el análisis de residuos, de detectores que, en cromatografía monodimensional, no se considerarían realmente adecuados para este fin (Ramos, 2009).

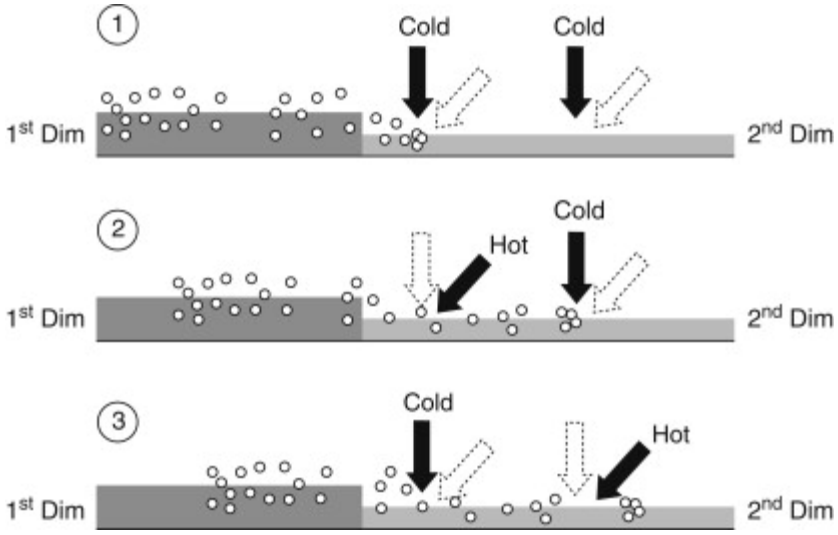

Figura 1.2.4. Representación esquemática del funcionamiento de un modulador criogénico de cuatro jet en dos etapas (Semard, 2009).

Los detectores empleados en GCXGC deben permitir la adecuada detección de los picos estrechos generados en la segunda dimensión, con anchuras típicas entre 50 a 600 ms (Ramos, 2009). Deben ser por tanto detectores que permitan velocidades de adquisición rápidas (al menos 100 Hz) o que adquieran datos en continuo. Entre los más comunes, sobre todo en las primeras etapas de la técnica, se encuentran el detector de ionización de llama (FID), el detector de nitrógeno-fósforo (NPD) o el microdetector de captura electrónica (micro-ECD). El acoplamiento con espectrómetros de masas ha sido más complicado debido a la limitada velocidad de adquisición de datos de la mayor parte de los espectrómetros comunes. Considerando que la velocidad de la mayor parte de cuadrupolos está en torno a 15-20 Hz en modo SCAN, su uso en combinación con GCxGC sólo sería posible, en el menor de los casos, para el registro de un intervalo muy reducido o seleccionado (SIM) de masas. La excepción es el espectrómetro de masas de tiempo de vuelo (ToF MS), que permite velocidades de adquisición superiores a los 200 Hz y, por tanto, adecuadas para el correcta detección y registro de los picos obtenidos mediante GCxGC.

1.2.2.3. Acoplamiento entre cromatografía de gases y espectrometría de masas

La MS es una potente técnica analítica basada en la ionización de los analitos, su posterior separación en función de su relación masa/carga (*m/z*) en un analizador y finalmente el registro de los fragmentos formados por un detector. El acoplamiento entre la GC y la MS presenta la ventaja frente a otras técnicas de detección de aportar información estructural de moléculas , además de la posibilidad de poder cuantificar.

En el análisis por MS, cuando los compuestos llegan a través de la columna hasta la **cámara de ionización** son normalmente ionizados por el impacto de electrones acelerados, por lo general, a 70 eV o mediante ionización química. Posteriormente, los iones son enfocados hacia el analizador, donde se separan los fragmentos cargados en función de su relación *m/z* permitiendo su posterior detección. Los principales analizadores utilizados en combinación con GC son los cuadrupolares (qMS), en el que los iones formados viajan a través del espacio comprendido entre cuatro barras equidistantes y son separados en función de su relación *m/z* debido al potencial de radiofrecuencia y de corriente continua aplicado a las mismas. Respecto a la GCxGC, el analizador de MS más empleado es, como ya se ha indicado, el **ToF MS,** que separa los iones de acuerdo con la energía cinética requerida para alcanzar el detector mientras se desplazan a lo largo de una distancia conocida. Los analizadores ToF-MS pueden adquirir a velocidades de 20 a 500 espectros de masa por segundo, lo que, por un lado, permite la reconstrucción apropiada de picos estrechos generados en la segunda dimensión del GCxGC y, por otro, facilita la adecuada deconvolución de posibles picos superpuestos.

La **interpetración de los espectros de masas** es un punto crítico del análisis químico que permite identificar los iones fragmentos de los espectros de masas, permitiendo la potencial elucidación de la estructura e identificación del compuesto o de su fórmula química. Para la identificación, siempre que es posible, se hace uso de librerías de espectros de masas (o espectrotecas). Ejemplos de espectroteca comerciales serían las biliotecas de espectros del Instituto Nacional de Estándares y Tecnología (NIST, Gaithersburg, Md., E.E.U.U.) (Stein, 2017) o la Wiley. Estas bibliotecas pueden ser comparadas con los espectros de masas obtenidos mediante EI a 70 eV a estudio mediante el uso de algoritmos.

A pesar de la gran capacidad de identificación alcanzada por las amplias bibliotecas de espectros y los diferentes algoritmos usados, en muchos casos la similitud estructural de las moléculas hace que sea difícil su identificación inequívoca mediante el uso exclusivo de estas herramientas. En estos casos, es fundamental el estudio detallado de los espectros de masas de los compuestos de interés ..

1.2.3. Caracterización analítica del método propuesto

La caracterización de un método ha de tener en cuenta todas sus etapas. Así, en el caso de un método cromatográfico, habrá que considerar el procedimiento de muestreo, la preparación de la muestra, la separación cromatográfica, la detección de los compuestos separados y la evaluación de la información analítica generada.

Los criterios de caracterización más frecuentemente considerados son la precisión, la exactitud, la linealidad, el límite de detección, el límite de cuantificación, la selectividad y la robustez. La elección de todos o algunos de los citados criterios dependerá de las características y objetivos del método de análisis a validar.

- La **precisión** es el grado de dispersión entre una serie de medidas obtenidas de múltiples repeticiones de una muestra bajo las condiciones de análisis establecidas. Se puede considerar a dos niveles distintos: repetitividad, cuando expresa la precisión en las mismas condiciones de operación y en cortos periodos de tiempo, y reproducibilidad, cuando las condiciones son distintas en cuanto a día, equipo, analista, etc. (Bressolle, 1996)
- **Exactitud** es la concordancia entre el valor analítico obtenido mediante el método y el valor verdadero de concentración del compuesto.
- **Linealidad** es el intervalo de concentración del analito en el que la respuesta es función lineal de su concentración.
- El **límite de detección (LOD)** es la cantidad o concentración mínima de sustancia que puede ser detectada con fiabilidad por un método analítico determinado. En cromatografía se suele calcular como 3 veces la desviación estándar del ruido de la línea base.
- **Límite de cuantificación (LOQ)** es la mínima cantidad o concentración de analito que se puede cuantificar con precisión y exactitud, de manera reproducible, lo que en cromatografía corresponde a 10 veces la desviación estándar del ruido de la línea base.
- **Selectividad** es la capacidad de un método analítico para obtener una respuesta debida únicamente al analito de interés y no a otros componentes de la muestra (Bressolle, 1996).
- **Robusted** es la medida de su capacidad para permanecer inafectado por pequeñas variaciones en las condiciones experimentales (Bressolle, 1996; Taverniers, 2004).

1.3 Biliografía

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2. Objetivos, metodología y plan de trabajo

2. Objetivos, metodología y plan de trabajo

2.1. Objetivos

El **objetivo general** de esta tesis, enmarcado dentro del proyecto del Plan Nacional CTQ2012-32957, **es el desarrollo de nuevos métodos de análisis mediante técnicas cromatográficas multidimensionales basadas en los acoplamientos GC-Q MS y GCxGC-ToF MS para la resolución de problemas de diversa índole en los campos alimentario y ambiental.** Con este fin, se plantean una serie de casos de estudio concretos que precisan mejoras, bien en relación a la etapa preparación de muestra, bien en la resolución cromatográfica o en relación a la elucidación estructural de los compuestos de interés.

Así, para conseguir este objetivo general, se plantearon los siguientes objetivos parciales según los tipos de muestra:

- Desarrollar nuevos métodos para la extracción selectiva de carbohidratos bioactivos (prebióticos y ciclitoles) a partir de muestras vegetales.
- Caracterizar por GC-Q MS mezclas complejas de ciclitoles y sus glicósidos. Aplicar estos avances para el análisis de estos compuestos en extractos vegetales.
- Caracterizar mezclas complejas reales de carbohidratos y glicósidos bioactivos mediante GC-Q MS y GCxGC-ToF MS.
- Caracterizar de forma exhaustiva la composición de lixiviados de envases de plástico empleados para la conservación de alimentos mediante GCxGC-ToF MS.
- Evaluar la composición en carbohidratos de bajo peso molecular y lípidos de la materia orgánica del suelo mediante GC-Q MS y GCxGC-ToF MS.

2.2. Metodología y plan de trabajo

Para alcanzar los objetivos parciales mencionados, se planteó un plan de trabajo (**Figura 2.2.1**) que incluía tres grandes bloques: (i) extracción y análisis de carbohidratos y glicósidos bioactivos de muestras vegetales mediante GC-Q MS y GCxGC-ToF MS (resultados presentados en la **Sección 3** de esta Memoria), (ii) caracterización de lixiviados de envases alimentarios mediante GCxGC-ToF MS (**Sección 4**), y (iii) caracterización de los lípidos y carbohidratos de bajo peso molecular de la materia orgánica del suelo (**Sección 5**).

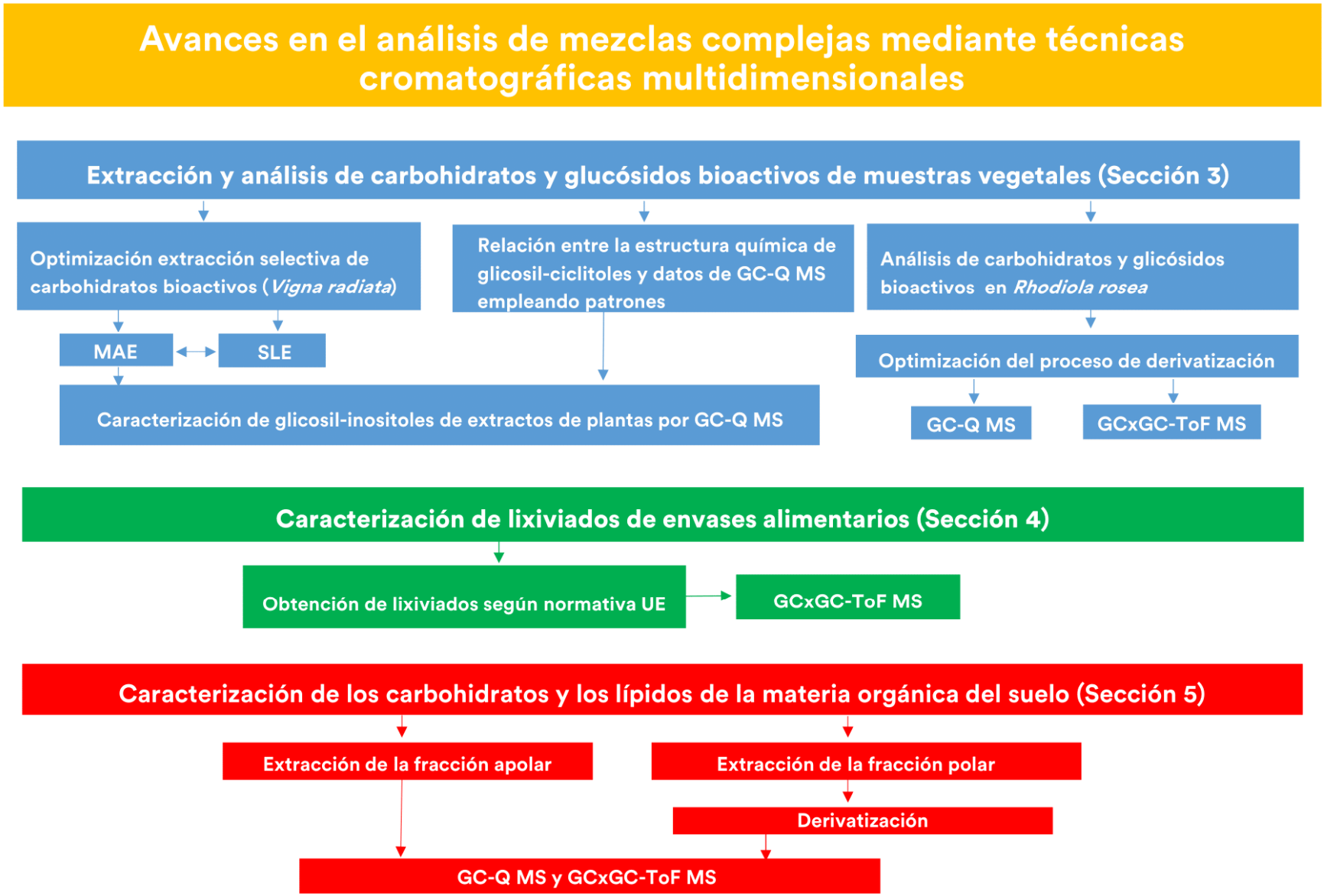

Figura 2.2.1. Plan de trabajo

A continuación, se detalla la metodología seguida para el desarrollo de cada una de las secciones previamente mencionadas:

- **Sección 3: Extracción y análisis de carbohidratos y glucósidos bioactivos de muestras vegetales**

    - Sección 3.1. Caracterización de glicosil-ciclitoles mediante GC-Q MS
        - Análisis por GC-Q MS de patrones de glicosil-ciclitoles derivatizados.
        - Establecimiento de relaciones entre la estructura química de glicosil-ciclitoles y los datos obtenidos por cromatografía de gases (índices de retención lineales) y por espectrometría de masas (abundancias de fragmentos *m/z* característicos).
        - Identificación/caracterización por GC-Q MS de nuevos glicosil-ciclitoles en muestras vegetales (hojas de *Coriaria* y legumbres)

    - Sección 3.2. Extracción selectiva y caracterización mediante GC-Q MS de glicosil-ciclitoles y otros carbohidratos bioactivos en extractos de *Vigna radiata*
        - Preparación de trimetilsilil oximas (TMSO) de los carbohidratos de semillas de *Vigna radiata*.
        - Caracterización de glicosil-ciclitoles y otros carbohidratos bioactivos mediante GC-Q MS. Elucidación de estructuras.
        - Optimización de las condiciones de extracción selectiva de los compuestos investigados mediante extracción sólido-líquido (SLE) y extracción asistida por microondas (MAE). Comparación de ambos métodos.
        - Análisis cuali- y cuantitativo de distintas muestras de semillas de *Vigna radiata*.

    - Sección 3.3. Identificación y caracterización de carbohidratos y glucósidos bioactivos de complementos de *Rhodiola rosea* mediante GC-Q MS y GCxGC-ToF MS
        - Optimización del proceso de derivatización de glucósidos de fenilalcanoides de *Rhodiola rosea*.
        - Extracción de carbohidratos y glucósidos bioactivos de raíz de Rhodiola.

- Optimización del método cromatográfico y caracterización de la metodología propuesta (intervalo de respuesta lineal, precisión, sensibilidad…)
- Aplicación del método desarrollado al análisis cuali- y cuantitativo de carbohidratos y glucósidos bioactivos mediante GC-Q MS y GCxGC-ToF MS. Caracterización de nuevos fenilalcanoides y carbohidratos de bajo peso molecular presentes en complementos alimenticios de *Rhodiola rosea.*

- **Sección 4: Caracterización de lixiviados de envases alimentarios mediante GCxGC-ToF MS**
  - Tratamiento de envases comerciales de polipropileno (PP) destinados a contener alimentos con simulantes alimentarios de diferente naturaleza seleccionados de acuerdo a la legislación vigente en la UE.
  - Optimización de las condiciones de análisis de los extractos mediante GCxGC-ToF MS.
  - Caracterización exhaustiva de los analitos presentes en los lixiviados de los envases alimentarios. Caracterización de compuestos no contemplados en la legislación vigente.

- **Sección 5: Caracterización de los carbohidratos de bajo peso molecular y los lípidos de la materia orgánica del suelo**
  - Obtención de extractos de hoja, hojarasca y suelo de bosques mediterráneos (enebro y pino) en disolventes orgánicos polares y apolares.
  - Análisis cuantitativo por GC-Q MS de los extractos obtenidos (previa derivatización de los extractos polares).
  - Análisis cualitativo por GCxGC-ToF MS de los extractos polares y apolares de hojas. Identificación de nuevos compuestos.

3. Extracción y análisis de carbohidratos y glucósidos bioactivos de muestras vegetales

3. Extracción y análisis de carbohidratos y glucósidos bioactivos de muestras vegetales

3.1. Prefacio

3.1.1. Ingredientes bioactivos: carbohidratos y glucósidos

El *Institute of Medicine's Food and Nutrition Board* de E.E.U.U. estableció en 1994 el término de alimentos funcional como "cualquier alimento o ingrediente alimenticio que proporcione un beneficio para la salud más allá de los nutrientes tradicionales contenidos en dicho alimento" (Thomas, 1994). Desde entonces se reconoce que los alimentos no solo proporcionan una nutrición básica, sino que también pueden prevenir enfermedades y contribuir a tener una buena salud y longevidad. Estos efectos sobre la salud pueden ser debidos directamente a ellos o deberse a los metabolitos producidos por el huésped o la microbiota intestinal.

Recientemente la obtención de diferentes ingredientes bioactivos para su uso alimentario ha generado gran interés con vistas al desarrollo de nuevos alimentos funcionales (Rodriguez, 2006). Este interés es aún mayor si los ingredientes bioactivos se obtienen a partir de productos naturales o, incluso, de matrices alimentarias. Son muchos los ingredientes bioactivos considerados hoy en día en el mercado de los alimentos funcionales; entre ellos, destacan los carbohidratos y glucósidos bioactivos.

- **Carbohidratos bioactivos**

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

- **Inositoles y derivados**

Los inositoles libres son ciclitoles que se caracterizan por presentar un anillo de 6 carbonos con un grupo hidroxilo en cada uno de ellos (1,2,3,4,5,6-ciclohexano hexanol). Hay un total de nueve isómeros que se diferencian por la disposición axial o ecuatorial de los grupos hidroxilo que presentan. La nomenclatura utilizada para designarlos es: *cis*-, *epi*-, *allo*-, *neo*-, *myo*-, *muco*-, D-*chiro*-, L–*chiro*- y *scyllo*- (Angyal, 1959) (ver **Figura 3.1.1.**).

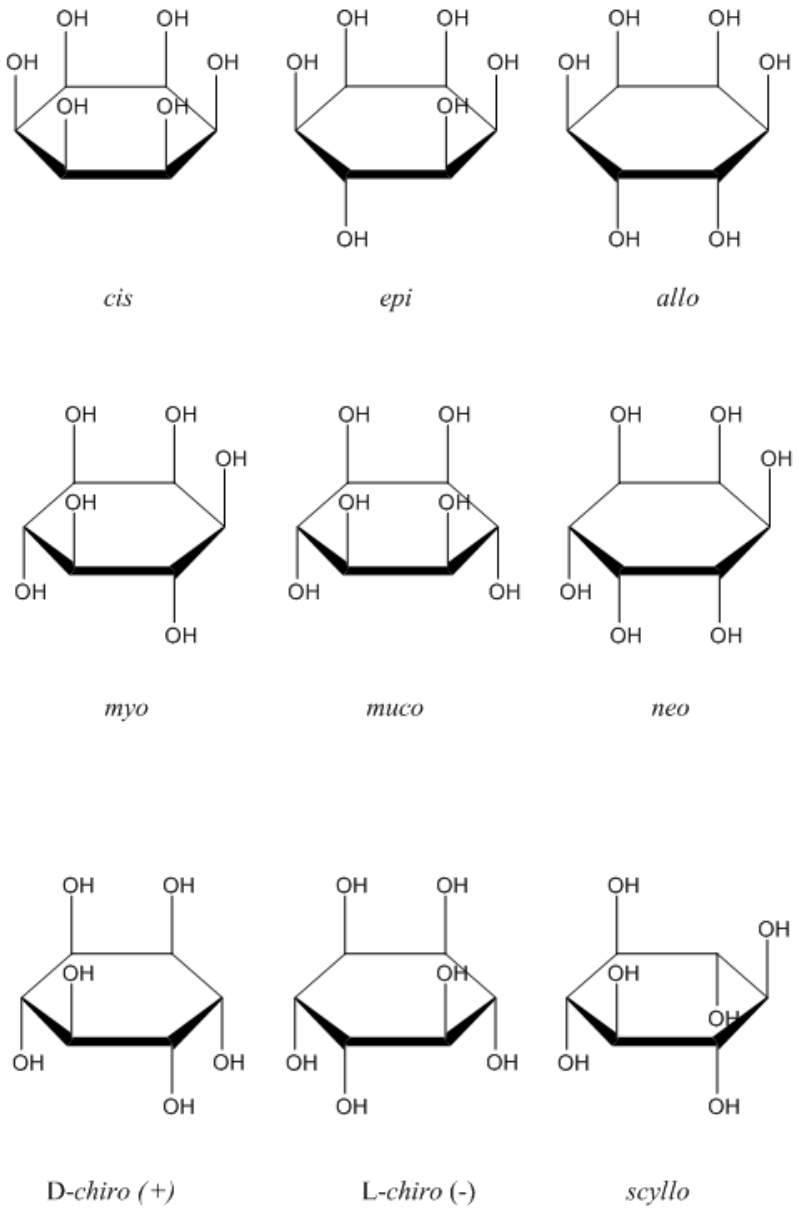

Figura 3.1.1. Estructura de los inositoles

Los inositoles libres pueden encontrarse en alta concentración en plantas como *Araceae, Leguminosae o Moraceae* (Obendorf, 1997; Watson, 2001), en las que llegan a alcanzar valores por encima del 30% de la cantidad total de carbohidratos solubles en algunas semillas (Horbowicz, 1994).

El *myo*-inositol es el más común y abundante en la naturaleza (Ruiz-Aceituno, 2013), encontrándose en forma de fosfolípidos en el reino animal y en forma de fosfatos en las plantas. En estas últimas, también se encuentran ampliamente distribuidos el D-(+)- y el L-(-)-*chiro*-inositol, principalmente en sus formas de metil-éteres [D-(+)-pinitol y L-(-)-quebrachitol]. Sin

embargo, inositoles como el *epi*-, *allo*- y *cis*-inositol solamente pueden ser obtenidos mediante síntesis (Anderson, 1972).

Los derivados de inositoles presentes en la naturaleza más frecuentes son los glicosil-inositoles y los glicosil-metil-inositoles, los cuales presentan uno o varios monosacáridos unidos al ciclitol. La **Tabla 3.1.1** recoge los nombres y estructuras de algunos de los glicosil-ciclitoles más comunes en la naturaleza. Un ejemplo es el fagopiritol, que está constituido 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. Otro ejemplo es el galactopinitol, que se caracteriza por estar compuesto por pinitol (3-*O*-metil-D-*chiro*-inositol) 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).

Unos de los alimentos más ricos en inositoles, tanto libres como glicosilados, son las legumbres. El D-chiro-inositol es especialmente abundante en la soja (Kim, Kim et al. 2005), mientras que el pinitol lo es en la algarroba (Baumgartner, 1986). Otros metil-inositoles como el ononitol o metil-scyllo-inositol son característicos de la alfalfa y el frijol mungo (Vigna radiata), respectivamente (Campbell, 1984). Los fagopiritoles (glicosil-chiro-inositoles) se encuentran en concentraciones relativamente altas en soja, altramuz, lentejas y garbanzos (Quemener, 1983); mientras que el galactinol es un glicosil-inositol mayoritario en alfalfa (Horbowicz, 1994) y lentejas (Quemener, 1983).

Los inositoles se han asociado principalmente con la resistencia a la insulina, y presentan la ventaja respecto de los fármacos hipoglucemiantes convencionales de no causar efectos secundarios gastrointestinales o hepáticos (Fonteles, 1996). Esto se debe a su enlace α-galactosídico, que los hace indigeribles por los seres humanos (Zheng, 1999). Se ha encontrado que la ingestión de inositoles y sus derivados por sujetos sanos induce una mejora en los parámetros del metabolismo de los carbohidratos (Bañuls, 2016). Por este motivo, estos compuestos se han propuesto para tratar trastornos tales como la diabetes mellitus, la obesidad, la aterosclerosis, la hipertensión, el síndrome de ovario poliquístico o el tratamiento de niños prematuros con dificultad respiratoria (Hallman, 1992; Ostlund, 1996; Nestler, 1999; Kim, 2005).

Tabla 3.1.1. Clasificación de inositoles más frecuentemente encontrados en la naturaleza.

	Glicosil-			**Glicosil-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					
***D-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)			

El *myo*-inositol se caracteriza por ejercer un importante papel en la utilización de la grasa. Además, promueve el crecimiento y mejora la conductancia nerviosa en diabéticos (Holub, 1986). Este compuesto es uno de los precursores de fosfatidilinositol, el cual forma parte de las membranas celulares y actúa como segundo mensajero en diferentes procesos metabólicos, como la activación de la serotonina. Así, las alteraciones en el metabolismo del *myo*-inositol se han relacionado con neuropatías como la depresión o la ansiedad, además de con la diabetes mellitus y el fallo renal crónico (Clements, 1980; Nemets, 2001). Diferentes estudios han comprobado valor terapéutico del *myo*-inositol en el tratamiento de la bulimia nerviosa (Gelber, 2001), la depresión y los trastornos obsesivo compulsivos (Nemets, 2001). Otras propiedades observadas en el *myo*-inositol es que disminuye la mortalidad por fallo respiratorio, y que reduce los posibles daños en la retina y pulmones de recién nacidos prematuros con síndrome de dificultad respiratoria (Hallman, 1992). Este compuesto es también empleadoen la industria cosmética, ya que mejora la oxigenación del tejido cutáneo (Charrier, 2004).

El D-*chiro*-inositol presenta propiedades similares a la insulina, ya que favorece la captación de la glucosa hacia el interior de las células musculares, contribuyendo así a la disminución de la glucosa en sangre (Yap, 2008), siendo efectivo contra la insulinoresistencia (Ostlund, 1996). Igualmente se han observado efectos positivos en el tratamiento de dolencias como el ovario poliquístico asociado a la hiperinsulinemia y la resistencia a la insulina (Nestler, 1999). Otras referencias apuntan a que el D-*chiro*–inositol, junto a otros inositoles como el *allo*-, *cis*-, *epi*-, *muco*-, *neo*- y *scyllo*-inositol, pueden útiles en el tratamiento de dislipemias, hipercolesterolemia o enfermedades cardiovasculares, ya que presentanpropiedades fisiológicas mejoradas con respecto a las del hexaniacinato de *myo*-inositol (Hendrix, 2008).

También se han atribuido diferentes propiedades a los metil-inositoles. Un ejemplo de ello es el pinitol, que mimetiza la acción hipoglucemiante de la insulina al estimular la captación de la glucosa por parte de tejido muscular y adiposo e inhibe la liberación de glucosa del hígado (Kim, 2005). Asimismo, disminuye los niveles de ácidos grasos plasmáticos y en 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, 1996) . También se le han atribuido propiedades antioxidantes y crioprotectoras, compartidas con otros metil-inositoles, como quebrachitol, ononitol o D-1-O-metil-muco-inositol (Orthen, 2000).

En relación con los glicosil-inositoles, está descrito el uso de fagopiritoles para el tratamiento del ovario poliquístico y de la diabetes mellitus tipo 2,debido a la similitud de la estructura de

estos compuestos con uno de los mediadores de la insulina que se encuentra de manera deficiente en dichos pacientes (Obendorf, 2002). .

- **Prebióticos**

Ciertos carbohidratos de bajo peso molecular presentan resistencia a la digestión en el tracto gastrointestinal superior, estómago e intestino delgado. Esto permite que lleguen íntegros al colon, donde sirven de nutrientes para ciertos microorganismos beneficiosos (probióticos), los cuales producen diversos productos bioactivos, como ácidos grasos de cadena corta que presentan propiedades anti-inflamatorias y anticancerígenas. Paralelamente, el potenciamiento del crecimiento de microbiota beneficiosa conduce a la exclusión competitiva de microorganismos patógenos del colon (Aluko, 2012).

Los prebióticos se definen como "ingredientes que producen 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, lo que confiere beneficios para la salud del hospedador" (Roberfroid, 2010). Las bifidobacterias y los lactobacilos son ejemplos de bacterias del colon que tienen el potencial de mejorar la salud del anfitrión. Los alimentos clasificados como prebióticos deben demostrar que no se descomponen en el estómago ni se absorben en el tracto gastrointestinal (Gibson, 1995).

Aunque existen muchos carbohidratos prebióticos que se obtienen por reacciones de síntesis (como los β-galactooligosacáridos o la lactulosa), muchos prebióticos se encuentran de forma natural en una gran variedad de plantas. Así, por ejemplo, las semillas de soja son una fuente importante de oligosacáridos como la rafinosa. Los oligosacáridos derivados de este carbohidrato se denominan α-galactooligosacáridos (α-GOS) u oligosacáridos de la familia de la rafinosa (Corzo, 2015). Otros carbohidratos prebióticos muy comunes son la inulina y los fructooligosacáridos (FOS) (Huebner, 2007).

- **Glucósidos bioactivos**

Según la IUPAC (Unión Internacional de Química Pura y Aplicada), un glucósido es ": cualquier molécula en la cual un glúcido se enlaza a través de su carbono anomérico, mediante un enlace *O*-glucosídico o un enlace *S*-glucosídico, a otro compuesto de diferente naturaleza

química". Por tanto, para que la molécula se califique como glucósido, el glúcido o glicona (mono- u oligosacárido) debe estar enlazado a una molécula que no sea otro glúcido (aglicona).

Los glucósidos desempeñan funciones importantes en los organismos vivos, si bien sus propiedades varían dependiendo de su estructura química. Los glucósidos se clasifican en función de esa estructura, principalmente atendiendo a la aglicona, siendo ésta parte de la molécula la más importante y útil en bioquímica y farmacología. En esta Memoria, nos centraremos en los glucósidos de fenilalcanoides, por sus numerosas propiedades bioactivas

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- **Glucósidos de fenilalcanoides**

Los fenilalcanoides son metabolitos secundarios presentes en plantas, bacterias y hongos. Los más comunes son los fenilpropanoides, compuestos derivados de fenilalanina y/o tirosina formados a partir de ácidos cinámicos o *p*-cumáricos (Seigler, 2012). En concreto, los glucósidos de fenilpropanoides son compuestos naturales presentes en algunas plantas utilizadas en medicina tradicional como la *Rhodiola, Eleutherococus, Silybum* o *Melissa* (Kurkin, 2003). A estos compuestos se les han atribuido propiedades bioactivas, tales como actividad antibacteriana, antiviral, analgésica, antiespasmódica, neuroprotectora, citostática, antiinflamatoria y antioxidante (Kim, 2001; Akbay, 2002; Ohno, 2002; Díaz, 2004).

Algunos de los glucósidos de fenilpropanoides más bioactivos son los glucósidos del alcohol cinamilo: **rosín** (cinamil-O-β-D-glucopiranósido, **rosavín** (cinamil-(6-O-α-L-arabinopiranosil)-O-β-D-glucopiranosido) y **rosarín** (cinamil-(6-O-α-L-arabinofuranosil)-O-β-D-glucopiranosido), también conocidos en su conjunto como rosavinas (Figura 3.1.2.). A estos compuestos se les han atribuido diferentes propiedades antioxidantes, neuroestimulantes, antidepresivas, nootrópicas, anticancerígenas, neurotrópicas, inmunoestimulantes y hepatoprotectoras (Kurkin, 2003; Kurkin, 2003; Kurkin, 2006; Bany, 2008).

Otros glucósido de fenilalcanoide destacable es el **salidrósido** (p-hidroxifeniletil-O-β-D-glucopiranosido; Figura 3.1.2.). Este compuesto es un glucósido del tirosol al que se le han atribuido propiedades contra la neurotoxicidad inducida por el amiloide-β generado en cuadros patológicos de Alzheimer (Zhang, 2016). El efecto neuroprotector del salidrósido se ha asociado con la estimulación del fosfatidilinositol-3-quinasa (Zhang, 2016). También se han atribuido a este compuesto efectos protectores contra lesiones pulmonares fibróticas, propiedades antidepresivas, anti-inflamatorias y antioxidantes (Tang, 2016).

3.1.2. Extracción selectiva y fraccionamiento de carbohidratos y glucósidos bioactivos

Para poder obtener extractos enrriquecidos en carbohidratos o en glucósidos bioactivos a partir de muestras naturales o de interés alimentario es necesaria la aplicación de técnicas de extracción que permitan la recuperación cuantitativa y lo más selectiva posible de estos compuestos de interés de una forma rápida, económica y eficaz.

Tratamientos previos a la extracción incluyen el secado y triturado de la muestra, tal y como se describió en la Introducción de esta Memoria. Una vez homogeneizada la muestra, se procede a la extracción de los compuestos de interés, en general mediante SLE. Este proceso se suele acelerar mediante agitación mecánica (Burbano, 1995) o la aplicación de ultrasonidos (Kong, 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 durante la extracción oscilan entre la ambiental hasta la de ebullición de los disolventes extractantes, que suelen ser polares, en particular agua, etanol o metanol, o mezclas de los mismos (Obendorf, 2000; Martínez-Villaluenga, 2004; Kim, 2005; Fuentes-Alventosa, 2009; Li, 2012). Como ya se indicó, la SLE suele involucrar grandes volúmenes de disolventes orgánicos y presenta además otras limitaciones, como los largos tiempos de extracción. Estas limitaciones han contribuido a aumentar en los últimos años el interés por evaluar el potencial en este campo de estudio de otras técnicas alternativas de extracción en las que este proceso se acelere mediante la aplicación de algún tipo de energía externa. A este grupo de técnicas de extracción pertenecen la MAE. Esta técnica se ha aplicado para la extracción de diferentes carbohidratos de alto peso molecular con una gran variedad de estructuras, como pectinas de frutas (Bagherian, 2011; Bélafi-Bakó, 2012; Li, 2012; Maran, 2013; Holck, 2014), celulosa de remolacha (Fishman, 2011), inulina de brácteas de alcachofa (Ruiz-Aceituno, 2016), galactomananos y arabinogalactanos de granos de café (Passos y Coimbra 2013), xilanos de fibra de maíz (Benkő, 2007), polisacáridos sulfatados del alga *Fucus vesiculosus* (Rodriguez-Jasso y col. 2011) y carragenanos de algas *Hypnea musciformis* (Vázquez-Delfín, 2014). Sin embargo, su aplicación para la extracción de carbohidratos de bajo peso molecular ha sido mucho más limitada. De hecho, hasta donde sabemos, sólo se ha empleado para la extracción de inositoles (*chiro*-, *scyllo*- y *myo*-inositol) de brácteas de alcachofa (Ruiz-Aceituno, 2016). Por tanto, resulta de interés el desarrollo de nuevos métodos de basados en MAE para la extracción de carbohidratos bioactivos en otras matrices alimentarias ricas en estos compuestos, como pueden ser las legumbres.

Sin embargo, uno de los problemas asociados a la extracción de carbohidratos bioactivos de fuentes vegetales es que, normalmente, estos compuestos se encuentran en la matriz en forma de mezclas complejas de compuestos con intervalos muy amplios y diferentes de concentración. De esta forma, la extracción de carbohidratos bioactivos lleva normalmente implícita la co-extracción de otros carbohidratos que pueden interferir en sus propiedades, lo que hace necesaria una etapa posterior de fraccionamiento entre ellos. Sin embargo, esta etapa no resulta trivial debido a la similitud de sus estructuras, la mencionada diferencia de las concentraciones a las que se encuentran en los productos naturales y la complejidad de las mezclas. El Anexo I incluye una revisión detallada de las principales técnicas empleadas para este propósito, así como la discusión de sus principales ventajas y limitaciones.

Como alternativa a estos métodos de fraccionamiento posteriores a la extracción, es posible plantearse la mejora de la **selectividad del proceso de extracción** de los carbohidratos bioactivos frente a los interferentes, lo que permitiría simplificar el proceso de preparación de muestra al hacer innecesario su fraccionamiento posterior. Es conocido que las diferencias de solubilidad de los carbohidratos en disolventes orgánicos dan lugar a la precipitación selectiva de algunos de ellos, facilitando así su separación. Sin embargo, las referencias bibliográficas en este campo son escasas. Hay algunos estudios que evalúan la solubilidad de cetosas bioactivas y aldosas en distintos alcoholes y mezclas hidroalcóholicas con objeto de poder separar ambos tipos de carbohidratos (Olano, 1979; Montañés, 2007). En general, la lactulosa y la tagatosa (cetosas) presentan mayor solubilidad que la lactosa y galactosa (aldosas) en estos disolventes, lo que permite su fraccionamiento.

Se han realizado también estudios para la extracción de inositoles del piñón empleando agua, alcoholes y mezclas hidroalcóholicas (Ruiz-Aceituno, 2014). Sin embargo, no se consiguió llevar a cabo una extracción selectiva de estos compuestos frente a los azúcares co-extraídos (glucosa, fructosa y sacarosa). Por tanto, sería de interés llevar a cabo más estudios empleando otras matrices, compuestos y condiciones.

3.1.3. Análisis de carbohidratos y glucósidos bioactivos por GC-MS y GCxGC-MS

En la bibliografía, se han descrito diversos métodos de análisis para la determinación de carbohidratos y glucósidos bioactivos. Estos métodos incluyen desde ensayos microbiológicos o enzimáticos (Angyal, 1959; Anderson, 1972; Kennedy, 1978) hasta métodos cromatográficos (Burbano, 1995; Tagliaferri, 2000; Sanz, 2004) o electroforéticos (Kong, 2008). Entre las técnicas cromatográficas de análisis, las más usadas son laGC y la LC y sus acoplamientos a MS. La GC es,

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

En cuanto a los glucósidos de fenilalcanoides, su análisis se ha llevado a cabo comúnmente mediante LC (Ganzera, 2001; Rodin, 2012). Sin embargo, el desarrollo de métodos por GC que permitieran el análisis simultáneo de los estos compuestos junto con los carbohidratos de bajo peso molecular sería de gran interés. No obstante, hay que indicar que tanto los carbohidratos como los glucósidos posee una alta polaridad, hidrofilidad y baja volatilidad, por lo que tienen que ser convertidos en derivados volátiles antes de poder ser analizados por GC.

- **Derivatización**

Los métodos clásicos de derivatización de carbohidratos consisten en reemplazar todos los átomos de hidrógeno activo por sustituyentes no polares. Los derivados de metilo, trifluoroacetilo, trimetilsililo y tert-butildimetilsililo son los derivados más populares, permitiendo el análisis por GC de sacáridos de hasta un cuarto grado de polimerización, aunque el desarrollo de columnas más resistentes a las altas temperaturas ha permitido el análisis de carbohidratos de 1400 Da (dos Santos Pereira, 1999) e incluso de 2500 Da (Dos Santos Pereira, 1998).

La sililación es uno de los métodos de derivatización más utilizados debido a la estabilidad de los derivados y el alto rendimiento de la reacción (Brokl, 2010; Ruiz-Matute, 2010; Rodríguez-Sánchez, 2013). En el proceso de sililación de carbohidratos, tiene lugar la introducción de grupos dimetilsilil, trimetilsilil o t-butildimetilsilil en los grupos OH, SH o NH del carbohidrato para la formación de silil éteres o ésteres (Churms, 1990). Para llevar a cabo la derivatización, se disuelve la muestra en disolventes no acuosos, tal como la piridina o dimetilsulfósido. La reacción de sililación se puede llevar a cabo con una amplia variedad de agentes donadores, tales como el hexametildisilazano (HMDS), trimetilclorosilano (TMCS), trimetilsililimidazol (TMSI) y bis(trimetilsilil)-trifluoroacetamida (BSTFA); los cuales pueden utilizarse combinados entre sí para incrementar el efecto sililante (Sanz, 2007). A pesar de que la reacción es instantánea, algunos autores proponen entre 5 minutos (Holligan, 1971) y 30 minutos (Knapp, 1979) de equilibrio. De la misma manera,la temperatura de reacción puede oscilar entre la ambiental y los 45˚C. Los derivados resultantes suelen ser bastante estables y volátiles (Sherman, 1970).

En el caso de los azúcares no reductores, la sililación da lugar a un único pico cromatográfico. Sin embargo, los carbohidratos reductores pueden formar hasta cinco formas sililadas para cada compuesto, correspondientes a las diferentes formas tautoméricas. En matrices complejas, esto puede llegar a ser un gran inconveniente. Por ello, en estos casos, se suele aplicar la transformación del grupo carbonilo en una oxima antes del proceso de sililación, disminuyendo de esta forma a dos el número de picos cromatográficos observados, los correspondientes a los isómeros *syn* (*E*-) y *anti* (*Z*-) (Molnár-Perl, 1997).

Estos procesos de derivatización están bien establecidos para el análisis de carbohidratos neutros. Sin embargo, para azúcares con distintos sustituyentes (ácidos, básicos…) y glucósidos, la reacción no resulta trivial y es necesario llevar a cabo una optimización del proceso seleccionando los reactivos y condiciones más adecuadas.

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

Aunque la GC-MS proporciona información estructural sobre los compuestos analizados, en el caso de los carbohidratos, su similitud estructural, la existencia de múltiples isómeros, la falta de patrones comerciales apropiados y la ausencia de bases de datos específicas hacen 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, siendo necesario realizar un estudio detallado de las relaciones *m/z* de los iones característicos, que en muchas ocasiones involucran fragmentos minoritarios. 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, lo que ayuda a su identificación (Knapp, 1979; Fox, 2002).

- **Análisis por cromatografía de gases bidimensional–espectrometría de masas**

Como se comentó en la Introducción general de esta Memoria, la GCxGC-ToF MS permite un aumento importante del poder de separación frente a la GC. Hasta el momento, los estudios descritos en la bibliografía respecto al uso de esta técnica para análisis de carbohidratos o glucósidos de fenilalcanoides son muy limitados, o incluso inexistentes para caso de los glucósidos. La GCxGC-TOF MS se ha aplicado con éxito al análisis de enantiómeros de

monosacáridos (Myrgorodska, 2017), además de al estudio de disacáridos de la miel (Brokl, 2010) y frutas (Marsol-Vall, 2015).

Considerando estos antecedentes, el objetivo general de esta sección de la tesis se centró en el desarrollo de métodos para el análisis de carbohidratos y glucósidos bioactivos procedentes de judía munga (*Vigna radiata*) y *Rhodiola rosea*, respectivamente. En concreto, la **Sección 3.2.** está basada en el artículo titulado "Characterization of cyclitol glycosides by Gas Chromatography coupled to Mass Spectrometry" de Ruiz-Aceituno, **Carrero-Carralero**, Ruiz-Matute, Ramos, Sanz y Martínez-Castro publicado en la revista *Journal of Chromatography A*, 1484 (2017) 58-64, en el que se realiza un estudio de los espectros de masas obtenidos mediante GC-Q MS para glicosil-ciclitoles, con el fin de establecer relaciones entre la estructura química de estos compuestos y los datos obtenidos por GC (índices de retención lineales) y por MS (abundancias de fragmentos *m/z* característicos) que ayuden a la identificación de compuestos desconocidos en muestra reales. Los resultados obtenidos en esta sección se aplicaron también en la **Sección 3.3** para la identificación de nuevo glicosil-ciclitoles en judía munga. Esta sección recoge el trabajo titulado "Extraction and characterization of low molecular weight bioactive carbohydrates from mung bean (*Vigna radiata)*" de **Carrero-Carralero et al.** enviado a la revista *Food Chemistry* para su publicación. En este estudio, se optimiza un proceso de extracción selectiva mediante MAE de inositoles, glicosil-ciclitoles y α-GOS y los resultados obtenidos se comparan con los proporcionados por la SLE convencional. Por último, en la **Sección 3.4** se pone a punto, por primera, vez un método mediante GC-Q MS y GCxGC-TOF MS para el análisis de glucósidos de fenilalcanoides en complementos de raíz de *Rhodiola rosea*. Esta sección está basada en el artículo titulado "Gas chromatographic techniques for the comprehensive characterization of *Rhodiola rosea* food supplements" de **Carrrero-Carralero** et al. enviado a la revista *Journal of Chromatography A* para su publicación.

Considerando además el gran número de técnicas empleadas para el fraccionamiento de carbohidratos bioactivos, en esta sección se ha considerado de interés incluir un anexo (**Anexo 1**) que revisa las técnicas más comúnmente empleadas para este fin, valorando sus respectivas ventajas y limitaciones. Este anexo está basado en el Capítulo "Fractionation of Food Oligosaccharides" de Moreno, **Carrero-Carralero**, Hernández-Hernández y Sanz, incluido en el libro "Food Oligosaccharides: Production, Analysis and Bioactivity" (Ed. Wiley Blackwell, 2014).

3.2. Characterization of cyclitol glycosides by Gas Chromatography coupled to Mass Spectrometry

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Journal of Chromatography A, Volume 1484, 2017, Pages 58-64,

https://doi.org/10.1016/j.chroma.2017.01.001.

3.2.1. Introduction

Cyclitol glycosides are considered pseudosaccharides, constituted by a chain of one to four galactosyl residues (linked in the C6 position) and an inositol or a methyl inositol. These compounds are naturally present in vegetal kingdom, mainly in seeds (Bjergegaard, 2001). Its accumulation is associated with the acquisition of desiccation tolerance and longevity of seeds (Obendorf, 2012). They are especially abundant in Leguminosae which are extensively consumed as food, feed and pasture. Other edible seeds such as buckwheat (*Fagopyrum esculentum*) (Steadman, 2000) and pine nuts (*Pinus pinea*) (Ruiz-Aceituno, 2012) are also sources of these compounds.

Several bioactive properties, mainly associated with insulin resistance, have been attributed to cyclitols (e.g. *chiro*-inositol and pinitol) and their derivatives (e.g. fagopyritols). Thereby, these naturally occurring pseudosaccharides have been proposed for treating disorders such as diabetes mellitus, obesity and polycystic ovary syndrome (Steadman, 2000; Thomas, 2016).

Cyclitol glycosides are water soluble and either conventional solid liquid extraction (Obendorf, 2012) or advanced techniques such as pressurised liquid extraction (Ruiz-Aceituno, 2014) have been proposed for their effective extraction from natural sources. Different techniques such as nuclear magnetic resonance (Bernabe, 1993; Chien, 1996; Obendorf, 2000), liquid chromatography (Vidal-Valverde, 1993), gas chromatography (GC) and GC coupled to mass spectrometry (MS) (Beveridge, 1977; Schweizer, 1981; Ruiz-Aceituno, 2013), have been used for cyclitol glycoside instrumental analysis. Among these techniques, GC-MS can provide valuable structural information for the characterization of these compounds along with other soluble carbohydrates avoiding tedious fractionation processes.

Although GC relative retention times of several cyclitol glycosides can be found in the literature (Skøt, 1984; Obendorf, 2005; Obendorf, 2012), linear retention index (I^T) data are still scarce (Ruiz-Aceituno, 2012; Ruiz-Aceituno, 2014). Moreover, a systematic study of their mass spectra is also lacking.

The aim of this work is to provide I^T values and MS data of different cyclitol glycosides and correlate this information with their chemical structure in an attempt to establish criteria that can be used for identification of unknown compounds. The practical applicability of the proposed approach has been demonstrated by the characterization of several non-previously identified cyclitol glycosides in seeds such as *Cicer arietinum* (chickpea) and *Vigna angularis* (adzuki bean) and in leaves of *Coriaria myrtifolia* and *Coriaria ruscifolia*.

3.2.2. Materials and methods

3.2.2.1. Standards and samples

IUPAC recommendations for nomenclature of cyclitols have been followed through this paper.

Sugars (sucrose, galactosyl-(1→6)-galactose, raffinose, stachyose and verbascose) and free inositols (*chiro*-inositol, *scyllo*-inositol, *myo*-inositol) were acquired from Sigma (St. Louis, MO). Free methyl-inositols, like sequoyitol, quebrachitol and pinitol were from Extrasynthèse (Genay, France), Carbosynth (Berkshire, UK) and Sigma, respectively, whereas bornesitol and ononitol were not available as commercial standards and were extracted from grass pea and black-eyed pea, respectively (Ruiz-Aceituno, 2013). Galactinol was acquired from Sigma and other galactosyl-inositols, such as fagopyritol A1, fagopyritol B1, fagopyritol A2, fagopyritol B2 and fagopyritol A3 and di-galactosyl-*myo*-inositol (DGMI), were extracted from buckwheat (*Fagopyrum esculentum*) as previously described (Gui, 2013; Ruiz-Aceituno, 2013). Regarding galactosyl-methyl-inositols, lathyritol was obtained from grass pea (*Lathyrus sativus*), whereas galactopinitol A1, galactopinitol B1 and ciceritol, were extracted from chickpea (Ruiz-Aceituno, 2013); galactosyl-ononitol and digalactosyl-ononitol were identified in black-eyed pea (*Vigna unguiculata*) according to data reported elsewhere (Richter, 1997; Peterbauer, 2003).

Samples (adzuki bean, black-eyed pea, buckwheat, chickpea and grass pea) were obtained from local markets in Madrid (Spain) and were ground using a domestic mill (Moulinex, Barcelona, Spain) and sieved through a 500 µm sieve before extraction.

Leaves from *C. myrtifolia* and *C. ruscifolia* were kindly supplied by Dr. R. Morales from Real Jardín Botánico de Madrid (CSIC).

3.2.2.2. Extraction of cyclitol glycosides

Exhaustive extraction of cyclitol glycosides and other low molecular weight carbohydrates from seeds and leaves was achieved by three consecutive extraction cycles of 1 g sample with 10 mL of hot Milli-Q water (60 ˚C) for 2 hours under constant stirring as previously indicated (Ruiz-Aceituno, 2012; Ruiz-Aceituno, 2013). The three extracts were combined, filtrated through a Whatman No. 4 paper and kept at -20 ˚C until analysis.

3.2.2.3. GC-MS analysis

A two-step derivatization procedure (oximation + silylation) was carried out prior to GC-MS analysis [21]. This methodology gives rise to two peaks for reducing sugars and only one for non reducing sugars which results in a better GC resolution of cyclitol glycosides from other coextracted carbohydrates from plants. In brief, 0.5 mL of phenyl-β-D-glucoside (1 mg mL^{-1}) was added to 1 mL of standard solutions or plant 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). Trimethylsilyl (TMS) derivatives were obtained using 350 µL of hexamethyldisilazane (Sigma) plus 35 µL of trifluoroacetic acid (Sigma) at 45 ˚C for 30 min. After centrifugation, 1 µL of supernatant was taken for instrumental determination.

GC-MS analysis was carried out using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA) with an HT5 (5% phenyl (equiv.) polycarborane–siloxane) capillary column (25 m × 0.22 mm i.d. × 0.1 µm film thickness; SGE, Ringwood, Australia). Carrier gas was helium at 1 mL min^{-1}. Oven temperature was programmed from 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 320˚C. The mass detector was operated in electron impact (EI) mode at 70 eV, scanning the 50–650 *m/z* range. The transfer line and ionisation source were heated at 280 and 230˚C, respectively. HP ChemStation software (Agilent Technologies) was used for data acquisition and data treatment.

3.2.2.4. Statistical analysis

Stepwise linear regression was performed using Statistica 7.0 (StatSoft Inc.,Tulsa, OK, USA). Characteristic *m/z* ions of glycosyl-inositol and glycosyl-methyl-inositol and their corresponding ratios were considered as independent variables.

3.2.3. Results and discussion

3.2.3.1. Chromatographic data

Table 3.2.1. shows I^T values of thirteen cyclitol glycosides with different structures (derived from *chiro*- and *myo*-inositol and methyl-inositols and mono-, di- and triglycosyl compounds) on HT5 stationary phase. For all studied compounds, the glycosidic moieties were

always α-D-galactopyranose (abbreviated as galactosyl), α-D-galactopyranosyl-(1→6)-α-D-galactopyranose (abbreviated as digalactosyl), and α-D-galactopyranosyl-(1→6)-α-D-galactopyranosyl-(1→6)-α-D-galactopyranose (abbreviated as trigalactosyl). Some non-reducing sugars (sucrose, raffinose, stachyose and verbascose), commonly present in legumes, have been included for comparison purposes. It is worth noting that cyclitol glycosides are non-reducing compounds. Therefore, a single peak was obtained for every TMS derivative in GC analysis.

All studied compounds behaved in a similar way to saccharides: galactosyl-cyclitols (degree of polymerization (DP2) eluted between sucrose and raffinose, while digalactosyl-cyclitols (DP3) eluted between raffinose and stachyose. Fagopyritol A3 (DP4) eluted between stachyose and verbascose and consequently, other trigalactosyl-cyclitols were assumed to elute between these saccharides.

In general, galactosyl-cyclitols derived from *chiro*-inositol eluted earlier than those derived from *myo*-inositol, so exhibiting a similar behaviour to that of free inositols (I^T *chiro*-inositol: 1874, I^T *myo*-inositol: 2048, (Ruiz-Aceituno, 2013)). Moreover, TMS glycosyl-methyl-inositols eluted at earlier retention times than their corresponding TMS glycosyl-inositols, as expected according to their lower molecular weight and in a similar way to that observed for free methyl-inositols (*e.g.* I^T pinitol: 1742, (Ruiz-Aceituno, 2013)).

3.2.3.2. EI mass spectra

Typical full scan EI mass spectra obtained for TMS cyclitol glycosides and their chemical structures are presented in Anexo 1B. These mass spectra were characterised by *m/z* ions corresponding to both glycosidic and cyclitol moieties along with other fragments related to glycosidic linkages.

Mass spectra of all TMS cyclitol glycosides showed the triplet *m/z* ions 191/204/217 characteristic of the silylated pyranose rings. Relative abundances of these *m/z* ions in all the examined galactosyl-cyclitols were 191<217<204, similar to those of galactosyl disaccharides (although proportions of these ions can vary depending on the reducing sugar ring and on the position and configuration of glycosidic linkages in disaccharides (Kamerling, 1971)). However, a different trend was observed in MS profiles of free cyclitols which presented a *m/z* ion 204 lower than *m/z* 191 and 217. As a typical example of these profiles, **Figure 3.2.1.** shows the mass spectrum of galactinol (1A), galactosyl-(1→6)-galactose (1B) and *myo*-inositol (1C).

Table 3.2.1. Linear retention indices (I^T) on HT5 (5% phenyl (equiv.) polycarborane–siloxane) and mass spectrometric features (abundances of proposed *m/z* ratios) of glycosyl cyclitols. Some sugars have been included for comparison purposes.

Compound	Structure	t_R(min)	I^T	DP[a]	Ratios of *m/z* ions		Presence of *m/z* 375
					133/129	260/265	
Sucrose	α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside	31.0	2516	2			
Galactopinitol A1	O-α-galactopyranosyl-(1→5)-3-O-methyl-D-*chiro*-inositol	33.0	2633	2	1.07	1.54	Yes
Galactopinitol B1	O-α-galactopyranosyl-(1→2)-3-O-methyl-D-*chiro*-inositol	34.1	2719	2	0.84	1.79	Yes
Fagopyritol A1	O-α-galactopyranosyl-(1→3)-D-*chiro*-inositol	34.3	2740	2	0.30	0.04	No
Lathyritol	O-α-galactopyranosyl-(1→3)-1-O-methyl-D-*myo*-inositol	34.6	2760	2	0.80	1.63	Yes
Fagopyritol B1	O-α-galactopyranosyl-(1→2)-D-*chiro*-inositol	35.0	2801	2	0.26	0.05	No
Galactosyl-ononitol	O-α-galactopyranosyl-(1→3)-4-O-D-methyl-*myo*-inositol	35.1	2811	2	0.92	1.56	Yes
Galactinol	O-α-D-galactopyranosyl-(1→3)-D-*myo*-inositol	35.6	2874	2	0.33	0.12	No
Raffinose	O-α-D-galactopyranosyl-(1→6)-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside	40.1	3158	3			
Ciceritol	O-α-D-galactopyranosyl-(1→6)-O-α-D-galactopyranosyl-(1→5)-3-O-methyl-D-*chiro*-inositol	47.3	3489	3	0.77	1.34	Yes

Table 3.2.1. Continuación

Fagopyritol A2	O-α-galactopyranosyl-(1→6)-O-α-galactopyranosyl-(1→3)-D-*chiro*-inositol	49.1	3565	3	0.34	0.0	No
Digalactosyl-ononitol	O-α-galactopyranosyl-(1→6)-O-α-galactopyranosyl-(1→3)-4-O-D-methyl-*myo*-inositol	49.5	3580	3	0.64	2.08	Yes
Fagopyritol B2	O-α-galactopyranosyl-(1→6)-O-α-galactopyranosyl-(1→2)-D-*chiro*-inositol	51.1	3640	3	0.01	0.0	No
Digalactosyl-*myo*-inositol	O-α-galactopyranosyl-(1→6)-O-α-galactopyranosyl-(1→3)-D-*myo*-inositol	53.7	3734	3	0.21	0.0	No
Stachyose	O-[α-D-galactopyranosyl-(1→6)]$_2$-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside	62.1	3980	4			
Fagopyritol A3	O-[α-galactopyranosyl-(1→6)]$_2$-α-galactopyranosyl-(1→3)-D-*chiro*-inositol	72.4	4236	4	0.03	-*	No
Verbascose	O-[α-D-galactopyranosyl-(1→6)]$_3$-α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside	76.1	4305	5			

[a]Degree of polymerization; *m/z 260 and 265 ions not detected

Marked differences were observed among mass spectrum profiles of TMS cyclitol glycosides and disaccharides. The most noticeable ones were related to relative abundances of highest *m/z* ions (Figure 3.4.1 A and B). Whereas mass spectra of di- and trisaccharides are characterised by high relative abundances of certain *m/z* ions above 217 (Sanz, 2002; Brokl, 2009), cyclitol glycosides mass spectra showed relative low abundances of these particular *m/z* ions. Even the ion at *m/z* 361, which is characteristic of the glycosidic linkage, and it is rather abundant in saccharides (Figure 3.4.1 B) and other glycosides, shows a low relative abundance in cyclitol glycosides (i.e., < 19%; Figure 3.4.1 A). This feature as well as the general mass spectrum profile could be useful to differentiate cyclitol glycosides from sugars.

In order to distinguish between cyclitol glycosides derived from inositols and from methyl-inositols, mass spectra of different free cyclitols were considered (Anexo 1C). As already reported (Sherman, 1970), *m/z* 265, 305, 318, 367 and 419 ions are characteristics of free inositols. Binder & Haddon (Binder, 1984) found that *m/z* ions at 89, 159, 247, 260, 375 and 449 were useful to differentiate between free methyl-inositols and free inositols (Binder, 1984). However, classification of cyclitol glycosides could not be carried out using most of these fragments due to: (i) the low relative abundances of some of the highest *m/z* ions (mainly *m/z* 419 and 449), which hamper their detection; (ii) *m/z* ions 89, 159 and 247 are also found in saccharides and they could come from the glycosidic moiety; and (iii) ion at *m/z* 260 can not be considered specific for TMS glycosyl-methyl-inositols as it is also present in low relative abundances in TMS glycosyl-inositols. On the other hand, ion at *m/z* 375, which is only present in glycosyl-methyl-inositols, could be useful to differentiate most of these compounds. Nevertheless, appropriated classification can not rely on a single *m/z* ion. Therefore, a survey of the mass spectra of the 13 TMS cyclitol glycosides was carried out in an attempt to identify other fragment ions that could be considered characteristic markers for these carbohydrates. As it was expected due to the structural similarity of these compounds, mass spectral differences were related to divergent fragment intensity of the several detected *m/z* ions. Therefore, ratios of the most characteristic ions (those related to fragmentation differences caused by the presence or absence of the *O*-methyl group in the cyclitol ring) were also considered as possible differentiation factors.

A stepwise linear regression considering the characteristic *m/z* ions and their corresponding ratios as independent variables was performed in order to have an objective tool for determining which fragments or fragment ratios are related with the structural features of TMS galactosyl-cyclitols (group descriptor: presence or absence of a methyl group) so helping to their

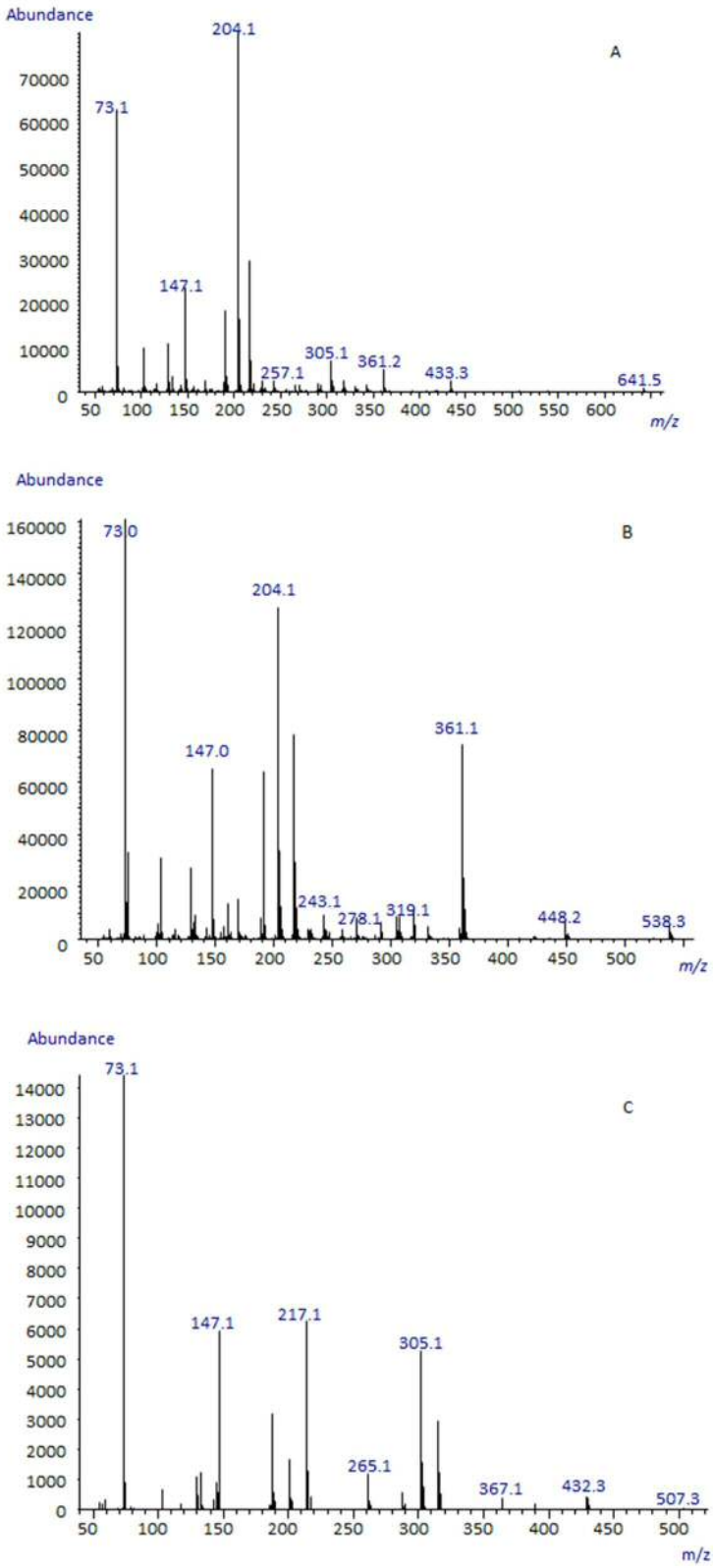

Figure 3.2.1. Mass spectra of TMS oxime derivatives of (A) galactinol, (B) galactosyl-(1→6)-galactose and (C) *myo*-inositol.

characterization. Two variables (ratios of *m/z* ions 133/129 and 260/265) were enough to correctly classify these compounds (R^2 =0.97, p-level< 0.0001). Ion at *m/z* 265 is characteristic of inositols ($(TMSiO)_2$-CH=O+$SiMe_2$), whereas ion at *m/z* 260 was always detected in methyl-inositols mass spectra; ion at *m/z* 133 (Me_2SiOH-Si+Me_2) is also characteristic of *O*-methyl group in the ring and ion at *m/z* 129 (Me_2SiO-CH=CH-CH=O^+) was present in saccharides mass spectra. Although slight variations in these ratios could probably be detected for other cyclitol glycosides, some general criteria allowing to distinguish between glycosyl-methyl-inositols and glycosyl-inositols could be stated from these results: (i) ratio of *m/z* ions 133/129 was close to 1 in TMS glycosyl-methyl-inositol derivatives, whereas this ratio was below 0.4 in inositol derivatives; and (ii) ratio of *m/z* ions 260/265 was always above 1 in TMS glycosyl-methyl-inositols, whereas it was 0 or close to 0 in glycosyl-inositols.

Differences in MS profile of cyclitol glycosides with different DP (i.e. 2, 3 and 4) were also evaluated. Although the effect of the length of saccharidic chain on the mass spectra seemed to be small, these compounds could be correctly classified by their retention data.

Regarding the influence of linkages, Obendorf *et al*. [10] found that ratios of *m/z* ions 305/318 and 318/319 were different for TMS fagopyritols A1 (0.9 and 2.6, respectively) and B1 (2.2 and 0.9, respectively) and were useful to distinguish one from each other. We have found similar results for these compounds; but also for the corresponding digalactosyl derivatives FPA2 (0.8 and 4.2, respectively) and FPB2 (2.2 and 1.4, respectively), and for FPA3 (0.5 and 10.3, respectively). In general, ratios of *m/z* ions 305/318 were lower than 1 for fagopyritols A (linkage 1→3) and above 1 for fagopyritols B (linkage 1→2). On the contrary, fagopyritols A showed higher ratios of *m/z* ions 318/319 than fagopyritols B. These conclusions could be useful to identify other glycosyl-inositols with similar glycosidic linkages.

3.2.3.3 Analysis of cyclitol glycosides in plants

In order to demonstrate the utility of the proposed GC and MS criteria in the identification of new cyclitol glycosides in real plants, extracts of adzuki beans, chickpeas and leaves of *C. myrtifolia and C. ruscifolia* were analysed. Some peaks, not previously identified, with mass spectra compatible with cyclitol glycoside structures were detected in these samples. **Table 3.2.2** shows the I^T values on HT5 stationary phase and the characteristic *m/z* ratios of these peaks in the different plants studied. Peaks were labelled as unknown and they were numbered in order of their elution in the different sample extracts.

Table 3.2.2. Linear retention indices (I^T) on HT5 (5% phenyl (equiv.) polycarborane–siloxane) and mass spectrometric features (i.e. abundances of proposed *m/z* ratios) of unknown compounds found in chickpea (*Cicer arietinum*), adzuki bean (*Vigna angularis*) and leaves of *Coriaria myrifolia and Coriaria ruscifolia.*

Code	t_R	I^T	DP[a]	Source	Ratios of *m/z* ions		Presence of *m/z* 375	Tentative assignation
					133/129	260/265		
U1	34.6	2763	2	Chickpea	0.95	–*	Yes	Isomer of galactosyl-pinitol
U2	34.7	2787	2	Adzuki	0.70	0.92	Yes	Isomer of galactosyl-ononitol
U3	35.3	2844	2	*Coriaria*	0.44	0.09	No	Isomer of galactinol
U4	35.6	2872	2	*Coriaria*	0.31	0.07	No	Galactinol
U5	35.9	2895	2	*Coriaria*	0.36	0.05	No	Isomer of galactinol
U6	47.8	3518	3	Adzuki	0.37	0.00	No	Isomer of DGMI
U7	47.9	3517	3	Chickpea	0.65	2.47	Yes	di-galactosyl-pinitol B
U8	48.4	3540	3	Adzuki	0.42	0.00	No	Isomer of DGMI
U9	53.7	3730	3	Chickpea	0.22	0.00	No	DGMI
U10	70.8	4198	4	Chickpea	0.71	2.40	Yes	tri-galactosyl-pinitol A

[a]Degree of polymerization; *m/z 265 ions not detected.

Figure 3.2.2. shows the GC-MS profiles of the TMS cyclitol glycoside eluting zones of adzuki bean extracts. Besides the previously identified galactosyl-ononitol, galactinol, di-galactosyl-ononitol and DGMI (peaks numbers 1, 2, 3 and 4, respectively) [20], three unknown cyclitol glycosides were detected in this legume. Peak U2 (**Figure 3.2.2**), with I^T value of 2787 and mass spectrum similar to galactosyl-ononitol, could probably correspond to 5-O-(α-D-galactopyranosyl)-4-O-methyl-*myo*-inositol previously isolated by Yasui [27] from this legume. Peaks U6 and U8 (**Figure 3.2.2**) showed mass spectra characteristic of glycosyl-inositols. Considering their I^T values (3518 and 3540, respectively, **Table 3.2.2**) these peaks were tentatively assigned as two di-glycosyl-inositols derived from *myo*-inositol (the only free inositol detected in adzuki beans), probably isomers of DGMI.

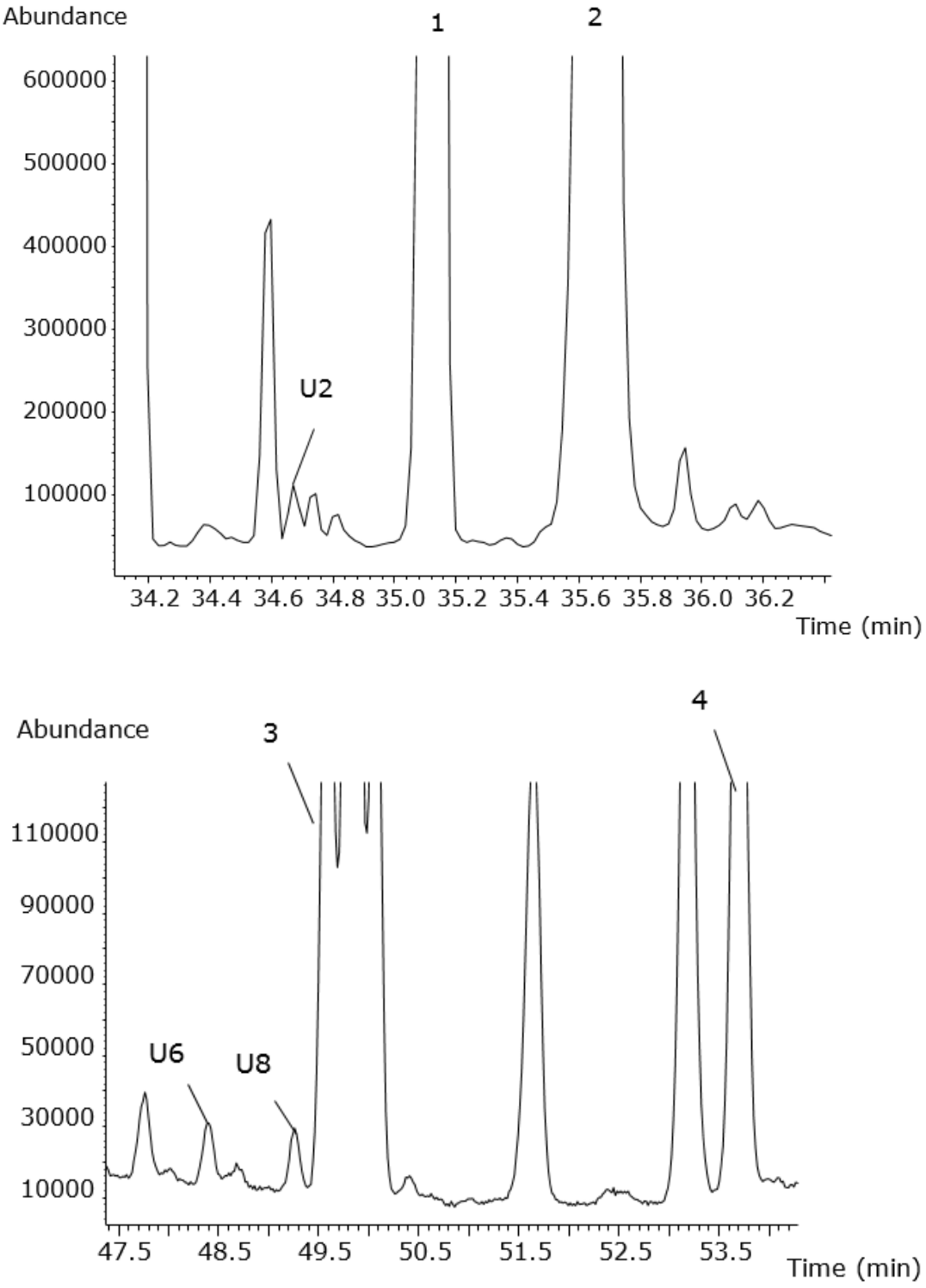

Figure 3.2.2. GC-MS profile of the cyclitol glycoside eluting zone of adzuki bean extract. (1) Galactosyl-ononitol; (2) Galactinol; (U2) Unknown 2; (U6) Unknown 6; (U8) Unknown 8; (3) Di-gal-ononitol; (4) DGMI. See Table 2 for tentative assignation of U compounds.

Different cyclitol glycosides were also detected in chickpea (**Figure 3.2.3**). Apart from those previously identified in the literature (Quemener, 1983; Ruiz-Aceituno, 2013), namely galactopinitol A1, galactopinitol B1, galactinol and ciceritol (peak numbers 1, 2, 3 and 4 in **Figure**

3.2.3), four unknown compounds, with I^T values corresponding to a cyclitol glycoside, two di-cyclitol glycosides (DP3) and a tri- cyclitol glycoside (DP4) (U1, U7, U9 and U10 in Figure 3.4.5), were detected. Mass spectra with similar features were observed for peaks U1, U7 and U10: ratios of *m/z* ions 133/129 and 260/275 were higher than 0.6 and 2.7, respectively. Moreover, *m/z* 375 ion was also present. These results would suggest that these peaks could correspond to galactosyl-methyl-inositols, which could be tentatively assigned as galactosides of pinitol, on the base of the high abundance of this methyl-inositol in chickpea. U7 eluted near ciceritol and showed a mass spectrum similar to this compound. Therefore, it could tentatively be identified as di-galactosyl-pinitol B (a regioisomer of ciceritol) which has been also tentatively identified in vetch (*Vicia villosa*) seeds (Szczeciński, 2000). To the best of our knowledge, this is the first time that the presence of this compound in chickpea is described. Peak U10 had also a mass spectrum similar to ciceritol and it could tentatively be identified as tri-galactosyl-pinitol A. This compound had been previously isolated from chickpea and identify by NMR and methylation analysis (Nicolas, 1984; Obendorf, 2012). However this is the first time that this compound is directly detected by GC-MS in chickpea extracts. Peak U9 showed *m/z* ratios characteristic of a glycosyl-inositol (low values of the *m/z* ratio 133/129 plus absence of *m/z* 260 and 375). This peak was assigned as DGMI by comparison with the corresponding standard (Table 3.4.1 and Anexo 1D).

Leaves of *C. myrtifolia and C. ruscifolia* were included in this study since they seemed to contain some cyclitol glycosides, compounds which have not previously been described in this plant genus. Figure 3.4.4. shows the GC-MS profile obtained for *C. myrtifolia* extracts previously subjected to the derivatization procedure. *myo*-Inositol was the only free cyclitol found in the *Coriariae* leaves. Three peaks (U3, U4 and U5) with mass spectra compatible with cyclitol glycosides were found in both samples. I^T values and characteristic *m/z* ratios of these peaks are shown in Table 2. U4 was identified as galactinol by comparison with the commercial standard (**Table 3.2.1**). The other two cyclitol glycosides, with I^T values of 2844 (U3) and 2895 (U5), had mass spectra similar to that of galactinol. These compounds could be assigned as glycosyl-inositols, probably derivatives of *myo*-inositol, since no other free inositol was detected in these extracts. To the best of our knowledge, this is the first time that galactinol and its two isomers are found in *Coriariaceae.*

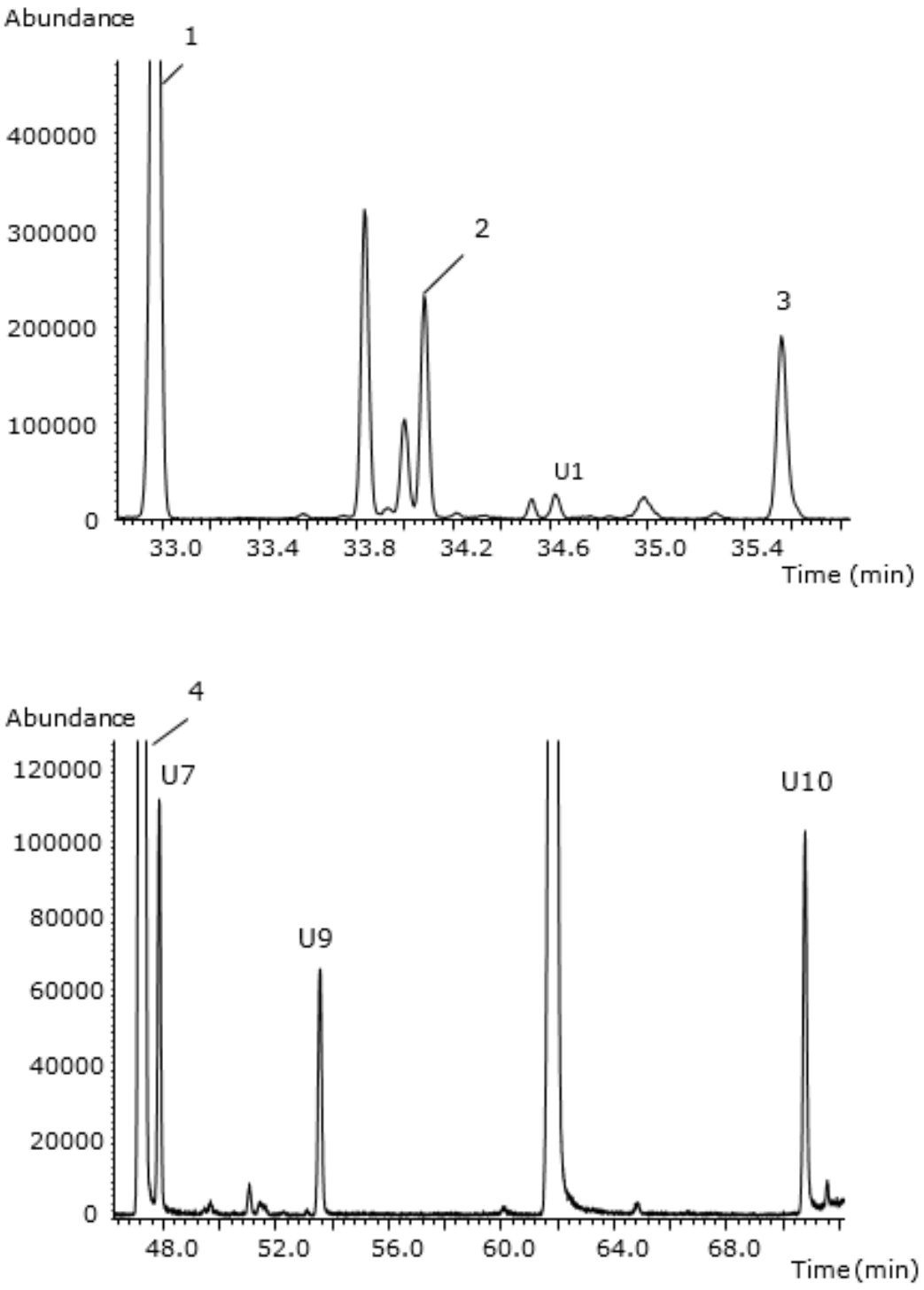

Figure 3.2.3. GC-MS profile of the cyclitol glycoside eluting zone of chickpea extract. (1) Galactosyl-pinitol A1; (2) Galactosyl-pinitol B1; (U1) Unknown 1; (3) Galactinol; (4) Ciceritol; (U7) Unknown 7; (U9) Unknown 9; (U10) Unknown 10. See Table 2 for tentative assignation of U compounds.

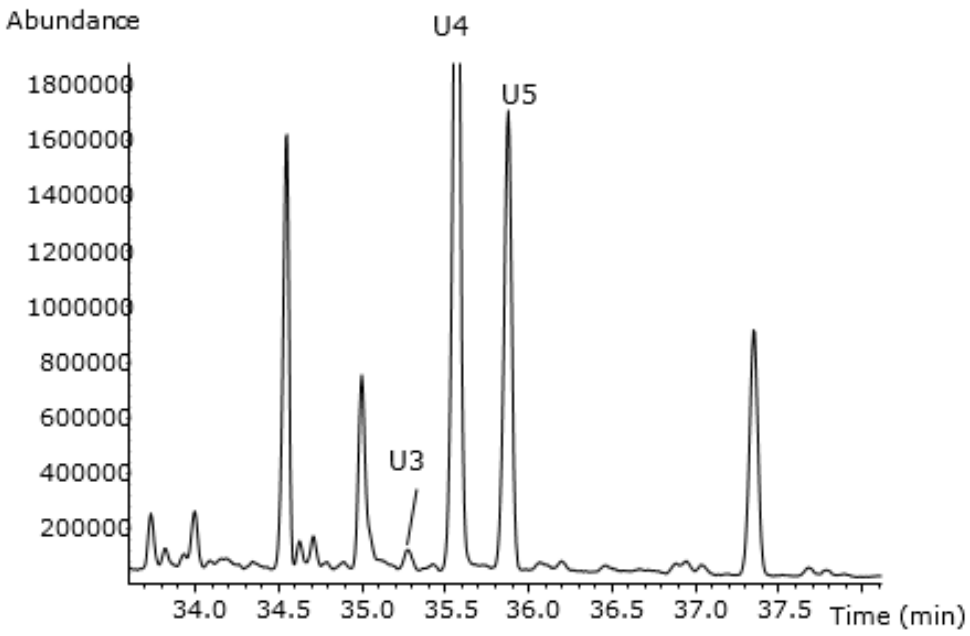

Figure 3.4.4. GC-MS profile of the cyclitol glycoside eluting zone of *C. myrtifolia* extract. (U3) Unknown 3; (U4) Unknown 4; (U5) Unknown 5. See Table 2 for tentative assignation of U compounds.

3.2.4. Conclusions

A detailed study of TMS galactosyl-cyclitols has been carried out by GC-MS. GC retention data (I^T) on a 5% phenyl (equiv.) polycarborane-siloxane column and relative abundances for characteristic *m/z* ratios have been correlated with galactosyl-cyclitol structures in an attempt to provide insight in the characterization (i.e. DP and class of cyclitol) of unknown compounds. Abundance ratios of *m/z* ions 133/129 and 260/265 and the presence or absence of *m/z* 375 ion were demonstrated to allow unequivocal classification of glycosyl-methyl-inositols and glycosyl-inositols. These data have allowed the tentative identification of several new cyclitol glycosides in chickpea, adzuki bean and *Coriariae* leaves as an example of the practical validity of the proposed classification criteria. Most of these cyclitol glycosides have been reported for the first time in the evaluated samples.

3.3. Extraction and characterization of low molecular weight bioactive carbohydrates from mung bean (*Vigna radiata*)

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3.3.1. Introduction

Mung bean (*Vigna radiata* L. Wilczek) is an important annual legume considered the hardiest of all legumes because of its capability to grow under a wide range of climatic conditions (Soucek, 2006; Tang, 2014). This pulse is native from Asia (mainly from India, Bangladesh, China and South East Asia), where it has been traditionally consumed in different forms (boiled, cooked, as sprouts, etc) (Anwar, 2007) and as component of many products (Raghuvanshi, 2011). Mung bean is widely exported to different countries. In particular, total European import of this pulse was about 20 thousand tonnes per year in the past five years.

Mung bean has many benefices for digestion, showing good digestibility and low flatulence (Gosal, 1983). Moreover, several properties such as antitumoral activity (Soucek, 2006), inhibition of angiotensin I-converting enzyme (Li, 2006), antioxidant activity (Randhir, 2007) and antimicrobial potential to inhibit the growth of the bacteria *Helicobacter pylori* (Mitchell, 2002; Randhir, 2007), have been attributed to mung bean. The consume of mung bean has also been associated with a small increase in blood glycemic index in humans, which makes this legume to be considered useful for diabetic patients (Randhir, 2007; Tang, 2014).

Mung bean is an excellent source of carbohydrates (65%) and proteins (24%) (*10*), but other compounds such as polyphenols, flavonoids, tocoferol, organic acids and lipids are also present in lower concentrations in this legume (Anwar, 2007; Randhir, 2007; Kanatt, 2011; Tang, 2014). The content of carbohydrates in mung bean is higher than in other legumes, with starch being the predominant one (42%). However, many bioactive carbohydrates such as -galactooligosaccharides (-GOS), namely manninotriose, raffinose, stachyose and verbascose (Åman, 1979), which are well-known prebiotics (Tang, 2014), and cyclitols such as *scyllo*-inositol, methyl-*scyllo*-inositol (Ueno, 1973), *myo*-inositol and galactinol (Åman, 1979; Schweizer, 1981), have also been described in this legume. Several bioactive proprieties have been attributed to these cyclitols (including glycosides and their derivates) such as antihyperglycemic (Bates, 2000), lipid-lowering (Choi, 2009), as well as antioxidant (Agarie, 2009) and hepatoprotective effects (Dhanasekaran, 2009). It is worth to note that both *scyllo*-inositol and methyl-*scyllo*-inositol can be considered as potential therapeutic agents against Alzheimer's disease (Tanaka, 2015; Thomas, 2016).

Solid liquid extraction (SLE) with polar solvents has been commonly applied to extract bioactive carbohydrates, incluiding inositols, from mung bean (Montañés, 2007; Nie, 2013). However, the use of advanced extraction techniques such as microwave assisted extraction (MAE) is currently gaining great attention as alternative to these well accepted but tedious and time-consuming SLE-based processes (Al-Suod, 2017). MAE has been demonstrated to be useful for the

extraction of high molecular weight carbohydrates (pectins, galactomannans, inulin, xylans, etc) from different natural matrices (Passos, 2013; Ruiz-Aceituno, 2016). However, to the best of our knowledge, up to now only one application of this technique for the extraction of low molecular weight carbohydrates (LMWC; (Ruiz-Aceituno, 2016)) can be found in the literature. In that work, the MAE process was optimized to simultaneously extract three free inositols (*chiro*-, *scyllo*- and *myo*-inositol) and inulin from artichoke bracts. In general, MAE provides high yields of bioactive carbohydrates in short times and using low solvent volumes (Ruiz-Aceituno, 2016). On the other hand, it should be mentioned that, in these treatments, non-bioactive carbohydrates such as glucose, fructose or sucrose, which can interfere in the bioactivity of obtained extracts, are usually co-extracted. Ion exchange chromatography (Camero, 2004) and simulated moving bed chromatography (Saska, 1996) have been proposed as efficient techniques for the fractionation of inositols and sugars. However, these techniques are expensive and time consuming. As a cost-effective alternative, a biotechnological procedure involving *Saccharomyces cerevisiae* has recently proposed for the selective fractionation of inositols and sugars in edible legume extracts (Ruiz-Aceituno, 2013).

Regarding carbohydrate analysis, gas chromatography couple to mass spectrometry (GC-MS) is usually the technique of choice considering its resolution power, sensibility, robustness and identification capabilities (Beveridge, 1977; Schweizer, 1981; Ruiz-Aceituno, 2013). However, considering the similar structures of carbohydrates, and the presence of isomeric compounds, like in the case of cyclitols, such identifications are not straightforward. A recent work published by our group (Ruiz-Aceituno, 2017) provided new insight between GC (I^T values) and MS data and chemical structure of different cyclitol glycosides. The established criteria are useful for identification of unknown compounds.

Therefore, in this work an exhaustive characterization of LMWC (including bioactive carbohydrates such as -GOS, cyclitols and cyclitol glycosides) of mung bean has been carried out by GC-MS for the first time. SLE and MAE have been optimized and compare for the selective extraction of these bioactive LMWC. Finally, MAE has been applied to extract these carbohydrates from mung beans of different geographical origins.

3.3.2. Materials and Methods

3.3.2.1 Standards and samples

Analytical standards of fructose, sorbitol, galactose, glucose, galactinol, *myo*-inositol, *scyllo*-inositol, maltose, maltotriose, maltotetraose, manninotriose, nystose, pinitol, raffinose,

stachyose, sucrose, and verbascose were obtained from Sigma Chemical Co. (St. Louis, US). Di-galactosyl-*myo*-inositol (DGMI) was extracted from buckwheat (*Fagopyrum esculentum*) as previously described (Ruiz-Aceituno, 2017). *Vigna radiata* beans from 6 different brands of different geographical origins [Taiwan (MBT1), Argentina (MBA1), Spain (MBS1 and MBS2) and China (MBC1 and MBC2)] were purchased at local markets in Madrid (Spain).

3.3.2.2. Extraction procedure

Dried *Vigna radiata* beans were milled until powder with a domestic grinder (Moulinex, Spain). The whole sample was sieved through a square mesh of 500 µm before extraction.

Solid liquid extraction (SLE)

Dried *Vigna radiata* sample (MBS1; 0.5 g) was extracted with 10 mL of the solvent [water (100%), methanol (100%), ethanol (100%), and mixtures of methanol:water (50%, v/v) and ethanol:water (25%, 50% and 75 %, v/v)] under constant stirring at 25˚C. Using the optimum solvent, the influence of the extraction time and temperature was investigated by analyzing extracts took at 5, 30, 60 and 120 min. In all cases, extracts were immediately centrifuged at 4400 *g* for 10 min at 4 ˚C and kept in a freezer at -18 ˚C until analysis. Otherwise specified, all experiments were performed in triplicate.

Microwave assisted extraction (MAE)

A MARS 6 (CEM, NC, USA) MAE instrument was used in the study. Microwave power was set at 1250 W in all instance. Mung bean sample (MBS2) was placed in 100 mL Green Chem vessels (CEM) and dissolved in 10 mL of the selected solvent. The effect of three independent factors (temperature, time and amount of sample) on the extraction of bioactive carbohydrates was evaluated following a Box–Behnken experimental design using StatGraphics Centurion XV software (Statistical Graphics Corporation, Rockville, MD, USA). A total of 15 experiments were carried out in randomized order. The experimental ranges used for the different parameters were: temperature (T) 50 - 120 ˚C, time (t) 3-30 minutes, and amount of sample (s) 0.01-1.00 g.

The quadratic model proposed was:

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

where β_0 is the intercept, β_i are the first-order coefficients, $\beta_{i,i}$ the quadratic coefficients for i^{th} factors, $\beta_{i,j}$ are 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: R_i, cyclitol amount (mg g^{-1} dry sample); R_a, α-GOS amount (mg g^{-1} dry sample), and R_c, non-bioactive sugar amount (mg g^{-1} dry sample). The experimental conditions that independently maximized R_i and R_a, and minimized R_c were obtained from the fitted models by multiple linear regression (MLR). A desirability function (R_D) was also optimized to provide MAE conditions that simultaneously maximize different responses; this function takes values between 0 (completely undesirable value) and 1 (completely desirable or ideal response).

Under optimized conditions, the effect of three consecutive extraction cycles was also evaluated. Obtained extracts were immediately cooled down on ice, centrifuged at 4400 *g* for 10 min at 4 ˚C and kept in a freezer at -18 ˚C until analysis. The optimized method was applied to the treatment of mung bean samples from different origins.

3.3.2.3. GC-MS analysis

A two-step derivatization procedure (oximation + silylation) was carried out prior to GC-MS analysis. Mung bean extracts (2 mL) were mixed with 0.1 mL of a 70% ethanolic solution of phenyl-β-D-glucoside (1 mg mL^{-1}; internal standard) and dried under vacuum (38-40˚C). Oximation was done using 2.5% of hydroxylamine chloride in pyridine (350 µL) at 75 ˚C for 30 min; after this, the silylation of the carbohydrates was carried out using hexamethyldisilazane (350 µL) and trifluoroacetic acid (33 µL) at 45 ˚C for 30 min. Under these conditions, two peaks corresponding to the *syn* (*E*) and *anti* (*Z*) forms per reducing sugar are obtained, whereas non-reducing carbohydrates (i.e. cyclitols, cyclitol glycosides, sucrose and oligosaccharides of raffinose family), which do not form oximes, give a single peak. After derivatization, 1 µL of the derivatized mixture was injected into the GC-MS equipment.

For GC-MS analyses, a 7890A gas chromatograph coupled to a 5975C quadrupole mass spectrometer detector (Agilent Technologies, Palo Alto, CA, USA) with a HT5 (5% phenyl (equiv.) polycarborane–siloxane) capillary column (25 m × 0.22 mm i.d. × 0.1 µm film thickness; SGE, Ringwood, Australia) was used. Helium at 1 mL min^{-1} was employed as carried gas. The oven temperature was programmed from 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 320 ˚C. The MS detector was operated in the electron impact (EI) mode at 70 eV, scanning the 50–650 *m/z* range. The transfer line was set at 280 ˚C and the ionization source at 230 ˚C. For data acquisition and treatment, the HP ChemStation software (Agilent Technologies) was used.

Carbohydrates were identified by comparison of their corresponding linear retention indices (I^T) and mass spectra with those of available standards and/or previous data from literature (Ruiz-Aceituno et al., 2017). Tentative identification of new cyclitol glycosides was done on the basis of their I^T and MS data.

Quantitative data were obtained by the internal standard method. Standard solutions were prepared over the expected concentration range (0.1 - 1 mg mL^{-1} for each compound) in mung bean extracts to calculate each corresponding response factor relative to phenyl-β-D-glucoside. Those compounds whose standards were not available were assigned with a response factor of 1.

3.3.3. Results and Discussion

3.3.3.1 Identification of LMWC present in aqueous Vigna radiata extracts

Figure 3.3.1 shows the GC-MS profile of a Milli-Q water extract (2 h extraction at 25˚C under constant stirring) from mung bean seeds (sample MBS1) obtained by SLE. Up to 45 LMWC were detected in this extract (see Table **3.3.1** for peak assignment and I^T values of the different compounds detected). Detected LMWC included mono- and disaccharides, oligosaccharides (up to degree of polymerization 6) and cyclitols.

Regarding monosaccharides, glucose, galactose and fructose were detected in water extracts; sugar acids such as galacturonic acid and glucaric acid and linear polyalcohols such as sorbitol were also found. The presence of sucrose, maltose and non-reducing -GOS from raffinose family, namely raffinose, stachyose, verbascose and ajugose, previously reported in the literature (Åman, 1979; Kotiguda, 2006), were confirmed in these extracts by comparison with their corresponding standards. Reducing oligosaccharides, such as maltotriose and maltotetraose, other non-identified di-, tri- and tetrasaccharides, diglycosyl-glycerol and triglycosyl-glycerol were also detected. Manninotriose which was reported by Åman (Åman, 1979) has not been found in these samples.

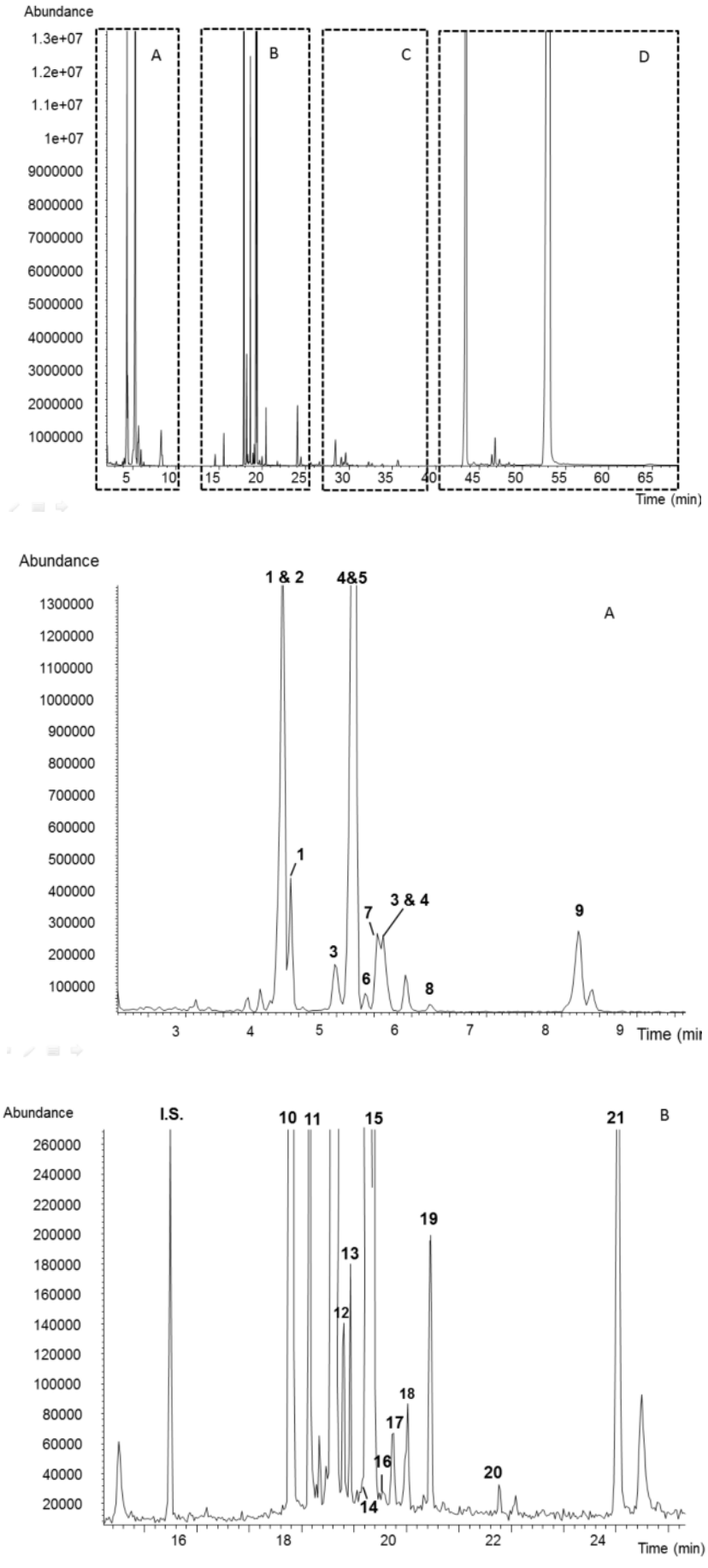

Figure 3.3.1. Continuation

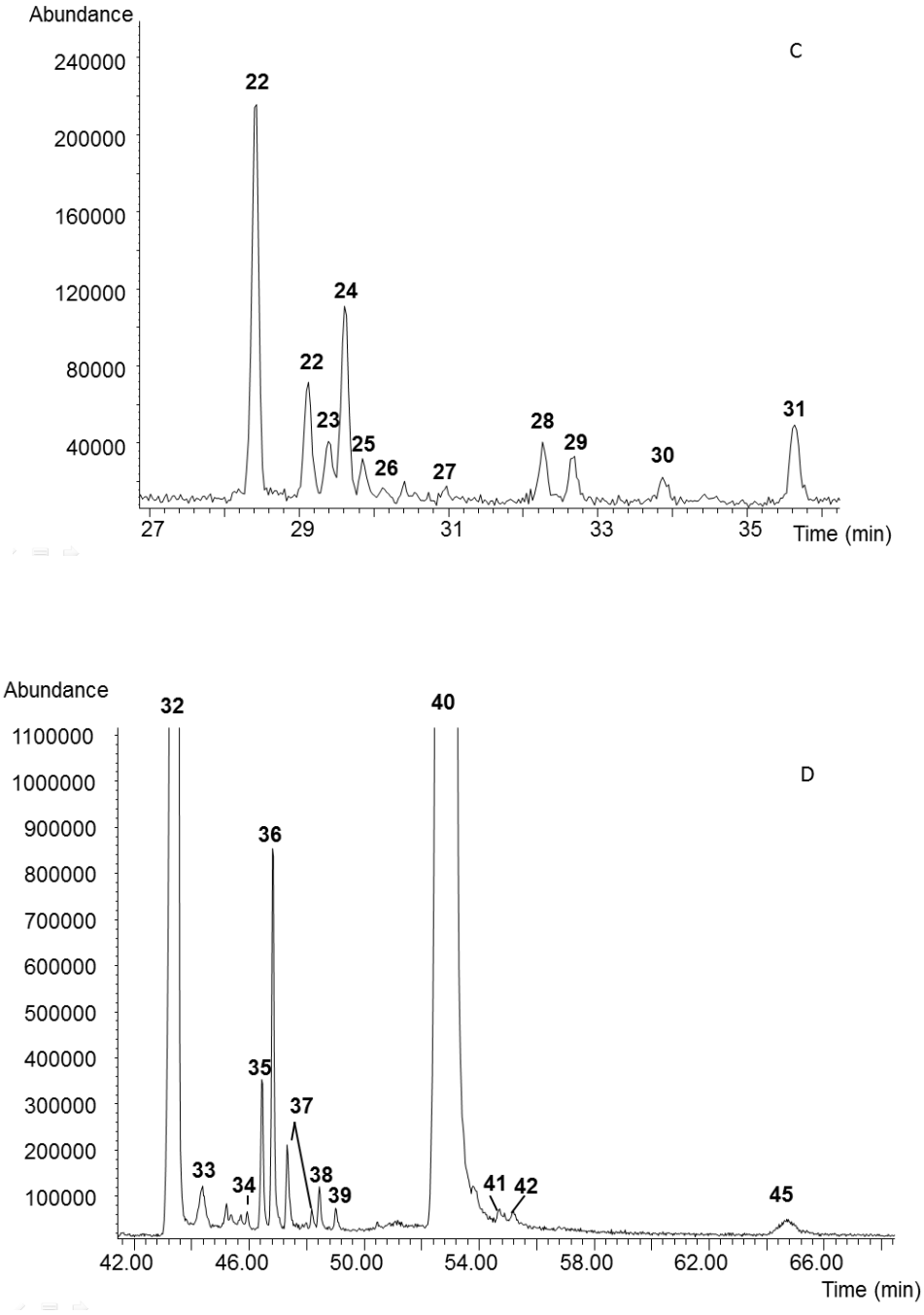

Figure 3.3.1. GC-MS profile of TMSO carbohydrates extracted by SLE from *Vigna radiata* (sample MBS1) using water as solvent. See Table 3.5.1 for peak assignation. Internal standard (I.S.)

Table 3.3.1. Low molecular weight carbohydrate concentration (mg g^{-1} dry weight; standard deviations are shown in parenthesis) and retention indices (I^T) of mung bean extracts (sample MBS1) obtained using SLE with different solvents.

Peak number	Carbohydrate	I^T	Water	Methanol	Ethanol	Methanol:water (1:1)	Ethanol : water (1:1)	Ethanol : water (3:1)
1	**Fructose**	1826/ 1846	**1.03** (0.06)	**0.007** (0.002)	**0.001** (0.001)	**0.05** (0.02)	**0.032** (0.007)	**0.017** (0.008)
2	**Sorbitol**	1835	**0.8** (0.7)	**0.002** (0.001)	-	**0.009** (0.002)	**0.04** (0.02)	**0.012** (0.005)
3	**Galactose**	1893/ 1941	**3.0** (0.9)	**0.14** (0.08)	**0.033** (0.005)	**0.09** (0.02)	**0.2** (0.1)	**0.07** (0.02)
4	**Glucose**	1912/ 1941						
5	**Methyl-*scyllo*-inositol**	1911	**1.2** (0.4)	-	-	**1.7** (0.3)	**1.6** (0.3)	**1.2** (0.2)
6	**Galacturonic acid**	1923	**0.9** (1.5)	-	-	-	**0.18** (0.07)	**0.04** (0.01)
7	**Glucaric acid**	1933	**0.97** (0.08)	-	-	-	-	-
8	***scyllo*-Inositol**	1979	**0.08** (0.05)	-	-	-	**0.037** (0.007)	**0.013** (0.011)
9	***myo*-Inositol**	2055	**0.51** (0.07)	**0.015** (0.003)	-	**0.57** (0.09)	**0.48** (0.04)	**0.16** (0.02)
10	**Sucrose**	2517	**27,6** (9,5)	**2,7** (1.4)	**1.0** (0.3)	**13.6** (2.2)	**14.8** (2.2)	**5.4** (1.5)
11	**Pentosyl-hexose**	2549	**0.960** (0.677)	-	-	-	**0.011** (0.006)	**0.003** (0.001)
12	**Glycosyl-methyl-scyllo-inositol**	2606	**0.048** (0.003)	**0.015** (0.002)	**0.003** (0.001)	**0.047** (0.007)	**0.045** (0.005)	**0.029** (0.001)
13	**Glycosyl-methyl-scyllo-inositol**	2643	**0.10** (0.02)	**0.024** (0.003)	**0.005** (0.001)	**0.10** (0.01)	**0.09** (0.01)	**0.04** (0.01)
14	**Glycosyl-methyl-inositol**	2658	**11.4** (5.1)	**0.006** (0.001)	-	**0.08** (0.01)	**0.06** (0.03)	**0.013** (0.001)
15	**Maltose**	2697/2 711		-	-	-	**0.022** (0.006)	**0.007** (0.003)
16	**Glycosyl-methyl-inositol**	2724		**0.006** (0.001)	-	**0.019** (0.005)	**0.014** (0.004)	**0.006** (0.003)
17	**Galactosyl - inositol**	2775	**0.013** (0.009)	**0.002** (0.000)	-	**0.014** (0.002)	**0.007** (0.008)	**0.004** (0.005)
18	**Galactosyl - inositol**	2798	**0.028** (0.017)	-	-	**0.020** (0.007)	**0.018** (0.008)	**0.004** (0.003)
19	**Galactinol**	2832	**0.10** (0.04)	**0.08** (0.01)	**0.010** (0.002)	**0.11** **(0.01)**	**0.19** (0.01)	**0.13** (0.01)

Table 3.3.1. Continuation

20	**Diglycosyl-glicerol**	2959	**0.06** (0.01)	**0.015** (0.001)	**0.002** (0.001)	**0.013** (0.002)	**0.08** (0.01)	**0.015** (0.006)
21	**Raffinose**	3159	**0.7** (0.1)	**0.61** (0.02)	**0.11** (0.02)	**2.0** (0.3)	**1.5** (0.1)	**1.2** (0.2)
22	**Maltotriose**	3465/ 3500	**1.8** (0.4)	-	-	-	**0.021** (0.004)	**0.016** (0.001)
23	**Digalactosyl-methyl-scyllo-inositol**	3524	**0.02** (0.01)	**0.008** (0.002)	-	-	**0.033** (0.006)	**0.08** (0.01)
24	**Digalactosyl-methyl-scyllo-inositol**	3529	**0.20** (0.04)	**0.057** (0.008)	**0.001** (0.001)	**0.18** (0.02)	**0.100** (0.007)	-
25	**Digalactosyl-methyl-scyllo-inositol**	3539	**0.008** (0.005)	**0.002** (0.000)	-	-	**0.012** (0.003)	-
26	**Digalactosyl-inositol**	3552	**0.026** (0.005)	**0.003** (0.001)	-	**0.024** (0.004)	**0.017** (0.06)	-
27	**Digalactosyl-inositol**	3583	**0.011 (0.006)**	**0.001** (0.001)	-	**0.005** (0.001)	**0.010** (0.010)	-
28	**Trisaccharide**	3629	**0.08** (0.03)	**0.004** (0.001)	**0.004** (0.001)	**0.037** (0.006)	**0.023** (0.004)	-
29	**Digalactosyl-inositol**	3642	**0.02** (0.01)	-	**0.003** (0.002)	**0.022** (0.002)	**0.025** (0.004)	**0.007** (0.006)
30	**Tri-galactosyl-glycerol**	3678	**0.03** (0.01)	-	-	-	**0.025** (0.004)	**0.017** (0.002)
31	**Digalactosyl-*myo*-inositol (DGMI))**	3722	**0.05** (0.02)	**0.013** (0.004)	-	**0.030** (0.006)	**0.027** (0.004)	**0.021** (0.003)
32	**Stachyose**	3980	8.5 (0.1)	3.6 (0,1)	-	14.9 (1.5)	12.7 (2.7)	6.5 (1.3)
33	**Reducing tetrasaccharide**	4016	**0.09** (0.13)	-	-	-	-	-
34	**Tetrasaccharide**	4096	**0.5** (0.2)	**0.23** (0.07)	-	**0.6** (0.1)	-	-
35	**Trigalactosyl-methyl-scyllo-inositol**	4108	**0.07** (0.03)	**0.005** (0.002)	-	**0.006** (0.009)	**0.027** (0.004)	**0.028** (0.001)
36	**Trigalactosyl-methyl-scyllo-inositol**	4117	**2.4** (0.2)	-	-	-	**0.05** (0.01)	**0.043** (0.005)
37	**Maltotetraose**	4117/ 4154		-	-	**0.02** (0.02)	**0.2 (0.1)**	**0.12** (0.02)
38	**Trigalactosyl-inositol**	4167	**0.02** (0.01)	-	-	**0.06** (0.05)	**0.015** (0.003)	**0.007** (0.006)

Table 3.3.1 Continuation

39	**Trigalactosyl-inositol**	4187	**0.016** (0.005)	**-**	**-**	**-**	**0.014** (0.004)	**-**
40	**Verbascose**	4305	**54.8** (3.6)	**9.5** (3.3)	**0.32** (0.06)	**42.3** (6.7)	**62.5** (9.6)	**15.8** (2.4)
41	**Tetragalactosyl-methyl-scyllo-inositol**	4389	**0.028** (0.005)	**0.010 (0.003)**	-	**0.10** (0.03)	**0.036** (0.008)	-
42	**Tetragalactosyl-methyl-scyllo-inositol**	4407	**0.09** (0.02)	**0.013 (0.003)**	-	**0.12 (0.01)**	**0.050** (0.007)	-
43	**Reducing pentasaccharide**	4445	**0.8** (0.6)	**-**	-	**-**	-	-
44	**Reducing pentasaccharide**	4476	**0.3** (0.2)	**-**	-	-	-	-
45	**Ajugose**	4770	**1.1** (0.2)	**0.21** (0.08)	**-**	**0.8 (0.1)**	**0.6** (0.3)	**1.3** (1.7)
α-GOS			**65.2** (4.1)	**13.9** (3.6)	**0.44** (0.08)	**59.2** (8.5)	**77.3** (12.661)	**24.903** (5.629)
Free inositols			**1.793** (0.497)	**0.015** (0.003)	**0.000** (0.000)	**2.265** (0.400)	**2.1** (0.4)	**1.3** (0.2)

Regarding cyclitols, three free inositols, namely *scyllo*-inositol (I^T 1979), *myo*-inositol (I^T 2055) and *O*-methyl-*scyllo*-inositol (I^T 1911) were detected in this extract. The presence of these inositols in this legume has been previously described by Ford (1985) (Ford, 1985). However, to the best our knowledge, information concerning the presence of cyclitol glycosides in mung bean is very scarce in the literature and, in fact, only galactinol had been previously reported (Ueno, 1973; Åman, 1979; Ford, 1979). In this work, 20 compounds with mass spectrometric features characteristic of cyclitol glycosides were identified. Table **3.3.2** summarises relevant mass spectrometric features (i.e., characteristic *m/z* fragments and ratios of their abundances) of cyclitol glycosides (probably galactosides) found in this mung bean extract.

Table 3.3.2. Mass spectrometric features (i.e., characteristic *m/z* fragments and ratios of their abundances) of glycosyl-cyclitols found in mung bean extracts.

Peak	DP*	Ratios of *m/z* ions		Presence of *m/z* 375	Tentative assignation
		133/129	260/265		
12	2	1.27	3.60	Yes	Glycosyl-methyl-*scyllo*-inositol
13	2	1.49	3.00	Yes	Glycosyl-methyl-*scyllo*-inositol
14	2	1.20	**	Yes	Glycosyl-methyl-*scyllo*-inositol
16	2	1.53	4.23	Yes	Glycosyl-methyl-*scyllo*-inositol
17	2	0.21	0.00	No	Glycosyl-inositol
18	2	0.18	0.00	No	Glycosyl-inositol
19	2	0.28	0.00	No	Galactinol
23	3	1.49	2.75	Yes	Diglycosyl-methyl-*scyllo*-inositol
24	3	1.05	2.66	Yes	Diglycosyl-methyl-*scyllo*-inositol
25	3	1.34	**	Yes	Diglycosyl-methyl-*scyllo*-inositol
26	3	0.35	0.00	No	Diglycosyl-inositol
27	3	0.00	0.00	No	Diglycosyl-inositol
29	3	0.00	0.00	No	Di-glycosyl-inositol
31	3	0.26	0	No	DGMI
35	4	1.00	2.65	Yes	Triglycosyl-methyl-*scyllo*-inositol
36	4	0.91	3.00	Yes	Triglycosyl-methyl-*scyllo*-inositol
38	4	0.38	0.00	No	Tri-glycosyl-inositol
39	4	0.34	0.00	No	Triglycosyl-inositol
41	5	1.26	**	Yes	Tetraglycosyl-methyl-*scyllo*-inositol
42	5	1.16	**	Yes	Tetraglycosyl-methyl-*scyllo*-inositol

*Degree of polymerization; ** *m/z* 265, not detected.

Among the cyclitol glycosides detected in mung bean extract, eleven derivatives of methyl-inositols were tentatively identified on the base of mass spectrometry data, in particular the presence of the *m/z* ion at 375 and the relative abundances of *m/z* ions 133/129 and 260/265. As previously discussed by Ruiz-Aceituno et al. (2017), ratios of *m/z* ions 133/129 close to 1 can be found in TMS glycosyl-methyl-inositol derivatives. Meanwhile, values below 0.4 for these ratios are characteristic for inositol derivatives. Moreover, ratios of *m/z* ions 260/265 above 1 are characteristic of TMS glycosyl-methyl-inositols, whereas ratios of 0 or close to 0 are

characteristics of glycosyl-inositols. Considering that only *O*-methyl-*scyllo*-inositol has been found as free methyl-inositol, most probably these eleven compounds are derivatives of this cyclitol. According to their retention times, compounds 12, 13, 14 and 16 (I^T 2606, 2643, 2658 and 2724, respectively), were assigned as methyl-*scyllo*-inositol glycosides with substitutions at different positions. Cyclitol glycosides described in legumes are α-galactosides (Peterbauer, 2001); thereby, these compounds could tentatively be assigned as α-galactosyl-methyl-*scyllo*-inositols. Nevertheless, due to the symmetry of the methyl-*scyllo*-inositol ring, only three α-galactosides of this cyclitol are expected. The fourth one could then be a β-galactoside or a glucoside, as described in other plants (e.g. O-β-glucosyl-*scyllo*-inositol was isolated by Kamano et al. (Kamano, 1971) from *O. stenophylla*).

Meanwhile, compounds 23, 24 and 25 (I^T 3524, 3535 and 3641, respectively) could be identified as isomeric digalactosyl-methyl-*scyllo*-inositol derivatives; compounds 35 and 36 (I^T 4108 and 4117) could correspond to trigalactosyl-methyl-*scyllo*-inositol derivatives; and compounds 41 and 42 (I^T 4389 and 4407) could be tetragalactosyl-methyl-*scyllo*-inositol derivatives. To the best of our knowledge, the presence of methyl-*scyllo*-inositol glycosides in this legume has not been previously described.

Regarding glycosyl-inositol derivatives, galactinol (peak 19) was identified by comparison of its I^T value (2832) and mass spectrum with those of the corresponding standard. Compounds 17 and 18, with I^T values of 2775 and 2798 respectively, were identified as galactosyl-inositols, whereas compounds 26, 27, 29 and 31 (with I^T values of 3552, 3583, 3642 and 3722) were assigned as digalactosyl-inositols. This latter compound (analyte 31) was positively identified as DGMI by comparison of its I^T value and mass spectrum with those of the corresponding standard. Finally, compounds 38 and 39 (I^T 4167 and 4187) were tentatively assigned as trigalactosyl-inositols. In all cases, and as observed in a previous manuscript (Ruiz-Aceituno, 2017), glycosyl-methyl-inositol derivatives were found to eluted before glycosyl-inositol derivatives on the 5% phenyl (equiv.) polycarborane–siloxane stationary phase used for GC separation. Considering that both *myo*- and *scyllo*-inositols occur in free form in *V. radiata*, the glycosyl-inositols mentioned above could be derivatives of both *myo*- and *scyllo*-inositol. In this case, the latter should be expected at very low levels, since this cyclitol is a minor compound in this bean.

3.3.3.2 Optimisation of the SLE of LMWC from mung beans

As shown in section **3.3.3.1**, apart from α-GOS and cyclitol derivatives, several LMWC, such as glucose, fructose or maltose, were co-extracted. These compounds may (i) cause interference in the instrumental analysis of the bioactive analytes (e.g., maltose coeluted with a glycosyl-methyl-inositol), (ii) negatively affect the bioactivity of obtained extracts intended as functional foods, and (iii) promote an undesirable increase of the caloric content of these extracts. Therefore, with the purpose of reducing the content of interfering sugars in the obtained extracts and favour a selective extraction of the bioactive carbohydrates (i.e., cyclitols and -GOS), the use of different solvents during SLE under constant stirring for 2 h at 25 ˚C was evaluated.

Table 3.3.1 compares the concentrations (as mg g^{-1} dry sample) of the identified LMWC in sample MBS1 after SLE using different solvents, including water, methanol, ethanol, and several hydroalcoholic mixtures. Under the applied extraction conditions, water was the most effective solvent for the extraction of previously described analytes, while some of these compounds were not extracted using alcohols and hydroalcoholic mixtures. Regarding water extracts, verbascose (54.8 mg g^{-1} dry sample) and stachyose (8.5 mg g^{-1} dry sample) were the most abundant bioactive carbohydrates, followed by methyl-*scyllo*-inositol (1.20 mg g^{-1} dry sample) and ajugose (1.1 mg g^{-1} dry sample). Regarding cyclitol glycosides, the derivatives of methyl-inositol were more abundant (0.45 mg g^{-1} dry sample) than those of inositol (0.28 mg g^{-1} dry sample). However, high concentrations of non-bioactive carbohydrates (52.7mg g^{-1} mung bean) were also co-extracted. Moreover, some coelutions of these carbohydrates with methyl-*scyllo*-inositol (peak 5) and glycosyl-methyl-inositol derivatives (peaks 14, 16, 36 and 38) were observed. The similarity of the mass spectra of these compounds hindered their quantitation in these extracts (Table 3.5.1).

On the contrary, fewer carbohydrates and lower concentrations of those extracted were obtained when using both methanol and ethanol; therefore, these solvents were discarded and not further considered in the present study. The evaluated hydroalcoholic mixtures (methanol: water 1:1, v/v; and ethanol: water 1:1 and 3:1, v/v) showed to be more selective solvents than water for the extraction of the bioactive carbohydrates of interest. High concentrations of free inositols (2.07-2.26 mg g^{-1} dry sample), cyclitol glycosides (0.84-0.99 mg g^{-1} dry sample) and -GOS (59.3-77.2 mg g^{-1} dry sample) were obtained using, respectively, ethanol:water 1:1 and methanol:water 1:1 as extraction solvents, whereas lower concentrations of other non-bioactive carbohydrates (15.1-15.6 mg g^{-1} dry sample) were obtained. Moreover, cyclitol glycosides that coeluted with some carbohydrates in the water extract, could be successfully quantified in these

samples. Finally, an extra experiment involving ethanol:water 3:1 (v/v) as extractant was carried out with the aim of totally avoiding the extraction of the non-bioactive carbohydrates while preserving the efficient recovery of the bioactives. As it can be seen in Table 3.51, although lower concentrations of non-bioactive carbohydrates were obtained with this mixture (5.7 mg g^{-1} dry sample), the extraction of the targeted bioactive carbohydrates (free inositols, glycosyl-cyclitol derivatives and -GOS) was also lower (1.3, 0.43 and 24.9 mg g^{-1} dry sample, respectively). Considering these results and that ethanol is a greener solvent than methanol, the mixture ethanol:water 1:1 (v/v) was chosen for subsequent studies. On the other hand, the improved selectivity provided by this solvent made unnecessary the subsequent removal of coextracted non-bioactive carbohydrates typically required for water extracts (Ruiz-Aceituno, 2013).

Representative compounds of each category (α-GOS, free and glycosylated inositols and other non-bioactive LMWC) were selected as target compounds to determine the influence of the extraction time (evaluated in the 5, 30, 60 and 120 min range) and temperature (evaluated at 25˚C and 50˚C) in the SLE process. As shown in **Figure 3.3.2**, in general, concentrations (mg g^{-1} dry sample) of inositols (free and glycosylated) and α-GOS slightly either increased up to 1 h of extraction or kept constant during the whole extraction time evaluated. On the contrary, higher concentrations of some non-bioactive LMWC (i.e. glucose and galactose) were obtained at 2 h of extraction. Therefore, 1 h was chosen as optimal extraction time. The effect of temperature (50 ˚C) was also evaluated for the extraction of the target compounds, however, no notable differences in the concentration of both bioactive and non-bioactive LMWC relative to 25 ˚C were observed in this treatment.

3.3.3.3 Optimisation of the MAE of LMWC from mung beans

Considering the results obtained during mung bean treatment by SLE, 10 mL of ethanol/water 1:1 (v/v) were selected as solvent for MAE of sample MBS2. The influence of the three considered independent variables (*T, t* and *s*) on the efficiency of the MAE of α-GOS (R_a), inositols and glycosyl-inositols (*Ri*) and non-bioactive sugars (*Rc*) was evaluated. A total of 15 experiments were carried out in randomized order according to the Box–Behnken experimental design. Concentrations (mg g^{-1} dry sample) of the selected carbohydrates for the three categories previously considered for SLE, obtained for each experiment are shown in **Table 3.3.3**. R_a varied between 5.30 and 21.21 mg g^{-1} dry sample, *Ri* between 1.84 to 4.78 mg g^{-1} dry sample and *Rc* varied between 49.83 to 143.81 mg g^{-1} dry sample. In general, the lowest concentrations of all analyzed carbohydrates were obtained at 120˚C, probably due to the degradation of these compounds at this high temperature.

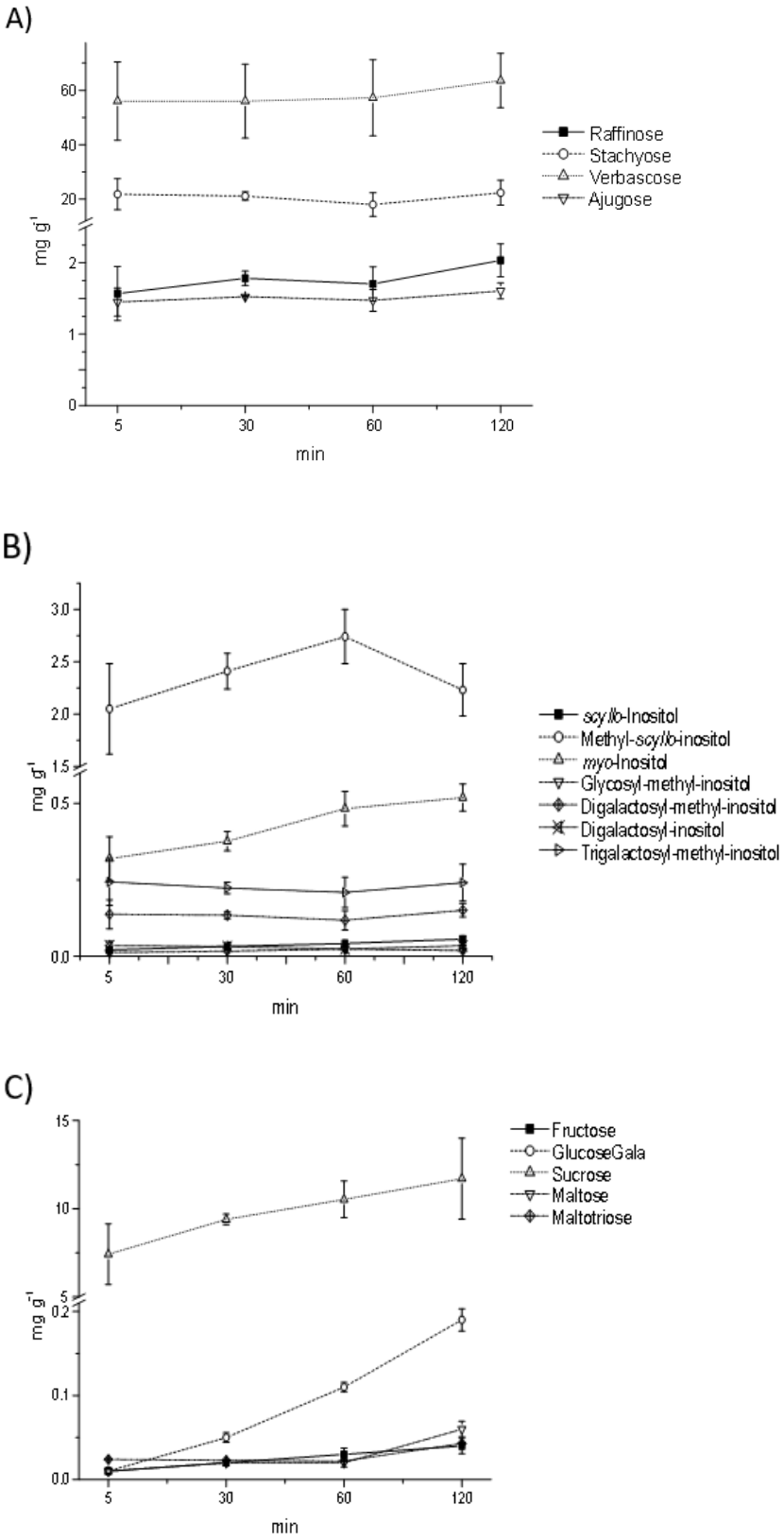

Figure 3.3.2. Content of the target analytes in the extracts by SLE (mg g^{-1} dry sample) at different extraction times. α-GOS (A), free inositols and Glycosyl-Inositols (B) and non-bioactive carbohydrates (C)

Table 3.3.3. Box–Behnken experimental design of the MAE of selected carbohydrates from mung bean (MBS2). R_i, cyclitol amount (mg g^{-1} dry sample); R_a, α-GOS amount (mg g^{-1} dry sample) and R_c, non-bioactive sugar amount (mg g^{-1} dry sample).

Experiment No	t	T	s	R_c	Ri	R_a
1	3	85	0.1	5.30	2.22	69.79
3	16.5	120	1	7.95	1.84	49.83
4	30	120	0.55	8.27	2.74	130.44
5	16.5	120	0.1	6.17	2.93	83.10
6	3	120	0.55	8.42	2.29	80.27
7	16.5	50	1	12.81	3.20	107.25
8	30	50	0.55	15.70	4.17	143.81
9	16.5	50	0.1	7.43	3.59	81.78
10	3	50	0.55	16.85	4.78	121.48
11	3	85	1	14.10	2.96	123.83
12	16.5	85	0.55	19.14	4.49	125.81
13	16.5	85	0.55	14.86	3.30	114.67
14	30	85	0.1	8.45	3.59	99.01
15	30	85	1	12.66	3.76	108.23
16	16.5	85	0.55	21.21	4.24	127.47

Response surface methodology was used to calculate the coefficients of the different R_a, R_i and R_c in the model proposed and to estimate the statistical significance of the regression coefficients. In the case of the α-GOS, T and s^2 were the most significant coefficients (R_a = 109.032 - 0.395*T + 164.113*s – 149.194*s^2; $P < 0.10$), whereas T, T^2 and s^2 were the most significant to maximize the concentration of inositols (R_i = 2.465 + 0.030*T + 3.970*s - 0.0003*T^2 – 3.609*s^2; $P < 0.10$). The optimal conditions were 50 ˚C, 3 min, and 0.55 g of dry sample in terms to maximize the extraction of bioactive carbohydrates (including both α-GOS and glycosyl-inositols). Regarding R_c model, the most significant coefficients ($P < 0.05$) to minimize the concentration of non-bioactive carbohydrates were T, s, T^2 and s^2 (R_c = -9.575 + 0.427*T + 37.232*s -0.003*T^2 – 28.754*s^2); according to this model, the optimal conditions were 120 ˚C, 16.5 min and 0.1 g of dry sample.

Finally, a multiple response that simultaneously maximized R_a and R_i and minimized R_c was considered. Optimal operating parameters (R_D = 0.85) were 50 °C, 3.6 min and 0.5 g of dry sample when using 10 ml of solvent. Under these conditions, three sequential extraction cycles were carried out with the aim of achieving an exhaustive extraction of targeted carbohydrates. Results of this experiment demonstrated that 90 % of total extracted compounds were already recovered from the beans in the first extraction, while 10% were found in the second extraction and only trace levels of some of the most abundant compounds were found in the third

extraction cycle. Consequently, two sequential extraction cycles were used for subsequent studies.

3.3.3.4. Comparison el SLE and MAE methods

The efficiency of the two optimized extraction methods, i.e., conventional SLE and MAE, concerning the recovery of α-GOS, cyclitols and other carbohydrates from mung bean (sample MBS2) was then compared. Results are shown in **Table 3.3.4**. In general, similar concentrations of all carbohydrates were obtained by both methods. Only some differences were observed for specific compounds which were better extracted by MAE, like raffinose (1.87 mg g^{-1} dry sample by SLE and 5.1 mg g^{-1} dry sample by MAE) and a diglycosyl-methyl-inositol (0.13 mg g^{-1} dry sample by SLE and 0.45 mg g^{-1} dry sample by MAE). On the contrary, the highest concentration of some non-bioactive carbohydrates were obtained using SLE, such as for glucose+galactose (0.12 *vs* 0.05 mg g^{-1} dry sample by SLE and MAE, respectively).

Table 3.3.4. Comparison of the SLE and MAE efficiency for the extraction of selected compounds from MBS2 under optimal conditions.

		Analyte content (mg g^{-1})	
Peak number	**Target analyte**	**SLE (50˚C, 1 h, 2 cycles)**	**MAE (50˚C, 3 min, 2 cycles)**
1	Fructose	0.034 (0.008)	0.042 (0.005)
3 & 4	Galactose & Glucose	0.12 (0.01)	0.054 (0.005)
5	Methyl-*scyllo*-inositol	3.0 (0.2)	4.1 (0.2)
8	*scyllo*-inositol	0.046(0.001)	0.05 (0.01)
9	*myo*-inositol	0.5 (0.1)	0.5 (0.1)
10	Sucrose	11.6 (1.1)	12.2 (1.4)
13	Glycosyl-methyl-*scyllo*-inositol	0.026 (0.004)	0.07 (0.02)
15	Maltose	0.25 (0.05)	0.31 (0.06)
21	Raffinose	1.9 (0.3)	5.1 (0.6)
22	Maltotriose	0.025 (0.004)	0.051 (0.004)
23	Digalactosyl-methyl-*scyllo*-inositol	0.13 (0.03)	0.45 (0.07)
26	Digalactosyl-inositol	0.03 (0.01)	0.023 (0.008)
32	Stachyose	19.7 (0.1)	16.3 (2.1)
35	Trigalactosyl-methyl-*scyllo*-inositol	0.20 (0.05)	0.19 (0.02)
40	Verbascose	62.9 (21.1)	74.0 (6.1)
45	Ajugose	1.4 (0.7)	0.9 (0.6)

It is worth to highlight that MAE yielded these concentrations by using the same amount of mung bean as for SLE (0.5 g) and the same volume of solvent (10 mL), but with the advantage of using shorter extraction times (2 cycles of 1 h in SLE *vs* 2 cycles of 3 min in MAE). Therefore, MAE treatment was selected for the analysis of mung bean samples from different geographical origin.

3.3.3.4. Application of the optimized MAE method to the analysis of mung bean of different origins

The optimized MAE method was applied to the extraction of bioactive carbohydrates from commercial mung beans from additional different geographical origins [Taiwan (MBT1), Spain (MBS2), Argentina (MBA1) and China (MBC1 and MBC2)]. As it can be seen in **Table 3.3.5**, different concentrations of bioactive carbohydrates were observed for the different samples investigated. In general, mung bean sample from Argentina showed the lowest concentrations of bioactive compounds (α-GOS, 68.2 mg g^{-1} dry sample; free inositols, 4.0 mg g^{-1} dry sample; and glycosyl-inositols, 1.9 mg g^{-1} dry sample). Meanwhile, MBS2 showed the highest concentration of α-GOS (96.3 mg g^{-1} dry sample), MBT1 the highest concentration of free inositols (7.8 mg g^{-1} dry sample) and MBC2 the highest concentration of glycosylyl-cyclitols (5.2 mg g^{-1} dry sample). In all studied samples, verbascose was the most abundant carbohydrate, with concentrations varying between 50.4 to 74.0 mg g^{-1} dry sample, followed by stachyose (concentrations in the range 11.5-15.4 mg g^{-1} dry sample). On the other hand, galactinol was the most abundant glycosyl-inositol (0.4-1.1 mg g^{-1} dry sample), whereas some diglycosyl-methyl-*scyllo*-inositols (e.g. peak 24) and triglycosyl-methyl-*scyllo*-inositols (e.g. peak 36) were also found at relatively high concentrations (0.23-0.8 mg g^{-1} dry sample and 0.3-1.3 mg g^{-1} dry sample range, respectively).

3.3.4. Conclusions

This study reports a detailed characterization of LMWC in mung bean samples from different geographical origins. Several glycosyl-inositols (probably from both *myo*- and *scyllo*-inositol) and glycosyl-methyl-inositols not previously described have been detected in the investigated mung bean extracts. Abundance ratios of *m/z* ions 133/129 and 260/265 and the presence or absence of *m/z* 375 ion have allowed the characterization of these compounds on the base of their respective mass spectra. Moreover, a feasibility of SLE and MAE for the selective extraction of cyclitols (free and glycosylated) and α-GOS against other high content

Table 3.3.5. Concentration (mg g-1) of LMWC extracted from mung beans of different geographical origins.

Peak number	Carbohydrate	MBA1	MBC1	MBC2	MBT1	MBS2
1	Fructose	0.058 (0.009)	0.08 (0.02)	0.08 (0.03)	0.14 (0.01)	0.042 (0.005)
2	Sorbitol	0.054 (0.005)	0.07 (0.01)	0.04 (0.02)	0.056 (0.003)	0.031 (0.004)
3, 4	Galactose & Glucose	0.056 (0.003)	0.08 (0.01)	0.04 (0.01)	0.06 (0.01)	0.054 (0.005)
5	Methyl-*scyllo*-inositol	3.4 (0.3)	4.6 (0.7)	5.9 (1.0)	6.9 (1.9)	4.1 (0.2)
6	Galacturonic acid	0.6 (0.1)	0.31 (0.04)	0.3 (0.1)	0.3 (0.1)	0.36 (0.07)
7	Glucaric acid	0.55 (0.04)	0.59 (0.06)	0.8 (0.1)	1.14 (0.07)	0.40 (0.05)
8	*scyllo*-inositol	0.062 (0.005)	0.07 (0.01)	0.10 (0.01)	0.12 (0.02)	0.056 (0.007)
9	*myo*-inositol	0.55 (0.04)	0.65 (0.08)	0.61 (0.06)	0.7 (0.1)	0.55 (0.05)
10	Sucrose	10.3 (0.3)	10.7 (1.8)	13.9 (1.7)	17.5 (2.3)	12.2 (1.4)
11	Pentosyl-glucoside	0.04 (0.02)	0.05 (0.04)	0.032 (0.007)	0.026 (0.006)	0.021 (0.003)
12	Glycosyl-methyl-*scyllo*-inositol	0.10 (0.02)	0.17 (0.02)	0.23 (0.02)	0.23 (0.04)	0.155 (0.003)
13	Glycosyl-methyl-*scyllo*-inositol	0.22 (0.02)	0.31 (0.03)	0.44 (0.06)	0.31 (0.04)	0.28 (0.02)
14	Glycosyl-methyl- *scyllo*-inositol	0.23 (0.04)	0.18 (0.06)	0.34 (0.05)	0.31 (0.06)	0.28 (0.06)
15	Maltose	0.18 (0.03)	0.31 (0.08)	0.43 (0.14)	0.27 (0.17)	0.31 (0.06)
16	Glycosyl-methyl- *scyllo*-inositol	0.04 (0.01)	0.03 (0.01)	0.08 (0.03)	0.07 (0.02)	0.07 (0.02)
17	Galactosyl-inositol	0.025 (0.004)	0.040 (0.007)	0.09 (0.01)	0.058 (0.006)	0.045 (0.006)
18	Galactosyl-inositol	0.02 (0.01)	0.016 (0.002)	0.024 (0.004)	0.024 (0.005)	0.021 (0.003)
19	Galactinol	0.4 (0.3)	0.8 (0.1)	1.1 (0.1)	1.0 (0.1)	0.8 (0.1)
20	Di-glycosyl-glicerol	0.26 (0.02)	0.30 (0.03)	0.45 (0.03)	0.39 (0.05)	0.330 (0.004)
21	Raffinose	4.0 (0.3)	6.6 (0.6)	6.3 (0.8)	5.4 (0.3)	5.1 (0.6)
22	Maltotriose	0.031 (0.003)	0.057 (0.006)	0.097 (0.005)	0.06 (0.02)	0.051 (0.004)
23	Digalactosyl-methyl-*scyllo*-inositol	0.045 (0.004)	0.04 (0.02)	0.036 (0.008)	0.024 (0.003)	0.041 (0.007)
24	Digalactosyl-methyl-*scyllo*-inositol	0.23 (0.04)	0.50 (0.09)	0.8 (0.1)	0.63 (0.05)	0.45 (0.07)
25	Digalactosyl -methyl- *scyllo*-inositol	0.012 (0.004)	0.014 (0.002)	0.019 (0.004)	0.02 (0.01)	0.011 (0.003)
26	Digalactosyl -inositol	0.03 (0.01)	0.02 (0.01)	0.014 (0.002)	0.018 (0.006)	0.023 (0.008)
27	Digalactosyl -inositol	0.046 (0.005)	0.059 (0.002)	0.071 (0.005)	0.05 (0.02)	0.057 (0.004)
28	Trisaccharide	0.08 (0.01)	0.096 (0.005)	0.113 (0.007)	0.08 (0.03)	0.108 (0.006)
29	Digalactosyl-*scyllo*-inositol	0.013 (0.002)	0.023 (0.004)	0.047 (0.008)	0.04 (0.01)	0.022 (0.003)
30	Trigalactosyl-glycerol	0.038 (0.003)	0.046 (0.006)	0.056 (0.007)	0.051 (0.009)	0.03 (0.02)
31	DGMI	0.033 (0.004)	0.06 (0.02)	0.08 (0.02)	0.08 (0.03)	0.05 (0.01)
32	Stachyose	13.2 (1.3)	11.5 (1.6)	15.4 (1.9)	13.4 (1.3)	16.3 (2.1)
35	Trigalactosyl-methyl- *scyllo*-inositol	0.12 (0.02)	0.27 (0.06)	0.37 (0.09)	0.37 (0.07)	0.19 (0.02)
36	Trigalactosyl-methyl- *scyllo*-inositol	0.3 (0.1)	0.9 (0.2)	1.3 (0.4)	1.0 (0.2)	0.65 (0.07)
37	Maltotetraose	0.15 (0.03)	0.24 (0.04)	0.27 (0.01)	0.24 (0.05)	0.226 (0.005)
38	Trigalactosyl-inositol	0.004 (0.007)	0.030 (0.01)	0.07 (0.03)	0.05 (0.02)	0.022 (0.008)
39	Trigalactosyl -inositol	0.024 (0.006)	0.04 (0.02)	0.04 (0.01)	0.04 (0.02)	0.033 (0.005)
40	Verbascose	50.4 (9.9)	54.5 (1.7)	62.2 (2.7)	54.9 (13.0)	74.0 (6.1)
45	Ajugose	0.6 (0.2)	1.5 (0.3)	2.0 (0.9)	0.9 (0.2)	0.9 (0.6)
Total α-Gos		**68.2 (11.6)**	**74.1 (4.3)**	**85.9 (6.4)**	**74.5 (14.8)**	**96.3 (9.4)**
Total free inositols		**4.0 (0.3)**	**5.3 (0.8)**	**6.6 (1.1)**	**7.8 (2.0)**	**4.7 (0.25)**
Total glycosyl-cyclitols		**1.9 (0.6)**	**3.5 (0.7)**	**5.2 (0.9)**	**4.3 (0.8)**	**3.2(0.5)**
Total non-bioactive carbohydrates		**12.4 (0.6)**	**13.0 (2.1)**	**16.7 (2.3)**	**20.4 (2.9)**	**14.2 (1.7)**

non-bioactive LMWC also present in the samples has been evaluated. Ethanol water 1:1 (v:v) was found to be a selective solvent for the extraction of bioactive carbohydrates. After careful optimisation of the several parameters affecting the efficiency of the extraction with both techniques, MAE provided the most satisfactory results. To the best of our knowledge, this is the first time that MAE has been used for the extraction of these bioactive carbohydrates from legumes, in particular from mung bean. MAE has proved to be a good alternative to conventional SLE to obtain extracts enriched in bioactive carbohydrates. The developed procedure could be considered as a valuable greener alternative to more time-consuming methodologies in use base on SLE for the enrichment of food ingredients with industrial interest.

3.4. Gas chromatographic-based techniques for the characterization of low molecular weight carbohydrates and phenylakanoid glycosides of *Rhodiola rosea* food supplements

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3.4.1. Introduction

Rhodiola genus belongs to the Crassulaceae family and it is mainly cultivated worldwide to obtain dietary supplements (Marchev, 2016), which usually consist of alcoholic extracts from *Rhodiola* roots, traded alone or in combination with other plants (Platikanov, 2008).

Rhodiola is defined as an adaptogen, showing a favourable influence on a diversity of physiological functions (Chiang, 2015; Nabavi, 2016). These properties have been described in different species of the *Rhodiola* genus (Lee, 2000; Wiedenfeld, 2007; Yoshikawa, 2008; Choudhary, 2015); however, *R. rosea* is the most appreciated for its beneficial activities as antioxidant, anti-hypoxic, immunomodulatory, cardioprotective and neuroprotective, among others (Grech-Baran, 2015; Marchev, 2016). Recently, hypoglycemic and hypolipidaemic activities have also been attributed to *R. rosea* (Niu, 2014; Mao, 2017); supplements from this plant could potentially be therapeutic agents for diabetes mellitus treatments.

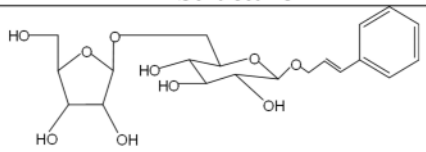
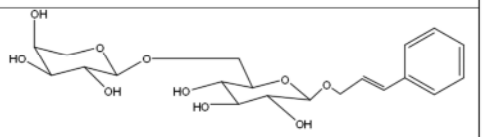
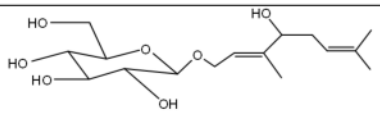
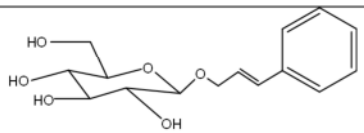
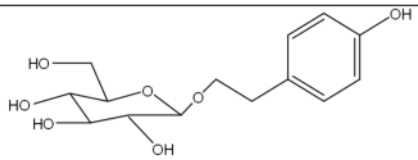
The presence of different compounds in *Rhodiola* roots such as polyphenols, terpenes, phenylalkanoids, carbohydrates and glycosides has been reported to be related to the biological activities of this plant (Kurkin, 2003; Nabavi, 2016; Mao, 2017). Several manuscripts have been devoted to the analysis of polyphenols, terpenes and phenylalkanoids in *Rhodiola* extracts (Ali, 2008; Avula, 2009; Zhou, 2014). Regarding the last compounds, a global estimation by high performance liquid chromatography (HPLC) of selected bioactives [mainly the phenylpropanoid glycosides rosarin, rosavin and rosin, which are reported as 'rosavins', and their relationship with salidroside (**Table 3.4.1**)] is usually considered to evaluate *Rhodiola* bioactivity. Nevertheless, each of these active compounds shows a specific physiologic effect and their individual determination is highly recommended.

Studies regarding *Rhodiola* roots carbohydrate composition are scarce and mainly aimed to the estimation of total and reducing sugars (Mao, 2017), or the rough determination of polysaccharides (Cai, 2012) and free monosaccharides (Perinskaya, 2014). Therefore, there is a need to develop analytical methodologies that allow the detailed study of the low molecular weight carbohydrate (LMWC) composition of *R. rosea* extracts for the required characterization of these food supplements prior to their consumption.

Considering the advantages in terms of resolution, sensitivity and identification capability provided by gas chromatography coupled to mass spectrometry (GC-MS), this technique is an interesting choice for the comprehensive characterization of the complex mixtures of LMWC and phenylalkanoid glycosides (PAG) present in *R. rosea* supplements. Moreover, the use of comprehensive two-dimensional gas chromatography coupled to time-of-flight mass spectrometry (GC×GC-ToF MS) could significantly improve the peak capacity and average

sensitivity over monodimensional GC (1D GC), resulting in an improved characterisation of less abundant compounds. Although GC-MS has been mainly used to analyse the volatile composition of essential oils from *R. rosea* roots (Evstatieva, 2010), to our knowledge, no previous reference has addressed the study by these GC-based techniques of the PAG present in *Rhodiola* root supplements or the comprehensive characterization by GC×GC of the LMWC in this particular type of matrix.

Table 3.4.1. Nomenclature and chemical structure of rosavins.

Common name	IUPAC name	Structure
Rosarin	**(2E)-3-Phenyl-2-propen-1-yl 6-O-α-L-arabinofuranosyl-β-D-glucopyranoside**	
Rosavin	(2E)-3-Phenyl-2-propen-1-yl 6-O-α-L-arabinopyranosyl-β-D-glucopyranoside	
Rosiridin	**(2Z)-4-Hydroxy-3,7-dimethyl-2,6-octadien-1-yl hexopyranoside**	
Rosin	**(2E)-3-Phenyl-2-propen-1-yl β-D-glucopyranoside**	
Salidroside	**2-(4-Hydroxyphenyl)ethyl β-D-glucopyranoside**	

At the sight of the above exposed, the main objective of the present study was the exhaustive characterization of LMWC and PAG of *R. rosea* root supplements by developing and applying GC-MS and GC×GC-ToF MS methodologies with a view to provide a deeper understanding of the composition of these food supplements. To this aim, efficiency of the derivatization procedure, mandatory for GC analysis of these compounds, was first evaluated.

3.4.2. Materials and Methods

3.4.2.1. Standards and samples

Analytical standards of 2,7-anhydro-*β*-D-*altro*-heptulose, arabinose, arabitol, cellobiose, fructose, galactose, glucose, 1-kestose, mannose, D-*altro*-2-heptulose (sedoheptulose), sucrose, *myo*-inositol, gentiobiose, raffinose, ribose, xylitol, and xylose were obtained from Sigma-Aldrich (St. Louis, USA). Rosin, rosarin, rosiridin, rosavin and salidroside were purchased from Chengdu Biopurify Phytochemicals Ltd. (Sichuan, China). Leaves from *Coriaria myrtifolia* and *Coriaria ruscifolia* were supplied by Dr. R. Morales from Real Jardín Botánico of CSIC (Madrid, Spain).

A total of eight *Rhodiola rosea* root supplements were analysed: Z1 – Z6 were kindly provided by Biosearch Life (Madrid, Spain); C1 and C2 were purchased from Lamberts Plus (Lamberts Española SL, Madrid, Spain) and Solgar España (Las Rozas, Madrid, Spain), respectively. As a reference, a *R. rosea* root sample (R1) was also analysed. **Table 3.4.2** shows the concentrations of 'rosavins', as indicated in the labels of the containers, of the different *Rhodiola* root supplements under study.

Table 3.4.2. Percentages of "rosavins" (rosin, rosarin and rosavin) experimentally measured by GC-MS and indicated in labels of *Rhodiola rosea* supplements.

%	C1	C2	Z1	Z2	Z3	Z4	Z5	Z6
Values in labels	1	3.2	3.5	10.1	4.1	8	3.2	3
Experimental values (SD)	1.24 (0.35)	2.51 (0.29)	4.20 (0.21)	11.60 (1.31)	3.68 (0.28)	7.38 (1.51)	4.12 (0.53)	3.54 (0.32)

3.4.2.2. Extraction procedure

Commercial supplements C1 and C2 (1 g) and Z1-Z6 (0.3 g) were dissolved into 10 mL of ethanol (Scharlau, Barcelona, Spain): water (70:30, v/v). Water of ultra-pure quality (18.2 MΩcm) was produced in house using a Milli-Q Advantage A10 system from Millipore (Billerica, MA, USA).

Rhodiola roots (R1) were ground using a mill (Moulinex, Barcelona, Spain) and sieved (< 500 µm) before extraction. One gram of ground root sample was extracted with 10 mL of ethanol: Milli-Q water (70:30, v/v) for 2 hours under constant stirring. Three successive extractions from the same sample were carried out and extracts thus obtained were further combined.

All extracts were filtrated through Whatman No. 4 filter paper (Sigma-Aldrich) and kept in the dark at -20 ˚C until analysis.

3.4.2.3. Analysis

3.4.2.3.1. Derivatization procedure

Rhodiola root extracts (1 mL) were mixed with 0.5 mL of a 70% ethanolic solution of phenyl-β-D-glucoside (1 mg mL^{-1}), employed as internal standard, and then dried under vacuum at 38–40 ˚C before derivatization. Trimethylsilyl oximes (TMSO) were chosen as derivatives considering that the formation of oximes before trimethylsilylation reduces the number of possible tautomers of reducing sugars to only two forms (the *syn* (*E*) and *anti* (*Z*) isomers), whereas non-reducing compounds were converted into trimethylsilyl ethers (Brokl, 2009). Oximes were formed using 2.5% hydroxylamine chloride in pyridine (350 µL) after heating at 75 ˚C for 30 min (Ruiz-Matute, 2010). Different silylation reagents [hexamethyldisilazane (HDMS), trimethylsilylimidazole (TMSI) + trimethylchlorosilane (TMCS)], and silylation temperatures (22 and 45˚C) were considered in the optimization of the derivatization procedure. Efficiency of this process, not previously evaluated for phenylpropanoid glycosides, was also determined.

After derivatization, 0.2 mL of hexane and 0.2 mL of Milli-Q water were added to the mixture to remove the excess of derivatization reagents, and 1 µL of the upper layer was further injected into the corresponding GC-based system.

3.4.2.3.2. GC-MS analysis

GC–MS analyses of derivatized samples were carried out in a 6890A gas chromatograph coupled to a 5975C quadrupole mass detector (both from Agilent Technologies, Palo Alto, CA, USA), using He at 0.8 mL min^{-1} as carrier gas. Analyses were performed on a ZB-5 (5% phenylmethylsiloxane) capillary column (25 m x 0.25 mm i.d., 0.25 µm film thickness; Phenomenex, Madrid, Spain). Different oven temperature programs were evaluated for separation of target compounds (see section 3.4.3.2.1.). Injection port temperature was set at 300 ˚C and injections were made in the split (1:20) mode. The mass spectrometer was operated in electron impact (EI) mode at 70 eV, scanning the 35–700 *m/z* range. Interface and source temperatures were 280 ˚C and 230 ˚C, respectively. Data acquisition was done using HPChem Station Rev. A.07.01 software (Agilent Technologies).

Qualitative analysis was based on the comparison of experimental mass spectra with those of standards, and was confirmed by using linear retention indices (I^T). Compounds for which commercial standards were not available were tentatively identified on the basis of their mass spectral information.

Quantitation was carried out by the internal standard method. Standard solutions of target compounds over the expected concentration range in the samples under study (0.01-0.25 mg mL^{-1}) were used to calculate the response factor (RF) relative to phenyl-β-D-glucoside (internal standard). Linearity for these calibration curves was evaluated from their correlation coefficients. Concentrations of compounds for which commercial standards were not available were estimated assuming a response factor equal to 1. Unless otherwise specified, all analyses were carried out in triplicate.

Precision of the method was measured on the basis of intra-day precision, calculated as the relative standard deviation (RSD, %) of n = 5 independent analyses of a mixture including phenylpropanoid glycosides (rosin and rosavin) and LMWC (glucose and *myo*-inositol) standards, selected as representative of these types of compounds. Limits of detection (LOD) and quantitation (LOQ) for these standards were calculated as three and ten times the standard deviation of the noise, respectively.

3.4.2.3.3. GC×GC-ToF MS analysis

The GC×GC-ToF MS equipment consisted of a 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus 4D ToF MS system (LECO Corp., St. Joseph, MI, USA), which was operated in EI mode at 70 eV. The column set employed included an HP-8 (8% phenyl polysiloxane-carborane; 30 m x 0.25 mm i.d., 0.25 µm film thickness) and a BPX-50 (50% phenyl / 50% methylpolyxiloxane-carborane; 1.9 m x 0.10 mm i.d., 0.10 µm film thickness) as first (^{1}D) and second (^{2}D) dimensions, respectively. Both columns were purchased from SGE (Melbourne, Australia). The system was equipped with a secondary oven for independent temperature program of the ^{2}D column. A quad-jet dual-stage modulator using liquid nitrogen was used for cryofocusing of analytes eluting from ^{1}D column and reinjection into the ^{2}D column.

All experiments were carried out under a constant pressure regime and helium was used as carrier gas (0.9 mL min^{-1}). The injection was carried out in the split mode (split ratio 1:10), at 275 ˚C. Temperature programs were for ^{1}D oven: 70 ˚C (0.2 min), at 4 ˚C min^{-1} to 290 ˚C (45 min) and for the ^{2}D oven: 90 ˚C (0.2 min), at 4 ˚C min^{-1} to 300 ˚C (45 min). The modulator was programmed to track the main oven with an off-set of 30 °C. The temperature of the interface and ion-source

was set at 250 ˚C. Mass spectra were collected in the 40–700 *m/z* range at an acquisition rate of 100 Hz. Data were recorded and processed using the LECO Chroma TOF 4.2 software. As in GC-MS, analyte identifications were considered to be tentative when based on experimental MS data and when authentic standards were not available for further confirmation.

3.4.3. Results and discussion

3.4.3.1. Optimal derivatization conditions of *R. rosea* phenylpropanoid glycosides

Silylation is a well-established procedure for derivatization of carbohydrates as it provides these compounds with the required volatility and stability for their GC analysis (Ruiz-Matute, 2011). On the contrary, derivatization of phenylpropanoid glycosides is not straightforward and evaluation of the reaction efficiency needs to be evaluated for the different experimental conditions assayed. The formation of per-trimethylsilyl (TMS) ethers is generally carried out using individual silylation agents such as TMSI, HMDS or TMCS, or combinations of these, and an aprotic solvent at different temperatures for different reaction times (Knapp, 1979). Phenylpropanoid glycoside standards (rosin, rosarin and rosavin) were initially converted into their TMS derivatives using HMDS as silylation agent and trifluoroacetic acid (TFA) as an effective catalyst for silylation (Brobst, 1966). However, under these experimental conditions, a non-reproducible derivatization (RSD up to 50%) of rosin, rosarin and rosavin was observed, probably due to a partial degradation of target compounds during silylation under these acidic conditions. A similar behaviour has been previously observed by Boldizsar *et al.* (Boldizsar, 2013) during derivatization of apigenin-2-*O*-apiosyl-glucoside using these reagents. Therefore, TFA was removed from the procedure and HMDS was replaced by a mixture of TMSI (a stronger silylation agent) and TMCS (1:1; v/v), according to Troyano et al. (Troyano, 1991). Under these conditions, a complete (average recovery, 98 %) and reproducible (average RSD, 6 %) derivatization of phenylpropanoid glycosides was achieved after 30 min. Different silylation temperatures (22 and 45˚C) were also evaluated; however, noticeable differences were not observed for any of them; 22 ˚C being chosen for further experiments. Finally, and due to the presence of a number of reducing carbohydrates in the samples under study, a previous oximation step was also considered to simplify the chromatographic profiles obtained. By the TMSO procedure, both reducing aldoses and ketoses showed only two peaks (*syn* (*E*) and *anti* (*Z*) isomers,) whereas non-reducing sugars, inositols and phenylalkanoid glycosides gave rise to only a single peak.

3.4.3.2. Qualitative analysis of *R. rosea* root samples

3.4.3.2.1. GC-MS

The GC-MS method was optimized to achieve the best separation of all extracted compounds (including the most volatiles) in the minimum analysis time. Different initial oven temperatures (80, 120 and 135 ˚C) were assayed; the best results were obtained at 120 ˚C. Moreover, different ramps to rise the oven temperature from 120 to 300 ˚C (10 min) were experimentally evaluated to obtain an appropriate separation between phenylpropanoid glycosides and other coextracted compounds. While coelutions between some peaks corresponding to glycosides and carbohydrates (e.g. between rosiridin and sucrose, resolution (R_s) < 0.5) were observed using 10 ˚C min^{-1}, better results were achieved at 5 ˚C min^{-1} and 3 ˚C min^{-1} (e.g. R_s between rosiridin and sucrose equal to 0.75 and 0.9, respectively). Analysis time was 46 min using 5 ˚C min^{-1} and 70 min using 3 ˚C min^{-1}. Therefore, and to fulfil the required compromise between resolution and analysis time, a ramp of 5 ˚C min^{-1} was selected as optimum for further studies.

As an example, **Figure 3.4.1** shows the GC-MS profile of the *R. rosea* root sample R1 obtained under optimal experimental (derivatization and separation) conditions. Similar GC-MS profiles were obtained for the different *R. rosea* supplements under study, and only differences in the relative abundances of the detected compounds were observed. Up to 37 compounds were identified in these samples, including phenylpropanoids, carbohydrates, acids, alcohols and sterols (see **Table 3.4.3** for peak identification). Several of these compounds have been previously reported in *R. rosea* samples analysed by other techniques such as HPLC (Avula, 2009; Rodin, 2012).

Table 3.4.3. Concentration (mg g^{-1}) of the different compounds determined by GC-MS in *R. rosea* root supplements.

Peak No.	Assignation	I^T	C1	C2	Z1	Z2	Z3	Z4	Z5	Z6	R1
1	**Glycerol**	1277	0.50 (0.02)	1.40 (0.04)	2.80 (0.24)	3.42 (0.44)	0.629 (0.002)	0.093 (0.001)	0.76 (0.01)	0.57 (0.01)	0.26 (0.14)
2	**Threose**	1522	0.083 (0.001)	0.18 (0.01)	0.79 (0.06)	0.71 (0.04)	0.15 (0.01)	-	0.136 (0.003)	0.08 (0.01)	0.09 (0.01)
3	**Tyrosol**	1576	0.47 (0.02)	0.73 (0.08)	1.70 (0.14)	1.61 (0.07)	0.81 (0.01)	0.090 (0.001)	1.84 (0.02)	1.27 (0.02)	1.39 (0.24)
4	**Ribose**	1648	0.112 (0.001)	0.097 (0.003)	0.039 (0.004)	0.031 (0.003)	0.038 (0.004)	0.22 (0.01)	0.25 (0.01)	0.25 (0.02)	0.23 (0.11)
5	**Xylitol**	1734	0.12 (0.01)	0.12 (0.01)	0.14 (0.01)	0.17 (0.01)	0.073 (0.004)	0.042 (0.001)	0.034 (0.001)	0.031 (0.002)	0.17 (0.04)
6	**Arabitol**	1748	0.26 (0.02)	0.37 (0.03)	1.16 (0.07)	1.18 (0.08)	0.33 (0.01)	0.038(0.001)	0.62 (0.05)	0.60 (0.01)	0.26 (0.03)
7	**Xylose**	1787; 1791	0.11 (0.01)	0.20 (0.01)	0.28 (0.01)	0.29 (0.01)	0.23 (0.02)	0.46 (0.01)	0.36 (0.04)	0.33 (0.03)	tr*
8	**Arabinose**	1789; 1796	0.314 (0.004)	0.80 (0.03)	1.32 (0.10)	1.56 (0.09)	0.63 (0.04)	3.49 (0.13)	0.474 (0.001)	0.45 (0.02)	2.50 (0.56)
9	**Pentose**	1812; 1825	0.33 (0.02)	1.84 (0.14)	2.94 (0.13)	3.04 (0.06)	0.31 (0.01)	0.028 (0.001)	0.25 (0.03)	0.24 (0.01)	0.16 (0.02)
10	**Hexitol**	1918	-	-	-	-	-	-	-	-	3.44 (0.73)
11	**Lactic acid**	1932	0.13 (0.01)	0.46 (0.01)	0.45 (0.02)	0.49 (0.01)	0.20 (0.01)	0.48 (0.09)	0.26 (0.01)	0.26 (0.01)	tr
12	**2,7-Anhydro-β-D-altro-heptulopyranose**	1962	0.77 (0.04)	7.16 (0.49)	8.07 (0.25)	8.27 (0.15)	1.41 (0.03)	0.30 (0.01)	2.26 (0.18)	2.01 (0.07)	5.38 (0.72)
13	**Gallic acid**	1963	4.17 (0.24)	8.42 (0.15)	22.53 (0.81)	22.51 (1.16)	14.79 (0.07)	4.62 (0.17)	6.43 (0.06)	6.16 (0.28)	1.81 (0.14)
14	**Mannitol**	1970	0.81 (0.03)	1.28 (0.06)	4.09 (0.17)	4.13 (0.14)	1.25 (0.05)	0.15 (0.01)	1.92 (0.16)	1.88 (0.03)	0.66 (0.17)

Table 3.4.3. Continuation

15	**Sorbitol**	1978	0.34 (0.02)	0.24 (0.01)	0.502 (0.001)	0.54 (0.04)	0.13 (0.01)	0.029 (0.002)	0.319 (0.001)	0.30 (0.03)	-
16	**Fructose**	1983; 1991	6.35 (0.05)	11.72 (0.95)	21.77 (0.17)	21.30 (0.49)	6.05 (0.18)	0.97 (0.06)	12.94 (1.05)	13.36 (0.57)	9.50 (1.45)
17	**Glucose + Mannose + Galactose**	2028-2060	7.19 (0.16)	17.01 (1.10)	22.45 (0.14)	22.45 (0.14)	8.79 (0.20)	22.19 (0.47)	23.38 (1.61)	8.79 (0.20)	14.80 (3.82)
18	***myo*-Inositol**	2130	0.18 (0.01)	0.24 (0.02)	0.44 (0.02)	0.46 (0.01)	0.186 (0.005)	0.034 (0.001)	0.50 (0.04)	0.47 (0.01)	0.82 (0.09)
19	**Heptitol**	2162	tr	tr	-	-	-	-	-	-	1.61 (0.41)
20	**Coriose**	2231; 2235	11.90 (0.71)	17.99 (0.30)	27.42 (1.36)	25.93 (2.41)	15.41 (1.19)	3.05 (0.08)	22.33 (1.88)	23.27 (0.99)	10.34 (2.00)
21	**Heptose**	2264; 2270	1.768 (0.003)	3.62 (0.12)	7.97 (0.21)	7.75 (0.24)	1.68 (0.09)	0.35 (0.02)	11.44 (0.91)	10.98 (0.29)	13.53 (0.07)
22	**Sedoheptulose**	2278; 2294	51.53 (0.60)	79.17 (0.14)	133.96 (2.12)	125.35 (1.98)	59.71 (1.50)	13.21 (0.32)	216.88 (13.41)	210.19 (2.41)	131.72 (16.78)
23	**Glycosyl-glycerol**	2362	0.118 (0.003)	0.18 (0.07)	0.13 (0.01)	0.17 (0.02)	0.096 (0.004)	-	0.227 (0.005)	0.197 (0.003)	0.50 (0.13)
24	**Benzyl glucopyranoside**	2430	0.29 (0.06)	0.69 (0.05)	1.37 (0.03)	1.22 (0.07)	0.40 (0.01)	0.153 (0.004)	0.45 (0.01)	0.29 (0.02)	2.76 (0.04
25	**Rosin**	2690	1.36 (0.33)	2.18 (0.06)	5.11 (0.31)	15.17 (1.34)	3.13 (0.34)	4.61 (0.15)	2.97 (0.24)	2.38 (0.40)	6.85 (0.52)
26	**Rosiridin**	2700	13.89 (2.96)	21.51 (0.56)	40.96 (3.86)	118.33 (5.16)	30.29 (1.81)	66.40 (14.37)	29.48 (3.50)	30.41 (18.23)	29.95 (2.74)
27	**Sucrose**	2707	0.70 (0.09)	3.63 (0.29)	10.80 (0.02)	10.66 (0.22)	8.79 (0.24)	1.91 (0.01)	26.00 (2.09)	27.59 (0.84)	15.40 (1.00)
28	**Salidroside**	2815	9.32 (1.83)	14.50 (1.02)	21.50 (11.91)	79.00 (5.70)	23.26 (2.37)	20.63 (1.90)	30.34 (2.38)	26.72 (3.77)	9.37 (0.78)
29	**Arabinosyl-1,6-glucose**	2812; 2852	4.82 (0.01)	8.89 (0.22)	13.99 (0.76)	14.56 (0.87)	6.01 (0.18)	9.03 (0.53)	11.11 (0.70)	11.09 (0.21)	13.54 (1.31)

Table 3.4.3. Continuation

30	**Cellobiose**	2871; 2891	1.03 (0.09)	8.56 (0.40)	-	-	3.30 (0.01)	-	0.84 (0.01)	0.52 (0.04)	3.87 (0.28)
31	**Gentiobiose**	2910; 3018	0.50 (0.02)	1.72 (0.03)	1.36 (0.04)	1.20 (0.08)	11.99 (0.01)	17.21 (0.50)	17.72 (0.23)	0.81 (0.01)	1.12 (0.13)
32	**Benzyl pentosyl-hexoside**	3198	0.27 (0.03)	0.27 (0.08)	0.44 (0.032)	0.46 (0.04)	0.37 (0.01)	0.46 (0.03)	0.41 (0.02)	0.41 (0.03)	-
33	**Rosarin**	3364	1.85 (1.23)	2.14 (1.72)	6.19 (0.25)	11.96 (1.06)	5.31 (0.52)	10.35 (1.67)	13.66 (14.32)	4.49 (1.18)	3.13 (0.34)
34	**beta-Sitosterol**	3372	tr	tr	-	-	-	-	-	-	0.59 (0.10)
35	**Rosavin**	3436	9.17 (1.94)	16.73 (1.09)	30.68 (1.55)	88.89 (10.73)	28.36 (1.90)	58.80 (13.31)	24.53 (3.59)	28.52 (15.84)	9.31 (1.87)
36	**Trisaccharides**	3501-3516	0.44 (0.10)	5.50 (0.45)	0.18 (0.03)	0.12 (0.01)	10.60 (0.51)	-	0.194 (0.005)	0.148 (0.001)	1.52 (0.23)
37	**Rosavin isomer**	3525	0.61 (0.01)	0.83 (0.01)	0.75 (0.01)	0.74 (0.05)	0.74 (0.02)	0.105 (0.001)	0.89 (0.13)	0.67 (0.08)	0.65 (0.09)

*: traces

Regarding phenylpropanoid glycosides, rosin (I^T = 2690), salidroside (I^T = 2815), rosarin (I^T = 3364) and rosavin (I^T = 3436) eluted as well-resolved peaks by using the previously optimized GC-MS method and were easily identified (MS data are provided in **Anexo 2 A**). A small peak eluting after rosavin (I^T = 3525) showed a mass spectrum [relative abundances of *m/z* ions at 117 (100), 204 (53), 217 (35), 259 (59), 349 (16)] similar to that of this phenylpropanoid and it was tentatively identified as an isomer of this compound. Other glycosides such as rosiridin (I^T = 2700) and benzyl glucopyranoside (I^T = 2430) already described in *R. rosea* (Ganzera, 2001; Huang, 2012) were also detected. Several minor peaks eluting between 32 and 41 min with *m/z* ions at 259 and 349 (characteristic of the pentosyl-hexosyl moiety of rosarin and rosavin) or with *m/z* ion at 331 (present in rosin) were also detected. In order to get insight into the identification of these minor components of *R. rosea* supplements, the use of GC×GC-ToF MS technique was explored for the first time in this paper under the experimental conditions described in Section 3.4.2.3.3..

Regarding carbohydrates, the presence of glucose, fructose, *myo*-inositol and sucrose, previously reported in the literature for this root (Perinskaya, 2014), was confirmed with GC-MS data experimentally obtained for commercial standards. Besides, many other sugars from different classes were also detected. The most abundant peaks, with I^T values of 2278 and 2294, showed a similar mass spectrum [*m/z* ions at 205 (56), 217 (25) and 319 (100)], compatible with that of an heptulose (see **Anexo 2 A** for the whole mass spectrum). These peaks were identified as the *E* and *Z* isomers of sedoheptulose by comparison with experimental retention and mass spectral data for the corresponding standard. Sedoheptulose was first detected in *Sedum spectabile* Bor. (La Forge, 1917), and it is known to play an important role in the cyclic regeneration of D-ribulose for carbon dioxide fixation in plant photosynthesis (Benson, 1951). The presence of this carbohydrate in *Rhodiola rosea* roots has been previously mentioned, without providing supporting analytical data, by Perinskaya and Sakanyan (Perinskaya, 2014) in a review on the current state and prospects of drugs based on rhizomes and roots of *R. rosea*.

Two peaks with I^T 2231 and 2235 and mass spectra [ions at *m/z* 73 (100), 147 (42), 217 (55), 307 (65) and 423 (5)] compatible with a 3-heptulose, were identified for the first time in this plant as the *E* and *Z* isomers of coriose. Identification of this natural 3-heptulose was carried out by comparison of I^T and mass spectra of these peaks with data experimentally obtained in the laboratory for *C. myrtifolia* and *C. ruscifolia* leaf extracts analysed under identical conditions.

This carbohydrate had also been previously reported by other authors in fruits, leaves, stems and roots of other *Coriaria* species (Okuda, 1968).

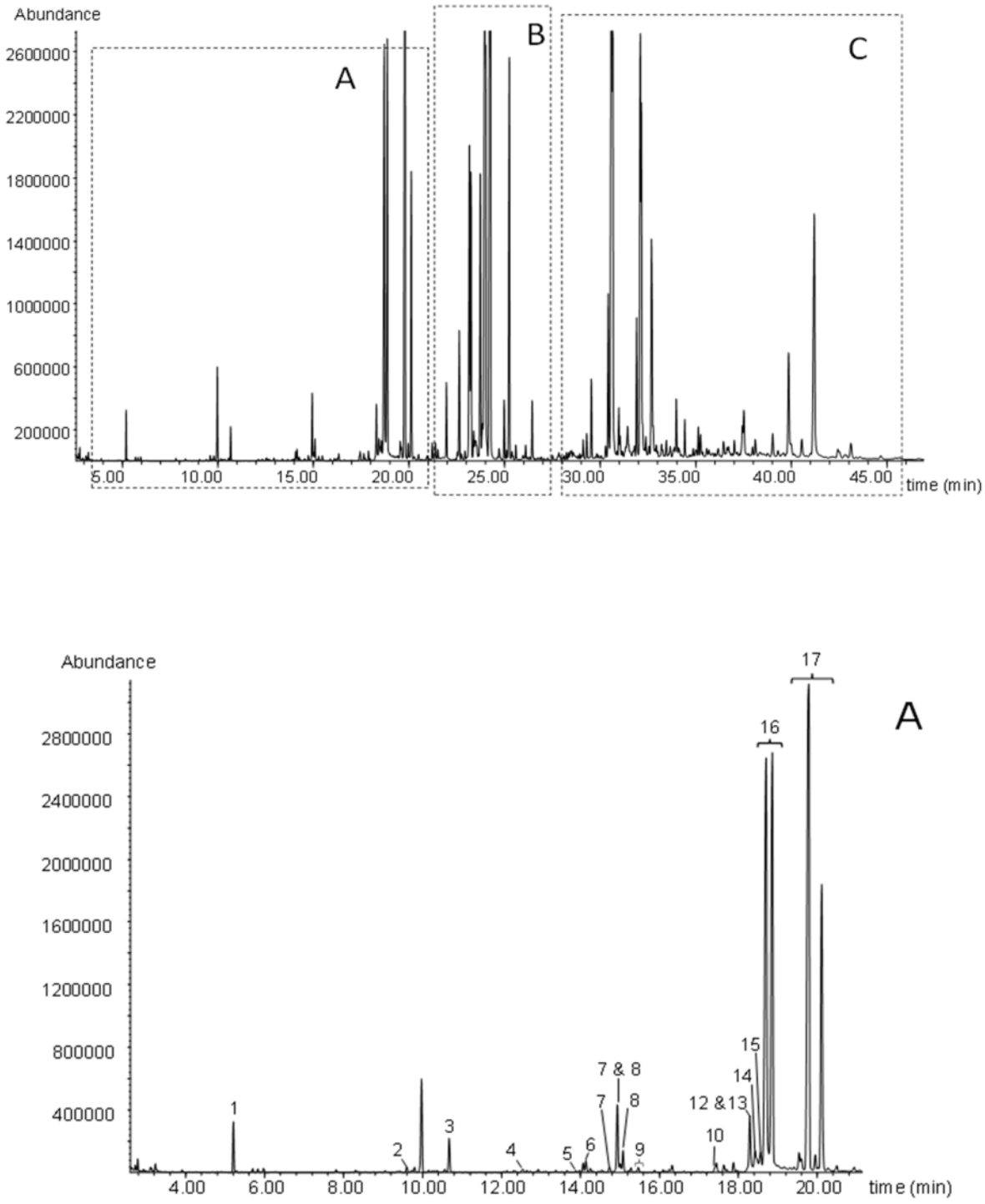


Figure 3.4.1. Continuation

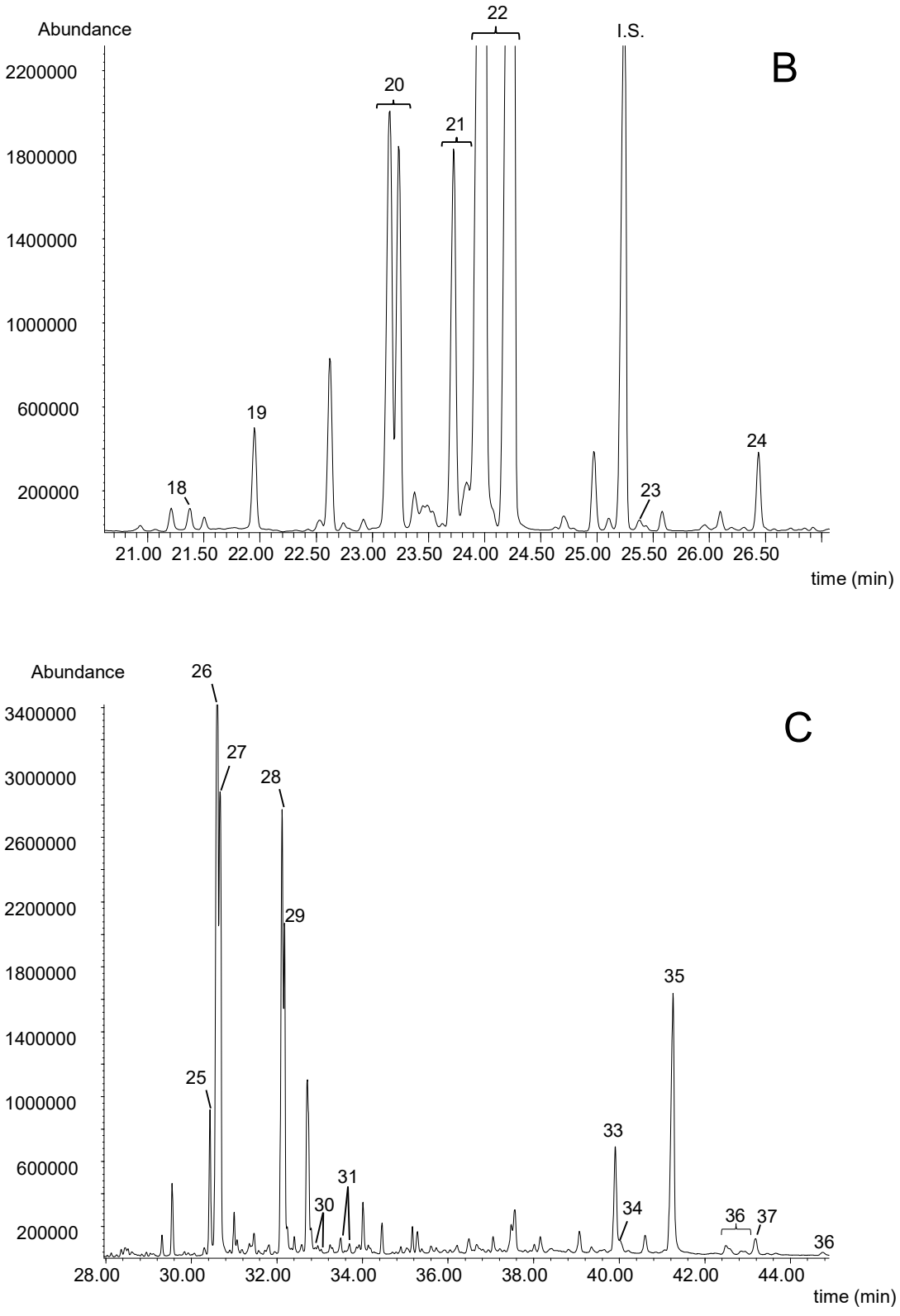


Figure 3.4.1. Gas chromatographic-mass spectrometric profile of *Rhodiola rosea* root sample (R1). For peak assignations, see Table 2. I.S.: internal standard.

Moreover, two peaks with I^T 2264 and 2270 and characteristic *m/z* ions at 205 (44), 217 (33), 307 (16) and 319 (62) were also tentatively assigned as *E* and *Z* isomers of an heptose, whose identification could not be fully confirmed. A peak with I^T value of 1962 and characteristic *m/z* ions at 204 (82), 217 (38), 317 (4), 332 (5) and 375 (2) was assigned as 2,7-anhydro-*β*-D-*altro*-heptulopyranose by comparison with experimental data for the corresponding commercial standard. An heptitol (I^T = 2162) was also detected in relatively small amounts. The presence of different seven-carbon sugars has been reported in several plant families such as Euphorbiaceae, Aquifoliaceae, Leguminosae and Apiaceae (Ogata, 1972; Okuda, 1974; Soria, 2009); however, to the best of our knowledge, this is the first time that these seven-carbon monosaccharides (except for sedoheptulose) have been detected and comprehensively characterized in *Rhodiola* genus.

Short chain carbohydrates such as a threose, several pentoses (ribose, xylose and arabinose) and polyalcohols (xylitol, arabitol, sorbitol, mannitol) were found at low abundances. Cellobiose and gentiobiose, which are disaccharides with glycosidic linkage in *β* position, similar to glycosides previously reported in *R. rosea* (Tolonen, 2003), were also detected.

Peaks with I^T values of 2812 and 2852 and characteristic *m/z* ions at 191 (17), 204 (40), 217 (47), 259 (74), 349 (29), 422 (1), typical of an aldopentosyl-(1→6)-aldohexose, were assigned to the *E* and *Z* isomers of arabinosyl-1,6-glucose, probably with α linkage, considering that this is the disaccharide present in rosavins.

Up to four non-reducing trisaccharides with I^T values between 3501 and 3516 were also detected. By comparison with commercial standards, the first of these peaks was identified as raffinose, whereas the peak with I^T of 3513 was assigned to 1-kestose.

Acids (gallic acid and lactic acid) and alcohols (glycerol, tyrosol, glycosyl-glycerol) were also present in *Rhodiola rosea*. Although water is not an appropriate solvent for the extraction of lipids, beta-sitosterol (I^T = 3372) was clearly identified in the chromatographic profile of the root extract (sample R1) here analysed.

3.4.2. GC×GC-ToF MS

Figure 3.4.2 shows the GC×GC contour plot (Total Ion Current, TIC) of the derivatized *R. rosea* root extract (sample R1). Inserts A, B and C correspond to the TIC, while inserts D1 and E1 correspond to *m/z* 259 and insert D2 to *m/z* 331. Considering the prevalence of compounds with

characteristic *m/z* ion 361 (typical of glycosidic linkage), this ion was also selected to comprehensively characterize this sample (Figure 3.4.3, inserts D3 and E2).

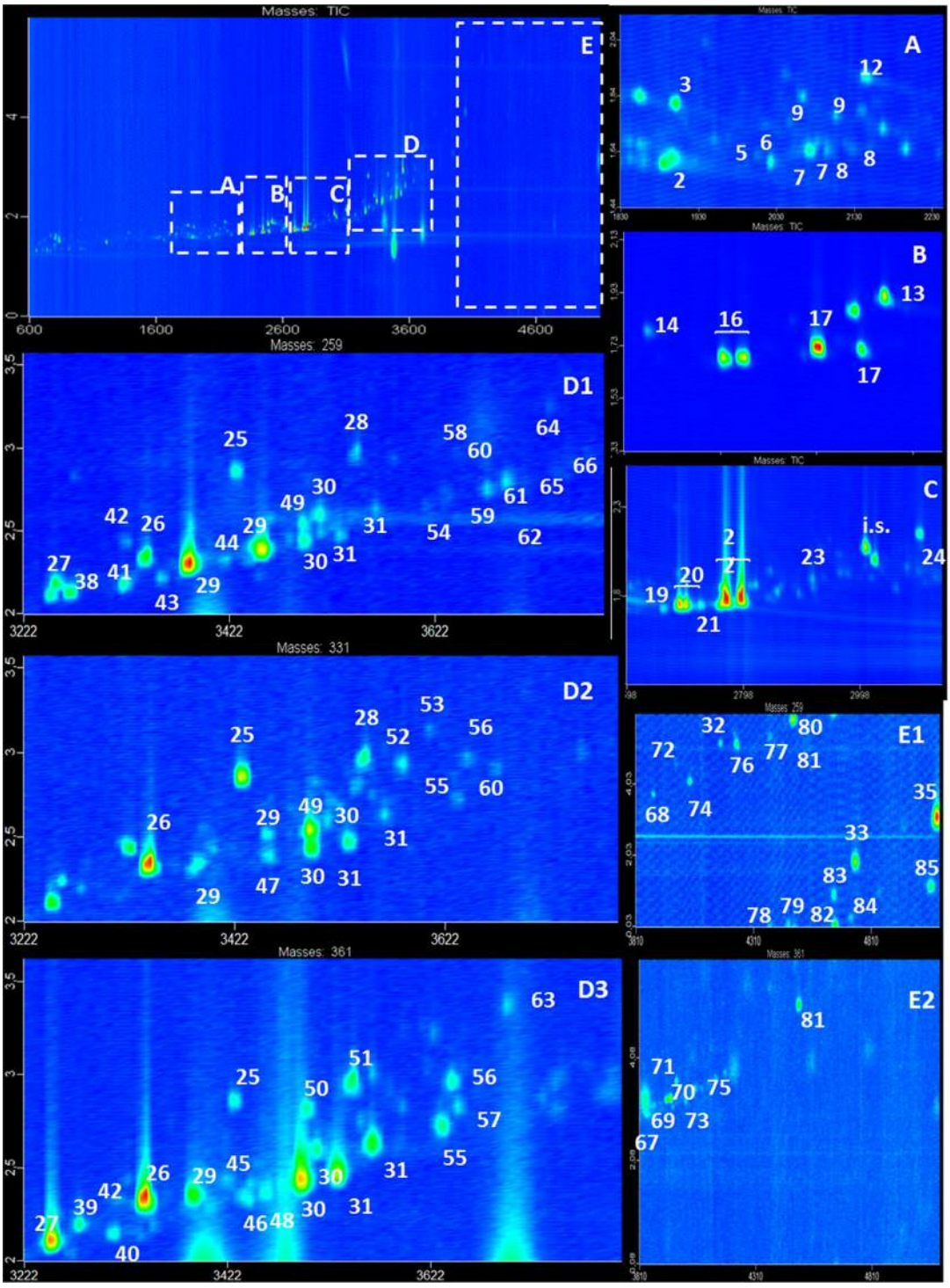


Figure 3.4.2. GC×GC contour plot (TIC) of the derivatized *Rhodiola rosea* root sample (R1). Inserts A, B and C correspond to TIC, while inserts D1 and E1 correspond to *m/z*: 259, insert D2 to *m/z* 331 and inserts D3 and E2 to *m/z* 361. Peak numbers refer to those of Tables 2 and 3.

In addition to the thirty seven compounds previously detected by GC-MS (including target glycosides: salidroside, rosin, rosarin, rosavin and rosiridin), forty eight new compounds were characterized/tentatively identified by GC×GC-ToF MS. Retention times in the first and second dimensions (tr_1 and tr_2) and characteristic *m/z* fragments of these new compounds are shown in **Table 3.4.3** (full mass spectra of peaks 38-85 are provided in **Anexo 2 B**).

Table 3.4.3. Tentative assignation of compounds detected by GC×GC-ToF MS in *Rhodiola rosea* root samples under study. PAG (phenylalkanoid glycoside), PH (pentosyl-hexose), HH (hexosyl-hexose), HX (hexoside), T (trisaccharide).

Peak No.	^1t_R (s)	^2t_R (s)	Characteristic *m/z*	Tentative identification
38	3264	2.13	204/217/259	PH
39	3276	2.21	204/361	HH
40	3312	2.19	204/361	HH
41	3318	2.19	204/217/259/349	PH
42	3318	2.43	153/204/217/259/331	PAG
43	3354	2.23	204/217/259/349	PH
44	3420	2.33	204/259/349	PH
45	3420	2.42	204/217/319/361	HH (sophorose *E*)
46	3444	2.40	204/217/259/319/361	HH (sophorose *Z*) + PH
47	3456	2.53	131/204/217/331/259	PAG
48	3462	2.35	204/217/259/319/361	PH + HH
49	3492	2.55	131/204/217/331/361	PAG
50	3498	2.83	217/282/361	HX
51	3564	3.01	217/281/361	HH
52	3582	2.93	103/204/217/331	PAG
53	3606	3.14	103/204/331	PAG
54	3612	2.65	204/217/259	PH
55	3630	2.74	204/217/361	HH
56	3642	2.95	204/217/361	HH
57	3648	2.82	204/217/315/361	HX
58	3666	3.15	131/204/217/259	PAG
59	3672	2.76	204/217/259/349	PH
60	3672	2.87	131/191/204/217/331	PAG

Table 3.4.3. Continuation

61	3690	2.77	204/217/259/349	PH
62	3690	1.66	204/217/271/361	HH
63	3696	3.37	179/204/361	HX
64	3732	3.22	204/217/259	PH
65	3732	2.56	204/217/259	PH
66	3750	2.95	131/204/217/361	HH
67	3828	3.29	204/217/361	T
68	3882	3.67	204/217/259	T (with at least one pentosyl unit)
69	3924	3.27	204/217/361	T
70	3942	3.29	204/217/319/361	T (1→2 linkage)
71	3966	3.64	204/217/337/361	Diglycosyl-glycerol
72	3978	4.58	204/217/259	T (with at least one pentosyl unit)
73	4008	3.24	204/217/361	T
74	4038	4.12	91/204/217/259	PAG (phenyl methyl- -L-arabinosyl-(1→6)- -D-glucoside)
75	4044	3.48	204/217/319/361	T (1→2 linkage)
76	4236	5.13	217/259	T (with at least one pentosyl unit)
77	4374	5.35	217/259/349	T (with at least one pentosyl unit)
78	4380	0.05	133/204/217/259	PAG
79	4458	0.11	105/204/217/259	PAG (2-phenylethyl - vicianoside)
80	4476	5.88	204/217/259/349	T (with at least one pentosyl unit)
81	4488	5.14	204/217/361/437	T (non-reducing)
82	4650	0.11	204/217/259/349	T (with at least one pentosyl unit)
83	4650	0.91	133/204/217/259	PAG
84	4722	0.27	131/217/259	PAG
85	5058	1.18	131/204/217/259/349	PAG

Peaks with characteristic *m/z* ion at 331 but absence of *m/z* 117 were detected. These compounds could correspond to phenylalkanoid glycosides (PAG) with an aglycon (*m/z* 103: peaks 52 and 53, *m/z* 131: peaks 47, 49 and 60 and *m/z* 153: peak 42) of different structure than rosin. Moreover, peaks with *m/z* ion at 259 and different *m/z* ions at 91 (peak 74), 105 (peak 79), 131 (peaks 58, 84 and 85) or 133 (peaks 78 and 83) were also detected, probably corresponding to PAG with a pentosyl-hexose unit and different aglycons than rosavin or rosarin. Among them, peak 79 could be tentatively assigned as 2-phenylethyl β-vicianoside (2-phenylethyl *O*-α-L-arabinopyranosyl-(1→6)-*O*-β-D-glucopyranoside) and peak 74 as phenyl methyl-α-L-arabinosyl-(1→6)-β-D-glucoside (see mass spectra in **Anexo 2 B**); both compounds had been previously detected in *R. rosea* by HPLC by Avula et al. (Avula, 2009).

Peaks with *m/z* 259 and absence of the characteristic ions for aglycons previously mentioned (peaks 38 and 41, 43 and 44, 46 and 48, 54 and 59, 61 and 65) were tentatively assigned as isomers *E* and *Z* of pentosyl-hexoses (PH) with different monomeric units and/or linkages; however, due to the absence of commercial standards their identification was not possible. Other peaks with *m/z* ion at 361, characteristic of glycosidic linkages, were detected and tentatively assigned as hexosyl-hexoses (HH) or hexosides (HX) (**Figure 3.6.2**). Among them, peaks 45 and 46 showed a mass spectrum compatible with glucosyl-glucoses with 1→2 glycosidic linkage (characterized by the high abundance of *m/z* ion at 319). Considering that other disaccharides with glycosidic linkage in β position (cellobiose and gentiobiose) have also been detected in this sample, these peaks could be tentatively assigned as the *E* and *Z* isomers of sophorose. Moreover, trisaccharides were also detected some of them including at least a pentosyl unit (characteristic *m/z* ion at 259; inserts E1 and E2 in **Figure 3.6.2**). Similar to disaccharides, peaks 70 and 75 (with an abundant *m/z* ion at 319) could correspond to the *E* and *Z* isomers of a trisaccharide with a 1→2 glycosidic linkage, whereas peak 71 could be assigned as diglycosyl-glycerol (characteristic *m/z* ion at 337) and peak 81 as a non-reducing trisaccharide (characteristic *m/z* ion at 437)

3.4.3 Quantitative analysis of *R. rosea* root supplements

Once the qualitative composition of *R. rosea* root samples was determined, quantitation of their main compounds by GC-MS was carried out.

Linearity, intra-day precision (RSD, %) and sensitivity data for a standard mixture including rosin, rosavin, glucose and *myo*-inositol were calculated (**Table 3.4.4**). Response was linear over the concentration range assayed (0.01-0.25 mg mL^{-1}). LOD and LOQ values were in the low µg mL^{-1}

range, the lowest values were obtained for *myo*-inositol (0.13 and 0.46 µg mL^{-1}, respectively), whereas the highest were found for rosavin (5.9 and 19.9 µg mL^{-1}, respectively). Good intra-day precision values were obtained for all standards considered (RSD ranging 3.4-6.9 %).

Table 3.4.4. Analytical parameters of the GC-MS method used for characterization of *R. rosea* root supplements.

Compound	Calibration curve	R^2	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	Intraday precision (RSD, %)
Glucose	y = 1.618 x - 0.0256	0.9932	0.25	0.84	6.5
***myo*-Inositol**	y = 1.6941 x - 0.0160	0.9940	0.13	0.46	6.9
Rosin	y = 0.8611 x - 0.0790	0.9996	1.8	6.1	4.9
Rosavin	y = 0.5807 x - 0.0098	0.9975	5.9	19.9	3.4

Table 3.4.3 shows the concentrations of the main compounds detected in the different R. rosea root supplements as well as in reference R. rosea root sample R1. In all cases, rosiridin was the most abundant glycoside (13.9-118.3 mg g-1) followed by rosavin (9.2-88.9 mg g-1). In general, the lowest concentrations of phenylpropanoid glycosides were found in the commercial samples (C1 and C2), whereas Z2 was the richest sample in these bioactive compounds. These data were in good agreement with 'rosavins' values indicated in the supplement labels (Table 3.4.5). As previously reported in the literature (Saunders et al., 2014), rosarin concentrations were lower than those of rosavin and similar to those of rosin in the analysed supplements. Concentrations of these bioactive compounds in the root sample (R1) were between the values found in the supplements and similar to those described in the literature (Saunders, 2013). Differences in 'rosavins' concentrations in the supplements could be attributed to the geographical origin, cultivation and harvesting practices and extraction processes, among others (Kolodziej, 2013; Saunders, 2013). Moreover, activity of Rhodiola supplements is usually evaluated as a ratio of 'rosavins' and salidroside concentrations. In this study, salidroside varied between 9.3 and 79.0 mg g-1; the lowest concentrations were determined in the commercial supplements C1 and C2 and in the root sample (R1). R. rosea extracts used in pre-clinical and clinical studies have been standardized to a minimum of 3% rosavins and 0.8–1.0% salidroside (Perfumi, 2007). All the analyzed supplements meets this requirement, except for supplement C1.

Table 3.4.5. Concentration (mg g^{-1}) of the different compounds determined by GC-MS in *R. rosea* root supplements.

Peak No.	Assignation	I^T	C1	C2	Z1	Z2	Z3	Z4	Z5	Z6	R1
1	**Glycerol**	1277	0.50 (0.02)	1.40 (0.04)	2.80 (0.24)	3.42 (0.44)	0.629 (0.002)	0.093 (0.001)	0.76 (0.01)	0.57 (0.01)	0.26 (0.14)
2	**Threose**	1522	0.083 (0.001)	0.18 (0.01)	0.79 (0.06)	0.71 (0.04)	0.15 (0.01)	-	0.136 (0.003)	0.08 (0.01)	0.09 (0.01)
3	**Tyrosol**	1576	0.47 (0.02)	0.73 (0.08)	1.70 (0.14)	1.61 (0.07)	0.81 (0.01)	0.090 (0.001)	1.84 (0.02)	1.27 (0.02)	1.39 (0.24)
4	**Ribose**	1648	0.112 (0.001)	0.097 (0.003)	0.039 (0.004)	0.031 (0.003)	0.038 (0.004)	0.22 (0.01)	0.25 (0.01)	0.25 (0.02)	0.23 (0.11)
5	**Xylitol**	1734	0.12 (0.01)	0.12 (0.01)	0.14 (0.01)	0.17 (0.01)	0.073 (0.004)	0.042 (0.001)	0.034 (0.001)	0.031 (0.002)	0.17 (0.04)
6	**Arabitol**	1748	0.26 (0.02)	0.37 (0.03)	1.16 (0.07)	1.18 (0.08)	0.33 (0.01)	0.038(0.001)	0.62 (0.05)	0.60 (0.01)	0.26 (0.03)
7	**Xylose**	1787; 1791	0.11 (0.01)	0.20 (0.01)	0.28 (0.01)	0.29 (0.01)	0.23 (0.02)	0.46 (0.01)	0.36 (0.04)	0.33 (0.03)	tr*
8	**Arabinose**	1789; 1796	0.314 (0.004)	0.80 (0.03)	1.32 (0.10)	1.56 (0.09)	0.63 (0.04)	3.49 (0.13)	0.474 (0.001)	0.45 (0.02)	2.50 (0.56)
9	**Pentose**	1812; 1825	0.33 (0.02)	1.84 (0.14)	2.94 (0.13)	3.04 (0.06)	0.31 (0.01)	0.028 (0.001)	0.25 (0.03)	0.24 (0.01)	0.16 (0.02)
10	**Hexitol**	1918	-	-	-	-	-	-	-	-	3.44 (0.73)
11	**Lactic acid**	1932	0.13 (0.01)	0.46 (0.01)	0.45 (0.02)	0.49 (0.01)	0.20 (0.01)	0.48 (0.09)	0.26 (0.01)	0.26 (0.01)	tr
12	**2,7-Anhydro-β-D-altro-heptulopyranose**	1962	0.77 (0.04)	7.16 (0.49)	8.07 (0.25)	8.27 (0.15)	1.41 (0.03)	0.30 (0.01)	2.26 (0.18)	2.01 (0.07)	5.38 (0.72)

Table 3.4.5. Continuation

13	**Gallic acid**	1963	4.17 (0.24)	8.42 (0.15)	22.53 (0.81)	22.51 (1.16)	14.79 (0.07)	4.62 (0.17)	6.43 (0.06)	6.16 (0.28)	1.81 (0.14)
14	**Mannitol**	1970	0.81 (0.03)	1.28 (0.06)	4.09 (0.17)	4.13 (0.14)	1.25 (0.05)	0.15 (0.01)	1.92 (0.16)	1.88 (0.03)	0.66 (0.17)
15	**Sorbitol**	1978	0.34 (0.02)	0.24 (0.01)	0.502 (0.001)	0.54 (0.04)	0.13 (0.01)	0.029 (0.002)	0.319 (0.001)	0.30 (0.03)	-
16	**Fructose**	1983; 1991	6.35 (0.05)	11.72 (0.95)	21.77 (0.17)	21.30 (0.49)	6.05 (0.18)	0.97 (0.06)	12.94 (1.05)	13.36 (0.57)	9.50 (1.45)
17	**Glucose + Mannose + Galactose**	2028-2060	7.19 (0.16)	17.01 (1.10)	22.45 (0.14)	22.45 (0.14)	8.79 (0.20)	22.19 (0.47)	23.38 (1.61)	8.79 (0.20)	14.80 (3.82)
18	***myo*-Inositol**	2130	0.18 (0.01)	0.24 (0.02)	0.44 (0.02)	0.46 (0.01)	0.186 (0.005)	0.034 (0.001)	0.50 (0.04)	0.47 (0.01)	0.82 (0.09)
19	**Heptitol**	2162	tr	tr	-	-	-	-	-	-	1.61 (0.41)
20	**Coriose**	2231; 2235	11.90 (0.71)	17.99 (0.30)	27.42 (1.36)	25.93 (2.41)	15.41 (1.19)	3.05 (0.08)	22.33 (1.88)	23.27 (0.99)	10.34 (2.00)
21	**Heptose**	2264; 2270	1.768 (0.003)	3.62 (0.12)	7.97 (0.21)	7.75 (0.24)	1.68 (0.09)	0.35 (0.02)	11.44 (0.91)	10.98 (0.29)	13.53 (0.07)
22	**Sedoheptulose**	2278; 2294	51.53 (0.60)	79.17 (0.14)	133.96 (2.12)	125.35 (1.98)	59.71 (1.50)	13.21 (0.32)	216.88 (13.41)	210.19 (2.41)	131.72 (16.78)
23	**Glycosyl-glycerol**	2362	0.118 (0.003)	0.18 (0.07)	0.13 (0.01)	0.17 (0.02)	0.096 (0.004)	-	0.227 (0.005)	0.197 (0.003)	0.50 (0.13)
24	**Benzyl glucopyranoside**	2430	0.29 (0.06)	0.69 (0.05)	1.37 (0.03)	1.22 (0.07)	0.40 (0.01)	0.153 (0.004)	0.45 (0.01)	0.29 (0.02)	2.76 (0.04
25	**Rosin**	2690	1.36 (0.33)	2.18 (0.06)	5.11 (0.31)	15.17 (1.34)	3.13 (0.34)	4.61 (0.15)	2.97 (0.24)	2.38 (0.40)	6.85 (0.52)
26	**Rosiridin**	2700	13.89 (2.96)	21.51 (0.56)	40.96 (3.86)	118.33 (5.16)	30.29 (1.81)	66.40 (14.37)	29.48 (3.50)	30.41 (18.23)	29.95 (2.74)

Table 3.4.5. Continuation

27	**Sucrose**	2707	0.70 (0.09)	3.63 (0.29)	10.80 (0.02)	10.66 (0.22)	8.79 (0.24)	1.91 (0.01)	26.00 (2.09)	27.59 (0.84)	15.40 (1.00)
28	**Salidroside**	2815	9.32 (1.83)	14.50 (1.02)	21.50 (11.91)	79.00 (5.70)	23.26 (2.37)	20.63 (1.90)	30.34 (2.38)	26.72 (3.77)	9.37 (0.78)
29	**Arabinosyl-1,6-glucose**	2812; 2852	4.82 (0.01)	8.89 (0.22)	13.99 (0.76)	14.56 (0.87)	6.01 (0.18)	9.03 (0.53)	11.11 (0.70)	11.09 (0.21)	13.54 (1.31)
30	**Cellobiose**	2871; 2891	1.03 (0.09)	8.56 (0.40)	-	-	3.30 (0.01)	-	0.84 (0.01)	0.52 (0.04)	3.87 (0.28)
31	**Gentiobiose**	2910; 3018	0.50 (0.02)	1.72 (0.03)	1.36 (0.04)	1.20 (0.08)	11.99 (0.01)	17.21 (0.50)	17.72 (0.23)	0.81 (0.01)	1.12 (0.13)
32	**Benzyl pentosyl-hexoside**	3198	0.27 (0.03)	0.27 (0.08)	0.44 (0.032)	0.46 (0.04)	0.37 (0.01)	0.46 (0.03)	0.41 (0.02)	0.41 (0.03)	-
33	**Rosarin**	3364	1.85 (1.23)	2.14 (1.72)	6.19 (0.25)	11.96 (1.06)	5.31 (0.52)	10.35 (1.67)	13.66 (14.32)	4.49 (1.18)	3.13 (0.34)
34	**beta-Sitosterol**	3372	tr	tr	-	-	-	-	-	-	0.59 (0.10)
35	**Rosavin**	3436	9.17 (1.94)	16.73 (1.09)	30.68 (1.55)	88.89 (10.73)	28.36 (1.90)	58.80 (13.31)	24.53 (3.59)	28.52 (15.84)	9.31 (1.87)
36	**Trisaccharides**	3501-3516	0.44 (0.10)	5.50 (0.45)	0.18 (0.03)	0.12 (0.01)	10.60 (0.51)	-	0.194 (0.005)	0.148 (0.001)	1.52 (0.23)
37	**Rosavin isomer**	3525	0.61 (0.01)	0.83 (0.01)	0.75 (0.01)	0.74 (0.05)	0.74 (0.02)	0.105 (0.001)	0.89 (0.13)	0.67 (0.08)	0.65 (0.09)

*: traces

Tyrosol, a bioactive phenylethanoid, was detected in all samples, Z5 (1.8 mg g^{-1}) showed the highest concentration of this compound followed by Z1 and Z2 (1.7 and 1.6 mg g^{-1}, respectively).

Regarding carbohydrates, sedoheptulose was the most abundant compound (51.5-216.9 mg g^{-1}) in all analysed samples, except for Z4 which showed the lowest concentration of this seven-carbon carbohydrate (13.2 mg g^{-1}). Coriose was also detected at relatively high concentrations (3.0-27.4 mg g^{-1}) in most of the samples analysed; Z1 was the supplement showing the highest concentration. The heptose detected in these samples (peak 21, Table 2) was more abundant in the root sample (R1, 13.5 mg g^{-1}) followed by the Z6 sample (11.0 mg g^{-1}), whereas Z1 and Z2 were the supplements with the highest concentrations (8.1-8.3 mg g^{-1}) of 2,7-anhydro-β-D-*altro*-heptulopyranose. Heptitol could only be quantified in root sample R1 (1.6 mg g^{-1}).

Considering the low abundance of mannose and galactose in all the samples analysed and the low chromatographic resolution (R_s < 1) of these peaks with those of glucose, these compounds were quantified together, their concentration laying in the range 7.1-26.4 mg g^{-1}. Low concentrations of pentoses and polyalcohols were also found in *R. rosea* samples; concentrations of arabinose and xylose were higher in the Z4 sample, whereas arabitol and xylitol were predominant in the Z1 and Z2 supplements. Total trisaccharide concentrations, including raffinose, 1-kestose and two non-identified trisaccharides, are also shown in **Table 3.4.5.** Low levels of these trisaccharides were found in all the samples, except for Z3 and C2 supplements which showed concentrations of 10.6 and 5.5 mg g^{-1}, respectively.

3.4.4. Conclusions

In this work GC-MS and GC×GC-MS methods have been developed for the simultaneous characterization of LMWC and PAG of *Rhodiola rosea* supplements, after a previous derivatization step. The high resolution provided by GC-MS allowed the improved qualitative and quantitative determination of individual phenylalkanoid glycosides (e.g. rosin, rosavin, rosarian, salidroside), carbohydrates, acids, alcohols and sterols in a single chromatographic run. In particular, several seven-carbon monosaccharides such as coriose and 2,7-anhydro-β-D-*altro*-heptulopyranose and disaccharides such as cellobiose and gentiobiose have been detected and quantified for the first time in these supplements. The combined use of this technique with GC×GC-ToF MS allowed the tentative identification of additional co-extracted compounds (up to 85) from this natural source, including different carbohydrates (pentosyl-hexoses, hexosyl-hexoses, etc) and phenylalkanoid glycosides. To the best of our knowledge, this is the first time that an exhaustive characterization of low molecular weight carbohydrates and phenylalkanoid

glycosides of *Rhodiola rosea* root supplements has been carried out by these gas chromatographic-based techniques.

3.5 Bibliografía

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4. Caracterización de lixiviados de envases alimentarios mediante GCxGC-ToF MS

4. Caracterización de lixiviados de envases alimentarios mediante GCxGC-ToF MS

4.1 Introducción

Los alimentos pueden entrar en contacto con una gran variedad de artículos y materiales durante su producción, procesado, almacenamiento y preparación previos a su consumo por la población. Todos estos productos se engloban bajo la denominación genérica de materiales en contacto con alimentos (Food Contact Materials, FCM) e incluyen todo lo que en algún momento entra en contacto con el alimento, puede llegar a entrar en contacto con el alimento, o puede llegar a ser transferido en condiciones de uso normal o previsible a los distintos componentes de una comida. De esta forma, el nombre FCM haría referencia a materiales que están tanto en contacto directo como indirecto con la comida, a los utensilios empleados para cocinar, la vajilla o los materiales de embalaje, pero también a los instrumentos empleados para procesar alimentos y los contenedores empleados para su transporte y almacenamiento. Dada la variedad de materiales involucrados en la producción, procesado y almacenaje de alimentos (plásticos, papel, metal, maderas, lacas, adhesivos, tintas de impresión, etc.), los FCM representan un campo de estudio amplio y complejo. En el caso particular de los FCM plásticos, hay que tener en cuenta que para su producción se emplean una gran variedad de sustancias químicas (Pocas, 2007).

Los materiales plásticos no sólo contienen los polímeros que dan lugar al plástico correspondiente. En casi todos los casos, los productores crean fórmulas (el plástico compuesto) que contienen diferentes aditivos para mejorar el rendimiento y las propiedades durante el procesado del material (moldeo por inyección, extrusión, soplado, moldeo por vacío, etc.), o durante su etapa de uso y envejecimiento. De esta forma, además de los monómeros del polímero correspondiente, los plásticos contienen otros compuestos de distinta naturaleza, como aditivos, plastificantes, estabilizantes, disolventes o pigmentos, entre otros. Dada la toxicidad de algunos de estos productos químicos y su capacidad para migrar desde los plásticos a los alimentos que contienen, la EU establece que la seguridad de los FCM debe ser evaluada de acuerdo a lo establecido en el artículo 3 del Reglamento No. 1935/2004 de la EU (Council, 2004) con el fin de salvaguardar la salud del consumidor. Dicho reglamento establece que los FCM producidos de acuerdo a la normativa europea deben ser fabricados siguiendo prácticas que eviten cualquier posible transferencia de dichos compuestos a los alimentos, con el fin de salvaguardar la seguridad de los mimos y evitar cambios en su composición o se deteriorar su sabor u olor.

4.1.1. El proceso de migración

La migración se define como el fenómeno que ocurre cuando las sustancias químicas presentes en un polímero migran a la superficie del material polimérico o a un medio en contacto con él. La migración puede ocurrir desde la superficie del material polimérico hacia el alimento, desde el interior de la superficie de los materiales hacia el alimento o a través del material hasta el alimento. El proceso de migración se considera relevante desde el punto de vista toxicológico para aquellos compuestos con un peso molecular inferior a 1000 Dalton (Da), ya que son los que tendrían capacidad para atravesar las barreras biológicas una vez ingresan en el cuerpo humano (EFSA, 2008).

En general, la migración de sustancias químicas presentes en los envases de plástico hacia los alimentos es un proceso no deseado, ya que algunos de estos compuestos pueden ser tóxicos o proporcionar un sabor u olor desagradable a la comida. La excepción sería el caso de los denominados envases inteligentes (Nerin, 2013).

La migración está controlada principalmente por dos procesos: la difusión y la partición. Ambos procesos fueron discutidos en la sección de Introducción de esta memoria.

En un polímero, el grado de migración de un componente del plástico hacia el medio que lo rodea (alimento) depende de varios factores. Las propiedades fisicoquímicas del migrante, del material plástico y del alimento (por ejemplo, contenido de agua, grasa o su acidez) son algunos de los factores que más afectan al proceso de migración. Como ya se ha apuntado, la migración de sustancias orgánicas desde el material polimérico depende de su tamaño. Las moléculas pequeñas, como los monómeros y los disolventes residuales, migrarán de manera rápida, ya que su pequeño tamaño facilita su movimiento a través de la matriz del polímero. Además, algunos de estos compuestos son gases a temperatura ambiente, lo que facilita aún más su migración. Sería, por ejemplo, el caso del formaldehido o el etileno. Muchos de los aditivos empleados en la formulación de plásticos tienen pesos moleculares en el intervalo 200-2000 Da. Algunos han sido diseñados con pesos moleculares deliberadamente altos con el fin de asegurar una tasa de migración baja. Sin embargo, la síntesis de otros productos atendiendo a este principio conlleva un elevado coste, por lo que en la práctica suele ser muy limitada. Sería el caso de los plastificantes o los retardantes de llama (no permitidos en FCM), que en general tienen pesos moleculares relativamente pequeños. Otros requisitos para evitar la migración es que la solubilidad del aditivo en el polímero debe ser alta y que no mostrar tendencia a migrar al líquido (o alimento) en contacto con el polímero; ambos aspectos, sin embargo, dependen también de las propiedades fisíco-químicas del alimento. La concentración inicial de la sustancia química en

el polímero es, por motivos obvios, otro factor determinante en el proceso de difusión. También afectan la cristalinidad del polímero (amorfo, semicristalino…), su estructura superficial y espesor.

Por otra parte, hay otra serie de factores no directamente relacionados con la naturaleza del FCM o el migrante en sí, y que también afectan de manera significativa el proceso de migración. Uno de los más importantes es la temperatura de exposición, ya que afecta a la solubilidad y movilidad de los potenciales migrantes. Otros aspectos relevantes en sentido son el tiempo de almacenamiento (o uso, en el caso de los contenedores de plásticos) y / o de contacto con el alimento.

Con el fin de salvaguardar la salud de los consumidores, la legislación vigente establece que, en todos los casos, la evaluación de la migración de sustancias de FCM debe hacerse contemplando el peor de los escenarios posibles y, con este fin, establece una serie de ensayos estandarizados adaptados a los diferentes casos de estudio (Union, 2011). Los datos obtenidos a partir de estos ensayos de migración son los que se emplean para realizar posteriormente la correspondiente evaluación de riesgos.

4.1.2. Legislación referente a plásticos

La legislación europea relativa a FCM se basa en la Regulación No. 1935:2004 (Council, 2004), que establece los requisitos a cumplir por los materiales destinos a entrar en contacto con los alimentos y por las nuevas sustancias de uso autorizado.

Dado que el plástico es el material de embalaje de mayor cota de mercado y uno de los FCM más importantes, este material ha sido objeto de una legislación específica en la EU, existiendo además legislaciones y normas específicas en ciertos países. La Regulación europea No. 10/2011 es la que establece los requisitos a cumplir por los materiales plásticos (Union, 2011). Incluye también datos relevantes referentes al uso de más de 150 en plásticos destinados a FCM, define las condiciones específicas a aplicar en las distintas pruebas de migración, los simulante alimentarios y establece los límites de migración global (Overall Migration Limit, OML; fijado en 10 mg dm^{-2} de superficie de contacto) y específica (Specific Migration Limits, SML) para determinados aditivos. Es importante destacar que esta Regulación establece que, dada la complejidad de las mezclas que pueden migrar desde el FCM al alimento y la variedad de la composición de muchos alimentos, los ensayos de migración deben desarrollarse empleando preferentemente disoluciones de simulantes alimentarios en vez de alimentos. Esta medida

tiene por objeto no sólo estandarizar las condiciones aplicadas en los estudios de migración, sino simplificar el análisis químico. En este sentido, hay que tener en cuenta que la composición final de los plásticos, y por tanto de las mezclas de compuestos que puede llegar a migrar desde el FCM, suele ser desconocida, no sólo porque se desconocen las posibles impurezas que acompañan a los productos añadidos en el proceso de síntesis del material, sino porque su formulación suele ser propiedad de la industria que los produce.

Las propiedades químicas y composición de los distintos simulantes alimentarios recogidos en la legislación vigente son variados, ya que deben representar a los distintos tipos de alimentos, por ejemplo, hidrofílico (de base acuosa), lipofílico (con grasa) o anfifílico. La legislación europea (Union, 2011) incluye tablas en las que los alimentos aparecen clasificados en distintas categorías y especifica el simulante alimentario asignado a cada una de ellas (**Tabla 4.4.1**.)

Los test de migración se realizan poniendo el material evaluado en contacto con el simulante apropiado. Las condiciones experimentales de la prueba, como el tiempo y la temperatura, se seleccionan teniendo en cuenta el uso final del artículo, respetando el principio de peor caso posible y, en consecuencia, seleccionando la temperatura más alta posible y el período de tiempo de exposición más largo, para incluir el peor uso final razonablemente previsible. A modo de ejemplo, la **Tabla 4.1.2.** resume algunas las condiciones de tiempo y temperatura a emplear en determinados estudios de migración.

Tabla 4.4.1. Lista de simulantes alimentarios establecidos en el Anexo III de Regulación EU No 10/2011 (Union, 2011).

Código del simulante	**Simulante**	**Tipo de alimento/comida**
	Agua	Alimentos hidrofílicos
A	10% etanol (v/v)	Alimentos hidrofílicos
B	3% ácido acético (w/v)	Alimentos hidrofílicos y capaces de extraer compuestos hidrofílicos y que tienen un pH < 4,5
C	20% etanol (v/v)	Alimentos hidrofílicos y capaces de extraer compuestos hidrofílicos, alimentos con un contenido el alcohol > 20%, y aquellos que tienen un contenido en grasas tal que hacen que aumenta su carácter lipofílico
D1	50% etanol (v/v)	Alimentos con un contenido el alcohol > 20%, y lácteos
D2	Aceite vegetal (o isooctano)	Alimentos grasos y alimentos que contienen grasas libres en su superficie
E	Óxido de poli(2,6-difenil-fenileno), tamaño de párticula 60-80 mesh, tamaño de poro 200 nm	Alimentos secos

4.1.3. Polipropileno

El polipropileno (PP) es un polímero termoplástico. Es unos los materiales plásticos cuyo mercado ha crecido de manera más rápida en los últimos años. En la actualidad, el mercado del polipropileno ocupa el segundo puesto por volumen de mercado en el campo de los polímeros, lo que representa una demanda equivalente al 25% del mercado. Su baja densidad (ahorro de peso), alta rigidez, resistencia al calor, inercia química, transparencia y la posibilidad de reciclarlo han contribuido a ampliar enormemente su campo de aplicación en la industria. El polipropileno es hoy día empleado con fines de embalado y etiquetado, en la industria textil (por ejemplo, en cuerdas, ropa interior térmica y alfombras), en artículos de papelería, para la fabricación de piezas de plástico de varios tipos, en equipos de laboratorio, altavoces y en componentes de la industria del automóvil, por mencionar algunos ejemplos.

Tabla 4.1.2. Criterios de selección de tiempo y temperatura a emplear para determinados test de migración de acuerdo a la normativa vigente (Union, 2011). El tiempo y la temperatura pueden ser seleccionados de manera independiente.

Tiempo de contacto en el peor escenario posible	**Tiempo del ensayo**	**Temperatura de contacto en el peor escenario posible**	**Temperatura del ensayo**
t ≤ 5 min	5 min	T ≤ 5 °C	5 °C
5 min < t ≤ 0,5 h	0,5 h	5 °C < T ≤ 20 °C	20 °C
0,5 h < t ≤ 1 h	1 h	20°C < T ≤ 40 °C	40 °C
1 h < t ≤ 2 h	2 h	40 °C < T ≤ 70 °C	70 °C
2 h < t ≤ 6 h	6 h	70 °C < T ≤ 100 °C	100 °C o T de reflujo
6 h < t ≤ 24 h	24 h	100 °C < T ≤ 121 °C	121 °C *
1 día < t ≤ 3 días	3 días	121 °C < T ≤ 130 °C	130 °C *
3 días < t ≤ 30 días	10 días	130 °C < T > 150 °C	150 °C *
> 30 días	Tiene condiciones específicas indicadas en la norma	150 °C < T ≤ 175 °C	175 °C *
		T > 170°C	Ajustar la T a la T real en el punto de contacto con el alimento *

*: Esta temperatura se utilizará solo para los simulantes alimentarios D2 y E. Para aplicaciones que impliquen calentamiento bajo presión, el ensayo de migración podrá efectuarse bajo presión a la temperatura pertinente. Para los simulantes de alimentos A, B, C o D1, la ensayo puede sustituirse por un ensayo a 100 ° C o a la temperatura de reflujo con una duración cuatro veces superior a la seleccionado de acuerdo con las condiciones de tiempo indicadas en el lado izquierdo de la tabla.

El competitivo coste de producción de los plásticos basados en polipropieleno, combinado con la versatilidad de sus propiedades versátiles han hecho que este material sea también el tipo preferido para la preparación de distintos tipos de embalaje y contenedores destinados a estar en contacto con alimentos, incluyendo desde útiles y recipientes de cocina (por ejemplo, ollas, botes o utensilios como cucharones), a bolsas y films de conservación.

El polipropileno es un homopolímero de adición. Es decir, sólo contiene una unidad monomérica (el propileno) que se repite a lo largo de la estructura (**Figura 4.1.1.**).

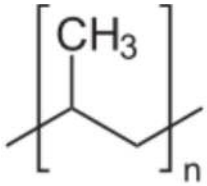
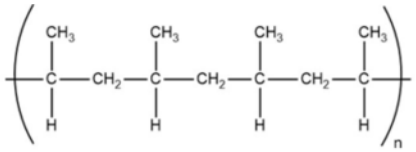


Figura 4.4.1. Estructura de la unidad monomérica del polipropileno.

La mayoría de los polipropilenos comerciales son isotácticos, es decir, presentan una orientación relativa regular de los grupos metilo (**Figura 4.1.2.A**). Este material tiene un nivel de cristalinidad intermedio (70-85%) entre el del polietileno de baja densidad (LDPE) y el polietileno de alta densidad (HDPE). La adición de entre un 5% y un 30% de etileno durante el proceso de polimerización se obtiene un copolímero que presenta una mayor resistencia a los impactos que el homopolímero de polipropileno. En ocasiones, se puede llegar a agregar un tercer monómero al proceso, el 1-buteno (Vasile, 2000).

En principio, el polipropileno se considera un material básicamente inertes y, en general, su uso en materiales, útiles o instrumentos que entran en contacto con los alimentos no representa un peligro ni para las personas que manipulan el plástico ni para aquellas que consumen dichos alimentos. Sin embrago, diferentes estudios han demostrado repetidamente que diferentes componentes de estos plásticos pueden, bajo determinadas condiciones, migrar hacia los alimentos que entran en contactos con ellos (Bradley, 2007). Estos potenciales migrantes incluyen monómeros residuales, polímero de bajo peso molecular (oligómeros) y, en general, cualquier tipo de aditivo o sustancias utilizadas en las formulación (Bradley, 2007), formada como consecuencia de la reacción de algunos de estos aditivos entre sí (Nerin, 2013), o generados como consecuencia de los procesos de esterilización a los que se suelen someter los plásticos antes de su uso en la industria alimentaria (Jonas, 2011; Biedermann, 2014)

A



B

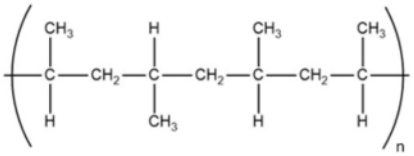


Figura 4.1.2. Estructura de los polipropilenos (A) isotáctico y (B) sindiotáctico.

Considerando estos antecedentes, y ante la evidencia de reducido número de investigaciones publicadas hasta la fecha en las que se empleara la técnica de GC×GC−ToF MS para la caracterización de lixiviados obtenidos a partir de FCM, en esta parte del estudio se decidió emplear esta técnica para la caracterización cualitativa de la fracción de componentes volátiles y semivolátiles presentes en diferentes alimentarios después de su aplicación al tratamiento de envases destinados a contener y almacenar alimentos en los hogares (tupperes) hechos de polipropileno. Los simulantes alimentarios se eligieron para cubrir un amplio espectro de alimentos comúnmente consumidos en las casas. Las condiciones del estudio de migración seleccionadas correspondían a las asignadas por la normativa vigente para uso prolongado del material, ya que se entendió que éste era el estudio que mejor se adaptaba a la evaluación realista de las condiciones de uso de estos recipientes en los hogares.

Los resultados más significativos de este trabajo se han resumido en la publicación que lleva por título “Characterization of (semi-)volatile compounds migrating into food simulants from polypropylene food containers by comprehensive two-dimensional gas chromatography−time-of-flight mass spectrometry” de **Carrero-Carralero et al**. y que se presenta a continuación.

4.2. Characterization of (semi-)volatile compounds migrating into food simulants from polypropylene food containers by comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry

C. Carrero-Carralero, J. Muñoz-Arnanz, M. Ros, S. Jiménez-Falcao, M.L. Sanz, L. Ramos

4.2.1 Introduction

Food packaging materials made of plastic protect food against external pollution. However, these food contact materials (FCM) are not completely inert (Bradley, 2007). Apart from monomers, different types of additives, stabilizers, plasticizers or cross-linking agents are frequently added to plastics during the polymerization process to improve the properties and durability of the final packaging material. Only the substances included in the list of authorized additives can be intentionally used in the manufacture of these FCM (Union, 2011). The list contains more than 150 substances. This list also assigns specific migration limits (SML) to substances with potential to migrate into food at concentrations that may either endanger human health or promote an unacceptable change in food composition or a deterioration in its organoleptic characteristics. However, despite these regulation actions, many studies have repeatedly reported on the capability of these and other components to migrate from plastic containers into food and food simulants (Bradley, 2007; Biedermann, 2014; Onghena, 2014). Migrating compounds include, among others, decomposition products, reaction and intermediate products, as well as other non-intentionally added substances (NIAS). But also compounds generated during sterilization processes (Jonas, 2011; Biedermann, 2014). The composition of these migrating substances cannot be predicted from the starting substances due to the frequent presence of by-products and neo-formed compounds (Nerin, 2013; Martinez-Bueno, 2017). In addition, the real composition of the different ingredients and materials used to manufacture the plastics usually remains unknown. Therefore, in practice, a real chemical characterization of the substances migrating from FCM into food can be accomplished by developing real migration experiments. For such an experiments, the use of simulant solutions is recommended due to the inherent complexity of most foodstuffs (Union, 2011).

It can be expect that an increasing number of non-identified compounds and NIAS will be detected in future due to the use of increasingly powerful analytical techniques (Nerin, 2013; Biedermann, 2014; Martinez-Bueno, 2017). Somehow surprisingly, up to now, the identification of these minor components have typically relied on the use of powerful mass spectrometry-(MS-)based detectors with high identification capabilities (i.e., high and ultra-high resolution MS) (Nerin, 2013; Martinez-Bueno, 2017). Meanwhile, the use also powerful separation-plus-detection techniques such as comprehensive two-dimensional gas chromatography combined with time-of-flight mass spectrometry (GC×GC−ToF MS) in this field has been much more limited and, in general, orientated to the characterization of the impurities or break-down products of specific (i.e., preselected) plastic components (Bradley, 2007; Biedermann, 2014). To our

knowledge, no study on the potential of GC×GC–ToF MS for the non-orientated (i.e., non-target) characterization of the lixiviates obtained by treating different polypropylene food containers with selected food simulant solutions have been reported has been reported by now in the literature. Therefore, the aim of this study was the exhaustive characterization of the (semi-)volatile fraction present in the extracts obtained when exposing four commercially available polypropylene food containers to selected food simulants: simulant A (distilled water), simulant B (acetic acid 3%, w/v), simulant C (ethanol 10%, v/v) and simulant D (ethanol 95%, v/v) for 10 days at 40 °C to simulate a long period of usage.

4.2.2. Materials and methods

4.2.2.1. Reagents and samples

Acetic acid, ethanol and isooctane were for residue analysis and acquired from Merck (Darmstadt, Germany). Anhydride sodium sulfate was from Panreac (Barcelona, Spain). Milli-Q water was obtained from a Millipore system unit (Bedford, MA, USA).

Three 250 mL square polypropylene food storage containers identified as authorized food contact material according to current legislation (Council, 2004) and intended for domestic use were acquired in local markets in Madrid (Spain). They belonged to different brands (identified throughout the study as A, B and C). Food storage containers A and B were manufactured in the EU, while C was manufactured outside the EU. The three containers offered a translucent appearance and were sold as appropriate for cold foods in refrigerators and freezers (up to -40 °C for brand A and up to -25 °C for brand C) and for microwave heating (up to 120 °C brand A and up to 90 °C brand C). No usage temperature range was specified in the case of brand B. These food containers had stickers (A, B and C) and a surrounding paperboard (B) in which the some usage characteristics were reproted.

The fourth investigated polypropylene food storage container was a one piece disposable plastic box used to transport and deliver prepared food and, in principle, had properties similar to those of other three investigated containers. No usage temperature range was specified on this food container either. The container was provided without stickers or paperboards. This food container was identified as brand D through the study.

4.2.2.2 Migration experiments

Distilled water, 3% (w/v) acetic acid (simulant B), 10% (v/v) ethanol (simulant A) and isooctane (simulant D2) representing aqueous, acidic, alcohol-containing and fat-containing foods, respectively, according to the European Regulation (Union, 2011), were used as food simulants. This migration solutions were identified throughout this study as simulants S.1-4, respectively. This European Regulation stipulates that food containers should be placed in contact with the selected food simulants at the worst conditions of temperature and contact time. Migration test were performed to evaluate long storage at room temperature or at lower temperatures. Thereby, according to the European Regulation (Union, 2011), migration tests were performed by treating the food containers with the corresponding food simulant at 40 °C for 10 days.

In a typical migration test, the four investigated food containers, pre-washed with Milli-Q water and air-dried, were filled up to ca. three quarts of their capacity (i.e., 200 mL) with the corresponding simulants and kept in the dark, closed, at 40°C for 10 days in a drying oven (Nahita, model 631/6; Madrid, Spain). Apart from the four investigated containers, each migration test included a reagent blank (i.e., 200 mL of the evaluated simulant placed in a glass crystallizing dish), which was subjected to the same treatment. Once the incubation period was completed, the simulant was removed, the container was rinsed with Milli-Q water, air dried and treated with another food simulant. The simulants treatment order was: water, 3% acetic acid, 10% ethanol and isooctane. The collected simulants were concentrated to incipient dryness and the final volume adjusted to 100 µL. Aqueous simulants were reconstituted in acetone before instrumental analysis and dried with sodium sulfate before instrumental analysis. Meanwhile, no solvent exchange was performed in the case of the isooctane extracts.

Otherwise specified, experiments were done in duplicate, i.e. two complete independent series of food containers were subsequently treated with the selected simulants yielding a total of 32 extracts and 8 reagent blanks.

4.2.2.3 Instrumental analysis

GC×GC–ToF MS analyses were performed on a Pegasus 4D (Leco Corp., St. Joseph, MI, USA). The instruments consists of a modified Agilent 6890 GC coupled to a ToF MS and equipped

with a split/splitless injector. Samples were injected in the hot split mode (1 µL at 275 °C) into a column set consisting of an HT-8 (30 m × 0.25 mm i.d.; 0.25 µm film thickness; 8% phenyl (equiv.) polycarborane siloxane) as first dimension column coupled to a BPX-50 (1.7 m × 0.1 mm i.d.; 0.1 µm film thickness; 50% phenyl 50% methylpolysilphenylene siloxane) as second dimension column. Both columns were purchased from SGE (Melbourne. Australia). The column set and initial experimental conditions were selected on the base of our previous experience in the analysis of complex mixtures (Bordajandi, 2008; Escobar-Arnanz, 2018), although reoptimised to avoid coelution among the target compounds and these and the co-extracted matrix components. Once optimized, the temperature of the main oven was programmed from 45 °C [60 °C in the case of the isooctane extracts] (2.5 min) to 190 °C at a rate of 20 °C/min and then to 300 °C (30 min; 35 min in the case of the isooctane extracts) at 3 °C/min. The second dimension oven was programmed to track the main oven but with an off-set of 30 °C, except for the final temperature, i.e. 75 °C [90 °C in the case of the isooctane extracts] (2.5 min) to 210 °C at a rate of 20 °C/min and then to 300 °C (30 min; 35 min in the case of the isooctane extracts) at 3 °C/min. Helium was used as carrier gas at a head-column pressure of 30 psi, which was maintained constant during the whole analysis. A nitrogen quad-jet dual-stage cryogenic modulator was used for sample focusing and injection in the second dimension column. The temperature of the modulator was set 40 °C above that of the main oven. A modulation period (PM) of 6 s with a 0.6 s hot jet pulse was used. The transfer line temperature was set at 275 °C.

The ion source temperature was set to 250 °C. Total ion monitoring was performed in the m/z 75-700 range; the energy of ionizing electrons was 70 eV and the voltage of the multiplier was 1670 V. The acquisition rate was set at 100 Hz in all instances. ChromaToF 4.2 was used for data acquisition and treatment. This software allowed automated baseline correction, peak deconvolution, and peak area and volume determination. The minimum required signal/noise (S/N) ratio for each detected peak was set at 100. A script function written in Visual Basic was used for automatic data filtering and identification of halogen-containing compounds on the base of characteristic m/z ions and clusters (Escobar-Arnanz, 2018). Different identification levels were differentiated during the analysis (Nerin, 2013). When authentic standard was available, positive identification of the peak detected in a sample was based on the mutual agreement of the retention time and mass spectrum with that of the corresponding standard analysed under identical experimental conditions. When an appropriate standard was not available, the tentative identification of the detected compound was based on the following multi-criteria approach (Escobar-Arnanz, 2018): (i) individual (i.e., manual) confirmation of preliminary assignment done by automatic peak finding by the software (spectra similarity

score, 750); (ii) identification of potential isomers belonging to the same class on the base of mass spectrum similarity with analytes positively identified and their clustering within the structured chromatograms generated by the column set used in the present study; (iii) positive match of the distribution patterns of the mass spectrum (m/z ratios) and positive comparison with those of commercial libraries and/or described in the literature ; and (iv) mass spectrum interpretation.

The limits of detections of the technique under optimized conditions, as determined for the standards analysed, were in ppb level (Escobar-Arnanz, 2018) , which was considered to allow proper detection for a large majority of the analytes present in the extracts at levels below the threshold of current regulation (Union, 2011).

4.2.3. Results and discussion

4.2.3.1. General overview

A preliminary inspection of the two-dimensional chromatograms obtained by GCxGC-ToF MS of the extracts obtained from the investigated food containers demonstrated that analytes with wide divergent volatilities and polarity were obtained in the four fractions investigated. The former spread out in the first dimension, while the later spread out in the second dimension. In any case, these analytes showed an appropriate separation from other co-extracted matrix components eluting at the bottom of the chromatogram, which illustrated the adequacy of the selected column set and the applied experimental conditions for the intended investigation. As somehow anticipated, differences were observed among the chromatograms obtained for the same food containers depending on the nature of the simulants applied (Figure S.1 in Anexo 3). Furthermore, although the same polymer was used to produce all studied food container, some significant differences existed among the chromatograms obtained for the same simulant depending on the food container analysed (see Figure 4.2.1. for a typical example). This observation agreed with previous findings reporting on the influence of the manufacturing procedure on the number, concentration and nature of the migrants detected in the simulant. All together evidenced the difficulties associated to this type of evaluations.

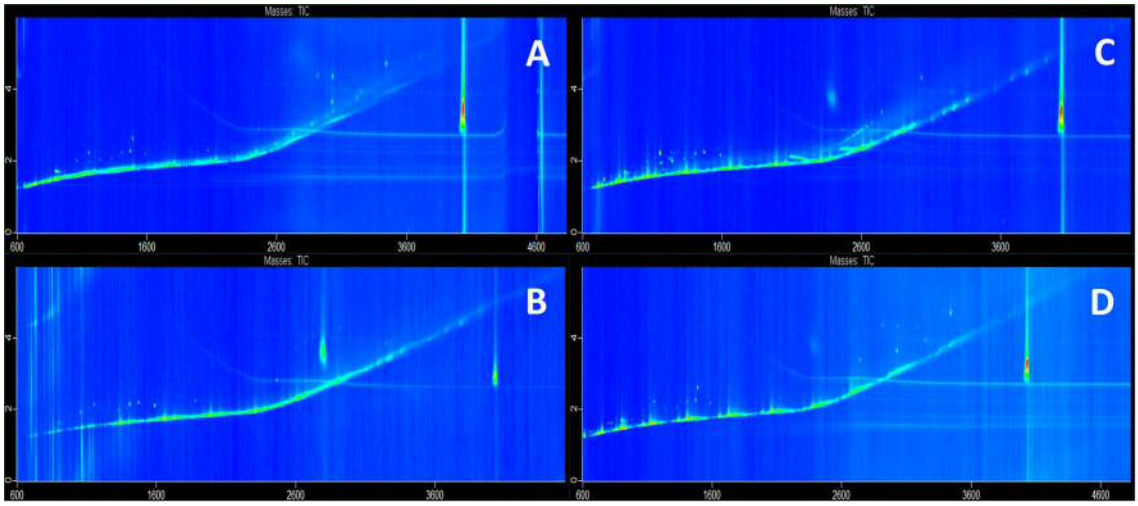


Figura 4.2.1. Comparison of the two-dimensional chromatograms obtained after treatment of the investigated food containers with isooctane (S.4).

In general, the simulants extracting the largest numbers of analytes from the investigated food container were 10% ethanol and isooctane. Among the investigated food containers, the one showing the largest number of migrants was brand D, followed by brands A, C and B. The total number of identified compounds for these food containers ranged between 187 (for brand D) and 77 (brand B). This general overview can also be read out from Table 4.2.1, where information concerning the most relevant analytes identified in the extracts obtained after treatment of the investigated food containers with the four assayed simulants are summarized. In total, 109 analytes were either positively or tentatively identified in the studied extracts. The complete list is presented in Table 4.2.1., were analytes have been sorted according to their first dimension retention time. For the sake of comparison, all analytes retention times have been referred to those obtained for the isooctane extracts. Only analytes showing an abundance ten times higher than that of the corresponding compound in the reagent blank (when detected) have been considered as positive in the samples and included the list.

The presence of migrating chemicals, and in particular NIAS, in these extracts is discussed in following sections according by their possible source.

Table 4.2.1. Analytes identified in the investigated extracts obtained by treatment of the food containers (brands A to D) with water (S.1), 3% acetic acid (S.2), 10% ethanol (S.3) and isooctane (S.4). Detection code: -, not detected at a level 10-times above that found in the corresponding reagent blank extract, +: positive analyte in the extract; ++: positive analyte present in the extract at high concentration.

Compound	^1t_R (s)	^2t_R (s)	*m/z*	Similarity	Brand A				Brand B				Brand C				Brand D			
					S.1	S.2	S.3	S.4	S.1	S.2	S.3	S.4	S.1	S.2	S.3	S.4	S.1	S.2	S.3	S.4
Benzoic acid	666	1.48	105, 120	980	++	++	++	-	++	+	-	-	++	++	++	-	++	++	++	-
4-Methylbenzaldehyde	708	1.38	91, 119	896	++	+	++	+	++	-	-	-	++	-	++	-	++	++	++	++
Naphthalene	726	1.54	102, 128	847	+	-	-	+	-	-	-	+	+	-	++	++	-	-	-	++
4-Methylbenzoic acid	744	1.57	119, 136	959	++	+	++	-	++	-	-	-	+	-	++	-	++	++	++	-
Dimethyl-benzaldehyde (isomer)	756	1.52	105, 133	944	-	-	-	-	++	-	-	-	++	++	++	-	++	++	++	++
Benzothiazole	758	1.64	108, 135	880	-	-	++	-	-	-	-	-	-	++	++	-	++	++	++	-
Isothiocyanatocyclohexane	768	1.55	83, 141	918	-	++	-	-	-	-	-	-	-	-	-	-	++	++	-	-
Cinnamaldehyde	798	1.62	103, 131	950	++	-	++	-	-	-	-	-	-	-	-	-	-	-	-	++
C1-Naphthalene	804	1.64	115, 142	957	-	-	-	++	-	-	-	+	-	-	-	++	-	-	-	++
Caprolactam	804	1.72	85, 113	939	++	++	++	-	++	+	-	-	-	-	-	-	-	-	-	-
C1-Naphthalene	822	1.68	115, 142	911	-	-	-	++	-	-	-	+	-	-	-	++	-	-	-	++
Eugenol	822	1.65	149, 164	945	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++
3,4-Dimethylbenzoic acid	840	1.74	105, 150	967	-	+	++	-	++	-	-	-	++	++	++	-	++	++	++	-
Biphenyl	858	1.74	153, 154	917	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+
2,4-Di-tert-butylphenol isomer	876	1.64	191, 206	793	-	-	++	+	+	-	-	-	-	-	++	+	-	-	++	-
Phthalic anhydride	882	1.76	104, 148	844	++	-	-	-	++	-	-	-	++	-	-	-	++	++	-	-
3-Methoxy-4-hydroxy-benzaldehyde	894	1.86	151, 152	874	+	-	++	-	+	-	-	-	-	++	++	+	+	++	++	-
C2-Naphthalene (dimethyl)	900	1.75	141, 156	859	-	-	-	++	-	-	-	++	-	-	-	++	-	-	-	++
2,4-Di-tert-butylphenol	900	1.73	191, 206	916	++	++	++	++	++	-	-	++	++	++	++	++	++	++	++	++
Phthalide	906	1.86	105, 134	905	-	-	-	-	++	++	-	-	++	++	++	-	+	++	++	-
C2-Naphthalene (dimethyl)	918	1.8	141, 156	901	-	-	-	++	-	-	-	+	-	-	-	++	-	-	-	++

Table 4.2.1. Continuation

Dimethyl phthalate	918	1.88	163, 179	732	-	-	++	-	+	++	-	-	-	+	-	-	-	-	-	-
2,6-Di-tert-butylbenzoquinone	918	1.65	177, 205, 220	810	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	+
Butylated hydroxytoluene	926	1.69	205, 220	821	-	++	++	-	-	++	-	-	-	+	++	+	+	++	++	-
Dodecanoic acid	930	1.69	171, 200	920	+	-	++	-	++	-	-	-	++	-	++	-	+	+	++	-
Hydroxymethyl phthalimide	936	2.12	104, 147	939	++	++	++	-	+	-	-	-	++	++	++	-	+	++	++	-
C2-Naphthalene (dimethyl)	942	1.8	141, 156	836	-	-	-	+	-	-	-	+	-	-	-	++	-	-	-	+
C3-Naphthalene	954	1.88	155, 170	779	-	-	-	++	-	-	-	-	-	-	-	++	-	-	-	-
Dimethyl terephthalate	954	1.85	163, 179	908	-	-	++	-	-	-	-	-	-	+	++	-	+	+	++	-
C1-Biphenyl	954	1.85	152, 168	914	-	-	-	+	-	-	-	-	-	-	-	++	-	-	-	-
Isoeugenol	954	1.88	164, 206	842	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++
2,6-di-tert-butyl-4-ethyl-phenol	960	1.75	219, 234	816	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-
C1-Biphenyl	966	1.87	152, 168	947	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Acenaphthene	996	1.98	153, 154	905	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-
Difluoroacetophenone isomer	1008	1.75	113, 141, 156	905	+	+	++	-	-	-	-	-	-	+	++	-	-	-	++	-
Dibenzofuran	1026	2.02	139, 168	903	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	+
Diethyl Phthalate	1056	2.03	149, 177	958	++	++	++	++	++	++	++	++	++	++	++	++	++	-	++	++
C2-Biphenyl	1068	1.97	167, 182	770	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-
Fluorene	1116	2.16	165, 166	864	-	-	-	++	-	-	-	-	-	-	-	++	-	-	-	++
2-(Methylthio)-benzothiazole	1134	2.32	148, 181	958	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-
C6-Naphthalene (trimethyl-propenyl)	1152	2.12	195, 210	785	-	-	-	++	-	-	-	-	-	-	-	++	-	-	-	++
Benzophenone	1152	2.25	105, 182	967	-	-	-	++	-	-	-	++	-	-	-	++	-	-	-	++
Tetradecanoic acid	1152	1.89	185, 228	946	+	-	++	-	++	-	-	-	++	-	++	-	-	-	++	-
C6-Naphthalene (diisopropyl)	1158	1.99	197, 212	895	-	-	++	++	-	-	-	-	-	-	-	++	-	-	-	++
C6-Naphthalene (diisopropyl)	1170	1.98	197, 212	917	-	-	++	++	-	-	-	-	-	-	-	++	-	-	-	++
C6-Naphthalene (trimethyl-propenyl)	1182	2.17	195, 210	819	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-
2(3H)-Benzothiazolone	1220	2.65	123, 151	934	-	-	-	-	-	-	-	-	-	-	++	-	++	-	-	-

Table 4.2.1. Continuation

C6-Naphthalene (diisopropyl)	1224	2.09	197, 212	892	-	-	++	++	-	-	-	++	-	-	-	++	-	-	-	++
C6-Naphthalene (diisopropyl)	1248	2.01	197, 212	964	-	-	++	++	-	-	-	++	-	-	-	++	-	-	-	++
C6-Naphthalene (trimethyl-propenyl)	1254	2.25	195, 210	723	-	-	-	-	-	-	++	-	-	-	-	++	-	-	-	-
tris(2-Chloroisopropyl)-phosphate (TCIPP isomer)	1332	2.2	277,279,290	802	-	-	+	-	-	-	+	-	-	-	++	-	-	-	++	-
3,5-di-tert-Butyl-4-hydroxybenzaldehyde	1332	2.1	219, 234	891	+	-	+	++	+	-	+	++	+	+	+	++	+	+	+	++
3,5-di-tert-Butyl-4-hydroxyacetophenone	1386	2.14	233, 248	846	++	-	-	+	+	-	-	+	+	-	-	++	+	-	-	+
Diisobutyl phthalate	1410	2.26	149,223	961	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
Hexadecanoic acid, methyl ester	1410	1.92	227, 239, 270	826	-	-	++	+	-	-	-	+	-	-	++	++	-	-	++	+
N-(2-Cyano-ethyl)-benzenesulfonamide	1422	2.46	141, 170	954	-	-	++	-	-	-	++	-	-	-	++	-	-	-	++	-
Phenanthrene	1434	2.61	152, 178	958	-	-	-	++	-	-	-	++	-	-	-	++	-	-	-	++
Hexadecanoic acid	1440	2.05	129, 256	893	++	+	++	-	++	-	-	-	++	++	++	+	++	++	++	-
Methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate	1470	2.3	277, 292	838	-	-	-	++	-	-	-	++	-	-	-	++	-	-	++	++
Dibutyl phthalate isomer	1485	2.31	149, 205, 223	949	-	-	++	-	-	-	-	-	-	++	-	-	-	-	-	-
3,5-di-tert-Butyl-4-hydroxyphenylpropionic acid	1512	2.49	263, 278	805	++	-	++	-	++	-	-	-	++	-	++	-	++	++	++	-
Dibutyl phthalate	1566	2.4	149, 205, 223	782	++	++	++	++	++	++	-	++	++	++	++	++	++	++	++	++
7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	1590	2.14	205, 261, 276	935	-	++	++	++	-	++	-	++	-	++	++	++	-	++	++	++
N,N-bis-(2-hydroxyethyl)alkyl(C13-C15)amine related	1602	1.95	130, 174, 327, 484	-	+	+	++	-	-	-	-	-	-	-	-	-	+	+	++	+

Table 4.2.1. Continuation

N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1614	1.96	130, 174, 328	-	++	+	++	+	-	-	-	-	-	-	-	-	+	+	++	+
Glycerol monostearate related	1638	2.07	117, 130, 259, 315	-	-	-	++	-	-	-	-	-	+	++	++	-	-	-	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1644	1.97	130, 174, 328	-	++	+	++	+	-	-	-	-	-	-	-	-	+	++	++	+
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1668	1.99	130, 174, 328	-	+	-	++	-	-	-	-	-	-	-	-	-	-	+	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1704	2.02	130, 174, 328	-	++	++	++	+	-	-	-	-	-	+	-	-	++	++	++	+
Octadecanoic acid, methyl ester	1728	2.05	255, 298	895	-	-	++	-	-	-	-	-	-	-	++	-	-	-	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1752	2.05	130, 174, 328	-	++	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
2-Mercaptobenzothiazole	1755	3.34	109, 167	929	-	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-
Octadecanoic acid	1764	2.2	241, 284	913	++	-	++	-	++	-	-	-	++	-	++	-	++	++	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1770	2.05	130, 174, 328	-	++	++	++	++	+	-	-	-	+	+	++	-	++	-	++	++
Oxybenzone	1794	2.84	151, 227	934	-	-	-	-	-	-	-	-	-	-	+	-	-	-	++	-
Unknown Cl-1	1800	3.06	243, 257, 259	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
Octadecanoic acid, ethyl ester	1830	2.08	269, 312	872	-	-	-	-	-	-	-	-	-	-	++	-	-	-	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1884	1.99	248, 342, 402	-	++	-	++	-	-	-	-	-	-	-	-	-	+	+	++	+
Hexadecenamide	1902	2.48	210, 253	-	-	-	-	-	-	-	-	-	-	-	++	-	-	-	-	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1908	2.06	130, 174	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-
4,4'-(1-Methylethylidene)bis-phenol (Bisphenol A)	1914	3.56	213, 228	895	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	1932	2.13	130, 174	-	++	+	++	+	++	-	-	-	++	++	++	-	++	++	++	+
Hexadecanamide	1932	2.4	212, 255	757	-	-	++	-	-	-	-	-	-	-	++	-	-	-	++	-

Table 4.2.1. Continuation

N,N-bis-(2-hydroxyethyl)alkyl(C13-C15)amine related	1962	2.1	130, 174	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	++	+
Glycerol monostearate/glycerol mono-oleate related	1968	2.22	117, 130, 343	-	-	-	++	-	++	+	-	-	+	++	++	-	-	++	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	2034	2.13	130, 174	-	++	+	++	+	-	-	-	-	-	-	-	-	-	+	+	++	+
Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	2046	2.23	239, 270, 330	779	-	-	++	-	-	-	-	-	++	++	++	++	-	-	-	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	2076	2.16	130, 174	-	++	-	++	-	-	-	-	-	-	-	-	-	-	-	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	2094	2.16	130, 174, 356	-	++	++	++	++	+	-	-	-	+	+	++	-	++	++	++	++
di-p-Tolyl sulfone related	2106	3.13	139, 246	904	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-
Unknown Cl-2	2184	3.38	277, 291, 293	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
di-p-Tolyl sulfone related	2190	3.12	139, 246	909	-	-	-	-	-	-	-	-	+	-	++	-	-	++	-	-
Di(2-ethylhexyl) adipate	2192	2.29	129, 241	855	-	-	++	++	++	++	++	++	-	-	++	++	++	-	++	++
Oleamide	2232	2.65	264, 281	881	-	-	-	-	-	-	-	-	-	++	++	-	-	++	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	2256	2.25	130, 174, 356	-	++	-	++	-	++	-	-	-	++	+	++	-	++	++	++	-
Octadecanamide	2268	2.54	240, 283	861	-	-	-	-	-	-	-	-	-	-	++	-	-	-	++	-
N,N-bis-(2-hydroxyethyl)alkyl (C13-C15)amine related	2280	2.47	224, 283	-	++	+	++	+	+	-	-	-	+	+	++	-	++	++	++	+
Glycerol monostearate/glycerol mono-oletate related	2286	2.32	117, 130, 371	-	++	++	++	-	++	++	-	-	++	++	++	-	++	++	++	-
Bis(2-ethylhexyl) phthalate	2466	2.91	149, 167, 279	776	-	++	++	++	-	++	-	++	-	++	++	++	-	+	++	-
Glycerol monostearate related (Unknown 292)	2586	2.8	117, 130, 399	-	++	++	++	-	++	++	-	-	++	++	++	-	++	++	++	-

Table 4.2.1. Continuation

Octadecyl 3,5-di-t-butyl-4-hydroxyhydrocinnamate (Irganox 1076)	2676	2.68	516, 531	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++
Glycerol monostearate related	2718	2.97	191, 415, 416	-	++	++	++	-	++	-	-	-	++	++	++	-	++	++	++	-
Dodecyl ester octadecanoic acid	3444	4.78	285, 453	841	-	-	-	++	-	-	-	-	-	-	-	-	-	-	++	++
Dodecyl ester octadecanoic acid related	3702	5.49	285, 467	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
Glycerol monostearate related	3720	0.48	343, 412, 468	-	-	+	++	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol monostearate related	3924	1.07	343, 399	-	-	-	++	-	-	-	-	-	-	-	-	-	-	-	-	-
Tris(2,4-di-*tert*-butylphenyl) phosphite (Irgaphos 168)	4032	3.35	441, 646	-	-	-	-	++	-	-	-	++	-	-	-	++	-	-	-	++
Octadecanoic acid, octadecyl ester related	4032	0.4	285, 481	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	++	-
Tris(2,4-di-tert-buthylphenyl)phosphate (Irgaphos 168 oxide)	>4825	3.79	648, 663	-	-	-	-	++	-	-	-	++	-	-	-	++	-	-	-	++

4.2.3.2. Identification of compounds present in the migration solutions

- **Antioxidant and their breakdown products**

Some of the most frequently detected NIAS in simulant studies are impurities (including by-products) introduced during the synthesis of the plastic material or breakdown products. The later are associated to degradation processes, which can affect the polymer itself or some of the additives included in the formulation to improve the physico-chemical properties of the final material. The main degradation pathways are the exposure of the polymer to high temperatures or high irradiation energies (Nerin, 2013). These processes can occur during the manufacture of the polymer (e.g., during thermal-mechanical processes), or as consequence of the exposure of the plastic to microwaves (Jonas, 2011) or irradiation for sterilization purposes (Biedermann, 2014; Yang, 2016). The analytes resulting from these degradation processes are typically molecules with a molecular weight lower than the original compound and, consequently, with a greater potential to migrate from the polymer. However, some of these processes have also reported to generated heavier oxidation products with capacity to migrate from the polymer (Yang, 2016).

The antioxidant Irgaphos 168 (tris(2,4-di-tert-buthylphenyl)phosphite) is an accepted additive for polymers (Union, 2011). Because of its recognized capability to migrate from plastic, the EU set a specific migration limit (SML) for this compound of 60 mg kg-1. However, current legislation did not set any type of SML for their two known degradation products, 2,4-di-tert-butylphenol and tris(2,4-di-tert-buthylphenyl)phosphate (Yang, 2016). Irgaphos 168 and its oxidized degradation product were clearly detected in the isooctane extracts obtained from the four food containers investigated. Meanwhile, 2,4-di-tert-butylphenol was detected in all studied extracts, with the only exception of those obtained by treatment of food container B with 3% acetic acid and 10% ethanol. In addition, an isomer of this compound was also found in food containers A, C and D, in particular in the alcoholic extract. Another antioxidant, Irganox 1076 (octadecyl 3,5-di-t-butyl-4-hydroxyhydrocinnamate), was detected in the isooctane fraction obtained from container D. The presence of methyl 3-(3,5-di-tert-butyl-4-hydroxyphenyl)propionate, a compound identified as product of the degradation of Irganox 1076 and/or Irganox 1010 (Onghena, 2014) in this fraction, but for all studied containers, would suggest that these antioxidants could have been included in the formulation of the investigated polypropylene materials, although the original compound only reached detectable levels in one of the extracts. The simultaneous presence in other extracts of compounds such as 2,6-di-tert-butylbenzoquinone (isooctane fraction of brands C and D) and 7,9-di-tert-butyl-1-

oxaspiro(4,5)deca-6,9-diene-2,8-dione (distributed in different fractions with the exception of water), also considered degradation products of these Irganox compounds (Simoneau, 2012; Onghena, 2014), would contribute to support the previous statement.

2,6-Di-tert-butyl-4-ethyl-phenol is an antioxidant used in plastics and rubber products to improve their temperature stability and discoloration resistance (Bolgar, 2015). This compound was only detected in the isooctane fraction of food container A, a partition behavior that could be associated to the hindered structure of this phenol. Other frequently used phenolic antioxidant, butylated hydroxytoluene, was detected in the acidic and alcoholic simulants from all investigated brands, while transformation product 3,5-di-tert-butyl-4-hydroxybenzaldehyde, were mainly found in the isooctane fractions.

- **Plasticizers**

Bis-(2-ethylhexyl) phthalate is one of the most commonly applied plasticizers in plastic products. Although its use in FCM is quite limited in current legislations (1.5 mg kg-1) (Union, 2011), this compound was positive identified in the acid and ethanol and isooctane lixiviates from nearly all containers. Different compounds has been identified as metabolites of bis-(2-ethylhexyl) phthalate (e.g., 2-ethylhexanoic acid, 2-ethylhexanol, phthalic acid, mono-2-ethylhexyl phthalate) (Pietrogrande, 2003), and so suggested as possible degradation products of this phthalate (Bradley, 2007). However, none of these compounds were found in the analysed migration solutions.

Dibutyl phthalate is another plasticizer frequently detected in plastic materials (Onghena, 2014). Although its SLM is even more restrictive than that of di-(2-ethylhexyl) phthalate (0.3 mg kg-1) (Union, 2011), this compound was positively identified in nearly all the analysed migration solutions (the only exception was the ethanolic one obtained from brand container B). In addition, an isomer of this phthalate was also detected in ethanolic and the acidic extracts from brand containers A and C, respectively.

Other phthalates with a high detection frequency in the investigated migration extracts investigated were the diethyl phathalate and the diisobutyl phthalate,which were detected in all analysed solutions. No legal SLM has been set in current EU legislation for these chemicals.

- **Cross-linking agents**

2-Mercaptobenzothiazole is incorporated during the manufacture of plastics in small amounts to aid during the vulcanization process. However, this compound, which was only detected in the aqueous lixiviate from food container A, can also formed by degradation of other additives (e.g., by hydrolysis of the accelerator N-(1,1-dimethylethyl)-2-benzothiazolesulfenamide) (Bolgar, 2015). Benzothiazole, which has been identified in the alcoholic solutions of brand containers A and C and in all aqueous colutions obatied from brand container D, is another by-product frequently detected in plastic migration studies.

Retarders are chemicals added to slow down the crosslinking process. Commonly used retarders include stearic acid, salicylic acid, benzoic acid and phthalic anhydride (Bolgar, 2015). The last two compounds have been detected in the investigated solutions. Both compounds were found to migrate into water and, to a less extension in the case of the phthalic acid, to the 3% acetic acid simulant.

- **Other additives**

N,N-bis-(2-hydroxyethyl)alkyl(C13-C15)amine is an additive used as antistatic during the manufacturing of polypropylene and with a legislated SML of 1.2 mg Kg-1 (expressed as tertiary amine). This compound was not found in any of the investigated migration solutions. However, up to 16 possible degradation products associated to this compound were tentatively identified in this study (compounds named as N,N-bis-(2-hydroxyethyl)alkyl(C13-C15)amine related in Table 1). These compounds were mainly detected in the water and ethanol simulants, in particular in containers A and D. In addition, 2,4-di-tert-butylphenol was widely distributed in all migration solutions (again, specialy in brands A, C and D). A number of these chemicals were previously identified by Bardley and Courlier (Bradley, 2007), who suggested that they are formed by thermolysis of the parent additive. To our knowledge, no extra information is avalable in the literature regarding the presence of these degradation products in polypropylene plastics.

Glycerol monostearate is a non-ionic surfactant authorized as external lubricant for plastics (Union, 2011). This compound is obtained by reaction of triglycerides with an excess of glycerol. Consequently, expected impurities include diglycerides, unreacted triglycerides, glycerol and fatty acids (i.e., stearic acid, tetradecanoic acid, hexadecanoic acid) and their esters. Glycerol monostearate was not found in the investigated extracts. However, up to six compounds associated to this chemical (named as glycerol monostearate related in Table 1) were tentatively

identified in all aqueous migration solutions obtained from containers A, C and D. Nevertheless, it must be accepted that for two of these compounds (those eluting at retention times in the first dimension of 1968 and 2286) could also be breakdown products of glycerol monooleate, another non-ionic surfactant and emulsifier accepted for plastic manufacturing (Bradley, 2007). Some of the acids (i.e., even acids from dodecanoic to octadecanoic acid) and their corresponding esters detected in the different migration solutions could be breakdown products of these glycerols. Nevertheless, some of these compounds are also be used as additives (mainly lubricants or heat stabilizers) during plastic manufacturing. The presence of some of these analytes and their alkylated derivatives has been previously reported in polypropylene intended for food contact (Onghena, 2014), while no reference regarding the identification of the previously indicated glycerol related compounds has been found.

Oleamide is an amide derived from the fatty acid oleic acid, whose use is accepted as lubricant during plastics production (Union, 2011). Oleamide was tentatively identified in the acidic and alcoholic simulants obtained from containers C and D, a result that agreed with previous observations (Kawamura, 2000). Interesting, several compounds related to this fatty acid (i.e., hexadecenamide, hexadecanamide and octadecanamide) were also detected in these extracts.

- **Contaminants**

Recycling of the plastic materials have dealt in certain occasions to contamination of the new products with chemical compounds coming from the previous packages or from the misuse of these packages by consumers before they were discarded (Coulier, 2007; Onghena, 2014). In other cases, contaminants have been associated to the use of printing inks, adhesives or substances added to colored the final material (Nerin, 2013). The food container considered in this study were translucent and colorless and did not have any type of printout, but they had stickers or paperboards with the use instructions.

Naphthalene is a relatively frequently detected compound in polypropylene food containers (Onghena, 2014). Some of its alkyl-derivatives, in particular 2-methylnaphthalene and 2,6-dimethylnaphthalene, have also been detected in plastic containers made with different plastic materials, although their presence in polypropylene-based products is apparently less frequent (Onghena, 2014). In the food containers investigated in this study, apart from naphthalene, up to 13 alkylated naphthalene derivatives were detected (Table 1). These derivatives included two C1-naphthalenes (i.e., methylated-naphthalenes), but also three C2-, one C3- and seven C6-naphthalene derivatives. Among the later, it was possible to differentiate the trimethyl-

propenyl-naphthalenes from other diisopropyl-like isomers on the base of their respective mass spectra (main fragments were obtained at m/z of 195 and 210 for the former, while the main fragments were at m/z 197 and 212 for the later) and their different position in the contour plot (Figure 4.2.2.). In general, most of these compounds were found in the isooctane fraction, a finding consistent with their non-polar nature. The isooctane fractions containing more alkyl-naphthalenes were those obtained from food containers C and A. The presence of diisopropyl-naphthalene has been reported in some migration studies involving plastic bottles and articles, including those made from propylene. The presence of this chemical, not regulated for plastics, has been associated with a possible migration from inks in the papers and boards reporting the brand and instruction of the food container. The simultaneous presence of benzophenone (also use for this purpose) in the isooctane extracts would contribute to support this idea.

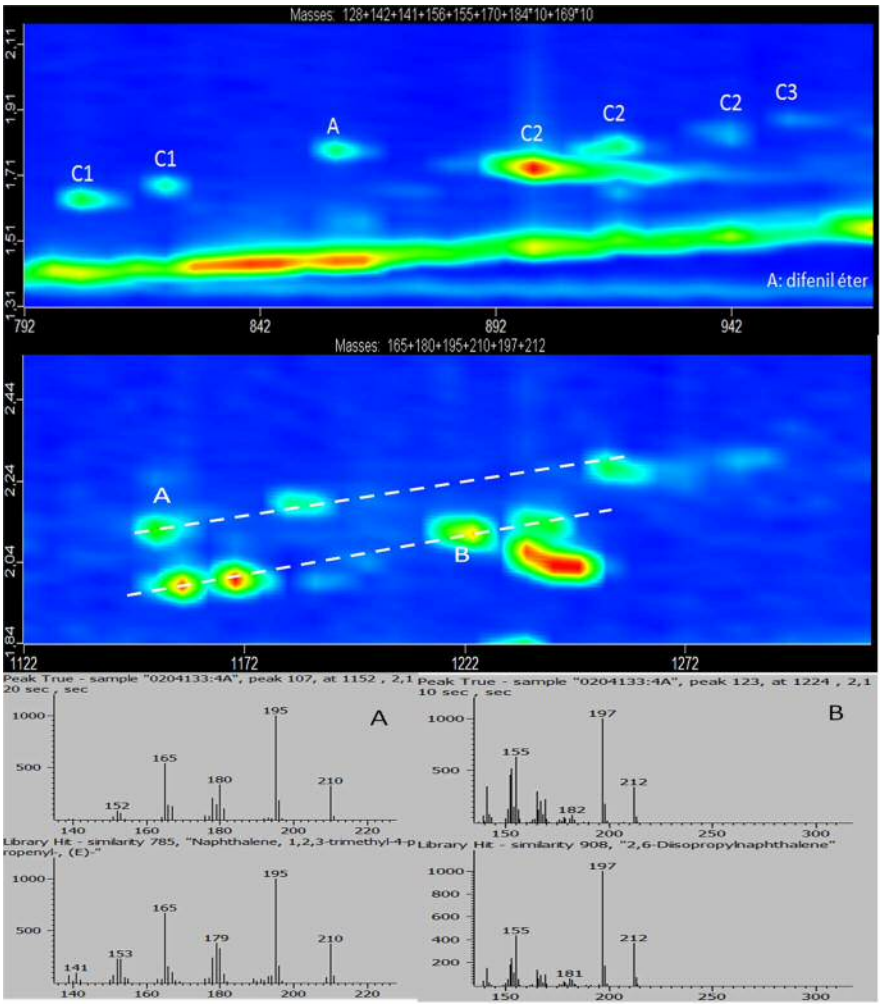


Figure 4.4.2. Typical structured elution profile of the C1- to C3- (upper contour plot) and C6-naphthalenes (lower contour plot) found in isooctane (S.4) simulant from food container A showing the separation elution on the second dimension of the (A) trimethyl-propenyl- and (B) diisopropyl-like naphthalene isomers.

Apart from this PAH-series, three other regulated PAHs were detected in the isooctane fraction, acenaphthene (in food container C), fluorene (in containers from brands A, C and D) and phenanthrene (in the four investigated containers). To our knowledge, none of these heavier PAHs have been previously detected in lixiviated from polypropylene food containers.

The application of a script function previously developed in our group for the automatic filtering and identification of halogen-containing chemicals (Escobar-Arnanz, 2018) confirmed that only a very limited number of analytes with these characteristics were present in the investigated simulants. Contaminants like PCBs or PBDEs were not present in the analysed extracts, although these microcontaminants have been recognized as toxic impurities potentially present in some recycled plastic materials (Bolgar, 2015; Ionas, 2016). However, three other compounds containing chlorines were found in some of the analysed ethanolic fractions. In particular, two non-identified chlorinated compounds were detected in extracts from brand A. Meanwhile, one organophosphorous flame retardant (OPFRs), tentatively identified as tri(2-chloroisopropyl)-phosphate, was found in this extracts in all investigated food containers.

Acknowledgments

Authors thank MINECO (projects CTQ2012-32957 and AGL2016-80475-R7. AEI/FEDER. UE). Fundación Ramón Areces and Community of Madrid (Spain) and European FEDER program (Project S2013/ABI-3028-AVANSECAL) for financial support.

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5. Caracterización de lípidos libres y carbohidratos de bajo peso molecular de la materia orgánica del suelo

5. Caracterización de lípidos libres y carbohidratos de bajo peso molecular de la materia orgánica del suelo

5.1. Prefacio

5.1.1. La materia orgánica del suelo

Se denomina *humus* al producto de transformación de restos orgánicos del suelo, como resultado de una amplia variedad de procesos naturales de degradación y modificación estructural, microbiana o abiótica, de la biomasa procedente plantas y de la fauna y microorganismos del suelo (Schnitzer, 1972; Stevenson, 1994; Kögel-Knabner, 2002).

La materia orgánica del suelo presenta, por tanto, una composición y estructura muy complejas, ya que a la gran variedad de biomacromoléculas precursoras se suma su posterior modificación por la acción de diversos organismos y procesos no-biológicos (interacciones organo-minerales, condensaciones abióticas, fotooxidación...). Como resultado de estas transformaciones de la materia orgánica del suelo, que dan lugar al humus se pueden diferenciar las denominadas *sustancias húmicas* y no-húmicas (ver **Figura 5.1.1**).

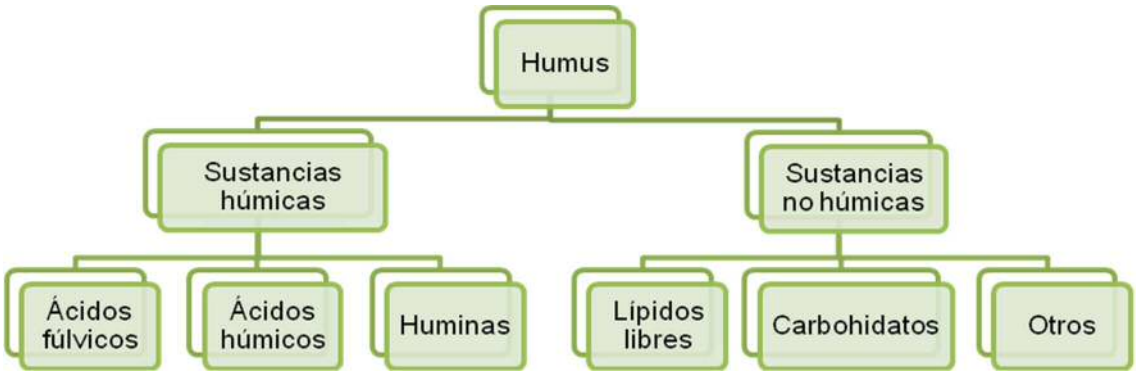

Figura 5.1.1. Clasificación de la materia orgánica del suelo

Las sustancias húmicas proceden de procesos de transformación a lo largo del tiempo de la parte no mineralizada de la materia orgánica incorporada al suelo, constituyendo alrededor del 65% del carbono total, en función de los distintos tipos de suelo (Tinoco, 2000). Esta fracción de la materia orgánica está compuesta por sustancias relativamente estables frente a la biodegradación (más de cien años de tiempo de residencia media en el suelo) y pesos moleculares muy elevados (en el intervalo 10^5—10^6 Da), de color oscuro y naturaleza mayoritariamente hidrofílica. Su clasificación se basa en criterios de solubilidad (Schnitzer, 1972). De este modo, entre las fracciones extraíbles por reactivos

alcalinos se pueden distinguir: ácidos fúlvicos (solubles en ácidos y en álcalis), ácidos húmicos (solubles en álcalis e insolubles en ácido) y huminas (insolubles a todos los valores de pH) (Almendros, 2008).

La fracción de sustancias no húmicas está constituida principalmente por carbohidratos, lignina, aminoácidos, péptidos, proteínas, ácidos nucleicos, ácidos grasos, ceras, resinas, pigmentos y otras moléculas de bajo peso molecular procedentes de la biomasa vegetal, animal y microbiana (Schnitzer, 1972). Esta fracción es relativamente biodegradable y presenta cortos periodos de residencia en los suelos. Dentro de este grupo se encuentran los lípidos libres o fracción lipídica. Es un grupo creado por conveniencia analítica y que incluye un conjunto heterogéneo de compuestos de diferente origen, estructura y propiedades. Estos compuestos se definen por su solubilidad en disolventes orgánicos o en sus mezclas (Dinel, 1990).

En general, la mayor parte de los estudios sobre la materia orgánica del suelo se han basado en establecer la naturaleza, origen y evolución de las sustancias húmicas, así como en el estudio de ciertas macromoléculas no húmicas, como es el caso de celulosas, hemicelulosas, ligninas, cutinas, suberinas, etc. (Gieseking, 1975; Stevenson, 1982). Sin embargo, los estudios relativos a la fracción lipídica son escasos, a pesar de estar presente en todos los tipos de suelos (Almendros, 1996; Tu, 2017). Asimismo, no existen prácticamente estudios que examinen de forma detallada la composición de la fracción de carbohidratos de bajo peso molecular del suelo (Medeiros, 2006).

5.1.2 La fracción de lípidos libres del suelo

El origen de la fracción de lípidos libres de los suelos es muy complejo, pues estos compuestos no solo proceden de gran diversidad de organismos, sino que las transformaciones de los lípidos biógenos en los suelos dan lugar a nuevos compuestos no existentes en los seres vivos. Mientras los carbohidratos y las proteínas experimentan generalmente una rápida hidrólisis para dar lugar a productos de bajo peso molecular solubles en agua, los compuestos alquílicos (ácidos grasos, alcanos…) o los lípidos cíclicos resisten mejor el ataque químico y microbiológico, manteniéndose intactos o transformándose en productos estables que tienden a preservarse (Kogel-Knabner, 1993). De hecho, una considerable fracción lipídica del suelo se encuentra constituida por moléculas que desempeñan, en las plantas superiores, importantes funciones alelopáticas (flavonoides, terpenoides…) que favorecen la colonización territorial, o presentan intensa acción antimicrobiana, o biocida respecto a organismos fitopatógenos del suelo (Stevenson, 1966; Lynch, 1976).

Por todo ello, la fracción lipídica del suelo presenta una gran importancia en el control de los procesos de biodegradación y humificación de la materia orgánica del suelo (Amblès, 1993) afectando a su

estructura macromolecular, al incorporarse a las sustancias húmicas y a los minerales de la arcilla, impermeabilizando los agregados, modificando las propiedades físicas del suelo, y estabilizando su estructura (Jambu, 1983; Fustec, 1985; Dinel, 1990). Por otra parte, la caracterización molecular de los lípidos presenta un gran valor diagnóstico en los estudios biogeoquímicos, permitiendo reconocer y cuantificar los flujos de carbono en los ecosistemas, así como evaluar los diferentes tipos de impactos ambientales (Bull, 2000). Por todo ello, el estudio de la fracción lipídica constituye una herramienta para la caracterización biogeoquímica de los diferentes ecosistemas, que facilita la identificación de los distintos grupos taxonómicos de organismos activos en el suelo, y permite la reconstrucción paleoambiental (Eganhouse, 1988; Almendros, 1996; Eglinton, 2013).

5.1.2.1 Principales componentes de la fracción lipídica del suelo

La fracción de lípidos del suelo normalmente constituye entre el 0,2 y el 4,0% del carbono total y está constituida principalmente por alcanos, ácidos grasos, hidroxiácidos, cetonas, esteroides, terpenoides y ésteres, en particular ceras (Stevenson, 1982; Dinel, 1990; Zelles, 1992; Stevenson, 1994). A continuación, se procede a describir los compuestos más importantes de dicha fracción:

Los ***hidrocarburos alifáticos saturados (alcanos)*** son compuestos presentes en la fracción lipídica de todos los suelos, y proceden principalmente del metabolismo de plantas y microorganismos. Los intervalos de átomos de carbono que predominan en los suelos son entre C_{15}–C_{35}, siendo frecuente el considerar que su origen es microbiano cuando hay predominio de alcanos de longitud de cadena inferior a 20 carbonos, en tanto que los alcanos de longitud de cadena por encima de 23 carbonos suelen proceder de ceras epicuticulares de plantas superiores (Simoneit, 1982). Los alcanos de síntesis biológica presentan tendencia al predominio de cadenas de número impar de átomos de carbono, pues su origen en organismos superiores suele tener lugar por medio de la descarboxilación de los correspondientes ácidos grasos. En los casos en que las series de alcanos no presentan esta tendencia a la imparidad, se piensa que su formación puede deberse a la actividad de microorganismos (Stránský, 1967; Tinoco, 2002). Por otra parte, la presencia de alcanos ramificados se considera debida a actividad bacteriana, como el caso de los alcanos *iso*- y *anteiso*- (Recio, 2010), mientras que los alcanos cíclicos o con gran número ramificaciones, que aparecen en forma de mezclas complejas de muy difícil separación cromatográfica, suelen indicar la presencia de hidrocarburos fósiles, como en el caso de suelos urbanos contaminados por residuos de combustibles (Simoneit, 1982).

Los ***ácidos alcanoicos*** o ácidos grasos son otros de los componentes más abundantes de la fracción lipídica del suelo, apareciendo en el intervalo C_{12}–C_{30}. Suelen presentarse en forma de cadenas con

número par de átomos de carbono, dominando el ácido palmítico (*n*-C_{16}) y el esteárico (*n*-C_{18}). Al igual que en el caso de los alcanos, el predominio de ácidos de menos de 20 átomos de carbono es indicadora de actividad microbiana, mientras que por encima de 23 carbonos señala un origen a partir del metabolismo de plantas superiores (Simoneit, 1982). La presencia de ramificaciones es generalmente un indicio de actividad bacteriana, así por ejemplo, los ácidos grasos *iso*- y *anteiso*- son indicativos de un activo metabolismo bacteriano, como se demostró en diferentes especies del género *Bacillus* (Tulloch, 1976; Goossens, 1986; Kaneda, 1991), donde se presentan en proporciones incluso superiores, a las de los homólogos lineales.

Las ***cetonas*** también pueden estar presentes en los suelos, aunque en general en menores proporciones. Su presencia es debida a procesos microbianos de oxidación de los correspondientes *n*-alcanos o *n*-alcoholes (Forney, 1971), así como a la *β*-oxidación y descarboxilación de los *n*-ácidos grasos incorporados al suelo con los restos vegetales (Cranwell, 1982). En suelos que contienen restos de plantas superiores se encuentran las *n*-alcan-2-onas en el intervalo de C_{17}–C_{35}, predominando las moléculas de número impar de átomos de carbono (Cranwell, 1982). Las cetonas comprendidas entre C_{23}–C_{31} son características de plantas inferiores, tales como briófitos del género *Sphagnum* y, en concreto, la heptacosan-2-ona (C_{27}) es usada como marcador especifico de este tipo de musgos (Nichols, 2007). Las cetonas isoprenoides, como la trimetilpentadecan-2-ona, son consideradas como productos de la degradación oxidativa del fitol de la molécula de clorofila y son, por tanto, indicadores de la presencia de organismos fotosintéticos (Brooks, 1978)

En la fracción lipídica de suelo se pueden encontrar diferentes ***céridos***, ésteres de ácidos y alcoholes de cadena larga, provenientes de los recubrimientos externos de las partes aéreas de las plantas (Tulloch, 1976); aunque también pueden proceder en cierta proporción de la actividad de microorganismos y artrópodos (Dinel, 1990). Los céridos presentes en los suelos presentan normalmente un número par de átomos de carbono y un peso molecular en el rango C_{36}–C_{52}, por lo que su identificación por cromatografía de gas suele requerir del uso de columnas y condiciones especiales para la separación de compuestos de elevado punto de ebullición.

Es común encontrar ***alcoholes grasos*** en los suelos, aunque siempre en menor abundancia que los alcanos y los ácidos alcanoicos. El intervalo generalmente encontrado es de C_{13}–C_{30}, predominando los alcoholes con número par de átomos de carbono. Suelen proceder del metabolismo de microorganismos cuando presentan menos de 20 átomos de carbono y del metabolismo de plantas cuando se encuentran por encima (Simoneit, 1982).

Los ***compuestos terpénicos*** son frecuentes, sobre todo bajo determinados tipos de vegetación que produce resinas. Los terpenos son especialmente abundantes en el reino vegetal, de tal forma que

muchos metabolitos terpénicos primarios realizan funciones de hormonas, como componentes de membrana, pigmentos fotoprotectores y transportadores de azúcares al interior de la célula para la síntesis de otros metabolitos. Paralelamente, tienen funciones de metabolitos secundarios implicados en la defensa de los vegetales frente herbívoros y patógenos, en la atracción de insectos polinizadores e interacciones alelopáticas (Goodwin, 1971; Gershenzon, 1994). Se nombran en base al número de moléculas de isopreno (2-metil-1,3-butadieno) presentes en su estructura, clasificándose en hemiterpenos (C_5), monoterpenos (C_{10}), sesquiterpenos (C_{15}), diterpenos (C_{20}), triterpenos (C_{30}) o tetraterpenos (C_{40}); ver **Figura 5.2.1**.

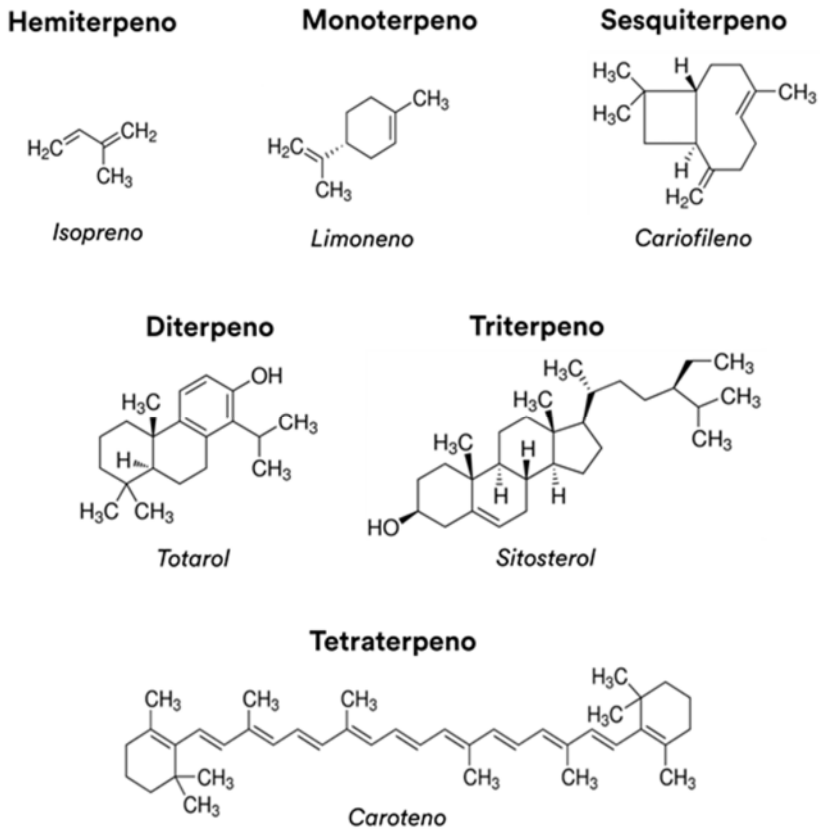

Figure 5.2.1. Ejemplos de diferentes estructuras de terpenos

Otros compuestos comunes en los suelos son los ***esteroides***, también derivados del isopreno, aunque que en este caso conforman estructuras tetracíclicas. Su utilidad como biomarcadores es debida a que estos compuestos, a pesar de estar sometidos a procesos de degradación en el suelo, conservan las características básicas de la molécula, lo que permite identificar su procedencia biológica (Otto, 2005).

Los ***hidroxiácidos y epoxiácidos grasos*** proceden de la degradación de los poliésteres vegetales (cutinas y suberinas). Son precursores alquílicos de gran importancia en la formación de sustancias húmicas (Nip, 1986; Wilson, 1987; Hatcher, 1988; Tegelaar, 1989; Almendros, 1991; Kögel-Knabner, 1992)

5.1.2.2. Fracción lipídica de los suelos mediterráneos

El clima es uno de los factores que más afecta a composición de la materia orgánica del suelo. El clima mediterráneo presenta marcados contrastes térmicos a lo largo del año y una pronunciada sequía estival, por lo que la disponibilidad hídrica representa uno de los factores más limitantes para los ecosistemas de estas regiones. Los suelos mediterráneos, por tanto, se caracterizan por estar sometidos regularmente a condiciones de sequía, seguidas de episodios de precipitaciones irregulares y de elevada intensidad. La alternancia climática de épocas de desecación drástica, junto con la acción cementante e insolubizadora de los carbonatos de la materia orgánica, así como la interacción con arcillas, favorece la condensación de compuestos orgánicos, la formación de agregados estables y la diferenciación de complejos patrones de microcompartimentación del suelo. Estos factores promueven la fijación de los lípidos a la matriz del suelo y su lenta transformación diagenética en formas resistentes a la biodegradación. Bajo estas condiciones, las fracciones lipídicas originalmente libres o directamente extraíbles con disolventes orgánicos, pasan a formas ligadas a las sustancias húmicas macromoleculares (ácidos húmicos, fúlvicos y huminas) (Almendros, 1996).

Por otra parte, la vegetación de los climas mediterráneos presenta adaptaciones específicas frente a la sequía temporal, produciendo abundantes cantidades de ceras y resinas, que acaban incorporándose a los suelos donde crecen. En este sentido, se ha descrito que en los suelos mediterráneos, la menor transformación cualitativa de los lípidos de las plantas tiene lugar en los ecosistemas donde hay un balance más elevado entre la humificación y la mineralización de la materia orgánica (Recio Vázquez, 2014). Esto parece sugerir que la diversificación molecular no está relacionada directamente con los procesos de biodegradación activa, sino con un aumento del tiempo de residencia del carbono orgánico en el suelo, que favorece la preservación selectiva de determinados lípidos de los suelos.

Los estudios relativos a la fracción lipídica de los suelos mediterráneos son escasos (Almendros, 1996; Recio Vázquez, 2014). Dichos estudios se han centrado principalmente en el análisis de suelos bajo bosques típicos mediterráneos constituidos por especies arbóreas planifolias de hoja perenne (*Quercus ilex*) o caduca (*Quercus pyrenaica*). Estos bosques suelen aparecer en combinación con especies de coníferas, como el pino (*Pinus* sp.) y el enebro o la sabina (*Juniperus* sp.), sobre todo en los casos en los que ha existido algún tipo de intervención humana. La composición lipídica en suelos de pino y enebro, que serán en concreto los descritos en el presente capítulo, se basan principalmente en los patrones de compuestos cíclicos, incluyendo di- y sesquiterpenos, además de algunos monoterpenos (Almendros, 1996). También se ha descrito la presencia de series homólogas de alcanos y ácidos grasos (Bull, 2000).

5.1.2.3. Relación entre composición lipídica de suelos mediterráneos y la aportación vegetal

El aporte vegetal es responsable en gran medida de la composición molecular de los lípidos libres del humus. Un estudio comparativo de dicha composición entre diferentes suelos permitiría recocer los diferentes patrones de transformación de los constituyentes de la vegetación común en ecosistemas afectados por diferentes condiciones ambientales (Almendros, 1996).

En el caso de la vegetación de pino y enebro, es conocido que los terpenos cíclicos mencionados en el anterior apartado son los componentes fundamentales de la fracción lipídica extraíble (Cabral, 2012; Mitić, 2017). La composición concreta puede diferir entre distintos individuos de una misma especie debido a factores entre los que los edáficos y climáticos suelen ser los más importantes.

Por este motivo, la determinación cuali- y cuantitativa de los lípidos de las hojas, que tras su descomposición en el suelo pasan a formar parte de la fracción lipídica de éste, es necesaria para un mejor conocimiento de los procesos de formación del humus.

Podemos definir la “hojarasca” como un horizonte del suelo donde han comenzado los procesos de humificación, pero las hojas aún mantienen su morfología original (Dickinson, 1974). Aunque dichos procesos son continuos, y no es posible fijar con precisión etapas intermedias en su desarrollo, es cierto que el estado “hojarasca” es fácilmente reconocible y se puede situar entre el estado inicial de “hoja” y el final de “humus”. La determinación de los lípidos libres de a hojarasca debería ser de gran utilidad para el conocimiento de los procesos de formación del humus en diferentes escenarios ambientales.

5.1.3. La fracción de carbohidratos de bajo peso molecular

Es conocido que los carbohidratos afectan a la estructura del suelo, a los procesos químicos, a la nutrición de las plantas y a la actividad microbiana (Medeiros, 2006). Las fuentes posibles de carbohidratos en el suelo son las plantas (fuente principal), la fauna edáfica y los microorganismos (Mehta, 1962). Dichas fuentes se encuentran interrelacionadas a través de un activo dinamismo: los carbohidratos procedentes de las plantas son una fuente de energía y carbono para los animales y microorganismos que habitan en el suelo, mientras que los microorganismos sintetizan hexosas primarias y las liberan al suelo (Cheshire, 1979). Por tanto, las relaciones entre los microoganismos y la concentración y composición de carbohidratos se pueden emplear para evaluar los estados más o menos avanzados de transformación y estabilización de la materia orgánica en el humus (Medeiros, 2006), que presentan gran importancia en relación con el potencial de secuestro de carbono del suelo.

Los estudios relativos a la fracción de carbohidratos de bajo peso molecular del suelo son muy escasos (Medeiros, 2006; Gunina, 2015). Los carbohidratos mayoritarios descritos en suelo son principalmente monosacáridos como la glucosa y fructosa, polialcoholes como el manitol y disacáridos como la sacarosa y la trehalosa (Medeiros, 2006). Las hojas de las coníferas como el pino o el enebro poseen un mayor contenido y una mayor variedad de carbohidratos de bajo peso molecular que el suelo (Assarsso, 1958; Gunina, 2015). Sin embargo, tampoco existen estudios exhaustivos de esta fracción.

5.1.4. Metodologías analíticas para el estudio de los carbohidratos de bajo peso molecular y los lípidos libres de suelo

Debido a la complejidad y heterogeneidad de los compuestos presentes en el humus del suelo, tanto para el análisis de lípidos libres y como de carbohidratos de bajo peso molecular del suelo, es necesario llevar a cabo un fraccionamiento previo que separe estas fracciones del resto de los componentes orgánicos (Davies, 1969; Augris, 1998; Grasset, 1998). En el caso de los carbohidratos, se realizan extracciones mediante SLE empleando disolventes orgánicos polares o agua (Medeiros, 2006; Gunina, 2015). En general, para el análisis de dichos carbohidratos de bajo peso molecular se ha empleado métodos colorimétricos para la determinación de azúcares totales (Deng, 1994), mientras que solo algunos estudios (Medeiros, 2006) han empleado la GC para la determinación de carbohidratos específicos, utilizando los métodos de sililación como los previamente descritos en el apartado 3.1.3 de esta Memoria.

En cuanto a los lípidos del suelo, esta fracción se extrae normalmente mediante tratamiento con disolventes no polares como el diclorometano, hexano, cloroformo, benceno o éter dietílico (Dinel, 1990), y usando temperaturas de extracción entre los 40–75 ˚C para evitar la alteración de los analitos. También se emplean disolventes orgánicos más polares, como metanol o etanol, aunque en menor medida (Amblès, 1991). Para la extracción de los lípidos del suelo se utiliza la tradicional SLE, extracción en continuo mediante Soxhlet (Almendros, 1996) o técnicas más avanzadas como la PLE (Weijers, 2006). Los lípidos extraídos con estos disolventes orgánicos, tras diferentes procesos de purificación o filtración, pueden ser analizadas directamente mediante GC-MS, utilizando columnas de metilpolisiloxano para llevar a cabo la separación (Almendros, 1996). A pesar de que la fracción de lípidos libres de hojas, hojarascas y suelos es muy compleja y requiere técnicas avanzadas para su análisis, en nuestro conocimiento, la GC×GC-ToF MS ha sido escasamente empleada hasta el momento para establecer su composición. Los estudios existentes que emplean esta técnica se basan en la determinación de la composición volátil del suelo aislada mediante SPME o SDE (Mateus, 2010), o en la determinación de contaminantes en el suelo (Danielsson, 2008).

Dada la complejidad de los suelos, los extractos de lípidos libres han sido generalmente caracterizados desde un punto de vista cualitativo, describiéndose las diferentes familias de compuestos presentes, pero sin tener en cuenta sus diferentes proporciones. Únicamente se han establecido relaciones de abundancia de los compuestos detectados (Almendros, 1996; De Blas, 2013). Estos estudios han permitido estudiar la evaluación diagenética de la fracción de los lípidos libres del suelo (Amblès, 1997), así como su relación con la composición mineral del suelo (Jambu, 1987). Se dispone también de información en la bibliografía sobre la concentración de lípidos totales (Dinel, 1990; Siem, 1999).

Considerando estos antecedentes, el **objetivo general** de esta sección de la Tesis se centrará en la caracterización exhaustiva, cuali- y cuantitativa, de la fracción de lípidos libres y carbohidratos de bajo peso molecular de hojas, hojarasca y suelos de bosques mediterráneos de pino y enebro. En concreto, en la **Sección 5.2** se realizó un estudio mediante GC-MS de la composición lipídica y de carbohidratos en suelos recolectados bajo dichas especies y tratados con disolventes tanto polares como apolares. Asimismo, se realizó una caracterización molecular de la composición de estos compuestos en las hojas y la hojarasca de los correspondientes bosques, considerándolas como un estado inicial e intermedio, respectivamente, en el proceso de humificación. Los resultados obtenidos de este estudio forman parte del artículo titulado “Quantitative GC-MS analysis of free lipids and low molecular weight carbohydrates from pine and juniper leaves, litter and soils” (Carrero-Carralero et al.), en evaluación para su publicación en una revista científica del campo.

Una vez establecida la compleja composición observada en los extractos apolares y polares de las hojas de estos bosques, se llevó a cabo un estudio sobre las posibilidades y problemas de la aplicación de GC×GC-ToF MS para el análisis de estas mismas muestras. Los correspondientes resultados constituyen la **Sección 5.3** de esta Memoria.

5.2. Quantitative changes in the molecular signature of free lipids and low molecular weight carbohydrates from pine and juniper at different stages of transformation in soil

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5.2.1. Introduction

Soil organic matter consists of a complex mixture of compounds widely deferring in terms of molecular weight, chemical structure and physical properties, which derive mainly from lignocellulosic plant biomass accumulated in the litter layer (Tu, 2017), but also from the activity of mesofauna, bacteria and fungi (Oades, 1993; Van Bergen, 1997). During the early stages of transformation of the organic matter accumulated on the soil, most soluble compounds are transported by water, while a small percentage remains into the soil surface as stable forms (Van Bergen, 1997; van Bergen, 1998). Although free organic compounds of low molecular weight are minor components of soils (from 0.2 to 4% of total carbon), this fraction may exert an important influence on many environmental processes and soil properties.

The lipid fraction of soils is a complex heterogeneous mixture including a wide variety of compounds soluble in organic solvents of low to medium polarity, which includes both complex condensed non-volatile components and simpler functional classes such as hydrocarbons, fatty acids, wax esters, ketones, hydroxyacids, terpenoids, steroids, acylglycerols, phospholipids, lipopolysaccharides, etc. (Stevenson, 1966; Dinel, 1990). These compounds display crucial functions in the ecosystem, such as forming hydrophobic coatings on the surface of soil aggregates, turning them more hydrophobic and avoiding both its physical disruption and the biodegradation of encapsulated organic matter preserved into these soil microcompartments (Jambu, 1995). On the other hand, the lipids could be considered as a molecular record of diagnostic compounds molecules providing valuable biogeochemical information on the structure and dynamics of the ecosystems (Almendros, 1996).

Low molecular weight carbohydrates (LMWC) in soil have also been scarcely studied. Although plants represent the main source of soil carbohydrates, microorganisms (fungi, bacteria, algae) and edaphic fauna also contribute to a lesser extent. In fact, these compounds also influence soil structure, chemical processes, plant nutrition and microbial activity (Medeiros, 2006).

Extraction of both free lipids and LMWC from the soil can be carried out easily by using organic appropriate solvents. In the case of lipids, hexane, ethyl ether, dichloromethane or methanol, or its admixtures have been used frequently (Stevenson, 1982), whereas polar solvents such as methanol, ethanol or water are used to extract carbohydrates. In any case, the chemical nature of the extracted compounds will vary according to the polarity of the solvent system used for the extraction (Bull, 2000).

The analysis of compounds present in free lipid and LMWC fractions has been mainly carried out by gas chromatography coupled to mass spectrometry (GC-MS) (Almendros, 1996; Bull, 2000; De Blas, 2013). This technique provides enough sensitivity and potential for structural identification for the

qualitative and quantitative analysis of these complex admixtures. However, accurate identification of individual lipid and carbohydrate molecules is not straightforward. Regarding quantitative analysis, previous exploratory studies have been based on the evaluation of the relative values of each compound to the total chromatographic area (Almendros, 1996; De Blas, 2013) although reliable quantitative analysis of these compounds has not yet been done.

Therefore, in this work a comprehensive qualitative and quantitative study of free lipids and LMWC of soils from two Mediterranean forests (*Juniperus communis* and *Pinus sylvestris* forests) from central Spain was carried out by GC-MS. These forests were selected considering their abundance in Spain and also their wide distribution along the world (Eilmann, 2006; Adams, 2008). Moreover, and in order to monitor the molecular transformations in the course of the selective biodegradations and accumulation of the soil organic matter fractions, the extractable fractions from the needles and litter from these two plant species was also analyzed.

5.2.2. Materials and Methods

5.2.2.1 Samples

Samples (needles, litter and soils) from forest ecosystems of *Juniperus communis* (common juniper) and *Pinus sylvestris* (pine) were collected at El Espinar (Segovia, Central Spain). Twigs with leaves were cut from the plants, while the soil samples were collected from the first 6 centimeters after removing the litter layer. Sampling was carried out at three different positions of each forest, separated at least 100 m one from the other. Samples were mixed to obtain representative pools of each species. All the samples were air-dried before the extraction procedure.

5.2.2.2. Analytical standards

Analytical standards of acenaphtenequinone, benzil, *chiro*-inositol, fructose, glucose, mannitol, *muco*-inositol, *myo*-inositol, perylene, phenanthrene, 1-phenyl-1-butanol, β-phenyl-D-glucoside, pinitol, sucrose and trehalose were acquired from Sigma Chemical Co. (St. Louis, US). Sequoyitol was acquired to Extrasynthese (Genay, France) and quebrachitol to Acros (Geel, Belgium). Methyl-1-*muco*-inositol was obtained from a honey sample as indicated by Sanz *et al.* (2004).

5.2.2.3. Extraction procedure

Needles were milled with a grinder, while soil and litter samples were ground in an agate mortar to obtain a fine and homogeneous powder. All the samples were sieved (< 500 µm) and stored under dry conditions and protected from direct light until analysis.

In order to obtain the non-polar extracts, 0.3 grams of leaves and 2 grams of litter and soil samples were suspended in 6 mL of dichloromethane; 20 µL of internal standards (1.6 mg mL^{-1}) were added to the sample solutions. Then, samples were ultrasonicated for 30 min in a bath and the extracts were filtered using silanized glass wool and centrifuged at 33 800 g for 5 min. The extracts were evaporated under vacuum and redissolved in 1 mL of dichloromethane.

The polar extracts were obtained from needles (0.3 g), litter and soil (2 grams each) using methanol (6 mL) as extraction solvent. Ultrasounds were applied for 30 min in order to enhance the extraction of the compounds, then the samples were left to sit for 30 min at room temperature. The extracts were filtered through Whatman No. 4 paper and centrifuged at 33 800 g for 5 min. The extracts were evaporated under vacuum and redissolved in 2 mL of methanol.

5.2.2.4. Derivatization procedure

Prior to GC-MS analysis of polar extracts, a derivatization procedure was carried out as indicated by Rodríguez-Sánchez et al., (2013). Methanol extracts (0.5 mL) were mixed with 0.1 mL of a 70% ethanol solution of phenyl-β-*D*-glucoside (1 mg mL^{-1}) which was used as internal standard. Samples were evaporated under vacuum and treated with 350 µL of 2.5% hydroxylamine chloride in pyridine at 75 °C for 30 min. Then, 350 µL of hexamethyldisilazane (HMDS) and 35 µL of trifluoroacetic acid (TFA) were added and kept at 45 °C for 30 min. The samples were centrifuged at 7000 *g* for 5 min at 5 °C, and 1 µL of the supernatant was injected onto the GC column. This two-step derivatization procedure (oximation + silylation) of carbohydrates allowed the reduction of signals corresponding to the reducing sugars, giving only two peaks (the *syn* (*E*) and *anti* (*Z*) isomers of the oxime) whereas only a single chromatographic peak was obtained for non-reducing sugars and inositols, corresponding to the *O*-persilylated derivative.

5.2.2.5. GC-MS analysis

The analyses were carried out in a 6890A gas chromatograph coupled to a 5973 quadrupole mass detector (both from Agilent Technologies), using He as carrier gas. A ZB-1MS (cross-linked methyl silicone) column (30 m × 0.25 mm i.d.; 0.25 µm film thickness) from Phenomenex (Torrance, CA) was used. For dichloromethane extracts, the oven was kept at 70 °C during 1.5 min, then heated to 290 °C at 6 °C min^{-1} and kept for 20 min. Injections were carried out in splitless mode (1 min) at 275 °C.

For methanol extracts, the oven temperature was programmed from 120 °C to 300 °C at 5 °C min^{-1} and kept for 20 min. Injections were carried out in split mode (1:50) at 300 °C.

The transfer line and ionization source were thermostated at 280 and 230 °C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV within the mass range *m/z* 35−650. Acquisition was done using MSD ChemStation software (Agilent Technologies).

Identification of compounds of non-polar extracts were based on the comparison of experimental I^T and mass spectra with those reported on the literature (Adams, 2007) and comparing with databases (Wiley, 1986; NIST, 2002). Compounds of polar extracts were identified by comparing I^T and mass spectra with those of the corresponding commercial standards. Compounds for which commercial standards were not available were tentatively identified on the basis of their mass spectra information.

Quantitation was carried out by the internal standard method. Standard solutions of target compounds over the expected concentration range in samples under study were prepared to calculate the response factor relative to internal standard (phenyl-β-D-glucoside for polar extracts and 1-phenyl-1-butanol and benzil for non-polar extracts). Concentrations of compounds from non-polar extracts and those for which commercial standards were not available in polar extracts were estimated assuming a response factor equal to 1. All analyses were carried out in triplicate. According to Foley and Dorsey (1984), limit of detection (LOD) was calculated as three times the signal to noise ratio (*S/N*), where *N* is five times the standard deviation of the noise, whereas the limit of quantitation (LOQ) was considered ten times this ratio. Intra-day precision (relative standard deviation, RSD) was calculated for specific compounds (typical from each group) from the results obtained for a leaves extract analysed in five different days.

5.2.2.6. Data analysis

To explain the origin of the variability of the concentration of the lipids in the samples the Shannon-Wiener diversity indices (*H'*) were calculated for the different groups (alkanes, fatty acids,

monoterpenes, sesquiterpenes and diterpenes) using the 'Species, diversity and richness' program (Version 2.6, designed by P.A. Henderson and R.M.H. Seaby, Pennington, Lymington, UK; 1998) .

5.2.3 Results and discussion

5.2.3.1. Analysis of non-polar extracts

Figure 5.2.1 shows the GC-MS profiles of dichloromethane extracts from needles, litter and soils from samples collected in forests of *Juniper communis* and *Pinus sylvestris,* respectively. In general, complex GC profiles were observed mainly in leaves and litter samples. Prior to the identification and quantitation of lipids in the samples, a selection of appropriate internal standards was required. Several standards chemically similar to the compounds to be analyzed but not naturally present in the samples and eluting in different regions of the chromatogram were selected: i.e., 1-phenyl-1-butanol, phenanthrene, benzil, acenaphtenequinone and perylene. Taking into account the complexity of the samples, coelutions of phenantrene and acenaphthoquinone with compounds naturally present in the samples were observed; therefore the use of these standards was discarded. Intra-day precision and LOD and LOQ were further evaluated considering specific compounds typical from each group (nonacosane, hexadecanoic acid, α-thujene, caryophyllene and abietatriene). Perylene eluted in a clean zone of the chromatogram, however, non-reproducible results were obtained when used as internal standard (RSD: 28–170%). Therefore, 1-phenyl-butanol and benzil were selected for the quantitative analysis of free lipids in the samples (RSD < 30%). LOD and LOQ on average were 1.3 μg g^{-1} and 4.8 μg g^{-1}, respectively.

Table 5.2.1 shows the concentrations (μg g^{-1} dry sample) of lipids found in the needles, litter and soil of pine and juniper forests. Up to 127 compounds were identified in these samples mainly corresponding to alkanes, fatty acids and cyclic compounds (terpenoids and steroids). Several compounds, including alcohols, ketones, acids, etc., were also detected and indicated in the Table 5.2.1. as 'other compounds'. Needles extracts showed the greatest lipid concentrations for both juniper (3.2 mg g^{-1}) and pine (5.4 mg g^{-1}). Only 28 free lipid compounds were found in pine soils; this sample also showed the lowest lipid concentration (58 μg g^{-1}). In general, among the different lipids detected, terpenoids were the most abundant in needles and litter of both juniper and pine, whereas alkanes were the predominant family in soils. In general, monoterpenes were less abundant than sesquiterpenes and diterpenes. Figure 5.2.2. shows the chemical structures of those lipids specific for juniper and pine forest (needles, litter and soil). These compounds could be useful biomarkers of these samples. Specific discussion of the different groups is done below.

Figure 5.2.3. shows the Shannon (*H'*) diversity indices obtained for major groups (alkanes, monoterpenes, sesquiterpenes and diterpenes) for both juniper and pine needles, litter and soils. A similar trend of *H'* values was observed for alkanes, sesquiterpenes and diterpenes; diversity increased from juniper needles (*H'*= 1.37 for alkanes, *H'*= 1.83 for sesquiterpenes and *H'*= 1.40 for diterpenes) to soil (*H'*= 1.91 for alkanes, *H'*= 2.17 for sesquiterpenes and *H'*= 1.56 for diterpenes). This increase could be due to the contribution of microbial products in soils and/or to diagenetic transformation leading to new lipid molecules. However, *H'* values of alkanes and diterpenes reached a maximum in pine litter (*H'*= 2.26 for alkanes, *H'* 2.90 for sesquiterpenes and *H'*= 1.71 for diterpenes), decreasing in pine soil (*H'*= 2.05 for alkanes, *H'*= 0 for sesquiterpenes and *H'*= 0.69 for diterpenes). This decrease in the diversity of these compounds point either to their selective biodegradation, or its condensation or fixation into macromolecular structures, or both, with the correspondingly decreased concentration of these molecules as free extractive compounds.

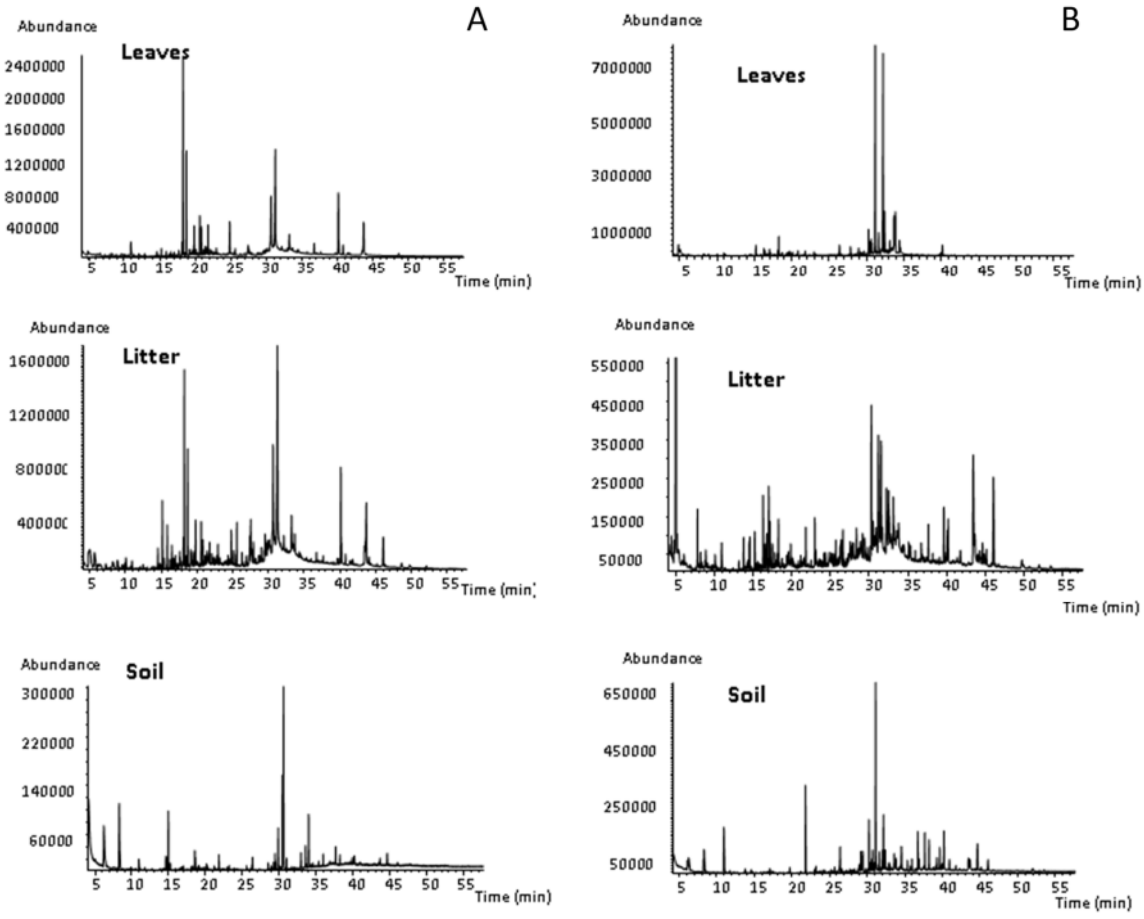

Figure 5.2.1. GC-MS profiles of dichloromethane extracts from needles, litter and soils from forests of *Juniperus communis* (A) and *Pinus sylvestris* (B).

Table 5.2.1. Concentration (μg g^{-1}) of lipids in dichoromethane extracts from needles, litter and soils from forests of *Juniperus communis* and *Pinus sylvestris*

Analyte		I^T	Juniper			Pine		
			Leaves	Litter	Soil	Leaves	Litter	Soil
Alkanes	Branched decane	998	16.2 (5.2)*	2.6 (2.3)	8.1 (1.3)	19.9 (21.6)	3.1 (0.5)	2.4 (0.5)
	Decane	1003	37.1 (25.6)	11.9 (0.8)	66.0 (53.4)	43.9 (40.1)	6.9 (1.5)	4.6 (1.0)
	Branched undecane	1093	17.6 (6.7)	4.5 (0.1)	6.7 (1.0)	20.1 (21.1)	3.4 (0.6)	2.9 (0.8)
	Undecane	1096	44.2 (32.3)	14.1 (0.1)	72.0 (52.5)	49.4 (42.8)	7.7 (1.9)	4.9 (1.2)
	Dodecane	1198	2.1 (1.0)	0.8 (0.1)	1.0 (0.3)	tr**	–	–
	Tridecane	1301	–	–	1.7 (0.1)	3.9 (0.1)	0.6 (0.1)	–
	Tetradecane	1403	–	–	–	15.1 (0.5)	0.41 (0.05)	–
	Pentadecane	1502	1.2 (0.5)	–	0.9 (0.2)	3.6 (2.9)	–	–
	Hexadecane	1600	–	–	1.30 (0.1)	3.1 (1.1)	–	–
	Heptadecane	1700	–	–	1.1 (0.1)	–	–	–
	Octadecane	1800	tr	–	1.0 (0.4)	–	0.42 (0.02)	–
	Nonadecane	1900	–	–	0.7 (0.3)	–	–	–
	Eicosane	2000	–	–	0.7 (0.3)	–	0.51 (0.01)	0.2 (0.1)
	Heneicosane	2101	3.77 (0.2)	3.2 (0.8)	0.71 (0.01)	–	2.6 (0.3)	0.5 (0.1)
	Docosane	2200	–	–	–	–	–	0.4 (0.1)
	Tricosane	2300	–	0.66 (0.1)	3.8 (1.6)	–	2.2 (0.2)	0.6 (0.1)
	Pentacosane	2501	–	1.2 (0.5)	2.3 (0.2)	4.6 (0.7)	1.9 (0.2)	1.3 (0.4)
	Hexacosane	2601	–	–	1.5 (0.1)	–	–	–
	Heptacosane	2700	–	2.1 (0.6)	4.32 (0.42)	7.8 (1.5)	3.8 (1.0)	2.7 (1.5)
	Octacosane	2799	–	–	1.6 (0.7)	tr	0.9 (0.1)	0.7 (0.3)
	Nonacosane	2897	11.4 (3.0)	4.2 (1.2)	13.8 (0.7)	6.8 (1.6)	10.7 (2.6)	11.1 (1.5)
	Triacontane	3001	–	0.8 (0.1)	1.10 (0.01)	tr	0.8 (0.1)	0.7 (0.6)
	Branched hentriacontane	3069	7.52 (1.8)	2.3 (0.4)	6.4 (4.2)	–	–	–
	Hentriacontane	3099	52.6 (25.9)	14.6 (5.1)	10.4 (0.4)	238.0 (120.3)	15.2 (6.2)	13.1 (10.1)
	Dotriacontane	3199	13.8 (7.6)	3.6 (1.7)	6.3 (6.2)	15.3 (3.6)	3.0 (2.2)	1.0 (0.1)
	Tritriacontane	3301	447.32 (235.6)	81.75 (44.9)	22.16 (13.5)	9.8 (2.4)	11.9 (4.5)	4.8 (2.9)
	Tetratriacontane	3401	12.7 (7.7)	5.2 (3.2)	–	–	–	–
	Pentatriacontane	3499	59.5 (40.4)	8.3 (4.4)	3.35 (0.01)	–	–	–
	Hexatriacontane	3600	5.5 (3.5)	4.06 (0.02)	–	–	–	–

Table 5.2.1. Continuation

Fatty acids		Hexanoic acid	961	8.9 (0.5)			176.0 (47.5)	–	–
		Oxononanoic acid	1452	–	–	–	52.9 (19.4)	–	–
		Dodecanoic acid	1545	16.3 (4.0)	–	–	59.8 (60.8)	–	–
		Tetradecanoic acid	1741	11.4 (0.6)	6.4 (3.5)	–	–	–	
		Pentadecanoic acid	1866	12.5 (5.5)	12.6 (5.8)	0.61 (0.05)	–	–	–
		Hexadecanoic acid	1940	90.0 (16.9)	5.7	–	53.1 (10.8)	–	–
		Heptadecanoic acid	2036	–	–	–	–	3.6 (1.8)	–
		Octadecanoic acid	2139	19.1 (6.4)	–	–	–	–	–
Cyclic compounds	**Monoterpenes**	Artemisatriene	933	–	–	–	26.2 (4.3)	–	–
		α-Thujene	942	39.60 (4.2)	66.1 (18.5)	3.81 (0.05)	466.2 (21.5)	0.56 (0.01)	0.53 (0.02)
		α-Pinene	948	43.8 (21.9)	83.4 (20.9)	–	168.3 (5.0)	–	–
		Camphene	952	–	–	–	67.11 (3.8)	–	–
		Monoterpene ($C_{10}H_{16}$)	971	6.0 (0.2)	1.9 (1.3)	–	–	–	–
		Sabinene	976	12.9 (1.6)	27.7 (3.5)	–	24.8 (4.7)	–	–
		2-*β*-Pinene	978	–	–	–	14.6 (3.6)	–	–
		p-Cymene	1011	10.0 (0.9)	tr	6.2 (3.6)	–	–	–
		Limonene	1020	15.0 (6.5)	14.0 (14.8)	–	–	–	–
		Monoterpene ($C_{10}H_{16}$)	1024	15.5 (1.8)	2.8 (2.9)	–	–	–	–
		Monoterpene ($C_{10}H_{16}$)	1050	10.9 (2.2)	19.6 (6.6)	–	–	–	–
		Sabinene hydrate	1079	6.2 (0.8)	1.9 (2.3)	–	–	–	
		p-Cymenene	1107	–	–	7.86 (0.04)	–	–	–
		α-Campholene aldehyde	1104	–	–	–	38.6 (8.5)	1.5 (0.8)	4.1 (0.7)
		Verbenol	1124	13.5 (2.3)	17.5 (17.5)	–	50.7 (5.3)	11.2 (3.6)	–
		α-Terpineol	1168	21.8 (14.7)	12.9 (7.6)	3.5 (0.1)	–	–	–
		Verbenone	1176	–	–	–	73.9 (8.1)	10.6 (5.3)	–
		Carvone	1213	9.7 (0.7)	2.0 (0.4)	–	–	–	–
		Monoterpene ($C_{10}H_{16}$)	1300	–	–	–	8.2 (2.4)	–	–
		Limonene glycol	1306	18.7 (0.4)	3.3 (0.1)	–	–	–	–

Table 5.2.1. Continuation

Cyclic compounds	**Sesquiterpenes**	*α*-Cubebene	1350	–	–	1.6 (0.3)	28.2 (1.8)	11.7 (5.1)	0.4 (0.2)
		Sesquiterpene ($C_{15}H_{24}$)	1352	–	–	0.7 (0.01)	–	–	–
		α-Ylangene	1372	–	–	2.1 (0.2)	11.6 (3.1)	3.8 (0.3)	–
		α-Copaene	1377	–	–	1.4 (0.4)	33.0 (0.8)	5.4 (1.2)	–
		β-Bourbonene	1385	4.2 (0.3)	1.5 (0.6)	–	35.9 (6.7)	12.9 (2.4)	–
		Junipene + sesquiterpene ($C_{15}H_{24}$)	1391	19.3 (1.6)	22.6 (9.7)	14.3 (0.6)	–	11.6 (2.9)	–
		Junipene (isomer)	1407	–	–	46.5 (0.1)	–	–	–
		di-*epi*-*α*-Cedrene	1414	–	–	7.7 (0.6)	–	–	–
		Caryophyllene	1418	30.4 (1.9)	82.4 (21.9)	8.4 (1.3)	269.7 (57.3)	20.2 (3.1)	–
		γ-Elemene	1429	17.8 (0.5)	13.5 (5.6)	6.5 (0.2)	–	–	–
		β-Copaene	1430	–	–	–	35.4 (18.7)	5.5 (0.5)	–
		Sesquiterpene ($C_{15}H_{24}$)	1441	–	–	–	11.5 (0.7)	4.31 (0.07)	–
		Sesquiterpene ($C_{15}H_{24}$)	1449	19.7 (1.7)	–	–	10.0 (6.4)	2.1 (0.2)	–
		α-Humulene	1454	–	–	–	52.9 (19.4)	2.9 (0.9)	–
		Muurola-4(14),5-diene	1459	–	–	–	28.2 (7.3)	2.0 (0.3)	–
		Germacrene *D*	1471	9.4 (0.8)	9.3 (3.9)	1.0 (0.2)	144.4 (19.6)	31.0 (1.5)	–
		Germacrene isomer	1477	–	–	–	112.1 (30.7)	4.6 (0.2)	–
		β-Selinene	1483	9.0 (7.2)	22.5 (10.4)	2.2 (0.2)	29.3 (5.6)	10.2 (0.1)	–
		trans-Cadina-1(6),4-diene	1488	–	–	–	41.7 (7.41)	9.3 (0.18)	–
		α-Muurolene	1495	7.7 (1.7)	–	–	64.8 (14.4)	18.4 (0.1)	–
		γ-Muurolene	1501	–	–	–	18.3 (9.3)	–	–
		Sesquiterpene alcohol	1504	–	–	–	128.5 (16.8)	4.8 (0.6)	–
		γ-Cadinene	1507	17.0 (3.2)	11.8 (4.3)	3.4 (0.4)	11.9 (0.01)	31.2 (1.1)	0.4 (0.2)

Table 5.2.1. Continuation

Cyclic compounds	**Sesquiterpenes**	*δ*-Cadinene	1516	–	–	–	119.5 (3.6)	18.1 (0.8)	0.27 (0.03)
		Sesquiterpene ($C_{15}H_{24}$)	1560	–	–	3.2 (0.7)	–	–	–
		Spathulenol	1565	52.8 (8.8)	31.7 (16.6)	3.6 (0.6)	144.3 (16.3)	7.2 (0.5)	–
		Caryophyllene oxide	1571	644.0 (16.2)	173.7 (57.5)	3.7 (0.7)	370.1 (32.8)	22.4 (0.9)	–
		Sesquiterpene alcohol	1584	–	–	–	7.7 (1.4)	–	–
		Humulene epoxide	1595	328.9 (15.6)	103.5 (36.4)	–	91.3 (1.0)	5.4 (0.7)	–
		Caryophylladienol II	1621	22.9 (2.0)	9.2 (5.3)	2.3 (0.3)	8.9 (2.5)	–	–
		Sesquiterpene alcohol	1626	–	–	–	73.1 (8.1)	5.2 (1.1)	–
		γ-Costol	1637	–	9.1 (0.5)	–	–	–	–
		Sesquiterpene alcohol	1638	–	–	0.6 (0.1)	56.6 (7.7)	–	–
		Sesquiterpene alcohol	1641	26.3 (5.9)	7.3 (4.7)	–	–	–	–
		Sesquiterpene alcohol	1653	119.8 (17.9)	59.4 (19.1)	–	70.7 (2.7)	–	–
		Sesquiterpene alcohol	1674	6.6 (1.4)	2.6 (0.2)	5.4 (1.7)	–	–	–
		Oplopanone	1707	120.1 (7.8)	41.1 (17.7)	–	107.3 (18.0)	4.0 (0.5)	–
		Spathulenol acetate	1791	11.5 (4.1)	1.9 (1.4)	–	–	–	–
		δ-Cadinene	1516	–	–	–	119.5 (3.6)	18.1 (0.8)	0.27 (0.03)

Table 5.2.1. Continuation

Cyclic compounds	**Diterpenes**	Dehydroabietal	1960	–	–	–	13.0 (0.8)	4.7 (1.4)	–
		Diterpene ($C_{20}H_{32}$)	1976	–	–	–	42.7 (37.3)	4.1 (1.4)	0.17 (0.03)
		Epimanoyl oxide	1981	12.3 (1.1)	10.0 (3.7)	2.4 (1.0)	11.2 (0.5)	2.9 (0.3)	–
		Pimaradiene	1987	22.7 (2.3)	38.5 (14.3)	3.8 (0.4)	–	–	–
		Abietatriene I***	2034	6.8 (0.3)	10.9 (2.8)	5.0 (0.9)	55.9 (4.1)	6.7 (0.6)	0.3 (0.1)
		Pimaradiene	2072	9.9 (1.9)	7.6 (1.9)	–	–	–	–
		Diterpene ($C_{20}H_{32}$)	2117	–	–	–	12.2 (3.1)	12.8 (6.1)	
		Abietatriene II	2176	–	–	–	tr	1.7 (0.2)	0.15 (0.01)
		Diterpene ($C_{20}H_{32}$)	2215	–	–	–	tr	0.94 (0.01)	–
		Totarol	2250	–	–	10.1 (4.5)	–	–	–
		Ferruginol I	2275	–	7.3 (4.4)	40.8 (10.3)	–	–	–
		Ferruginol II	2290	–	1.9 (1.3)	19.4 (18.8)	–	–	–
		Dehydroabietic acid I	2303	–	–	–	354.4 (98.8)	71.0 (28.4)	0.47 (0.01)
		Isopimaric acid	2331	74.8 (3.3)	100.9 (16.8)	50.1 (24.8)	128.4 (132.9)	10.4 (4.6)	–
		Diterpene	2371	–	–	–	318.3 (56.2)	72.5 (61.5)	0.99 (0.02)
		Dehydroabietic acid II	2401	–	–	–	375.9 (84.4)	45.4 (17.8)	0.65 (0.05)
		$C_{20}H_{28}O_2$	2562	12.7 (4.1)	12.8 (1.0)	2.3 (0.7)	–	–	–
	Triterpenes	*D:A*-Friedoolean-6-ene	3331	–	–	–	–	7.4 (2.8)	–
		α-Amyrin acetate	3358	–	–	–	–	9.3 (4.8)	–
	Steroids	Anthraergostatetraenol	2951	–	–	–	–	2.8 (1.6)	–
		Ergosta-4,22-dien-3-one	-	–	–	–	–	6.2 (0.1)	–
		-Sitosterol	3291	76.3 (20.1)	27.5 (0.1)	7.2 (3.3)	25.1 (3.3)	83.5 (31.4)	–
		Stigmast-4-*en*-3-one	3406	–	47.5 (5.4)	6.5 (3.7)	–	51.5 (24.6)	3.3 (0.4)

Table 5.2.1. Continuation

Other compounds	Benzoic acid	1135	–	–	–	31.6 (8.3)	–	–
	Octadienone,2,6,dimethyl	1151	9.1 (1.4)	–	–	–	–	–
	Benzaldehyde, 3,4,5-trimethoxy-	1554	1.6 (0.2)	–	–	–	–	–
	2-Pentadecanone, 6,10,14-trimethyl-	1828	35.0 (3.3)	20.9 (4.1)	–	82.8 (5.1)	–	–
	Heptadecanone	1829	39.9 (10.7)	–	48.2 (0.1)	–	–	–
	Xanthotoxin	2180	–	–	6.1 (2.2)	–	–	–
	Phenolic compound	2525	–	–	–	138.9 (21.6)	25.7 (7.8)	–
	Octacosanol	3092	347.7 (172.3)	48.7 (34.7)	4.0 (0.2)	227.9 (92.9)	7.0 (3.38)	1.4 (0.2)

* Standard deviation in parenthesis; **tr: traces; ***Roman numbers indicate different isomers

- ***n*-Alkanes**

Series of *n*-alkanes ranging from decane to tritriacontane in pine forest and up to hexatriacontane in juniper forest were detected. Some branched alkanes, with I^T values slightly lower than that of the corresponding linear alkanes, such as branched decane, undecane and hentriacontane, were also detected. It is worth to indicate that the highest concentration of *n*-alkanes was found in juniper leaves (732.5 μg g^{-1}), followed by pine needles (441.3 μg g^{-1}). Furthermore, juniper forests also characteristically contain high concentrations of alkanes in litter and soil (165.9 and 238.9 μg g^{-1}, respectively), whereas the soil under the pine forest showed the lowest number and concentration of these compounds (51.9 μg g^{-1}).

Regarding juniper forests, and from a qualitative point of view, the alkane profile of soil showed a great similarity to that of litter and needles; only some alkanes such as heptadecane, nonadecane and hexacosane are specific of soil (**Figure 5.2.2**.). However, quantitative differences were observed. Figure 5.2.4 shows the total concentrations of long chain (> C_{20}) and short chain alkanes (< C_{20}) in both juniper and pine forest. While juniper needles and litter were characterized by the predominance of long chain alkanes (83% and 80% of the total alkanes, respectively), juniper soil showed comparatively higher proportion of short-chain alkanes (78% of the total alkanes).

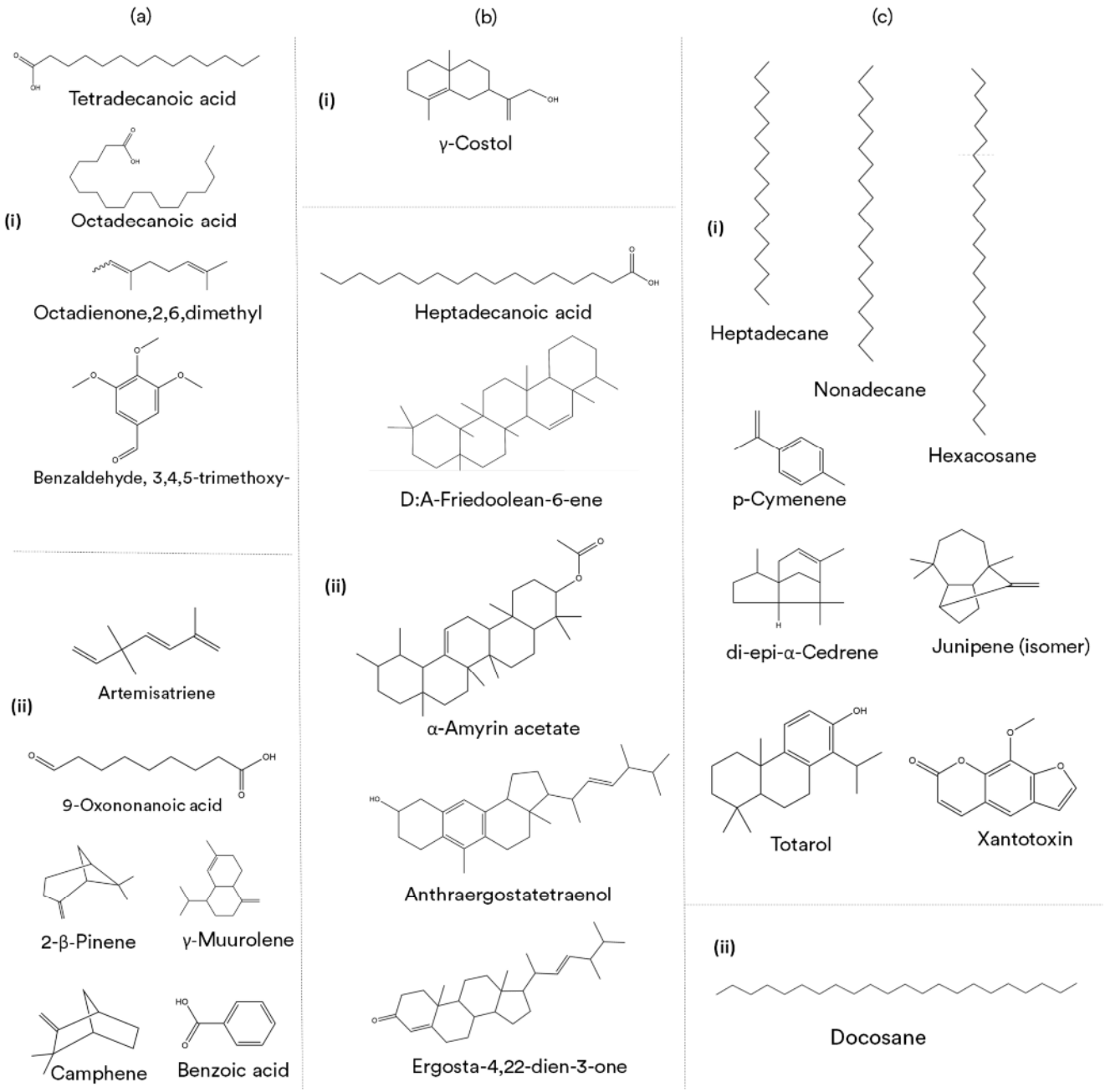

Figure 5.2.2. Classification of the chemical structures of specific lipids found in forests of juniper and pine. Columns from left to right are compounds (a) only present in leaves, (b) only present in litter and (c) only present in soil. Cell in columns represent from top to bottom represent (i) only present in juniper and (ii) only present in pine.

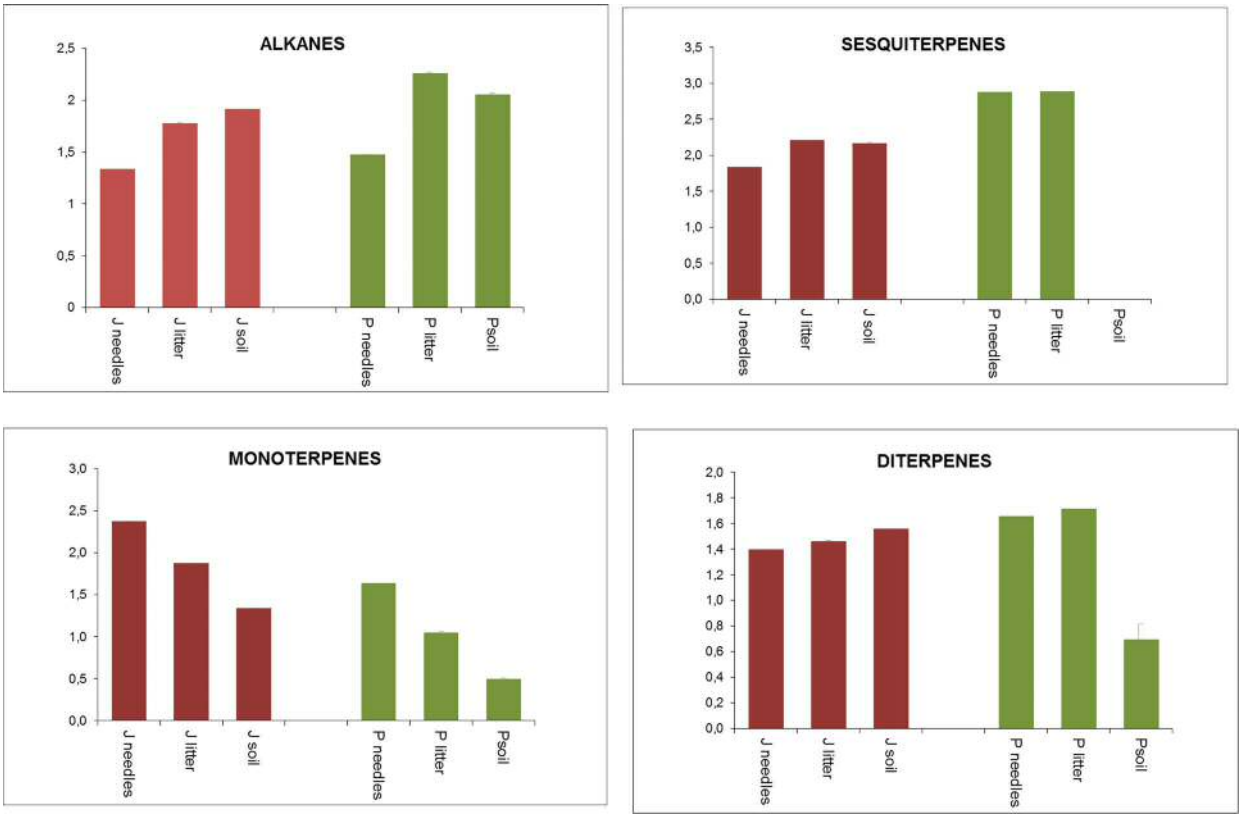

Figure 5.2.3. Shannon diversity indices obtained for alkanes, monoterpenes, sesquiterpenes and diterpenes in juniper (J) and pine (P) needles, litter and soils.

The predominance of individual alkanes for each sample is shown in **Figure 5.2.5.** Tritriacontane was the predominant alkane of juniper needles and litter with a concentration of 447.3 and 81.7 μg g^{-1}, respectively (Table 5.2.1). This alkane has been reported to be characteristic of *Juniperus communis* (Maffei, 2004) and other plants from the Cupressaceae family (Del Castillo, 1967). On the contrary, the most abundant alkane in juniper soil was undecane (72 μg g^{-1}) followed by decane (66 μg g^{-1}). This high predominance of short chain alkanes could be attributed to the degradation of long chain alkanes from needles and litter due to active microbial metabolism in soil (Moucawi, 1981; Fustec, 1985).

As in the case of juniper forest, long chain alkanes were more abundant than short chain alkanes in pine needles and litter. This distribution was also observed in soil under pine which showed a lower concentration of short chain alkanes (29%) than under juniper (**Figure 5.2.3.**). This could indicate that pine forests have comparatively low biogeochemical activity, and most of their alkanes are inherited from the plant (Simoneit, 1982). Comparing pine and juniper forest, as it can be observed (**Figure 5.2.4.**) the concentration of long chain alkanes was lower in pine needles than in juniper needles; on the contrary, short chain alkanes were more abundant in pine needles.

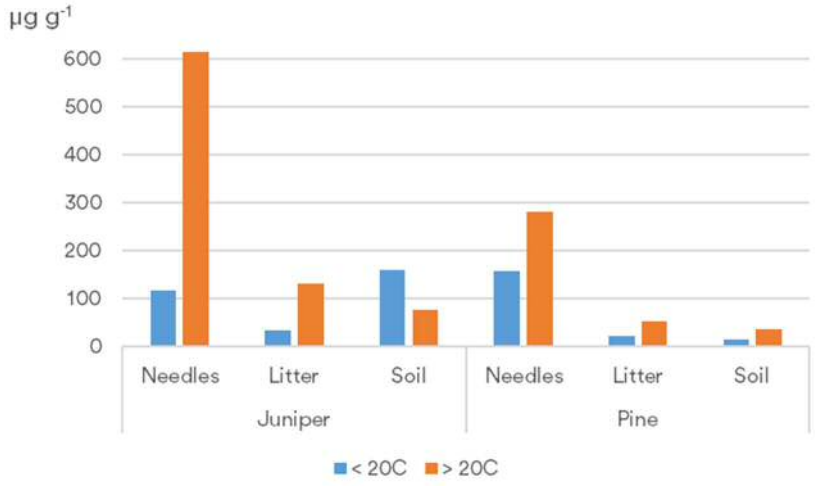

Figure 5.2.4. Total concentration (µg g^{-1}) of long chain (> C_{20}) alkanes and short chain (< C_{20}) alkanes of needles, litter and soils from juniper and pine forests.

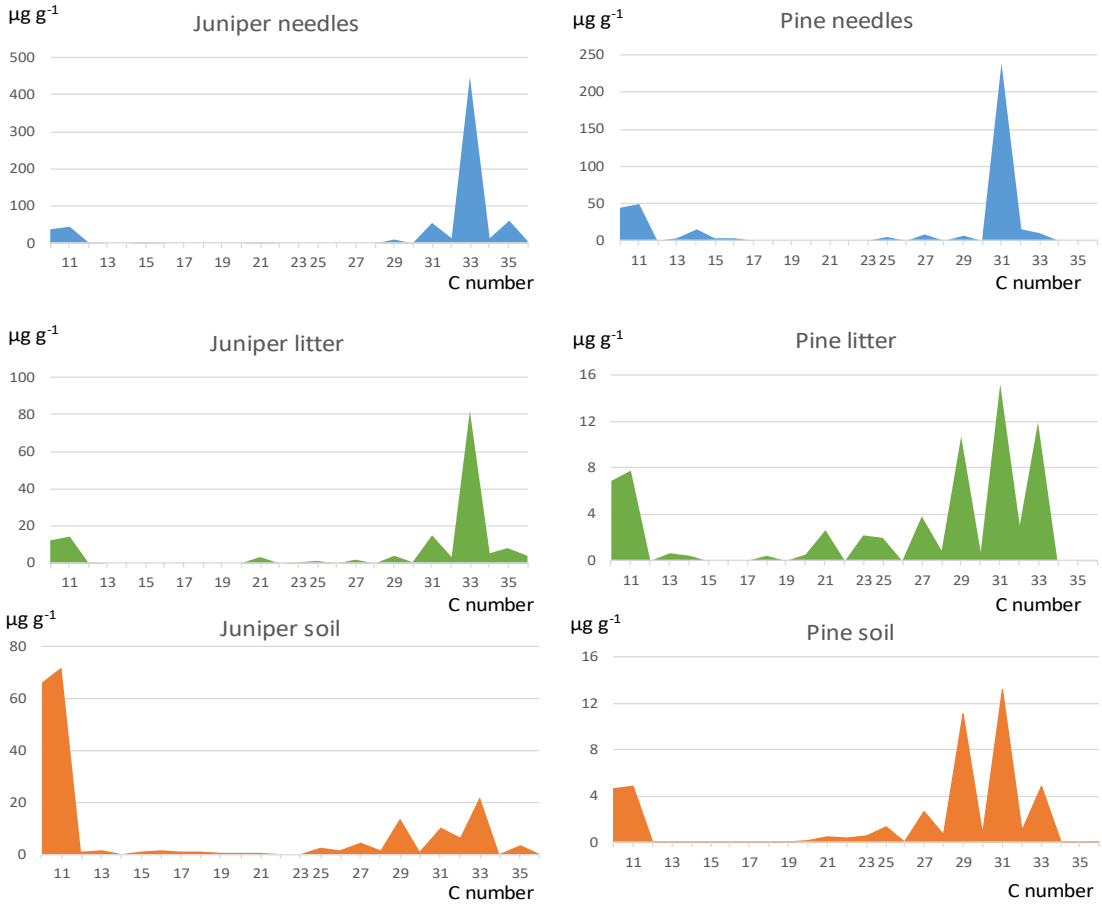

Figure 5.2.5. Homologous series showing the concentration (µg g^{-1}) of individual alkanes of needles, litter and soils from juniper and pine forests.

Pine forest was characterized by the predominance of hentriacontane in all the samples (concentrations of 238, 15.2 and 13.1 μg g^{-1} in needles, litter and soil, respectively) (Table 5.2.1). In general, total concentration of the alkanes with an odd number of C atoms was higher than that of alkanes with even number of C atoms (Figure 5.2.5.) pointing to a biogenic signature of alkanes inherited from epicuticular waxes from higher plants (Simoneit, 1982).

- **Fatty acids**

Up to 8 major fatty acids were detected in dichloromethane extracts (Table 5.2.1.). The highest concentration of fatty acids was found in pine needles (341.8 μg g^{-1}), followed by juniper leaves (158.2 μg g^{-1}). However, a higher variety of fatty acids was observed in the needles of juniper forests with the predominance of hexadecanoic acid (90 μg g^{-1}). This acid was not detected in juniper soil, where only pentadecanoic acid was found. The presence of tetradecanoic, pentadecanoic and hexadecanoic acids was also found in juniper litter at lower concentrations than in needles. Similarly to the alkane behavior, a higher concentration of short chain fatty acids was found in pine needles than in juniper leaves; however, unexpectedly, pine litter did not follow this distribution and only heptadecanoic acid was detected in this sample (**Figure 5.2.2.**).

- **Cyclic compounds**

Among cyclic compounds, terpenoids were the most abundant family found in pine and juniper forests. Different monoterpenes, sesquiterpenes, diterpenes and triterpenes including terpenic alcohols, acids, oxides and acetates were found in all analyzed samples (Table 5.2.1); in this work all these compounds are referred to as terpenes. Identification of these compounds was mainly carried out by their I^T values, taking into account that each terpene family showed diagnostic mass spectral features (e.g., the characteristic ions at *m/z* 93 and 136 of monoterpenes, at *m/z* 161 and 204 of sesquiterpenes, and at *m/z* 161 and 272 of diterpenes). Those compounds whose identification was not possible have been assigned as monoterpenes, diterpenes or sesquiterpenes in **Table 5.2.1** considering their typical mass spectra and their retention index.

The **Figure 5.2.6** shows the total concentration (μg g^{-1}) of monoterpenes, sesquiterpenes, diterpenes and triterpenes of needles, litter and soil of both juniper and pine forest. Sesquiterpenes were the most abundant terpenes in all samples, except for soil samples. Pine needles showed the greatest concentration of monoterpenes (864.7 μg g^{-1}), sesquiterpenes (2.0 mg g^{-1}), and diterpenes (1.3 mg g^{-1}). Triterpenes were only detected in low amounts in pine litter (16.7 μg g^{-1}). The lowest concentrations of terpenes were found in soil samples of both juniper (269.9 μg g^{-1}) and pine (7.6 μg g^{-1}).

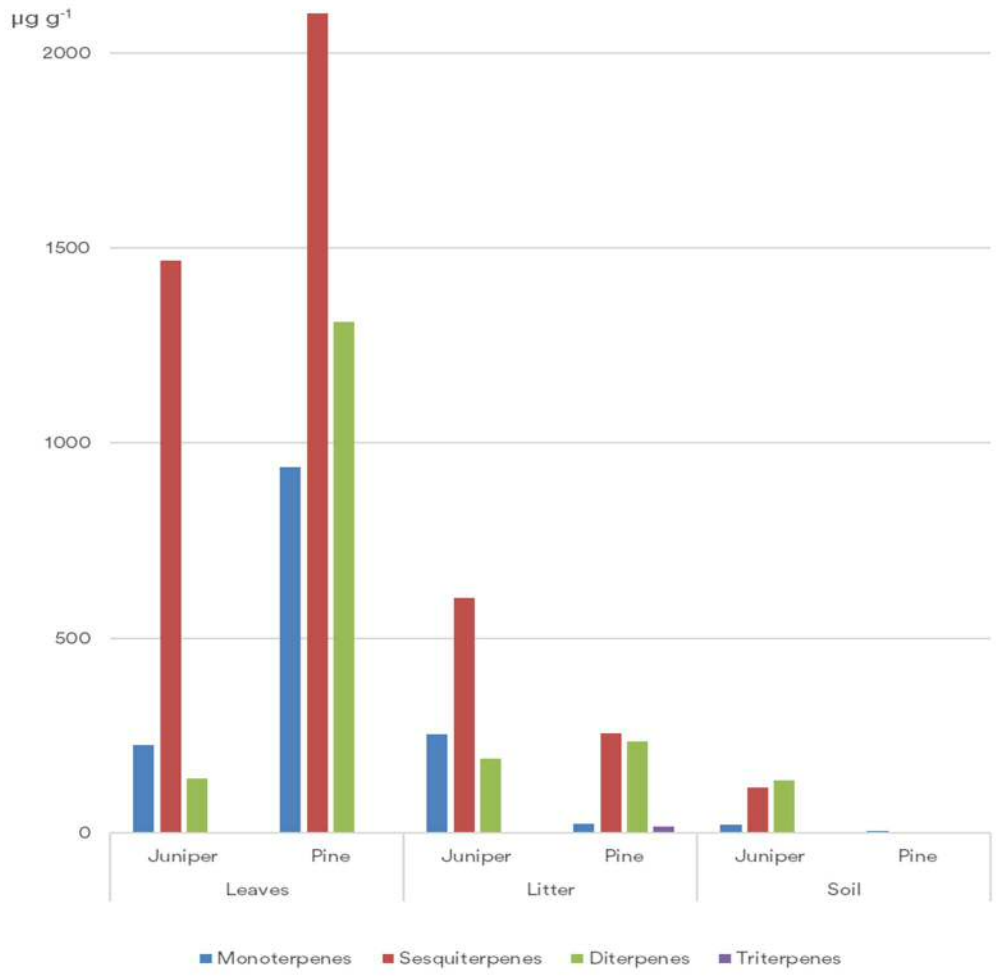

Figure 5.2.6. Total concentration (µg g^{-1}) of monoterpenes, sesquiterpenes, diterpenes and triterpenes in needles, litter and soil samples from juniper and pine forests

Regarding monoterpenes, up to 20 compounds were detected and characterized by their I^T values and mass spectra. Among them, some alcohols such as α-terpineol and verbenol were identified. Pine needles showed the greatest concentrations of monoterpenes; however, the concentrations of these compounds in pine litter and soil were particularly low, even lower than those found in juniper litter and soil. This pattern could be due to that most of this monoterpenes could not be in free forms, but physically entrapped into macromolecular lipid substances or in the soil organomineral matrix. In particular α-thujene was the most abundant monoterpene of pine needles (466.2 µg g^{-1}), followed by α-pinene (168.3 µg g^{-1}). Monoterpenoids such as limonene, sabinene and terpineol were only found in juniper forests, while α-campholene aldehyde and 2-β-pinene were only detected in pine forest.

Pine needles showed the highest concentration of sesquiterpenes, whereas very low concentration of these compounds were found in pine litter and soil. On the contrary, concentrations of sesquiterpenes in juniper soil were higher than those of pine soils, although their concentrations in leaves were

smaller. The same trend has been found in previous work (Almendros et al., 1996); sesquiterpenes had a tendency to be accumulated in soils under juniper forest, whereas small amounts have been found in soils under pine trees. Caryophyllene oxide was the most abundant sesquiterpene of pine needles (370.1 μg g^{-1}), followed by caryophyllene (269.7 μg g^{-1}). Regarding pine litter, *γ*-cadinene, germacrene *D*, caryophyllene oxide and caryophyllene were the most abundant. In pine soils only *γ*-cadinene, δ-cadinene and α-cubebene were detected. Cadinene-related molecules are ubiquitous in trees and they are the main odour constituents of some conifer woods, but also can be metabolites from several fungi (Rowe, 1989). They are also probably involved in polymerization reactions in the soil (Almendros, 1996).

The highest concentration of sesquiterpenes in juniper needles was found for caryophyllene oxide (644.0 μg g^{-1}) and for humulene epoxide (328.9 μg g^{-1}). Juniper litter showed greater concentrations of sesquiterpenes than pine litter; caryophyllene oxide (173.7 μg g^{-1}), humulene epoxide (103.5 μg g^{-1}) and caryophyllene (82.4 μg g^{-1}) were also de most abundant. Other sesquiterpenes such as spathulenol, β-selinene, *γ*-elemene β-bourbene, and *γ*-cadinene among others, were also detected in juniper needles and litter. The presence of these terpenes had been previously found in essential oils of this conifer (Adams, 1998; Pepeljnjak, 2005; Orav, 2010). Junipene isomers were found in juniper soils, these compounds are common in this kind of conifers (El-Sawi, 2007; Rezvani, 2010). Cedrene, a sesquiterpene characteristic of Cupressaceae family (Adams, 2008), was also found in soil under juniper forests.

Pine needles showed the highest concentrations of diterpenes (1.3 mg g^{-1}), followed by pine litter (266.4 μg g^{-1}). Only, low concentrations of diterpenes were found in pine soil (Figure 5.2.6.). While total diterpenes represented the 12% of the terpenoids of juniper needles and 18 % in juniper litter, this percentage was higher in juniper soil (50%). Juniper diterpenes belonged mainly to the chemical families of abietanes and pimaranes (**Figure 5.2.7.**), the latter ones were the most abundant in needles and litter and the abietanes were predominant in soils. Isopimaric acid, pimaradiene isomers and epimanoyl oxide were the pimaranes found in juniper needles, litter and soil; the first one was the most abundant pimarane found in these samples (75, 101 and 50 μg g^{-1}, respectively). Regarding abietanes, abietatriene was detected in the three juniper samples; however, ferruginol I and II were only detected in litter and soil samples, whereas totarol was only found in soil samples. These compounds are considered as diagnostic molecules of juniper forest (Almendros, 1996).

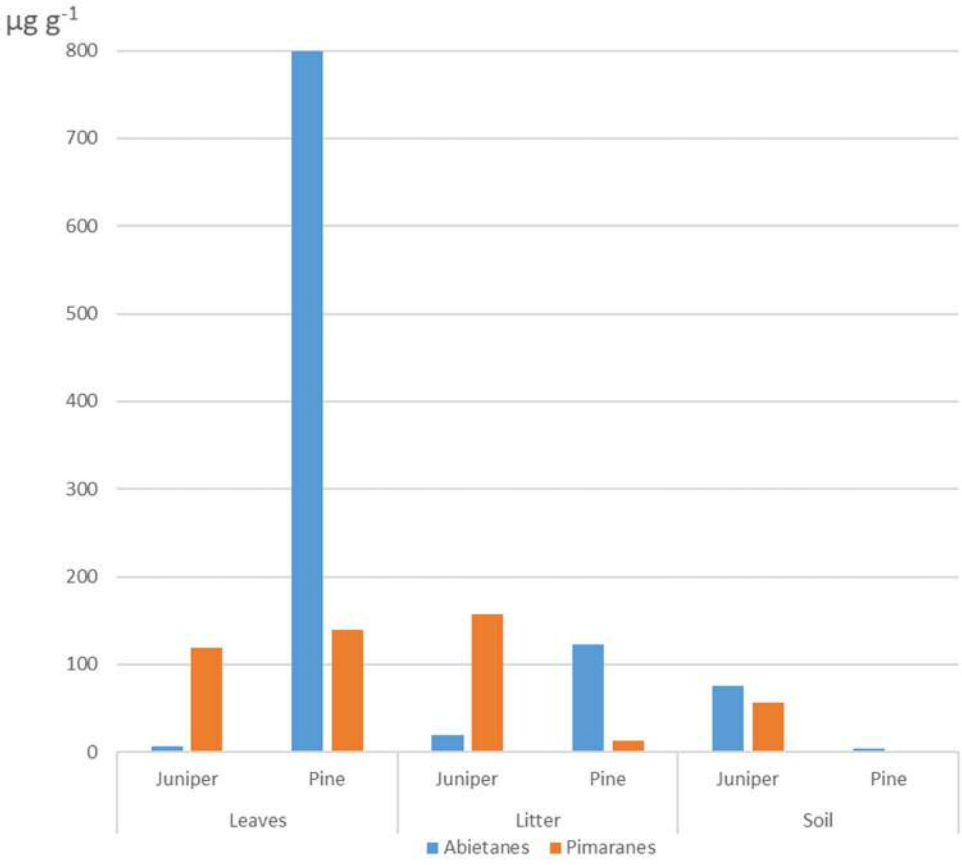

Figure 5.2.7. Diterpene composition (µg g^{-1}) of abietanes and pimaranes in the needles, litter and soil of both juniper and pine forest

The abietane diterpenes were more abundant in pine needles; among them, dehydroabietic acid isomers showed the highest concentrations (375.9 and 354.4 µg g^{-1}), whereas a notable reduction in the concentration of these compounds in litter (45.4 and 71.0 µg g^{-1}) was detected. These molecules have already been described as products from oxidation and thermal rearrangement of methyl levopimarate (Rowe, 1989) and have also been detected in pine soil (Almendros, 1988; Almendros, 1996).

Two triterpenes (friedoolean-6-ene and *α*-amyrin acetate) were also detected in pine litter at relatively low concentrations (7.4 and 9.3 µg g^{-1}, respectively).

Regarding steroids, *β*-sitosterol was the most abundant, and was detected in all samples, except in pine soil.

5.2.3.2. Analysis of polar extracts

Figure 5.2.8 shows the GC-MS profiles of the derivatized methanol extract of needles, litter and soil from juniper (A) and pine (B). Several peaks with characteristic mass spectra compatible with derivatized LMWC structures were detected. Moreover, cyclic acids (such as shikimic, quinic, pimaric and isopimaric acids) and fatty acids (such as hexadecanoic and octadecanoic acids) were also detected.

Regarding LMWC, monosaccharides, disaccharides and polyalcohols were identified. In particular, glucose, fructose and sucrose were detected in all extracts, except from that of pine soil. These carbohydrates are ubiquitous in plants (Assarsso, 1958). The presence of trehalose was also confirmed in these extracts by comparison with the corresponding standard. It is worth to point out that this compound was the only LMWC detected in pine soil.

Linear polyalcohols such as mannitol were detected in these samples; moreover, several cyclic polyalcohols were found. Different free inositols such as *myo*-inositols and methyl-inositols such as pinitol (3-*O*-metil-*D*-*chiro*-inositol), 1-methyl-*muco*-inositol, sequoyitol (5-*O*-methyl-*myo*-inositol) and quebrachitol (2-*O*-metil-L-*chiro*-inositol) were detected in both pine and juniper needles. *Muco*-inositol was also found in leaves of the last species. Presence of pinitol, sequoyitol and *myo*-inositol in pine needles has been previously reported in the literature (Ballou, 1953; Binder, 1984). Moreover, it is known that 1-methyl-*muco*-inositol is ubiquitous in the gymnosperms (Dittrich, 1972) and it probably comes from the epimerization of pinitol. However, Dittrich and Kandler (1972), which did not find this compound in Pinaceae family, stated that it could be a consequence of the absence of the enzyme which produces the conversion of pinitol into methyl-*muco*-inositol in these plants. In this work, 1-methyl-*muco*-inositol was found in pine needles at very low levels. Methyl-inositols are usually secondary metabolites of plants and they are not directly involved in their normal growth but these compounds play an important role in the defence against stressing environmental conditions (Al-Suod, 2017).

Different small peaks with mass spectra compatible with LMWC, mainly inositol derivatives, were also found in needles and litter of both pine and juniper. However, due to coelution problems their identification was not possible.

Table 5.2.2 shows the I^T values and concentrations (μg g^{-1}) of the LMWC and acids quantified in these extracts. The highest concentration of total LMWC was accounted for pine needles (116.3 μg g^{-1}) followed by juniper needles (111.3 μg g^{-1}). The concentration of these compounds was much smaller in litter and soils under both forest species. 1-Methyl-*muco*-inositol was the most abundant LMWC

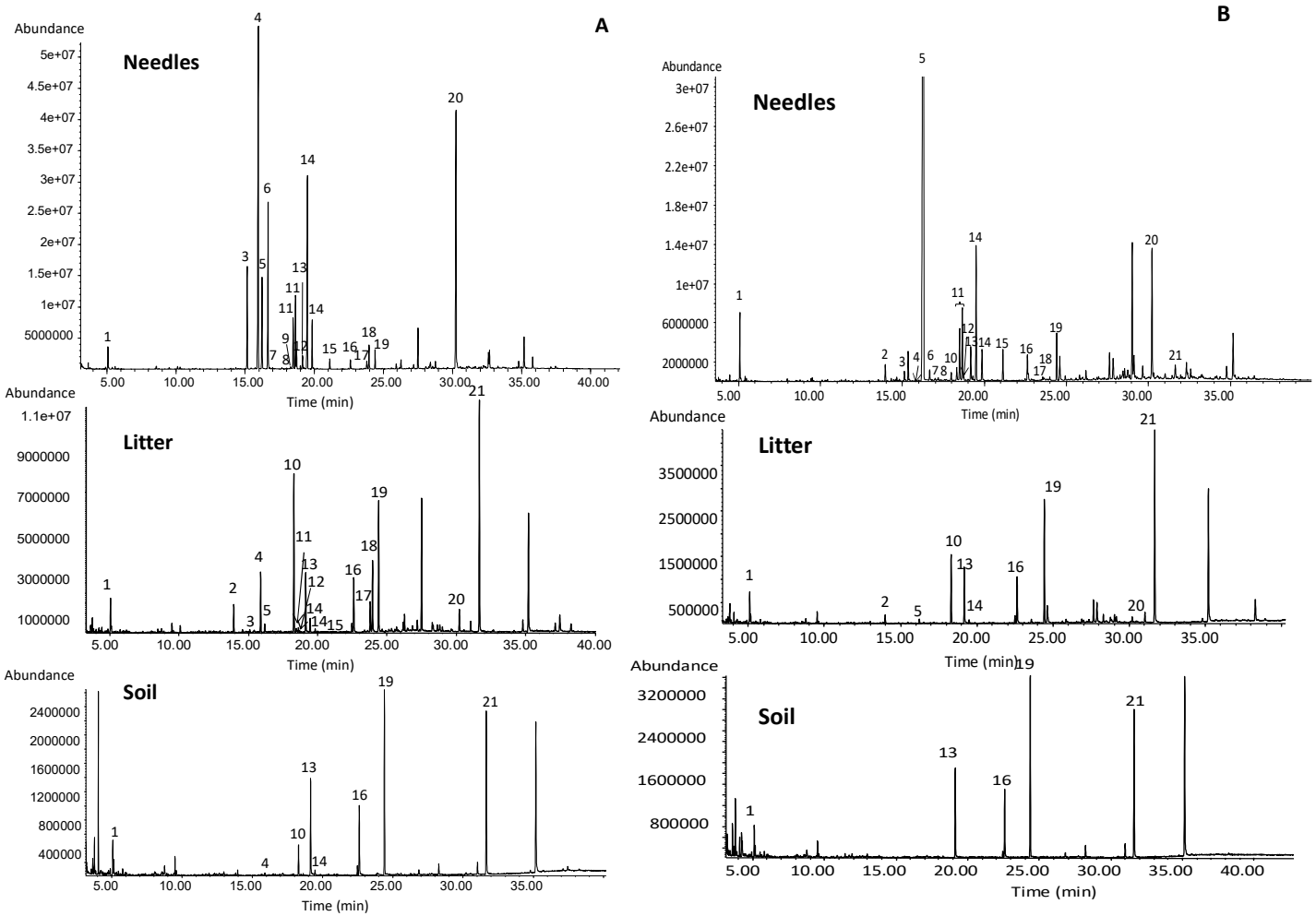

Figure 5.2.8. GC-MS profile of methanol extracts of needles, litter and soil under juniper (A) and pine (B) forests: 1: glycerol; 2: pentitol; 3: shikimic acid; 4: 1-methyl-*muco*-inositol; 5: pinitol; 6: quinic acid; 7: quebrachitol; 8: inositol; 9: *muco*-inositol; 10: mannitol; 11: fructose 1 and 2; 12: *chiro*-inositol + sequoyitol; 13: hexadecanoic acid; 14: glucose *E* and *Z*; 15: *myo*-inositol; 16: octadecanoic acid; 17: pimaric acid; 18: isopimaric acid; 19- phenyl-*β*-D-glucoside (internal standard); 20: sucrose; 21: trehalose.

Table 5.2.2. Concentration of LMWC (μg g^{-1}) in polar extracts of needles, litter and soils of juniper and pine forest.

	I^T	Juniper			Pine		
		Needles	**Litter**	**Soil**	**Needles**	**Litter**	**Soil**
Pentitol	1768	–	0.036 (0.004)*	–	0.45 (0.05)	0.016 (0.001)	–
1-methyl-*muco*-inositol	1867	57.7 (9.9)	0.054 (0.002)	0.0016 (0.0008)	0.0092 (0.0003)	–	–
Pinitol	1886	10.4 (2.1)	0.006 (0.001)	–	108.5 (27.5)	0.0049 (0.0002)	–
Quebrachitol	1914	0.053 (0.006)	–	–	0.013 (0.001)	–	–
Inositol	1960	0.022 (0.006)	–	–	0.019 (0.005)	–	–
***Muco*-inositol**	1976	0.030 (0.001)	–	–	–	–	–
***D*-Mannitol**	1994	0.25 (0.03)	0.234 (0.001)	0.027 (0.001)	0.44 (0.02)	0.116 (0.003)	–
Fructose	2004/2013	8.7 (0.5)	0.0057 (0.0001)	0.0015 (0.0001)	1.6 (0.3)	–	–
Sequoyitol + *chiro*-inositol	2018	1.43 (0.3)	0.0011 (0.0001)	–	0.08 (0.02)	–	–
Glucose	2061/2080	17.3 (0.8)	0.0128 (0.0001)	0.0031 (0.0001)	2.2 (0.3)	0.0048 (0.0004)	–
***Myo*-inositol**	2152	0.064	0.0006 (0.0001)	–	0.33 (0.05)	–	–
Sucrose	2736	15.4 (1.4)	0.022 (0.001)	0.0020 (0.0002)	2.20 (0.07)	0.009 (0.001)	–
Trehalose	2840	tr**	0.24 (0.03)	0.15 (0.02)	0.32 (0.01)	0.234 (0.004)	0.094 (0.003)
Total LMWC		111.05	0.61	0.19	116.28	0.38	0.09

* Standard deviation in parenthesis. ** tr: traces

Table 5.2.3. Concentration of acids (μg g^{-1}) in polar extracts of needles, litter and soils of juniper and pine forest.

	I^T	Juniper			Pine		
		Needles	**Litter**	**Soil**	**Needles**	**Litter**	**Soil**
Shikimic acid	1829	10.67 (1.36)	–	–	0.29 (0.02)	–	–
Tetradecanoic acid	1844	–	–	–	0.08 (0.01)	–	–
Quinic acid	1907	10.63 (2.38)	0.012 (0.001)	–	0.29 (0.01)	–	–
Hexadecanoic acid	2042	0.92 (0.03)	0.092 (0.002)	0.11 (0.01)	0.97 (0.05)	0.11 (0.01)	0.09 (0.01)
Octadecanoic acid	2239	0.12 (0.07)	0.093 (0.003)	0.081 (0.001)	0.76 (0.01)	0.10 (0.01)	0.06 (0.01)
Pimaric acid	2311	0.16 (0.01)	0.051 (0.002)	–	0.11 (0.01)	0.012 (0.001)	–
Isopimaric acid	2321	0.63 (0.08)	0.12 (0.01)	–	0.102 (0.002)	–	–
Eicosanoic acid	2437	–	–	–	–	0.012 (0.002)	–
Docosanoic acid	2637	–	0.015 (0.002)	–	–	0.010 (0.005)	–

detected in juniper needles, followed by glucose and sucrose. Pinitol was also found in relatively high concentrations in this sample (10.4 μg g^{-1}). It is worth to note that while the composition in leaves and litter of non-polar extracts was quite similar, polar extracts of juniper litter showed a LMWC signature more similar to that of the soil. Therefore, mannitol and trehalose were the most abundant LMWC of juniper litter (0.23 and 0.24 μg g^{-1}, respectively) and soil (0.03 and 0.15 μg g^{-1}, respectively). Regarding samples from pine forest, pinitol was the most abundant LMWC of pine needles, its concentration (108.5 μg g^{-1}) being markedly higher than that in juniper needles. As for juniper litter, trehalose and mannitol (0.23 and 0.12 μg g^{-1}, respectively) were the most abundant LMWC found in pine litter and, as previously commented, only low concentrations of trehalose (0.09 μg g^{-1}) were detected in pine soil.

Concentrations (μg g^{-1}) of cyclic and fatty acids found in juniper and pine needles, litter and soil are shown in Table 5.2.3. The highest concentrations of these compounds were found in juniper needles, mainly due to the high contribution of shikimic (10.67 μg g^{-1}) and quinic (10.63 μg g^{-1}) acids. Only hexadecanoic and octadecanoic acids were detected in soil samples. This trend was also observed in the study of fatty acids extracted using the non-polar solvent.

As conclusion, in this work, identification and absolute quantitation of 150 compounds (free lipids, LMWC and acids) has been carried out in needles, litter and soil of juniper and pine forest by GC-MS. To the best of our knowledge, this is the first comparative assessment of these molecular assemblages in the course of its transformation in the soils.

Acknowledgements

This work has been funded by Fundación Ramón Areces and by Ministerio de Economía, Industria y Competitividad (MINECO) of Spain (project AGL2016-80475-R). Authors also thank financial support from the Comunidad Autónoma of Madrid and European funding from FEDER program (project S2013/ABI-3028, AVANSECAL). C.C.C. thanks MINECO for a predoctoral contract and D.M. thanks CSIC, Fondo Social Europeo and Iniciativa de Empleo Juvenil for a contract.

5.3. Comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry for the analysis of polar and non-polar extracts of *Pinus sylvestris* and *Juniperus communis* needles

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5.3.1. Introduction

Pinus sylvestris (pine) and *Juniperus communis* (juniper) are two of the most typical forest of Mediterranean countries. In a previous work, 'lipids' [understood as a complex heterogeneous mixture of a wide variety of compounds soluble in organic solvents; (Dinel, 1990)] and low molecular weight carbohydrate (LMWC) composition of needles, litter and soils of forest of these species was evaluated by gas chromatography coupled to mass spectrometry (GC-MS) to monitor the molecular transformations in the course of the selective biodegradations and accumulation of the soil organic matter fractions (**Section 5.2**). While this technique was enough to evaluate litter and soil composition, very complex chromatographic profiles were observed for needle extracts (both polar and non-polar).

Comprehensive two-dimensional gas chromatography (GC×GC) has become a well-established technique for the analysis of complex mixtures (Tranchida, 2016). In this technique two columns with different stationary phases (such as polar and non-polar) are connected in series through a modulator. The use of two separation mechanisms generally results in a significant increase in peak capacity and the use of a modulation can provide an improvement in sensitivity. Coupling this technique to time-of-flight mass spectrometry (GC×GC-ToF MS) offers additional information for identification of unknown components.

Up to now, the use of GC×GC-ToF MS has been scarcely applied to the analysis of LMWC. Brokl *et al*. (2010) were the first who used this technique for the analysis of carbohydrates (Brokl, 2010). They successfully developed a new method to separate disaccharides of honeys and identified new compounds not previously described in this sample. Marsoll-Val *et al.* (2015) also proposed the use of this technique to determine the presence of α,α-trehalose and other disaccharides in apple and peach (Marsol-Vall, 2015). More recently, separation of a racemic mixture of monosaccharides has been successfully achieved by GC×GC-ToF MS using a β-cyclodextrin column in the first dimension (^{1}D) and a DB-Wax column in the second dimension (^{2}D). However, to the best of our knowledge this technique has not been used for the analysis of LMWC of plants.

The use of GC×GC-ToF MS is also limited for the analysis of non-polar extracts of plant leaves. It has been used to determine both volatile [extracted by solid-phase microextraction (SPME)] and non-volatile [simultaneous distillation-extraction (SDE)] composition of the needles of *Pinu*s spp. grew in Portugal. This technique allowed the tentative identification of around 200 pine components, including terpenes, aldehydes, ketones and esters (Mateus, 2010). Volatiles

emitted by needles of *Juniperus communis* have also been analysed by SPME and GC×GC-ToF MS (Cordero, 2010).

Therefore, in this work GC×GC-ToF MS has been used to characterize both polar and non-polar extracts of *Juniperus communis* and *Pinus silvestrys* needles, paying especially attention to their composition of alkanes, terpenes and low molecular weight carbohydrates. To the best of our knowledge, this is the first time that a simultaneous study of both, polar and non- polar extracts of juniper and pine needles by this technique is done.

5.3.2. Materials and methods

5.3.2.1 Samples

Needles of *Juniperus communis* (juniper) and *Pinus sylvestris* (pine) were collected at El Espinar (Segovia). Twigs with leaves were cut from the plants. Sampling was carried out at three different positions of each forest, separated at least 100 m one from the other. Samples were mixed to obtain representative pools of each species. All the samples were air-dried before the extraction procedure.

5.3.2.2. Standards

Analytical standards of sucrose, trehalose, *muco*-inositol, *chiro*-inositol, *scyllo*-inositol, *myo*-inositol, fructose, glucose, pinitol (3-O-methyl-D-*chiro*-inositol) and phenyl-β-D-glucoside were acquired from Sigma Chemical Co. (St. Louis, US). Sequoyitol (5-O-methyl-*myo*-inositol) was from Extrasynthese (Genay, France) and quebrachitol (2-O-methyl-*chiro*-inositol) from Acros (Geel, Belgium). 1-Methyl-*muco*-inositol was obtained from a honey sample as indicated by Sanz *et al.* (Sanz, 2004).

5.3.2.3. Extraction procedure

Needles were milled with a grinder to obtain a fine and homogeneous powder. All the samples were sieved (< 500 µm) and stored under dry conditions and protected from direct light until analysis.

In order to obtain the non-polar extracts, 0.3 g of leaves were dissolved in 6 mL of dichloromethane. Then samples were ultrasonicated for 15 min in a bath and extracts were filtered using silanized glass wool. Finally, 1 µL was injected in the GC×GC injection port.

Polar extracts were obtained from needles (0.3 g) using methanol (6 mL) as the extraction solvent. Ultrasounds were applied for 30 min in order to promote the extraction of the compounds and then, the samples were left to sit for 30 min at room temperature. Extracts were filtered through Whatman no. 4 paper and centrifuged at 4400 *g* for 5 min. Supernatants were evaporated under vacuum and redissolved in 2 mL of methanol.

5.3.2.4. Derivatization procedure

Prior to GC-MS analysis of polar extracts, a derivatization procedure was carried out as indicated by Sanz *et al.* (Sanz, 2002). Methanolic extracts (0.5 mL) were mixed with 0.1 mL of a 70% ethanolic solution of phenyl-β-D-glucoside (1 mg mL^{-1}) which was used as internal standard. Samples were evaporated under vacuum and treated with 350 µL of 2.5% hydroxylamine chloride in pyridine at 75 °C for 30 min. Then, 350 µL of hexamethyldisilazane (HMDS) and 35 µL of trifluoroacetic acid (TFA) were added and kept at 45°C for 30 min. Samples were centrifuged at 7000 *g* for 5 min at 5°C, and 1 µL of the supernatant was injected onto the GC column. This two-step derivatization procedure (oximation + silylation) of carbohydrates allowed the reduction of signals corresponding to the reducing sugars, giving only two peaks (the syn (*E*) and anti (*Z*) isomers of the oxime) while only a single chromatographic peak was obtained for non-reducing sugars and inositols, corresponding to the *O*-persilylated derivative.

5.3.2.5. GC×GC-ToF MS analysis

The GC×GC-ToF MS analyses were performed on an HP 6890 gas chromatograph (Agilent Technologies) coupled to a Pegasus 4D ToF MS (LECO Corp., St. Joseph, MI, USA), operating in EI mode at 70 eV. A ZB-1 MS column (100% methyl polysiloxane; 30 m x 0.25 mm *i.d.*, 0.25 µm film thickness; Phenomenex, Spain) was used for the first dimension (^{1}D) and a BPX-50 (50% phenyl / 50% methylpolyxiloxane-carborane; 1.7 m x 0.10 i.d., 0.10 µm film thickness; SGE, Melbourne, Australia) was selected for the second dimension (^{2}D). It is worth to point out that ^{1}D column was the same used in 1D GC analyses (Section 5.2). The system was equipped with a secondary oven for independent temperature program of the ^{2}D column. A quad-jet dual-stage modulator was used for cryofocusing of analytes eluting from ^{1}D column with liquid nitrogen.

All experiments were carried out under a constant pressure regime and helium was used as carrier gas (0.9 mL min^{-1}). The injection was carried out in the split mode (split ratio 1:20), at 250

˚C. Temperature programs were for ^{1}D oven: 80 ˚C (0.2 min), at 15 ˚C min^{-1} to 220 ˚C (15 min), at 4 ˚C min^{-1} to 300 ˚C (10 min) and for the ^{2}D oven: 100 ˚C (0.2 min), at 15 ˚C min^{-1} to 240 ˚C (15 min), at 4 ˚C min^{-1} to 300 ˚C (10 min). The modulator was programmed to track the main oven with an off-set of 30 ˚C. The temperature of the interface and ion-source was set at 250 ˚C. Mass spectra were collected in the 40–700 *m/z* range at an acquisition rate of 100 Hz. Data were recorded and processed using the LECO Chroma TOF 4.2 software. Analyte identifications were considered to be tentative when based on experimental MS data and when authentic standards were not available for further confirmation.

5.3.3. Results and discussion

5.3.3.1 Non-polar extracts

Figure 5.3.1. shows the GC×GC contour plot (fragments *m/z* 57, 83, 93 and 129) of the non-polar pine needle extract (A) and juniper needle extract (B). Around 100 analytes were tentatively identified, including alkanes (fragment at *m/z* 57), alcohols (fragment at *m/z* 83), terpenes (fragment at *m/z* 93) and fatty acids (fragment at *m/z* 129). As it can be observed, the GC×GC method clearly improved resolution of these compounds over previous 1D GC methodologies (**Section 5.2**) and several alkanes, fatty acids and alcohols not previously detected by 1D GC were now identified. While only 11 linear alkanes had been previously found in pine needles by 1D GC-MS (Section 5.2), alkanes from C_{12} to C_{33} were identified by GC×GC-MS. Alkanes from dodecane ($C_{12}H_{26}$) to pentatriaconte ($C_{35}H_{72}$) were also detected in juniper needle extract. Similarly, only 4 and 6 fatty acids were detected by 1D GC in pine and juniper needle extracts, respectively; however, fatty acids (FA) from heptanoic acid ($C_7H_{14}O_2$) to docosanoic acid ($C_{22}H_{44}O_2$) were identified by GC×GC-ToF MS. It is worth to note that fatty acids with an even number of carbons more predominant than those with an odd number. Linear alcohols from eicosanol ($C_{20}H_{42}O$) to triacontanol ($C_{30}H_{62}O$), except for henicosanol ($C_{21}H_{44}O$) and docosanol ($C_{22}H_{46}O$), were detected both in pine and juniper needles.

Regarding terpenes, monoterpenes, sesquiterpenes, diterpenes and triterpenes were detected. **Figures 5.3.2. and 5.3.3.** shows the GC×GC-ToF MS contour plots of the monoterpene and the sesquiterpene regions of the non-polar pine (A) and juniper (B) needles extracts, considering the high complexity of these elution zones.

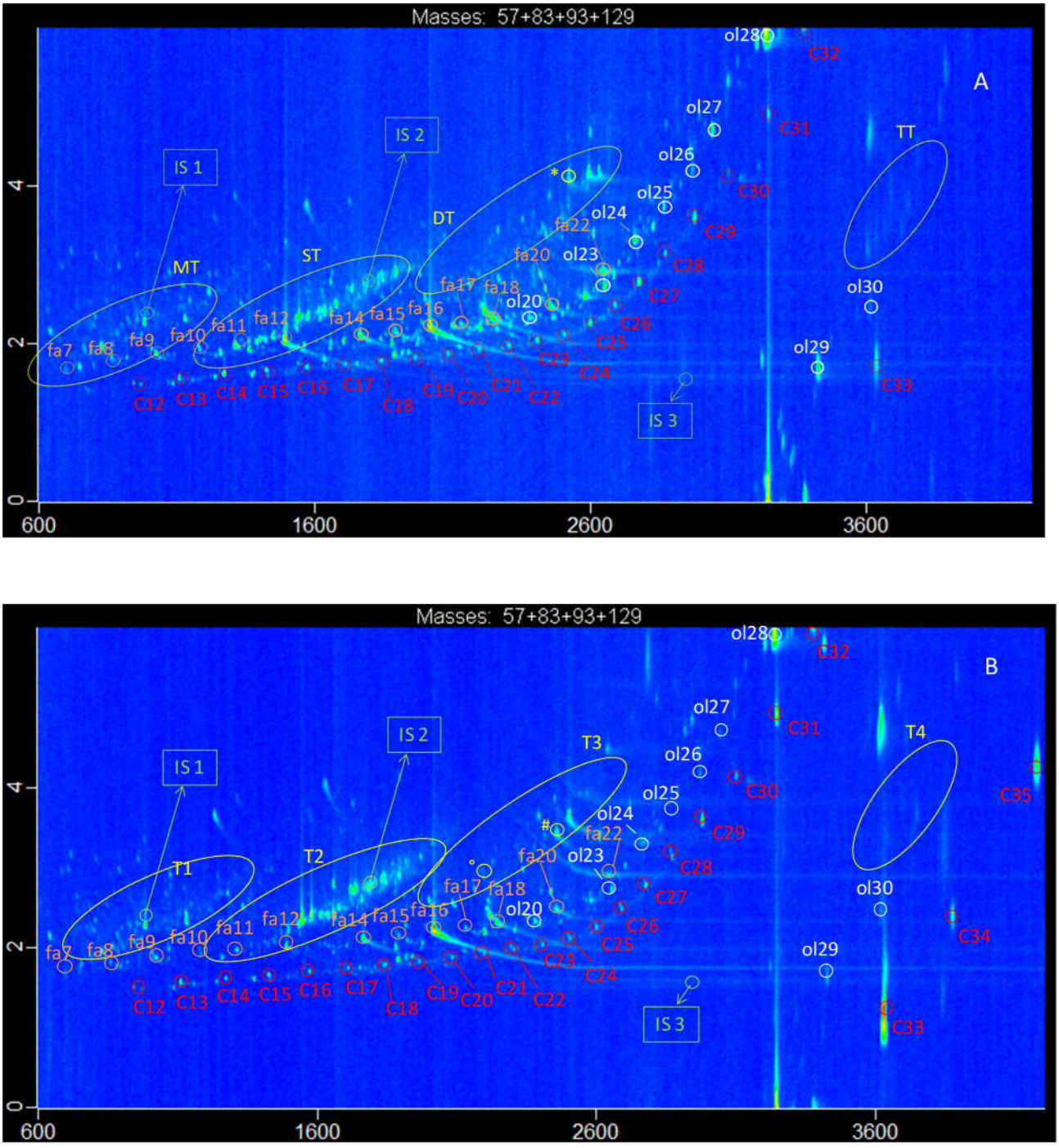

Figure 5.3.1. GC×GC contour plot (fragments *m/z* 57, 83, 93 and 129) of non-polar pine (A) and juniper (B) needles extracts. IS, internal standards: IS1, 1-phenyl-1-butanol; IS2, benzil; IS3, perylene. T, terpenoids regions: MT, monoterpenes; ST, sesquiterpenes; DT, diterpenes (* dehydroabietic acid, ° pimaradiene; # pimaric acid); TT, triterpenes.

Fragments at *m/z* 93 and *m/z* 136 were selected as characteristics of monoterpenes (Figure 5.3.2.). The greatest resolution power of GC×GC together with its enhanced sensitivity, allowed a more accurate identification of these compounds than by 1D GC and the characterization of new monoterpenes. As an example, myrtenal, which coeluted with α-terpineol in 1D GC, could be identified in juniper needles due to its higher retention time in the ^{2}D GC, allowing its separation from α-terpineol.

Sesquiterpenes (**Figure 5.3.3**.; characteristic fragments at *m/z* 161 + 204) was the most complex fraction, mainly in pine needle extract. These compounds were clearly separated by GC×GC from alkanes, acids and alcohols, although some coelutions were still observed among them. As an example of the enhanced resolution provided by GC×GC, calamenene which coeluted with α-muurolene in 1D GC was successfully separated in the second dimension, allowing its identification in pine needle extract. Moreover, another unknown sesquiterpene eluting between α-muurolene and γ-muurolene, which was not detected in 1D, was separated in GC×GC and characterized in both pine and juniper extracts.

5.3.3.2. Polar extracts

In a previous work (**Section 5.2**), some carbohydrates including cyclitols, mono- and disaccharides, were identified by 1D GC in pine and juniper needles. Moreover, different small peaks with mass spectra compatible with LMWC, mainly inositol derivatives, were also found in these samples. However, due to the presence of several coelutions in 1D GC their identification was not possible. **Figure 5.3.4.** shows the GC×GC contour plot (fragments *m/z* 117, 204, 217, 361, 318 and 305) of the previously derivatized polar pine (A) and juniper (B) needle extracts.

Several silylated fatty acids (from dodecanoic acid to octadecanoic acid; with characteristic *m/z* fragment at 117) were detected in both pine and juniper needle extracts. Moreover, other acids, such as shikimic and quinic, previously reported in these species (Cranswick, 1979), were successfully separated in this GC×GC analysis. Regarding resin acids, pimaric, isopimaric, dehydroabietic and 15-hydroxydehydroabietic acid were found in pine needles. These acids, except for dehydroabietic acid, were also detected in juniper needles, according to that reported by Basas-Jaumandreu *et al.* (Basas-Jaumandreu, 2015).

Two different regions, corresponding to monosaccharides (MS) and disaccharides (DS), were clearly distinguished in the GC×GC contour plot.

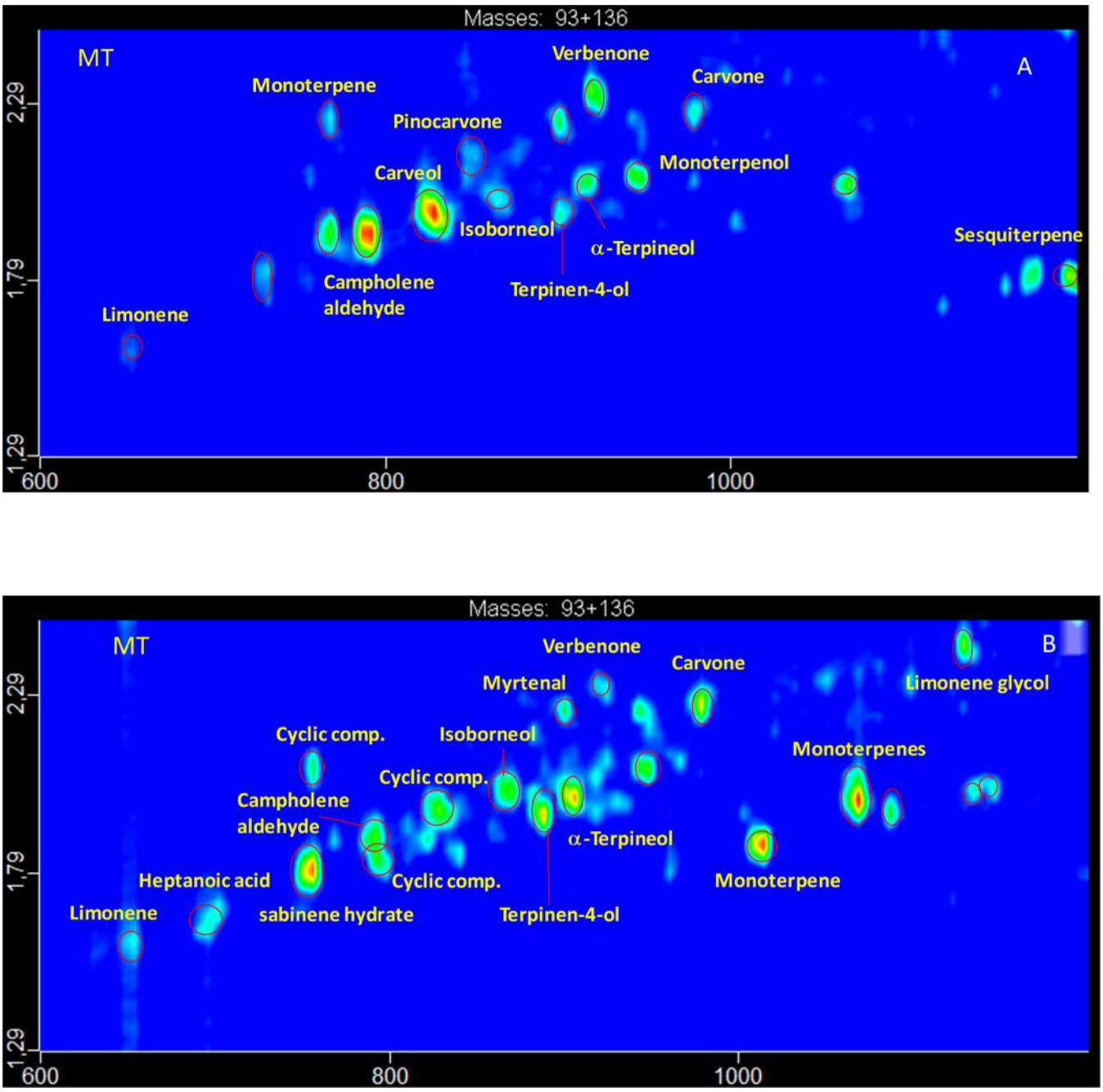

Figure 5.3.2. GC×GC contour plot of the monoterpene region (fragments *m/z* 93 + 136) of non-polar pine (A) and juniper (B) needle extracts.

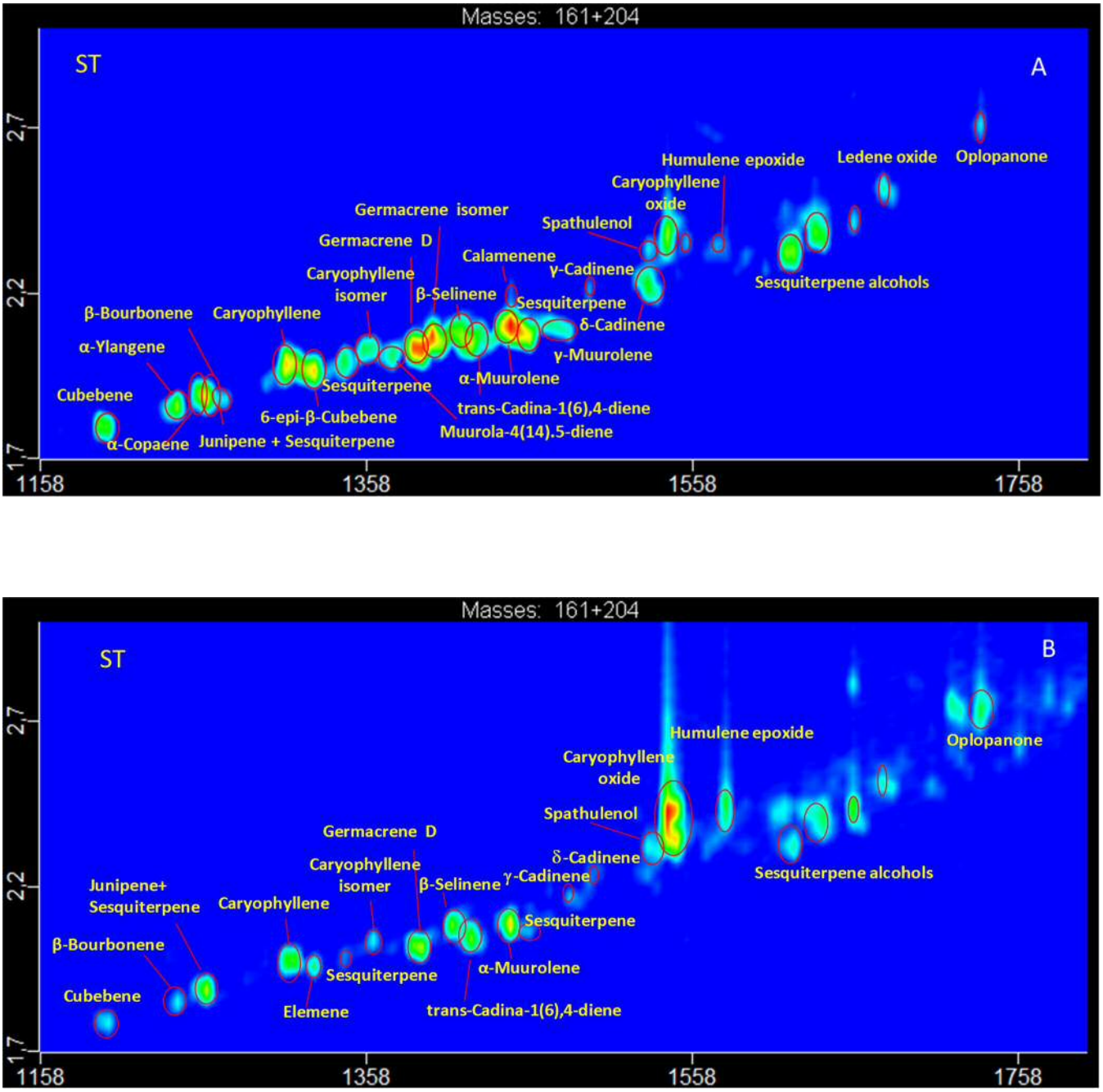

Figure 5.3.3. GC×GC contour plot of the sesquiterpene region (fragments *m/z* 161 + 204) of non-polar pine (A) and juniper (B) needle extracts.

Figure 5.3.5. shows the GC×GC-ToF MS contour plot of the monosaccharide regions. Ions at *m/z* 305 and 318 were selected as characteristic of cyclitols, whereas ions at *m/z* 307 and 319 were characteristic of ketoses and aldoses, respectively. Several pentitols, pentoses and the hexoses glucose, galactose and fructose were found in these extracts. The use of GC×GC-ToF MS allowed the identification of several cyclitols, some of them not previously reported in the literature, which could not be chromatographically resolved in 1D GC. Apart from those free inositols (characteristic fragments at *m/z* 265, 305 and 318) detected in 1D GC (**Section 5.2**; *chiro*- and *myo*-inositol), *scyllo*-inositol and an unknown inositol (peak 11) were detected in both juniper and pine needles. Peak 11 could tentatively assigned as *neo*-inositol by comparison of its retention data with that reported by McDonald *et al.* (McDonald IV, 2012). Moreover, similar to 1D GC analyses, *muco*-inositol was found in the juniper sample.

Several methyl-inositols (characteristic fragments at *m/z* 260, 305 and 318) were also detected in both species (Figure 5.3.5). Regarding pine needles, pinitol was the most abundant LMWC found in this sample; 1-methyl-*muco*-inositol and sequoyitol were also found. Presence of these compounds has been previously reported in *Pinus* sp. (Ballou, 1953; Binder, 1984). Similarly, these methyl-inositols, and according to 1D GC analyses, were detected in the juniper needle extract; both 1-methyl-*muco*-inositol and pinitol were the most abundant. It is worth to note that while sequoyitol and *chiro*-inositol coeluted in 1D GC, they were good resolved in GC×GC analyses. As previously discussed in **Section 5.2**, quebrachitol was also found in both samples. Other compounds assigned as methyl-inositols considering their mass spectra were detected in both juniper needles (peaks 9) and pine needles (peak 16). Peak 9 could be tentatively assigned as 1-methyl-*chiro*-inositol and peak 16 as ononitol (4-methyl-*myo*-inositol) by comparison of their retention times with those reported by Bidden and Haddon (Binder, 1984). Quercitol, a deoxy-inositol previously detected in *Quercus* sp. (Sanz, 2005), was also found in the juniper sample. To the best of our knowledge, this is the first time that the presence of these compounds is reported in these plants.

Figure 5.3.6 shows the GC×GC-ToF MS contour plot of the disaccharide regions (characteristic ions at *m/z* 204, 217, 259 and 361) of pine (A) and juniper (B) needles. A very complex chromatographic profile was observed; many carbohydrates from different classes were detected. As it could be seen in the Figure, organized chromatograms depending on the carbohydrate structure (the well-known roof tile effect) were observed.

Sucrose and trehalose were identified by comparison of their retention time and mass spectra with those of the corresponding standards; other disaccharides with structure of hexosyl-

hexoses were also detected (peaks labelled as 3); their identification was not possible due to the absence of commercial standards. Different peaks (labelled as 4) with characteristic ions at *m/z* 259 and 349, typical of pentosyl-hexoses were found. Moreover, several glycosyl-cyclitols (peaks 5 and 6) were detected. Mass spectra of these compounds were characterized by *m/z* ions of both glycosidic (*m/z* 191, 204 and 217) and cyclitol (*m/z* 265, 305 and 318) moieties and other fragments related to glycosidic linkages (*m/z* 361 with low abundance). Compounds with mass spectra characterized by ion at *m/z* 375 and a high ratio of ions at *m/z* 133/129 were assigned as glycosyl-methyl-inositols (as previously indicated in section 3.2), whereas more retained compounds with low abundance of fragment at *m/z* 133 and absence of ion at *m/z* 375 were tentatively identified as glycosyl-inositols. Several compounds (peaks labelled as 7) with characteristic *m/z* ions at 331 and 361, eluting at generally high retention times in ^{2}D (between 3 and 5 min), were detected. Compounds with these characteristic fragments have been previously detected in *Rhodiola rosea* (**Section 2.4**) and assigned as phenylalkanoids glycosides (PAG) with different aglycons. These compounds could also be present in pine and juniper needles. Due to the absence of commercial standards, these compounds could not be identified. Further studies to identify main peaks present in the samples would be conducted obtaining some standards either by synthesis reactions or by extraction from natural sources.

To the best of our knowledge, this is the first time that these LMWC have been detected in these samples.

This preliminary study represents a very promising approach for the characterization of the very complex profiles obtained for polar and non polar juniper and pine needle extracts.

Moreover, and considering the multiple coelutions observed in 1D GC, the enhanced resolution and sensitivity provided by the GC×GC-ToF MS methodology would be useful for the quantitation of targeted compounds, giving to more accurate quantitative results.

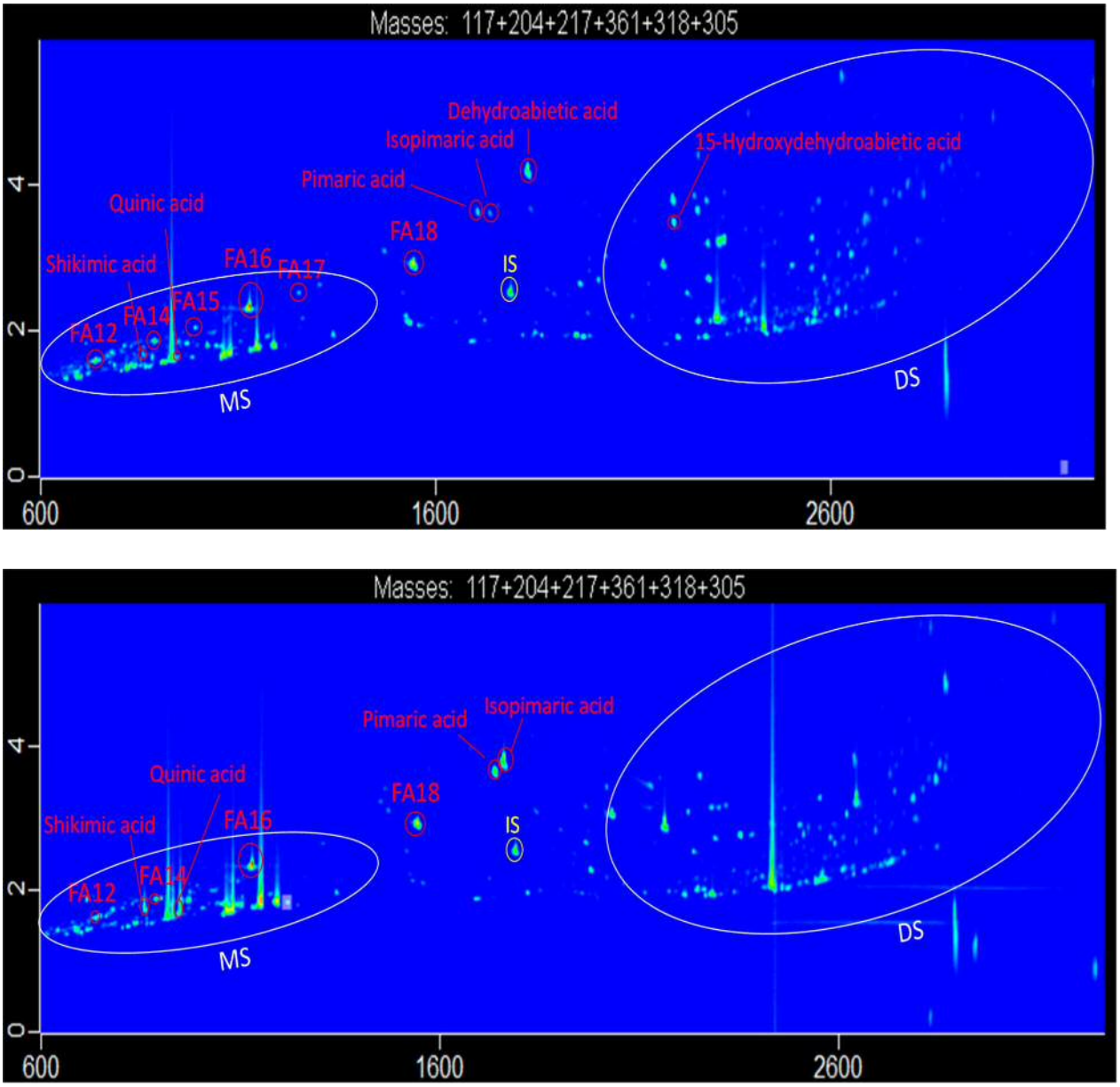

Figure 5.3.4. GC×GC contour plot (fragments *m/z* 117, 204, 217, 361, 305 and 318) of polar pine (A) and juniper (B) needles extracts. IS, phenyl-β-D-glucoside (internal standard); MS, monosaccharide regions; DS, disaccharide region; FA, silylated fatty acids.

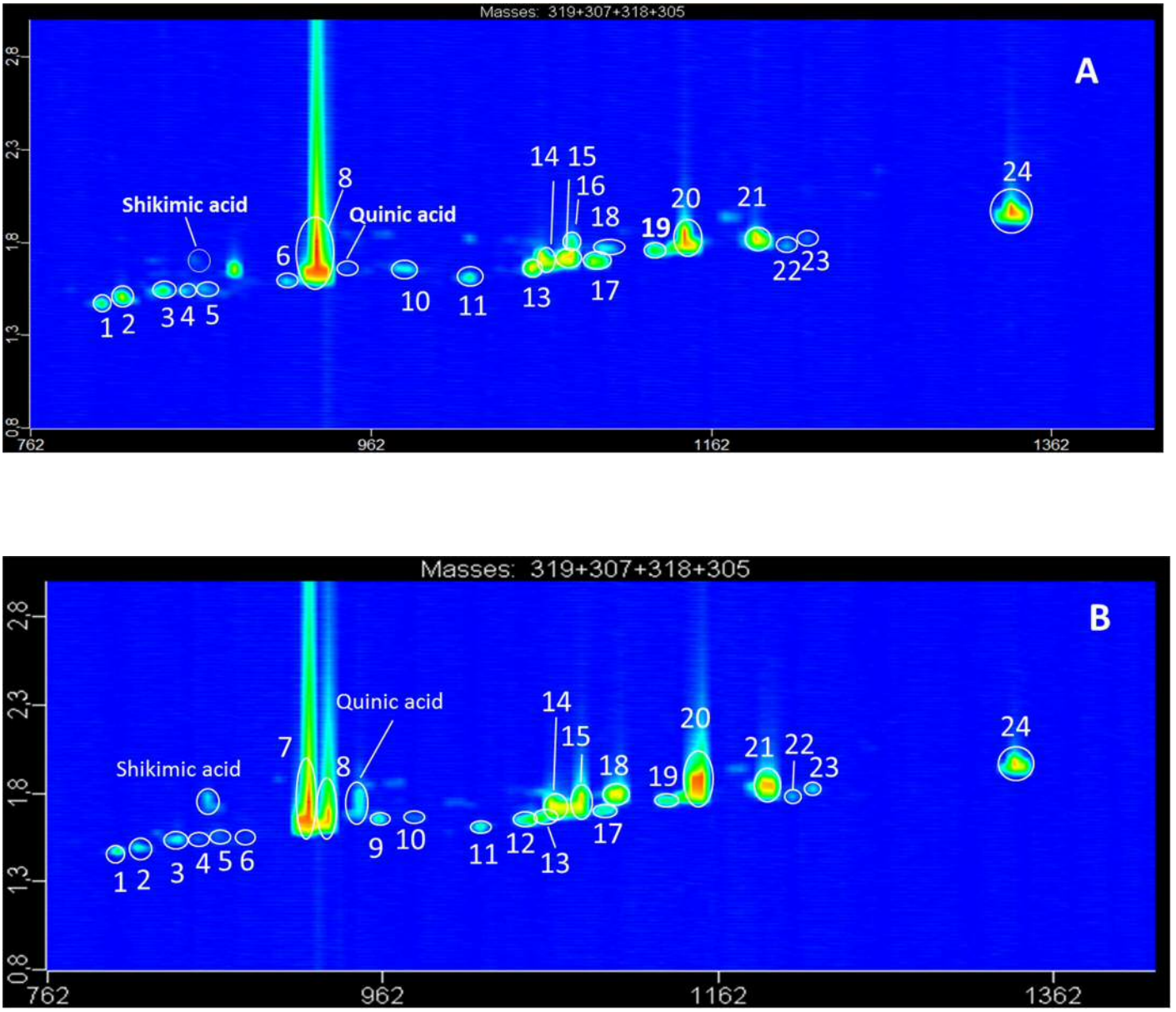

Figure 5.3.5. GC×GC contour plot (fragments *m/z* 305, 307, 318 and 319) of monosaccharide (MS) eluting region of polar pine (A) and juniper (B) needles extracts. 1, 2 and 3. Pentitols; 4 and 5. Pentoses; 6. Quercitol; 7. 1-Methyl-*muco*-inositol; 8. Pinitol; 9. Methyl-inositol; 10. Quebrachitol; 11. Inositol; 12. *muco*-Inositol; 13. Mannitol; 14. Fructose 1; 15- Fructose 2; 16. Methyl-inositol; 17. *chiro*-Inositol; 18. Sequoyitol; 19. Galactose *E*; 20. Glucose *E*; 21. Galactose *Z* + Glucose *Z*; 22. *scyllo*-Inositol; 23. Polyalcohol; 24. *myo*-Inositol.

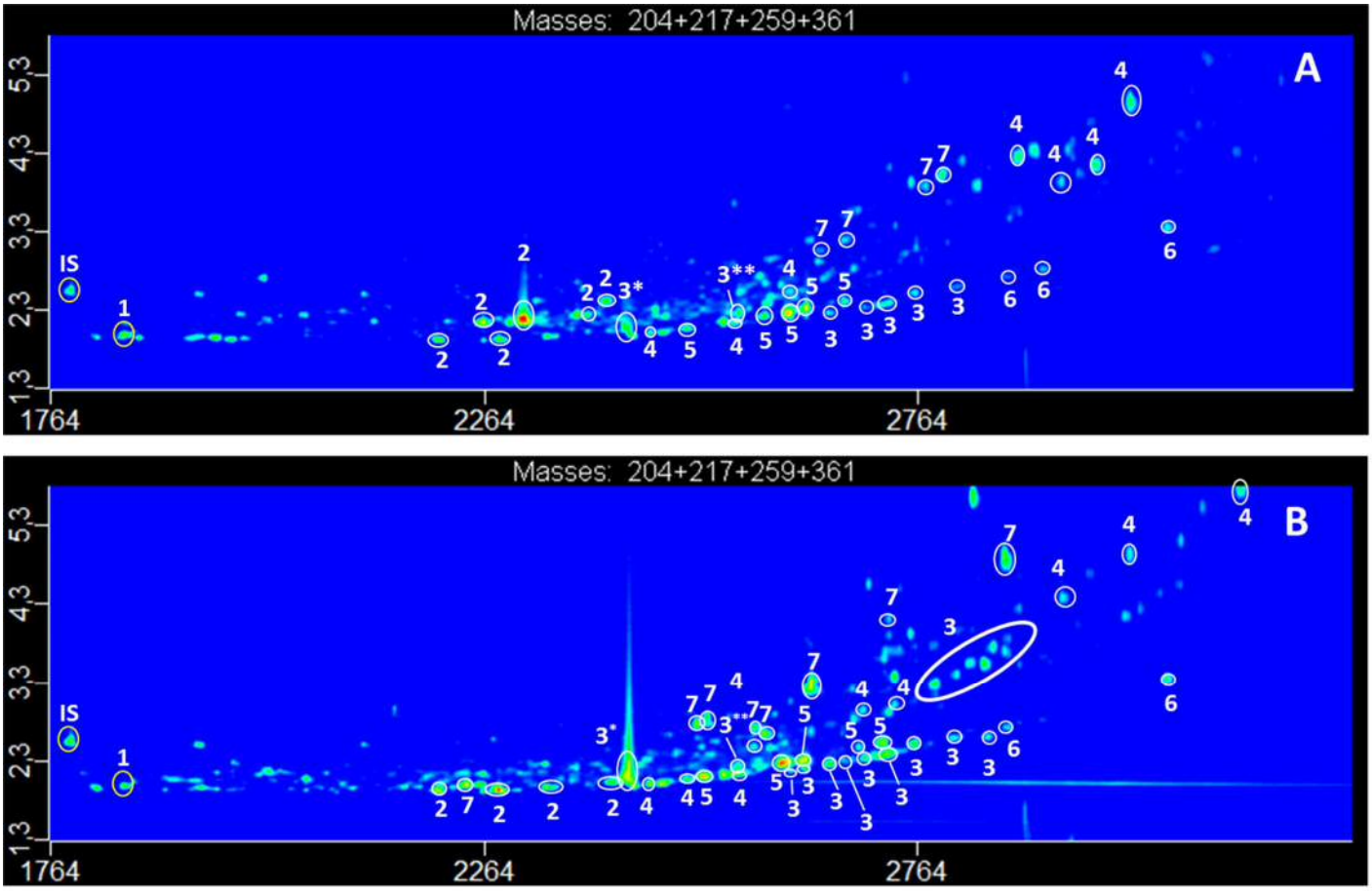

Figure 5.3.6. GC×GC contour plot (fragments *m/z* 204, 217, 259 and 361) of disaccharide (DS) eluting region of polar pine (A) and juniper (B) needles extracts. 1. Glycosyl-glycerol; 2. Glycosides; 3. Hexosyl-hexoses (*Sucrose; ** Trehalose); 4. Pentosyl-glucoses; 5. Glycosyl-methyl-inositols; 6. Glycosyl-inositols; 7.Glycosides with a characteristic ion at *m/z* 331.

Acknowledgements

This work has been funded by Fundación Ramón Areces and by Ministerio de Economía, Industria y Competitividad (MINECO) of Spain (project AGL2016-80475-R). Authors also thank financial support from the Comunidad Autónoma of Madrid and European funding from FEDER program (project S2013/ABI-3028, AVANSECAL). C.C.C. thanks MINECO for a predoctoral contract and D.M. thanks CSIC, Fondo Social Europeo and Iniciativa de Empelo Juvenil for a contract.

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6. Discusión integradora

6. Discusión integradora

En esta Memoria se han abordado diversos problemas en los campos alimentario y ambiental que precisan de la separación y caracterización precisas de los compuestos presentes en mezclas complejas empleando GC-Q MS y GC×GC-ToF MS. Se ha trabajado en el análisis de compuestos de muy distinta naturaleza (carbohidratos, fenilalcanoides, terpenos, ácidos grasos, alcoholes, toxinas, plastificantes…), la mayoría de ellos de carácter semi-volátil o de volatilidad baja. Se podría pensar que estas técnicas no son las indicadas para el análisis de algunos de estos compuestos, ya que la volatilidad y estabilidad térmica de los analitos investogados son requisitos imprescindibles para el uso de la GC. Sin embargo, el elevado poder de resolución de la GC, la gran sensibilidad y capacidad de identificación derivada del acoplamiento de esta técnica a la MS, y el desarrollo de métodos de derivatización para los compuestos que así lo requerían, han permitido dar respuesta de forma eficaz a los problemas planteados, contribuyendo, de esta manera, a aumentar el conocimiento actual en estos campos de estudio.

A lo largo de este trabajo se ha prestado también especial atención a la etapa de preparación de muestra. En este campo, los esfuerzos se han orientado no sólo a la puesta a punto de los métodos de derivatización antes mencionados, sino que también se han centrado en la optimización de procesos de extracción selectiva de los compuestos de interés. Incluso en la actualidad, la SLE suele ser la técnica de elección para la obtención de la mayoría de los compuestos investigados en este trabajo. Este hecho resulta en protocolos de tratamiento de muestra tediosos, muy manipulativos y con rendimientos no siempre satisfactorios. Por tanto, la búsqueda de técnicas alternativas de extracción más rápidas, eficaces, selectivas y viables desde el punto de vista económico resulta de gran interés. En relación con los estudios abordados en esta Tesis, la MAE demostró ser una técnica apropiada, ventajosa frente a la SLE y de gran potencial para la obtención de extractos enriquecidos en carbohidratos bioactivos (en concreto, glicosil-ciclitoles y α-GOS) al permitir la extracción selectiva de estos compuestos de matrices vegetales (judía munga), en el sentido de que posibilitaba (i) la eliminación de carbohidratos no bioactivos que, por su similitud estructural, coeluyen con los compuestos de interés durante su análisis por GC-Q MS; y (ii) la obtención de extractos enriquecidos en carbohidratos bioactivos, como potenciales ingredientes funcionales que permitan promover la salud del ser humano.

En cuanto al análisis de mezclas complejas de carbohidratos de bajo peso molecular, generalmente compuestas por un gran número de isómeros en distintas proporciones, la GC-Q

MS ha permitido la identificación y caracterización, por primera vez, de algunos de estos compuestos en alimentos (Sección 3.2, 3.3. y 3.4), plantas (Sección 3.2, 3.4, 5.2 y 5.3) y suelos (Sección 5.2). Las reglas que se han establecido para relacionar los datos de GC-MS y la estructura química de estos compuestos han permitido la caracterización de glicosil-ciclitoles no antes descritos, diferenciándose entre glicosil-inositoles y glicosil-metil-inositoles, en legumbres (garbanzo, judía adzuki y judía munga), hojas de plantas como coriaria, pino y enebro, y en hojarasca y suelo de estas dos últimas especies. Es de destacar también el gran potencial de la GC×GC-ToF MS para la determinación de estos compuestos, técnica muy poco empleada hasta ahora para el análisis de carbohidratos. Así, se ha descrito por primera vez la presencia de diversas hexosil-hexosas, pentosil-hexosas, heptosas y heptulosas, inositoles, metil-inositoles y/o glicosil-ciclitoles en plantas como la rhodiola, el pino y el enebro. Esta técnica multidimensional ha permitido también realizar un estudio exhaustivo sobre la composición en otros compuestos bioactivos, como son los glicósidos de fenilalcanoides, en suplementos alimenticios basado en extractos de raíz de *Rhodiola rosea*.

El control de la seguridad alimentaria es un tema de gran interés y actualidad por su repercusión e implicación social. Se trata de un tema amplio y variado y que, entre otros muchos aspectos, incluye la necesidad marcada por la legislación vigente de caracterizar los compuestos que pueden migrar desde los plásticos aceptados como FCM a los alimentos con los que entran en contacto. La complejidad de las formulaciones de estos materiales, cuya composición precisa suele ser desconocida, hace imprescindible el uso de técnicas de análisis instrumental capaces de proporcionar una elevada capacidad de resolución entre los componentes lixiviados y una adecuada sensibilidad, que haga posible la detección de algunos de los analitos tóxicos minoritarios que pueden estar presentes en estas mezclas complejas. En este trabajo, la GC×GC-ToF MS ha demostrado ser una herramienta muy útil para el análisis y caracterización cualitativa de la fracción semi-volatil presente en lixiviados obtenidos al tratar distintos envases de polipropileno empleados por los consumidores para la conservación de alimentos con simulantes alimentarios de distinta naturaleza en condiciones de uso prolongado (Sección 4).

En cuanto al análisis de los llamados “lípidos” del suelo (Sección 5.2 y 5.3), que incluyen terpenos, ácidos grasos, alcoholes, cetonas…, la GC-Q MS ha permitido llevar a cabo el análisis cuali- y cuantitativo de estos compuestos en bosques de pino y enebro, pudiéndose establecer relaciones con el proceso de transformación de estos compuestos desde la planta hasta su incorporación al suelo. Sin embargo, dada la complejidad de las muestras (sobre todo en el caso de las hojas), las coeluciones entre los compuestos de interés eran elevadas, dificultando su análisis. Por tanto, se planteó el empleo de la GC×GC-ToF MS. La mayor resolución

proporcionada por esta técnica permitió llevar a cabo identificaciones más precisas al resolver algunas de las coeluciones más relevantes detectadas con GC-QMS. El empleo de GC×GC-ToF MS para el análisis de las hojas, hojarasca y suelo de los bosques de pino y enebro abre nuevas vías de aplicación, tanto desde un punto de vista cualitativo como cuantitativo.

Los resultados obtenidos en esta Tesis Doctoral abren nuevas vías para la obtención de nuevos ingredientes funcionales, para el control de la seguridad de los alimentos y para el estudio de la biotransformación de los constituyentes del suelo. Además, los métodos desarrollados mediante GC-QMS y GC×GC-ToF MS pueden ser de aplicabilidad general a otras matrices.

7. Conclusiones / Conclusions

7. Conclusiones

1. Se han establecido relaciones entre las estructuras químicas de los derivados sililados de glicosil-ciclitoles y sus respectivos datos de GC-Q MS que han permitido la identificación/caracterización de diferentes compuestos no previamente descritos en muestras vegetales tales como garbazo, judía adzuki, coriaria y mungo verde.

2. Se ha propuesto un nuevo método de MAE para la extracción selectiva de carbohidratos bioactivos de bajo peso molecular (inositoles y prebióticos) de mungo verde (*Vigna radiata*), que, una vez optimizado, ha proporcionado un mayor rendimiento de extracción que el procedimiento convencional. Este procedimiento se considera una alternativa valiosa para su aplicación a la obtención de ingredientes bioactivos de fuentes naturales.

3. Se ha puesto a punto, por primera vez, metodologías de análisis basadas en GC-QMS y GC×GC-ToF MS para el análisis de carbohidratos de bajo peso molecular y glicósidos de fenilalcanoides en extractos de rhodiola (*Rhodiola rosea).* La mejora en la resolución aportada por estas técnicas, en combinación con los datos proporcionados por la MS, ha permitido la identificación/caracterización de nuevos compuestos pertenecientes a estas dos familias.

4. El método de GC×GC-ToF MS optimizado para el análisis de lixiviados de envases alimentarios ha permitido la caracterización exhaustiva de fracción (semi-)volátil de analitos que migran desde estos FCM a diferentes simulantes alimentarios, permitiendo la identificación de un total de 109 compuestos de distinta naturaleza, muchos de ellos no contemplados en la legislación vigente.

5. Se ha llevado a cabo la caracterización cuali- y cuantitativa de lípidos libres y carbohidratos de bajo peso molecular en hojas, hojarasca y suelos de pinos y enebros mediante GC-Q MS. La comparación de los patrones obtenidos para estos compuestos en cada uno de estos extractos ha permitido identificar el patrón de trasformación de estas sustancias a lo largo de su proceso de incorporación a los suelos.

6. La aplicación de GC×GC-ToF MS para el análisis de lípidos libres y carbohidratos de bajo peso molecular en las hojas de pino y enebro ha puesto de manifiesto el potencial de esta técnica para la caracterización exhaustiva de estos complejos naturales.

7. De manera genérica, se concluye que los trabajos desarrollados en el marco de esta Tesis Doctoral representan un avance en el estado general de los conocimientos en relación con las técnicas cromatográficas multidimensionales (en particular, GC-Q MS y GC×GC-ToF MS) al proponer soluciones a problemas dentro de los campos alimentario y ambiental.

7. Conclusions

1. The relationship between the chemical structure of trimethylsilyl derivatives of glycosyl-cyclitols and their respective GC-Q MS data have been established. These novel data allowed the identification/characterization of different compounds belonging to this chemical family not previously described in vegetable samples such as chickpea, adzuki bean, coriaria and mung bean.

2. A new method based on MAE has been optimised to allow the selective extraction of low molecular weight bioactive carbohydrates (inositol and prebiotic) from mung bean (*Vigna radiata*). The proposed methodology provided higher extraction yield than the conventional procedure and was considered a valuable alternative to obtain bioactive ingredients from natural sources.

3. The feasibility of GC-QMS and GC×GC-ToF MS have been explored for the first time for the analysis of low molecular weight carbohydrates and phenylalkanoid glycosides in rhodiola (*Rhodiola rosea*) extracts. The enhanced resolution power provided by these analytical techniques resulted in the identification/characterization of several new compounds belonging to these two chemical families.

4. GC×GC-ToF MS has also been used for the exhaustive characterization of the (semi-)volatile fraction of compounds able to migrate from polypropylene food containers into selected food simulants. The optimised methodology allowed the either positive or tentative identification of a total of 109 compounds of different nature, a number of them not included in the current legislation.

5. A comprehensive qualitative and quantitative characterization of free lipids and low molecular weight carbohydrates present extracts of pines and juniper leaves, litter and soils has been performed by GC-Q MS. The comparison of the patterns observed for

these compounds in the different evaluated extracts allowed to monitor their molecular transformation in the course of selective biodegradation and accumulation into soils.

6. The potential of GC×GC-ToF MS to unravel the composition of complex natural mixtures has been illustrated through exhaustive characterization of the free lipids and low molecular weight carbohydrates present in pine and juniper leaves.

7. Altogether, it is concluded that the several studies summarised in this PhD book contribute to the general state-of-knowledge regarding the use of multidimensional chromatographic techniques (in particular, GC-Q MS and GC×GC-ToF MS) regarding their application to food and environmental analysis by proposing successful solutions to selected relevant application problems.

8. Anexos

Anexo 1

Fractionation of Food Bioactive Oligosaccharides

F. J. Moreno, C. Carrero-Carralero, O. Hernández-Hernández, M. L. Sanz

Food Oligosaccharides: Production, Analysis and Bioactivity, First Edition.

JohnWiley & Sons, Ltd.

Supplementary Material

15 Fractionation of Food Bioactive Oligosaccharides

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15.1 Introduction

As indicated in previous chapters, bioactive carbohydrates, both naturally occurring and synthesized, consist of mixtures with different degrees of polymerization (DP), glycosidic linkages or monosaccharide unit composition. Moreover, those present in natural products can be part of complex samples constituted by other compounds including lipids, proteins, and so forth (Sanz and Martinez-Castro 2007).

Fractionation and purification procedures are required for both oligosaccharide production and analysis. Regarding the production of bioactive oligosaccharides, the removal of mono- and disaccharides fractions could be required to evaluate their functional properties (e.g. *in vitro* prebiotic activity), to obtain enriched bioactive fractions or to use them as food ingredients in specialized products for individuals with different disorders (diabetes, lactose intolerance, etc.), as well as in low calorie foods with a reduction of some mono- and disaccharides.

Fractionation of oligosaccharides based on their glycosidic linkages or monosaccharide composition can also be crucial for the elucidation of carbohydrate structures, and consequently, to gain insight into the structure/function relationship. Although analytical techniques have noticeably advanced over the past few years, chromatographic, spectrometric or spectroscopic tools are still not sufficient to achieve an exhaustive characterization of oligosaccharides present in complex mixtures.

The most commonly used purification steps are centrifugation, precipitation, and / or filtration. These wide-ranging procedures are commented in each corresponding chapter and they will not be discussed here since this chapter will focus on effective tools to fractionate specific bioactive oligosaccharides from complex carbohydrate mixtures. Nevertheless, some exceptions will be made, as could be the case of the continuous enzymatic production of galactooligosaccharides (GOS) and their separation from the recycled enzyme or the fractionation of xylooligosaccharides (XOS) from biomass, both accomplished by membrane filtration processes (section 15.2).

Fractionation of oligosaccharides is not a straightforward task due to the structural complexity, which also includes similarities in carbohydrate structures. The search for fractionation techniques that obtain high selectivity and efficiency, using small solvent volumes, environmentally friendly and inexpensive, is one of the main aims of both researchers and industries.

Food Oligosaccharides: Production, Analysis and Bioactivity, First Edition. Edited by Dr. F. Javier Moreno and Dr. María Luz Sanz.

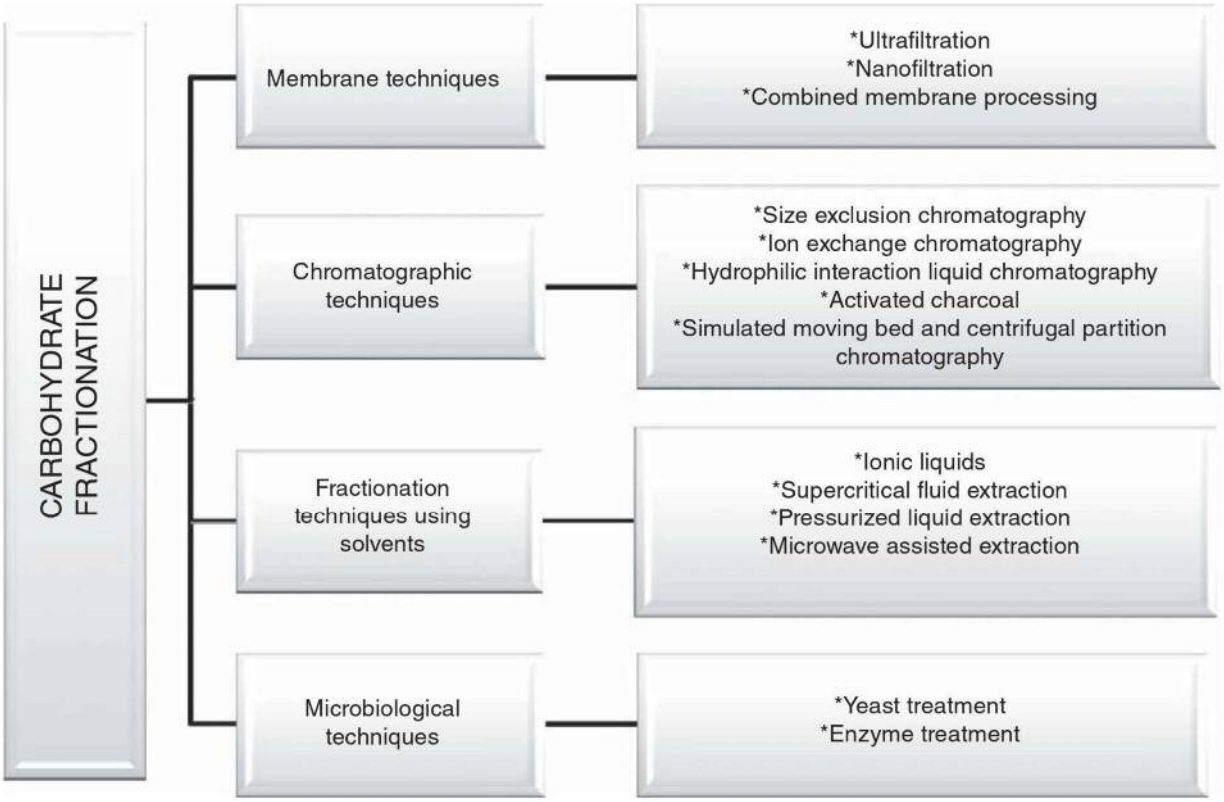

Figure 15.1 Scheme of the techniques used for the fractionation of food oligosaccharides.

This chapter deals with the different procedures used for the fractionation of bioactive carbohydrates for either producing functional ingredients or preparing samples for further analysis. As summarized in Figure 15.1, this chapter will cover not only advances in traditional methods but also novel techniques with potential for use in this field.

15.2 Membrane techniques

Membrane separation processes are normally driven by a pressure gradient in which the membrane fractionates components of a liquid mixture as a function of their solvated size and structure. Microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) are the standard technologies employed for purification and/or fractionation of bioactive components (Akin *et al.* 2012), differing mainly in the membrane pore size and operating pressure. Parameters such as membrane type, temperature, pressure, sample pretreatment, stirring, concentration and ionic environment influence the membrane filtration process. The industrial membrane configuration is usually cross-flow filtration, which means that the solution to be filtered is flowing across the membrane surface at a determined velocity while the filtrate is going through the membrane. Nevertheless, other operational modes, such as dead-end membrane filtration, as well as free and immobilized enzymes in a membrane reactor (in the case of enzymatically produced oligosaccharides) can also be used for the fractionation of carbohydrate mixtures (Pinelo *et al.* 2009). Membrane-based techniques can be considered as the most technically and economically feasible downstream strategy for the industrial manufacture of enzymatically modified oligosaccharides (Pinelo *et al.* 2009). Among their advantages over chromatographic purification techniques (section 15.3) are low energy requirements and operational complexity (Goulas *et al.* 2003).

Among the different membrane techniques, UF and NF processes are the most widely used to purify, concentrate and fractionate carbohydrates. Likewise, both types of membrane processes may also be used in a series to improve performance. Table 15.1 summarizes some of the most recent membrane-based applications for the fractionation of

Table 15.1 Some applications of membrane techniques for the concentration and purification of food bioactive oligosaccharides.

Target oligosaccharides	Starting substrates	Membrane technique	Membrane system	Operating conditions				Reference
				Effective area (cm^2)	T (°C)	P (bar)[a]	Permeate flux (L/h per m^2)[b]	
Lactulose	Lactulose syrup produced by the alkali isomerization of lactose	NF	Thin-film composite spiral-wound NF membrane module (NF2A-2540 MIL)	12 000	30	6–26	10–120	Zhang *et al.* (2011)
GOS	Enzymatic synthesis from lactose buffered solutions (continuous process)	UF	Laboratory-scale reactor system with a regenerated cellulose membrane (NMWCO 10 kDa)	50	45	0.75–2.75	27.75–47.35	Chockchaisawasdee *et al.* (2005)
GOS	Commercial mixture of GOS containing digestible mono- and disaccharides	NF	Stirred cell equipped with a flat sheet cellulose acetate membrane, NF-CA-50 (50% rejection of NaCl), and a thin film trilaminate polyethersulphone membrane, NF-TFC-50 (50% rejection of NaCl)	40	20–25	40	30–80	Goulas *et al.* (2003)
GOS	Enzymatic synthesis from whey	Combined UF, NF and RO	Polyethersulfone UF membranes (NMWCO 50 and 5 kDa), polyamide thin-film composite NF membrane with a microporous polysulfone supporting layer (9.8% rejection of NaCl), polypropylene thin-film composite RO membrane with a porous support (≥97% rejection of NaCl)	200 (UF) 25 (NF)	25	1.5 (UF) 10 (NF) 20 (RO)		Das *et al.* (2011)

(*continued*)

Table 15.1 (*Continued*)

Target oligosaccharides	Starting substrates	Membrane technique	Membrane system	Operating conditions: Effective area (cm^2)	Operating conditions: T (°C)	Operating conditions: P (bar)[a]	Operating conditions: Permeate flux (L/h per m^2)[b]	Reference
FOS (DPs ≥4)	Commercial mixture of FOS containing digestible mono- and disaccharides	UF	Stirred UF cell with an YM 1 regenerated cellulose membrane with a NMWCO of 1 kDa		4	3.0		Montilla *et al.* (2006)
FOS	FOS containing digestible mono- and disaccharides	NF	Forced-flow membrane reactor using five different tubular ceramic membranes of symmetric and asymmetric types	55.6	50	3.5	10–30[c]	Nishizawa *et al.* (2000)
FOS	Commercial powdered chicory rootstock	Combined UF and NF	A GR61PP UF membrane made of polysulfone (NMWCO 20 kDa) and a G10 NF membrane made of polyamide composite (30% rejection of NaCl)	3600	30 (UF) 25 (NF)	3.5 (UF) 4.0 (NF)	17–118 (UF) 11.2–82.0 (NF)	Kamada *et al.* (2002)
XOS	Liquors from almond shells autohydrolysis	UF	Bench-scale plant equipped with UF thin-film polymeric, flat sheet of polymeric membranes with a NMWCO of 1, 2.5, 3.5 and 8 kDa	12.57	25	2.6–9.0	1.1–55	Nabarlatz *et al.* (2007)
XOS	Liquors from rice husk autohydrolysis	NF	Small-scale pilot unit using a monolithic ceramic membrane made of TiO_2/ZrO_2 (95% rejection of monosaccharides)	2500	26	6–14	10–60	Vegas *et al.* (2006) Vegas *et al.* (2008)
XOS	Liquors from rice husk autohydrolysis	Combined UF and NF	Tubular ceramic membranes with NMWCO of 1 and 15 kDa.	220		8.0 (UF) 10.0 (NF) 4.0 (UF)		Gullón *et al.* (2010)

AXOS	Enzymatically produced arabinoxylan hydrolysates from wheat flour	UF	Stirred cell with UF membranes with NMWCO of 5, 10 and 30 kDa		Room temperature	4.0		Swennen *et al.* (2005)
Pectic oligosaccharides	High methylated citrus pectin and low methylated apple pectin	UF	Stirred cells with an Amicon PM 10 UF membrane with a NMWCO of 10 kDa	28.7	30			Olano-Martin *et al.* (2001)
Hemicellulose-derived oligosaccharides	Liquors from *Pinus pinaster* wood autohydrolysis	UF	Stirred cell with an UF regenerated cellulose membranes with NMWCO of 1 kDa	41.8		4.0		González-Muñoz *et al.* (2011)
Isomaltooligo-saccharides	Enzymatic synthesis from maltose buffered solutions	UF	Sandwich-structured enzyme membrane reactor with the enzyme immobilized between two sheets of UF membranes (PES/Pluronic F127 and PES/PEG)	287		1.0	26.53–209.06	Zhang *et al.* (2010)
Lactose-derived oligosaccharides	Goat Milk	Combined UF and NF	Two-stage tangential filtration process using multichannel tubular ceramic membranes made of ZrO_2-TiO_2 (NMWCO 50 kDa for UF and 1 kDa for NF)	94	30	0.9 (UF) 3.75 (NF)	35.1–73.4 (UF) 18.8–28.4 (NF)	Martinez Ferez *et al.* (2006a) Martinez Ferez *et al.* (2006b)
Soybean-derived oligosaccharides	Steamed soybean waste water from tofu processing	UF and either RO or NF	A polysulfone UF membrane (NMWCO 20 kDa), a spiral-type RO membrane NTR-7199 (99.3% rejection of NaCl) and an NF membrane NTR-7250 (60% rejection of NaCl)	4000 (UF) 17 000 (RO and NF)	50 (UF) 25 (NF)	2.0 (UF) 10–50 (RO) 5–30 (NF)	2.5–15 × 10^{6d}	Matsubara *et al.* (1996)

Notes: [a]Trans-membrane differential pressure. [b] Dependent on specific pressure applied in each assay. [c] $cm \cdot m^{-2} \cdot s^{-1}$. [d] $m^3\ s^{-1}$ per m^2.

bioactive oligosaccharides in foods. These approaches will be discussed in the following subsections according to the applied membrane separation technology.

Selective membrane chemistries can be used to separate specific sugars from mixtures of mono- and oligosaccharides. For example, one patent describes the use of borate-containing membranes and carriers for the selective removal of fructose from high-fructose corn syrup and related mixtures (Smith and Riggs 1998).

15.2.1 Ultrafiltration (UF)

Ultrafiltration is a well established membrane separation process for the purification of oligosaccharides from high molecular weight enzymes and polysaccharides. In general terms, there is a considerable number of studies addressing the fractionation of GOS, fructooligosaccharides (FOS) and xylooligosaccharides (XOS) by UF, which is in accordance with the amount of available data supporting their bioactivity. Thus, UF has been shown to be a very useful technique for the continuous enzymatic production of GOS using a cross-flow ultrafiltration membrane reactor. In brief, this strategy leads to the continuous removal of the GOS (along with water, some substrate and simple sugar by-products) while the enzyme is retained by the membrane and returned to the reactor. As an example, Figure 15.2 shows a laboratory-scale membrane-assisted reactor system. The main advantages of this continuous process are related to product inhibition (by eliminating the monosaccharide fraction which inhibits the β-galactosidase activity), contamination in multi-step processes, and the reuse of enzyme without the need to deactivate it, among others (Czermak *et al.* 2004).

Foda and Lopez-Leiva (2000) optimized the production of GOS from whey permeate containing different concentrations of lactose and β-galactosidase from *Kluyveromyces lactis* by using either laboratory scale (Amicon stirred cell, 41.8 cm^2 effective area) or pilot plant-scale membrane reactors. The largest yield of GOS (concentration of GOS/initial lactose concentration) was 31% for whey UF permeate, initially containing 20% lactose and 0.5% of the enzyme, using the pilot plant scale membrane reactor, consisting of a UF-hollow fiber Romicon module (PM-10), with a nominal molecular weight cut off (NMWCO) of 10 000 Da and an effective area of 0.5 m^2. A later study was carried out using the same enzyme and comparing the batch production in a stirred-tank reactor with the continuous production in a laboratory-scale reactor system fitted with a 10 kDa NMWCO composite regenerated cellulose UF membrane (Chockchaisawasdee *et al.* 2005). The continuous process provided better productivity of GOS than the batch process, which was attributed to its ability to maintain a permanent state, without loss of enzyme activity. In a similar study, Petzelbauer *et al.* (2002) used either a batch reactor or a continuous stirred-tank reactor coupled to a 10 kDa cross-flow UF module with polyethersulfone membranes, which were stable for more than 10 weeks at a temperature of up to 80 °C, for the production of GOS with two thermostable β-galactosidases from *Sulfolobus solfataricus* and *Pyrococcus furiosus*, respectively. A cut-off of 10 kDa was required to retain more than 99% of β-galactosidases, allowing their reuse for the continuous mode of operation. Comparing GOS production in batch and continuous mode of operation revealed that the amount of GOS produced by the β-galactosidase from *S. solfataricus* was significantly higher (between 3- and 1.3-fold) in continuous mode than in the batch reactor, probably due to a more efficient transfer of D-galactosyl residues to D-glucose and lactose,

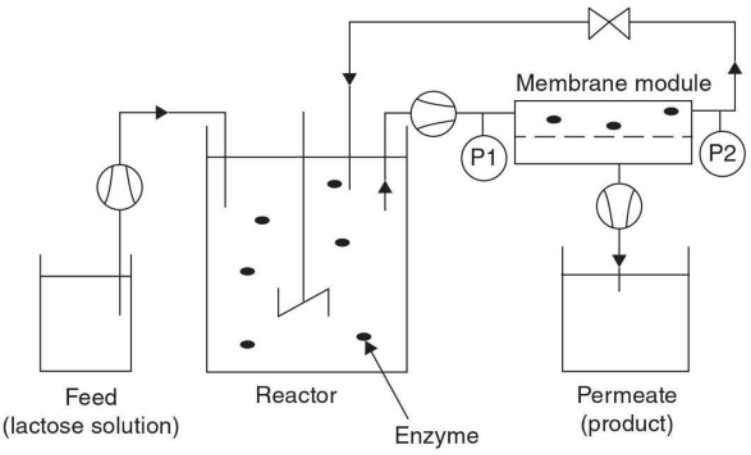

Figure 15.2 Laboratory-scale membrane-assisted reactor system. (Czermak, Ebrahimi, Grau, Ketz, Sawatzki, and Pfromm 2004. Reproduced with permission of Elsevier.)

and less secondary degradation of GOS in the former. In addition, differences in terms of composition of the individual GOS were also observed.

Czermak *et al.* (2004) described the continuous production of GOS from lactose in a continuous membrane-assisted reactor fitted with either a polymeric (NMWCO 50 000 Da) or a ceramic membrane (NMWCO 20 000 Da) by using different β-galactosidases, number of feed concentrations and average residence times. The variation of these parameters had a noticeable influence on the yield and composition of the GOS fraction. As a consequence, the maximum GOS concentration achieved was over 40% (w/w), with an average residence of 1 h and a feed lactose concentration of 31% (w/w). Pocedicova *et al.* (2010) compared GOS production using three different starting substrates (i.e., a buffered solution of lactose, recombined whey and UF-permeate) with similar concentrations of lactose (200 g/L) in batch and continuous membrane reactor with UF ceramic membrane (150 kDa). The highest GOS yields were obtained with recombined whey, followed by UF-permeate and lactose in buffer.

In addition to the use of native enzymes, UF also allows the option of using immobilized enzymes. Consequently, Matella *et al.* (2006) compared the production of GOS in a recycled free-enzyme UF and in a recycled immobilized-enzyme systems. Both systems showed very similar maximum GOS formation (20–22%, w/w) after 15–17 min of reaction with a β-galactosidase from *Aspergillus oryzae*. Ebrahimi *et al.* (2010) applied a novel two-stage integrated ceramic membrane reactor system to physically immobilize β-galactosidase from *K. lactis* for the continuous production of GOS as illustrated in Figure 15.3. The UF membrane had a NMWCO of 20 000 Da and consisted of a support layer (Al_2O_3) with large pores and a low-pressure decay and one separation layer (TiO_2) to control the permeation flux. This system was very efficient in producing GOS: the maximum formation exceeded 38% (w/w) when an average residence time of 23.5 min, a transmembrane pressure of 2 bar and an initial lactose concentration of 30% (w/w) were applied.

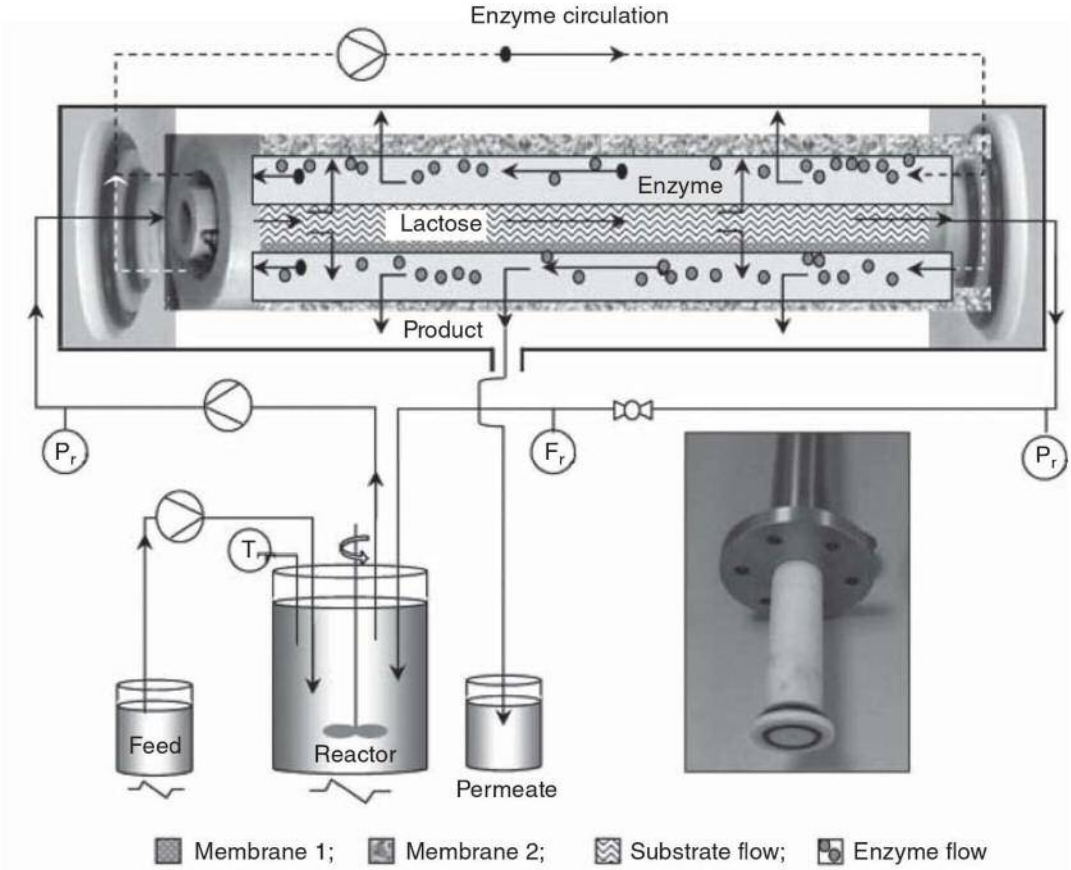

Figure 15.3 Two-stage integrated ceramic membrane reactor system to physically immobilize β-galactosidase from *K. lactis* for the continuous production of GOS. (Ebrahimi, Placido, Engel, Ashaghi and Czermak 2010. Reproduced with permission of Elsevier.)

Ultrafiltration in diafiltration mode with a regenerated cellulose membrane (NMWCO 1000 Da) was used for the successful fractionation of a commercial FOS solution (Raftilose P95) consisting of a mixture of oligosaccharides with DP from 2 to 7 (as previously mentioned in Chapter 4), as well as small amounts of fructose, glucose and sucrose (Montilla *et al.* 2006). Results indicated that diafiltration greatly reduced the concentration of mono-, di- and trisaccharides, whereas levels of FOS with DP larger than 4 significantly increased in the mixture. Likewise, the reduction of the mono- and disaccharide fraction in the diafiltered FOS was larger than the reduction of the trisaccharide concentration. In contrast, diafiltration mode using NMWCO 500 and 1000 Da membranes on the same system than above was not suitable for GOS fractionation (Hernández *et al.* 2009). These data suggests that not only the oligosaccharide size but also the structure and, consequently, the conformation might play a role in membrane fractionation.

Xylooligosaccharides derived from different sources (see Chapter 6 for more information about production and bioactivity) have also been purified by UF. Crude XOS require extensive purification processes before they can be used as functional food ingredients, due to the presence of large amounts of undesirable products, such as lignin-related phenolics, nonsaccharide fractions derived from extractions, digestible monosaccharides and their dehydration and condensation products, or ashes, among others. Moreover, XOS normally show a broad distribution of molar mass regardless of the reaction conditions (Nabarlatz *et al.* 2007). Therefore, membrane separation processes could be a suitable solution for the production of XOS with high purity and well-controlled molar mass distribution following their separation from higher molar mass products, as well as for the fractionation of XOS with different DP. In this context, Nabarlatz *et al.* (2007) used four commercial thin-film polymeric UF membranes with different NMWCO (i.e., 1.0, 2.5, 3.5 and 8.0 kDa) at pressures from 2.6 to 9 bar for the purification of XOS obtained from the autohydrolysis of almond shells. Results indicated that the selectivity towards the permeation of lignin-related product impurities over oligosaccharide-related products was better at low fluxes of permeate and with membranes of low NMWCO.

A two-step membrane process based on UF using 3 and 10 kDa membranes was employed for the fractionation of XOS derived from the enzymatic or acid hydrolysis of tobacco stalk (Akpinar *et al.* 2010). The first UF step which employed a 10 kDa NMWCO membrane was useful for removing the high molecular weight polysaccharides and proteins from the oligosaccharides, which had a recovery of 90% (w/w). The oligosaccharide syrup permeate was then subjected to a second UF step using a 3 kDa cut off membrane and whose retentate was mainly composed of XOS with a DP above 6, whereas the permeate contained mainly xylobiose and xylotriose, followed by xylotetraose and minor amounts of xylopentaose, xylose and high DP compounds. Overall, 71% (w/w) of XOS with a DP between 2 and 6 were present in the reaction hydrolyzate at the end of the two-step UF process. Finally, Wang *et al.* (2011) used UF with a commercial membrane with a NMWCO of 5 kDa to efficiently remove xylan from XOS.

In another study, UF and ethanol precipitation were compared for the separation and fractionation of arabinoxylooligosaccharides (AXOS) from their monomeric building blocks, arabinose and xylose, and polysaccharides (Swennen *et al.* 2005). One step UF using three commercial membranes with a NMWCO of 5, 10 and 30 kDa, as well as two step UF (10 and 30 kDa) processes were assayed. In general terms, although the fractions obtained by UF were more heterogeneous and polydisperse than the ones obtained with ethanol precipitation, AXOS fractions with similar a DP and degree of substitution were obtained with both methods.

Finally, UF separation has also been successfully used to purify oligosaccharides from different origins, such as pectic oligosaccharides (Olano-Martin *et al.* 2001), hemicellulose-derived oligosaccharides (González-Muñoz *et al.* 2011), chicory and dahlia fructans (Moerman *et al.* 2004), isomaltooligosaccharides (IMOS, Zhang *et al.* 2010), or lactose-derived oligosaccharides from goat milk (Martinez-Ferez *et al.* 2009).

15.2.2 Nanofiltration (NF)

Nanofiltration technology is a useful technique, especially, when UF does not offer sufficient rejection of low molecular weight solutes. Consequently, NF can be a better membrane technology to obtain higher concentrations of oligosaccharides than UF by increasing the rejection of mono- and, to a lesser extent, disaccharides. The main operating differences between NF and UF are the typical membrane pore size (2 nm versus 1–50 nm) and operating pressure (10–50 bar versus 1–15 bar). In addition, the NMWCO of NF membranes normally lies in the range of 200–1000 Da (Tsuru *et al.* 2000), combining UF and RO separation properties (Goulas *et al.* 2002).

Goulas *et al.* (2002) studied five different flat sheet membranes with the capacity to operate at maximum pressures between 20 and 35 bar in a cross-flow system for the fractionation of a model solution containing fructose, sucrose and raffinose, and of a commercial GOS mixture. Under optimal conditions, continuous diafiltration gave rise to satisfactory yield values for the GOS mixture (14–18% monosaccharides, 59–89% disaccharides and 81–98% oligosaccharides) indicating that the removal of monosaccharides was very efficient with only minor losses of the oligosaccharide content of the mixture. Furthermore, these authors established that the rejection factors of the sugars were largely dependent on pressure, sugar concentration of the feed and filtration temperature. Similar purification levels of nanofiltered GOS were obtained in a subsequent study performed by the same authors, whilst higher losses of di- and oligosaccharides were reported by applying UF (Goulas *et al.* 2003).

Feng *et al.* (2009) used four commercial spiral wound NF membranes for the fractionation of GOS mixtures at low pressure (up to 8 bar), in order to avoid fouling due to the compaction of the membrane layer when operating at high pressure (Martinez-Ferez *et al.* 2006a). Moreover, the main advantage of this type of membrane is the feasibility of scaling-up the filtration process, which could find immediate application in the industry. Under optimum conditions, these authors reported 90.5% and 52.5% rejection levels of monosaccharides and lactose, respectively, whereas an oligosaccharide purity of 54.5% was obtained (1.5 times of the raw material). Botelho-Cunha *et al.* (2010) observed a similar rejection pattern for NF fractionation of a GOS mixture at two different temperatures (25 and 40 °C). Thus, GOS-trisaccharides were totally retained and pressure-independent, while rejection coefficients of di- and monosaccharides increased when the effective transmembrane pressure was applied. Interestingly, these authors observed different rejection levels for lactose and GOS-disaccharides, indicating that rejection of neutral solutes by NF is not only dependent on simple sieving effects, but differences in the conformation carbohydrate due to the nature of the involved glycosidic linkages may also affect the selectivity of the NF process. Recently, Sen *et al.* (2012) have developed a rotating disk membrane bioreactor (RDMBR) to alleviate the problem of membrane fouling by giving rotational motion to the membrane. This novel bioreactor was successfully applied for the production of GOS by immobilized β-galactosidase on an NF membrane giving rise to a GOS yield and purity of 67.4% and 80.2%, respectively. These values were greater than those obtained in batch mode followed by diafiltration-assisted NF.

Nanofiltration in continuous diafiltration mode has also been successfully applied for the purification of lactulose syrup, removing more than 96.5% of NaCl and the chemical catalyst (H_3BO_3), with only an 11% loss of disaccharides (i.e., lactulose and lactose) (Zhang *et al.* 2011).

A forced-flow membrane reactor (FFMER) in which a β-fructofuronidase was immobilized on different porous ceramic NF membranes was applied for the continuous production of FOS (Nishizawa *et al.* 2000). Ceramic membranes were used because of their resistance to heat and chemical agents and also because their surface can be easily modified by silanization, allowing the enzyme to be immobilized. The saccharide composition of FOS was a function of the permeate flux, which was easily controlled by pressure. Overall, the FOS percentage in the saccharide composition with the FFMER system ranged from 54.2% to 55.9%, although this percentage was increased up to 57.2% by performing a two-stage NF. Li *et al.* (2004) also successfully applied NF for the purification of FOS in constant volume diafiltration (CVD) and variable volume diafiltration (VVD) modes, where the dilution water flux is not equal to that of the permeate, yielding in both cases FOS syrup purity above 90%. These results revealed that the relationship between FOS purity and yield was independent from the dilution ratio. These same authors determined in a later study by using an extended pore model that the factors which affected FOS transport inside an NF membrane were pressure gradient, steric hindrance and wall friction (Li *et al.* 2005). More recently, Kuhn *et al.* (2010) developed a process that included two NF stages with the same membrane to purify FOS also containing glucose, fructose and sucrose. The first stage was a diafiltration process, which served to reduce the effects of osmotic pressure and membrane fouling, and the resulting permeate was nanofiltered leading to FOS with purities above 90% and yields around 80%. Similar FOS yields were obtained by the same authors using a tangential membrane cell filtration in a diafiltration mode (Kuhn *et al.* 2011).

Purification of XOS has also been addressed by NF with a series of studies published during the last decade. Yuan *et al.* (2004) reported a pilot-plant production of XOS from corncob meal based on a downstream process characterized by a steaming treatment to extract xylan (optimum conditions were 160 °C and 1.5 h), followed by enzymatic hydrolysis during 5 h, flocculation, ion-exchange desalination and, finally, a NF step to purify and concentrate XOS offering

the advantages of energy-saving and partial removal of monosaccharides. Afterwards, charcoal adsorption and vacuum evaporation were also applied. The total yield of XOS based on the dry corncob meal was 16.9% and the XOS syrup contained 74.5% of xylobiose (DP 2) and xylotriose (DP 3). Vegas *et al.* (2008) compared the fractionation and purification of XOS from monosaccharides and other low molar mass materials obtained from rice husk xylan, with a polymeric tubular UF (NMWCO 4 kDa) membrane and a ceramic monolithic NF (1 kDa) membrane. Results showed that the UF membrane gave the best fractionation results, but lower recovery yields than the NF membrane. These authors determined that an increase in transmembrane pressure resulted in improved XOS recovery, at the expense of lower purity. Likewise, the final retentate from the NF process was further subjected to ion exchange and extraction with ethyl acetate, which led to XOS with purity over 91% and an overall yield of 71%. This downstream processing was previously optimized by the same research group (Vegas *et al.* 2006). Gullón *et al.* (2008) used NF for refining autohydrolysis liquors, prior to enzymatic hydrolysis with a commercial xylanase, and a subsequent ion-exchange step with an anionic resin to purify XOS from rice husks. Similarly, a processing strategy for XOS manufacture and purification from industrial solid wastes produced by malting industries (i.e., barley husks, spent grains and grain fragments) based on a double hydrothermal processing, double NF process (in diafiltration and concentration modes) with enzymatic hydrolysis and/or ion exchange and/or freeze drying has been developed (Gullón *et al.* 2011). Recently, Zhao *et al.* (2012) purified XOS from syrup by a discontinuous diafiltration process using a thin-film composite (TFC) spiral-wound NF membrane.

15.2.3 Combined membrane processing

The combination of several membrane separation technologies can be a powerful strategy to improve the yield and purity of oligosaccharide mixtures. Thus, application of NF is an attractive separation process for both desalting and concentration of UF permeate collected in the dairy industry, by ensuring the recovery of lactose in abundance for producing GOS (Rice *et al.* 2009). As examples, a two stage UF-NF process combined with tubular ceramic membranes with NMWCOs of 50 and 1 kDa, respectively, has been used to recover oligosaccharides in caprine milk, leading to a final retentate containing more than 80% of the original oligosaccharide content that is virtually free of lactose (less than 4%), protein and salts (Martinez-Ferez *et al.* 2006a; Martinez-Ferez *et al.* 2006b). Briefly, the first step was useful to separate oligosaccharides from proteins, and the resulting permeate was subsequently subjected to NF to elute salts and lactose.

Kamada *et al.* (2002) also demonstrated the effectiveness of combining UF and NF membrane processing to purify and concentrate FOS from chicory rootstock. Thus, after the first UF step carried out using a polysulfone membrane with a NMWCO of 20 kDa, 80.7% of the saccharides in the initial feed were recovered in the total permeate. The permeate was then subjected to NF by using four different membranes made of polyamide composite with nominal salt rejection of 65, 30, 10 and 3%, respectively. Among these membranes, the one with the nominal salt rejection of 30% was the most suitable for purifying FOS. Thus, under optimized conditions, mono- and disaccharides (mainly glucose, fructose and sucrose) were preferentially removed from the retentate while FOS with a DP $\geq$3 were retained and concentrated. The final composition of the NF permeate was as follows: 7.7% monosaccharides, 43.3% disaccharides and 42.7% oligosaccharides with DP ranging from 3 to 10.

Gullón *et al.* (2010) used UF membranes to successfully refine XOS produced by hydrothermal processing rice husks and to further purify them in combination with ion exchange. For the refining step, the autohydrolysis liquors from rice husks were ultrafiltered in diafiltration mode using a 1 kDa NMWCO membrane, then, nanofiltered (concentration mode) also with a 1 kDa NMWCO; the resulting retentate was subjected to an enzymatic treatment using commercial endoxylanases to reduce the average molar mass. The hydrolyzate was ultrafiltered in concentration mode with a 15 kDa NMWCO membrane and the permeate was further treated with an anionic exchange resin and, finally, freeze-dried. The percentages of recovery in the final product with respect to the starting material (i.e., autohydrolysis liquors) were: 4.9–14.1% for monosaccharides, 38% for glucooligosaccharides, 37.2% for XOS, 29% for arabinosyl moieties linked to oligosaccharides, 31.3% for acetyl groups, 26.7% for galacturonic acid equivalent and 2.3% for other nonsaccharide and nonvolatile compounds. In this context, membrane processing is normally combined with other purification steps (such as ion exchange or solvent extraction) to improve the efficacy of the fractionation of complex oligosaccharide mixtures. In another study, Rivas *et al.* (2012) also employed a combination of UF and NF using 5 and 1 kDa NMWCO regenerated

cellulose membranes within a more complex strategy scheme also involving double hydrothermal processing to produce bifidogenic oligosaccharides derived from wood mannan.

Combining a preliminary UF step with either RO or NF was also used to recover oligosaccharides from steamed soybean waste water in tofu (soybean protein curd) processing, giving rise to moderate yields (10% (w/v) and 22% (w/v), respectively) of total oligosaccharides (Matsubara *et al.* 1996).

The commercial low-glycemic sweetener Sucromalt is a mixture of fructose and glucooligosaccharides produced by alternansucrase (Carlson *et al.* 2009). A glucooligosaccharide mixture has been produced at the kg scale and the fructose was removed from Sucromalt by nanofiltration (Côté, personal communication). The resulting mixture was used for prebiotic feeding trials in chickens (Jacobs 2011). The oligosaccharide product currently marketed as Fibermalt is similarly composed of alternan oligosaccharides, and reportedly produced by "filtration techniques" (Pilling and Frohberg 2008).

Das *et al.* (2011) have reported the production of GOS from whey permeate with a purity of 77–78% using three-step membrane separation techniques: the first two were UF steps using 50 kDa and 5 kDa NMWCO polyethersulfone membranes, followed by NF in a diafiltration mode (TFC-SR2 membrane, 400 Da NMWCO). The UF permeate mainly comprised unreacted lactose and produced glucose, galactose and GOS. The NF step was carried out at 1 MPa transmembrane pressure (TMP) at 25 °C and was useful to fractionate GOS from mono- and disaccharides. These authors also indicated that the RO process used prior to hydrolysis was useful to remove some minerals from whey and might have increased the yield and efficiency of the whole process.

15.3 Chromatographic techniques

Size exclusion and ion exchange are by far the most used chromatographic techniques for the semi- or preparative fractionation of food bioactive oligosaccharides. Other techniques such as simulated moving-bed or activated charcoal which are characterized by their feasibility to be scaled up for industrial applications will also be described in this section.

15.3.1 Size-exclusion chromatography (SEC)

Size-exclusion chromatography (SEC) is a powerful technique that allows separation of oligosaccharides in a size-dependent mode when a solution flows through a packed bed of porous packing that is available in a wide range of pore volumes. As a consequence, separation of carbohydrates depends on the ratio of their molecular dimensions and the average diameter of the pores. In general, SEC has commonly been used for the separation of a homologous series of oligosaccharides. Semi-preparative or preparative high performance size-exclusion chromatography (HPSEC) can also be used for the fractionation of carbohydrates depending on the amount of carbohydrate required.

Gels operating at low temperatures (around 25–30 °C), such as dextran gels marketed under the trade name Sephadex (GE Healthcare, Uppsala, Sweden), agarose gels (trade name BioGel-A from Bio-Rad) and Sepharose (GE Healthcare) or polyacrilamide gels (BioGel-P from Bio-Rad), among others, have been used (Churms 2002). Occasionally higher temperatures (35–55 °C) can be applied on specific polysaccharide or dextran gels to decrease the interactions between the oligosaccharides and the gel matrix, improving the resolution, although the life of the packing becomes shorter. Water is the mobile phase chosen for the elution of carbohydrates.

Regarding detection, although several advances in analytical techniques have emerged over the last few years, differential refractometers are still the most common detectors used in SEC, mainly because of the advantages of coupling, direct detection of carbohydrates and their relatively low cost.

Several authors have used SEC to remove digestible carbohydrates (which account for around 45–50% of the product) from commercial GOS mixtures using polyacrylamide gels (Searle *et al.* 2010) or dextran gels (Shoaf *et al.* 2006; Huebner *et al.* 2007) to further study their antiadhesive and/or prebiotic activities. Different DP fractions were obtained and combined to create GOS mixtures with a composition equivalent to that of commercial products. Compared with other fractionation techniques (diafiltration, activated charcoal treatment and yeast treatment), SEC has proven to be the most appropriated to obtain different DP GOS fractions (up to DP8) with the highest purity (almost 100% for each DP fraction)

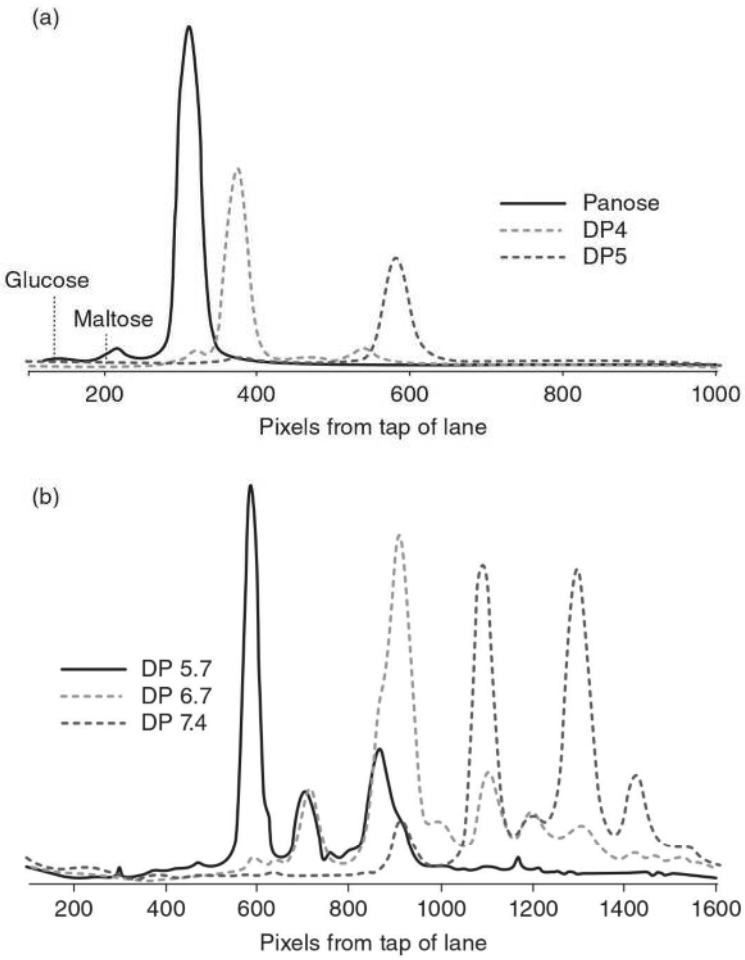

Figure 15.4 TLC densitogram of alternansucrase maltose-acceptor oligosaccharide fraction obtained after separation by SEC using Biogel-P2 and water as eluent. (a) single products of DP3-5. (b) Mixed products of DP5-8. (Sanz, Cote, Gibson and Rastall 2005. Reproduced with permission of American Chemical Society.)

and recovery (between 81–92%) (Hernández *et al.* 2009). Fractionation of food oligosaccharides of different nature, such as glucooligosaccharides (Sanz *et al.* 2005), gentiobiose- (Sanz *et al.* 2006), raffinose- (Hernández-Hernández, *et al.* 2011) and cellobiose-derived oligosaccharides (Ruiz-Matute *et al.* 2011), has been performed to evaluate how their molecular weight influences their effect on human gut bacteria. In general, the purest fractions were obtained for lower DP oligosaccharides (DP3-DP5), whilst fractions of higher molecular weight could not be completely purified, as can be observed in the TLC densitogram of the alternansucrase maltose-acceptor oligosaccharide fraction obtained after separation by SEC using a polyacrilamide gel (Figure 15.4, Sanz *et al.* 2005).

Size-exclusion chromatography is also used to purify naturally occurring oligosaccharides from different matrices such as FOS from garlic extracts (Zhang *et al.* 2012), neutral and pectic oligosaccharides from olive by-products (Lama-Muñoz *et al.* 2012), and so forth.

Different size exclusion columns connected in series have been used to guarantee the fractionation and purification of carbohydrates such as galactoglucomannans extracted from *Picea abies* (Lundqvist *et al.* 2003) or xylooligosaccharides (Sun *et al.* 2002).

Annular SEC is a system consisting of two concentric cylinders forming an annulus into which the stationary phase is packed. Finke *et al.* (2002) compared this system with fixed bed conventional gel chromatography for the fractionation of fructans, and the productivity (grams of carbohydrate separated per hour) of annular chromatography was 25-fold higher than that of the conventional method, thus, facilitating the fractionation at the preparative scale.

Overall, SEC is an effective technique for the fractionation of carbohydrates with high purity and yields; however, its main drawback is that it is a time consuming technique and target carbohydrates are recovered in high volumes of solvent which should be removed after the process. To overcome these problems, different approaches have been proposed, such as the development of rapid methods using different materials, for example Superdex-type columns (GE Healthcare) based on highly cross-linked porous agarose beds to which dextran has been covalently bonded (Knutsen *et al.* 2001), or the use of flash liquid chromatography (Flash LC), which uses glass columns and gas pressure-driven flow between 0 and 2 bar (Strum *et al.* 2012).

15.3.2 Ion exchange

Most food carbohydrates are weak electrolytes and, in consequence, they normally show little interaction in its natural form with ion-exchange resins in an aqueous medium (Sanz and Martínez-Castro 2007). However, acidic oligosaccharides, such as sialic or oligogalacturonic acids, are negatively charged at low pH and can be efficiently fractionated by anion-exchange chromatography on a semi-preparative or preparative scale. Thus, Smith *et al.* (1978) reported the large-scale isolation of seven sialyl oligosaccharide fractions from human milk by anion-exchange chromatography on a DEAE-cellulose column (45 × 1.5 cm) in acetic–pyridine buffers, pH 5.4. More recently, Finke *et al.* (1999) also fractionated human milk oligosaccharides (HMOs) into neutral and acidic oligosaccharides by anion-exchange chromatography on an AG 1-X2 column (30 × 4.4 cm) with 250 mM of ammonium acetate, pH 5.0 After desalting, the neutral and acidic fractions were further separated by SEC. Findings from this work underscored the great structural complexity of HMOs (see Chapter 1 for more information on HMOs).

Oligogalacturonic acids have been largely isolated by semi-preparative or preparative anion-exchange chromatography. These oligosaccharides are derived from the depolymerization of polygalacturonic acids, which are major components of cell wall polysaccharides (pectins) in plant tissues and can be present in dietary vegetables and fruits (the reader can find more information on this topic in Chapter 5). The preparative-scale isolation of oligogalacturonic acids has traditionally been performed by ambient-pressure, strong anion-exchange resins, including Dowex I-X8 (Nagel and Wilson 1969), AG 1-X8 (Dave *et al.* 1976), DEAE-Sephadex A-50 (Liu and Luh 1978), DEAE-Sephadex A-25 (Jin and West 1984; Suzuki *et al.* 2002), QAE-Sephadex (A-25-120) (Davis *et al.* 1986) and AG MP-1 (Doner *et al.* 1988). These methods are time consuming, labor intensive, and generally lack adequate resolution for oligogalacturonic acids with a DP larger than 7, although in some cases oligogalacturonic acids of up to DP 17 could be detected. To overcome these drawbacks, Hotchkiss *et al.* (1991) developed a faster and more efficient preparative HPLC method than those previously reported by using a weak anion-exchange aminopropylsilica gel column (25 × 2.14 cm) which allowed the successful isolation of oligogalacturonic acids up to a DP of 7 in gram quantities. Later, Spiro *et al.* (1993) purified oligogalacturonic acids with a DP between 10 and 15 (around 70 mg of each fraction) using Q-Sepharose (50 × 2.2 cm) fast-flow anion-exchange chromatography followed by semipreparative CarboPac PA1 (25 × 0.9 cm) high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The semipreparative CarboPac PA1 column was also used to isolate oligogalacturonic acids up to DP 5 (Zhan *et al.* 1998) and rhamnogalacturonan oligosaccharides (An *et al.* 1994; Schols *et al.* 1994; Zhan *et al.* 1998). Melotto *et al.* (1994) used the preparative CarboPac PA1 column (28 × 3.5 cm) successfully to isolate rhamnogalacturonan oligosaccharides. Later, Hotchkiss *et al.* (2001) reported the isolation of multi-milligram quantities of oligogalacturonic acids up to DP 20 by high performance anion-exchange chromatography utilizing a preparative CarboPac PA1 column (25 × 2.1 cm) and a nonlinear potassium acetate (pH 7.5) gradient. Guillaumie *et al.* (2006) also described the preparative separation of multi-milligram quantities of pectin oligogalacturonides with DP from 5 to 19 and purity above 95% by using a strong anion-exchange resin Source 15Q combined with ammonium formate buffer. A similar resin was used by Kabel *et al.* (2002) to separate neutral (acetylated) xylooligosaccharides from acidic (acetylated) xylooligosaccharides derived from hydrothermally treated Eucalyptus wood and brewer's spent grain in combination with SEC.

Ion-exchange chromatography need not be carried out at near-neutral pH. Many years ago, a method was developed to separate reducing from nonreducing sugars using a strong anion-exchange resin in the OH^- form (Roseman *et al.* 1952). This method is still of considerable value, and has recently been used for the large scale preparation of a cyclic tetrasaccharide from mixtures of linear, reducing oligosaccharides (Côté 2003). Such a method is very useful for the preparation

of nonreducing sugars such as trehalose, cyclic sugars, reduced polyols, and so forth, but the alkaline degradation of reducing sugars limits its applicability in other instances, since the process is carried out at very high pH.

Moving on to cation-exchange chromatography applications, these are normally employed to desalt solutions of oligosaccharides and data about fractionation of food bioactive oligosaccharides is scarce. Research on the ability of metal cations to form complexes with polyhydroxy compounds, including carbohydrates (Angyal 1973) has led to the development of separation methods based on these interactions. For example, Angyal *et al.* (1979) described the separation of several sugars and polyols on Dowex 50 on the Ca^{2+} form. This phenomenon has also been exploited to develop numerous commercial sugar-analysis columns based on ion-exchangers in the Ag^{+} form.

A method to separate stachyose, raffinose, sucrose, glucose, xylose and fructose on Dowex 50 in the K^{+} form was described a number of years ago (Saunders 1968). Vente *et al.* (2005) investigated the influence of Ca^{2+}, Na^{+} and K^{+} as the cations of the strong acid cation-exchange resin Dowex 50WX4-400 on the chromatographic removal of monosaccharides from disaccharides. Results indicated that K^{+} loaded resin had a stronger adsorption of sugars than the Na^{+} loaded resin. The lower retention of disaccharides was explained by the size exclusion mechanism, whereas difference in retention within the monosaccharide fraction was related to the number of equatorial-axial oriented sugar OH groups for complexation with the cation. These authors concluded that the K^{+} ion was the most suitable cation to separate glucose from oligosaccharides, whilst Ca^{2+} cation was the best choice to remove fructose from oligosaccharides. Early on, Keisuke and Tamura (1988) registered a patent based on a method for producing GOS wherein they used a preparative scale chromatography column with a strong acid cation-exchange resin, i.e. Unibead UBK-530 in Na-form, heated at 60 °C to purify the GOS. The separation of the components of the GOS mixture was driven by the molecular size exclusion effect, and fractions containing high concentrations of oligosaccharides, lactose, and monosaccharides were respectively collected from the eluates of the column. Sinclair *et al.* (2009) also adapted this preparative method to purify GOS, although they replaced the Na^{+} ion with a K^{+} ion.

15.3.3 Simulated moving-bed chromatography

Simulated moving-bed (SMB) chromatography was developed in the early 1960s (Broughton and Gerhold 1961) and ever since has been widely used by the sugar industry for the production of several mono- and oligosaccharides at the multi-ton scale (Heuer *et al.* 1998; Nicoud 1998; Schulte and Strube 2001). Briefly, SMB is a chromatographic technique based on a flow of liquid (mobile phase) moving countercurrent to a constant flow of solid (stationary phase). Countercurrent flow enhances the potential for separation and, hence, makes the process more efficient, with higher throughput and consumes less solvent (mobile phase) in comparison to traditional batch elution chromatography (Mihlbachler and Dapremont 2005). Given that providing a constant flow of solid is impractical in a production process, the solid instead is packed into several high pressure columns (in most cases 6–12) which are connected in a closed series and between each of them four valves are placed which can be individually opened and closed. A recycling pump inside the column ring delivers the mobile phase through all columns, whilst two additional pumps constantly introduce the feed and fresh eluent (inlet streams) and two pumps withdraw (outlet streams) the raffinate enriched with the less retained component and the extract enriched with the most retained components (Heuer *et al.* 1998; Schulte and Strube 2001). The inlet and outlet position is switched at regular time intervals in the direction of the liquid flow, mimicking an apparent solid flow in the opposite direction of the liquid flow, thereby simulating a countercurrent movement of columns. Adequately operating SMB chromatography requires computer simulations rather than empirical approaches, considering the great number of parameters, such as column diameter, column length, total column number, number of columns per section, inlet, outlet and recycle fluid flows and switch time interval, which have to be optimized (Schulte and Strube 2001).

Simulated moving-bed chromatography is very versatile because it is applicable to all kinds of chromatography (Geisser *et al.* 2005), although the adsorbents are usually a combination of size-exclusion and ion-exchange gels (Sanz and Martínez-Castro 2007). For example, Kishihara *et al.* (1989) developed a large scale separation of palatinose and trehalulose using a simulated moving-bed adsorber and a UBK 530 resin Ca-form as stationary phase. SMB chromatography was also used to efficiently separate lactose from HMOs using two different stationary phases: ion-exchange chromatography (MCI gel, Ca^{2+} form), as well as size-exclusion chromatography (HW40C gel) (Geisser *et al.* 2005). The IEC gel (MCI) was used for ligand-exchange chromatography, separating the molecules according to their size. Both

stationary phases were suitable for the separation of lactose, giving rise to similar results of relative lactose content in the extract and raffinate. Thus, with HW40C gel, the raffinate had <4% levels of lactose in comparison to the complex oligosaccharides (neutral and acidic oligosaccharides), whilst the extract showed >96% of the separated lactose. Using MCI gel, the content of lactose varied in the raffinate from 0 to 20% compared to HMOs, while in the extract it had a value of >96%. However, important differences were found in flow rates, lactose yield and, especially, in the stability of gels during their regeneration. The SEC gel provided lower flow rates and yields of lactose, and it was much more stable for the continuous SMB process. In contrast, the SEC gel did not undergo any change during regeneration and was much more suitable and comfortable to use for the separation of lactose from HMOs compared to the IEC gel. More recently, SMB chromatography was applied to three different sugar separations: raffinose-sucrose, fructose-glucose and xylose-glucose for comparison purposes with fractionation methods based on membrane cascades with different NF and UF membranes at pilot scale (Vanneste *et al.* 2011). These authors concluded that both fractionation methods could be suitable to successfully separate all studied applications. Likewise, two IEC resins in the K^+ form (Dowex 50W-X2 and Dowex Monosphere 99K/320) were studied for the purification of FOS (Nobre *et al.* 2010). Both IEC gels were useful to separate fructose, glucose, sucrose and FOS with different DP, although the Dowex Monosphere 99K/320 gel was found to be more suitable to work in the SMB system due to its greater resistance at high pressure.

15.3.4 Activated charcoal

The use of columns packed with charcoal (activated carbon) is a conventional method used for preparative separations of oligosaccharides, especially since Whistler and Durso (1950) fractionated different mixtures of mono-, di- and trisaccharide (i.e., raffinose) by using a mixture of equal amounts of Darco G-60 and Celite 535. Several carbon-based stationary phases for HPLC have been developed (Knox and Gilbert 1978), and, as commented in Chapter 21, graphitized carbon columns (GCC) are currently used for preparative separation of neutral linear oligosaccharides, *N*-linked oligosaccharides, chito-oligosaccharides, sulfated oligosaccharides, oligosaccharide alditols, cyclodextrins and glycopeptides (Koizumi 2002).

Retention of oligosaccharides on activated carbon is mainly driven by an adsorption mechanism and planar molecules are generally more retained than nonplanar ones (Koizumi 2002). These columns are especially suitable for the fractionation of mixtures that contain carbohydrates of different DP. Thus, by applying an activated charcoal treatment (Darco G60) to a typical mixture of GOS, monosaccharides and lactose were removed by eluting them with ethanol at low concentrations in water solutions (between 1% and 15%, v/v), whilst GOS were desorpted by eluting it with ethanol at a high concentration (50%, v/v) (Hernández *et al.* 2009). This treatment was revealed to be a rapid method to obtain considerable amounts of GOS at gram scale. However, the main disadvantages of activated charcoal treatments are that saccharides may not be sufficiently separated by size, leading to an incomplete removal of disaccharides and, if an attempt is made to obtain GOS with high purity, the yield could be poor. This was the case reported by Hernández *et al.* (2009) who obtained GOS of high purity with no presence of monosaccharides and lactose with 10% of ethanol, although the recovery of trisaccharides was very low. Akpinar and Penner (2008) successfully fractionated near gram quantities of pure cellooligosaccharides in the DP range of 3 to 7 with a charcoal celite column packed with equal amounts of Darco G-60 and celite 545 and using a water-ethanol gradient (0–45%). More recently, GCC Flash LC combined with real-time mass spectrometry detection has shown great potential to enrich and separate milligram quantities of specific human and bovine milk oligosaccharides, as well as to eliminate abundant saccharide components in milk, such as lactose and lacto-*N*-tetraose, and fractionate isomeric forms for more detailed biological assays (Strum *et al.* 2012). These results could not be achieved by size exclusion columns or amine columns.

15.3.5 Other chromatographic techniques

Although much less frequently used than those described above, this subsection briefly describes other chromatographic techniques that have been used for the semi- or preparative isolation of food bioactive oligosaccharides during the last decade.

Coulier *et al.* (2009) fractionated disaccharides and trisaccharides from a commercial prebiotic GOS mixture by preparative hydrophilic interaction liquid chromatography (HILIC), facilitating the subsequent characterization of these fractions by methylation analysis and NMR.

Cellulose column chromatography was a natural extension of paper chromatography, which had been used for many years in carbohydrate analyses. Researchers at Iowa State University were among the first to apply this method to the preparative chromatography of amylodextrins. Using powdered cellulose columns with various solvent mixtures, they were able to separate homologous saccharides up to DP 18 from acid hydrolyzates of starch (Thoma *et al.* 1959). Subsequently, FOS arising from the hydrolysis of levan have been separated using both carbon-Celite and cellulose chromatography (Tanaka *et al.* 1983). Akpinar *et al.* (2004) also demonstrated the potential of using cellulose stationary phases for the fractionation of water-soluble cellooligosaccharides. These authors indicated that the order of elution of this type of oligosaccharides (with DP from 3 to 6) was correlated with their relative solubility in aqueous ethanol solvents, as the higher DP cellooligosaccharides were less soluble and had longer retention times. Later on, Akpinar and Penner (2008) found that cellulose affinity/partition chromatography was the least time consuming and most economical method for the preparation of cellooligosaccharides of DP 4 and 5 as compared to charcoal-celite adsorption, SEC or cation-exchange chromatography.

Many studies have also been made to fractionate oligosaccharides into structurally distinct groups by affinity chromatography using immobilized lectin columns. However, these methods have been mainly applied to glycoconjugates, using lectins from different natural sources (lentils, peas, mushrooms, etc.). More information can be found in different reviews (Osawa and Tsuji 1987; Endo 1996).

Finally, the successful purification of FOS from a mixture also containing glucose, fructose and sucrose on zeolite fixed bed columns (using either one or two columns in a series) has been reported (Kuhn and Filho 2010).

15.4 Fractionation techniques using solvents

Although most of the methods and techniques indicated below are dedicated to the extraction of carbohydrates from different matrices, there are also some notable applications regarding carbohydrate fractionation.

15.4.1 Selective solvent solubility

It is well-known that differences in solubility of carbohydrates in organic solvents result in the selective precipitation of some of them, which can be easily separated from the extraction mixture. Nevertheless, literature in this field is scarce.

As mentioned in Chapter 9, ketoses such as tagatose and lactulose can be considered prebiotic and both are obtained by alkaline isomerization or by biological treatments of the corresponding aldose (galactose or lactose, respectively). Separation of ketoses from nonbioactive unreacted aldoses is required. Some studies have evaluated the solubility of these carbohydrates in different alcohols and water-alcohol mixtures (Olano 1979; Montañés *et al.* 2007). In general, lactulose and tagatose have been found to have higher solubility than lactose and galactose in these solvents at different temperatures (22, 30 and 40 °C); thermodynamic models were proposed to choose the best solvent to selectively purify these ketoses in mixture with other sugars (Montañés *et al.* 2007). However, these methods usually require high volumes of organic solvents and new alternatives are being developed.

15.4.2 Ionic liquids

Ionic liquids (IL) are organic salts with melting points lower than 100 °C, which consist of organic cations such as imidazolium, pyrrolidinium, pyridinium, and different inorganic or organic anions such as chloride, dycianamide, and so forth (Han and Row 2012). They have negligible vapor pressure, chemical and thermal stability, nonflammability, high ionic conductivity and wide electrochemical potential (Welton 1999). Bearing in mind these properties, ILs are considered efficient and environmentally friendly solvents in chemistry and are chosen as new alternatives to traditional organic solvents.

Ionic liquids are being extensively used in carbohydrate chemistry mainly for synthesis and catalysis (El Seoud *et al.* 2007). However, applications in food science are still scarce and mainly associated with the extraction of amino acids, proteins, azo dyes or contaminants among others (Ruiz-Aceituno *et al.* 2013).

A method based on the different sugar solubilities of fructose and glucose in ILs at room temperature has been patented and applied to the selective separation of these sugars in dates (Al Nashef *et al.* 2011). Although this procedure is not applied to the production of bioactive carbohydrates it can be used as a model for further investigation. 1,3-Dimethylimidazolium dimethylphosphate and 1-ethyl-3-methylimidazolium ethylsulfate were selected for separation of fructose and glucose, respectively. These ILs dissolve fructose and glucose in large quantities but at different proportions.

The purification of bioactive ketoses from aldoses using ILs as green solvents is currently under research (Carrero-Carralero, *personal communication*). The solubility of lactulose, lactose, tagatose and galactose in different ILS such as 1-ethyl-3-methylimidazolium dicyanamide, 1-hexyl-3-methylimidazolium chloride and 1-butyl-3-methylimidazolium methyl sulfate have been determined. In general, ketoses were more soluble than aldoses in ILs. These data could be used to selectively separate these carbohydrates and also to apply this method to fractionate different structural oligosaccharides.

Although ILs could be promising solvents for the fractionation of bioactive carbohydrates, more studies should be conducted to carefuly evaluate their toxicity before they are applied industrially. At this point, they should be treated the same as any other chemical for which there are limited toxicity and biodegradability data (Zakrzewska *et al.* 2010).

15.4.3 Supercritical fluid extraction (SFE)

Fractionation of carbohydrates by SFE is also based on the different solubility of carbohydrates in supercritical carbon dioxide (SC-CO_2), which is enhanced using polar co-solvents. Carbon dioxide changes to a supercritical state at relatively low pressures (73.8 bar) and temperatures (31.1 °C), and under these conditions, various properties of the fluid are placed between those of a gas and those of a liquid. In recent years SC-CO_2 with different co-solvents (isopropanol, methanol, alcohol/water mixtures, etc.) has been applied to selectively separate bioactive ketoses, such as tagatose from mixtures with galactose (Montañés *et al.* 2006) or lactulose from mixtures with different aldoses (Montañés *et al.* 2008). The efficiency in the recovery and purity of ketoses is highly dependent on the nature of the co-solvent. The modifier flow rate is the main factor influencing the recovery of sugars. Moreover, although a large increase in the amount of total carbohydrates extracted is achieved when more polar co-solvents are used, selective extraction of specific carbohydrates decreased, which accounted for the maximum amount of modifier added. Once the method is optimized, extraction purities higher than 90% of ketoses and recoveries higher than 75% are achieved (Montañés *et al.* 2006). However, when this method is applied to separate lactulose from a commercial mixture (Duphalac®; Solvay, Fr), also constituted by galactose, tagatose and lactose, the complete purification of lactulose in the extract was not achieved, considering the high amounts of galactose extracted (Montañés *et al.* 2008).

SC-CO_2 with different ethanol/water mixtures as co-solvents has also been applied to the fractionation of complex carbohydrate mixtures according to their DP. A two-step SFE procedure using ethanol/water as co-solvent and the most suitable extraction conditions (including temperature, pressure and co-solvent flow rate) allowed almost complete removal of monosaccharides and disaccharides from a mixture of prebiotic carbohydrates (GOS) (75% purity and 94% recovery) (Montañés *et al.* 2009). Another method including a third SFE step was also developed to obtain trisaccharides with high purity (Montañés *et al.* 2010). These processes, optimized at laboratory scale to fractionate mixtures of prebiotic carbohydrates, have been scaled-up to an industrial level evaluating their economic feasibility. These studies proved that the process could be considered profitable with a pay-out period of about 10 years (Montañés *et al.* 2012).

15.4.4 Pressurized liquid extraction (PLE)

As indicated in SFE, pressure can influence on the solubility of carbohydrates in different organic solvents. PLE systems work at high pressure and controlled temperatures and allow the use of solvents in subcritical conditions. Although these systems have been widely used for nonpolar analytes, to date limited applications have been developed for the fractionation of bioactive carbohydrates.

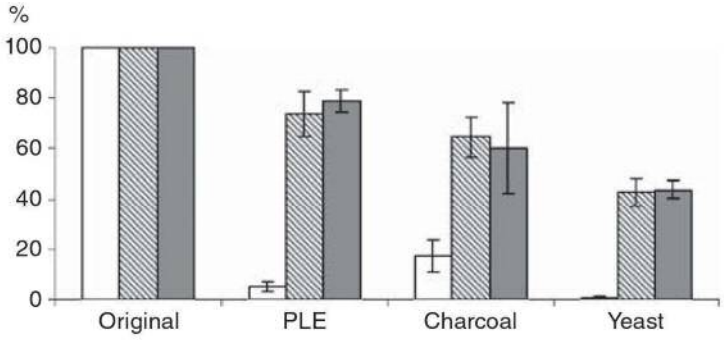

Figure 15.5 Recovery (% of the total content) of mono- (white), di- (hatched), and trisaccharides (solid) from citrus honey after PLE with adsorbent bed of activated charcoal, charcoal extraction and yeast treatment (n = 3). (Ruiz-Matute, Ramos, Martinez-Castro and Sanz 2008. Reproduced with permission of American Chemical Society.)

Different applications to extract and enrich carbohydrates using PLE and, mainly, subcritical water extraction (SWE) can be found in the literature (Rodríguez-Sánchez *et al.* 2010; Guan *et al.* 2010; Pronyk and Mazza 2012, Song *et al.* 2012). However, fractionation processes are limited. In this sense, a pressurized low polarity water (PLPW) extractor was successfully used to extract and separate hemicellulose, cellulose, lignin, and other phenolic compounds in flax shives. Hemicellulose and lignin were mainly separated at 170 °C, pH 3.0, with a flow rate of 2.5 mL/min. A two-stage PLPW extraction (the first stage to remove hemicellulose and the second stage for delignification) was used to improve the purity and yield of cellulose (Kim and Mazza 2009).

The fractionation of different cereals (triticale, durum wheat, feed barley, etc.) and oilseed (canola, mustard) straws using hydrothermal processing with PLPW (at 165 °C, with a flow rate of 115 mL/min and a solvent-to-solid ratio of 60 mL/g) has also been proposed (Pronyk and Mazza 2011). Separation of oligosaccharides and low molecular weight carbohydrates (liquid fractions) from polysaccharides (mainly glucans, galactans, mannans, etc.) was successfully achieved.

Separation of lactulose from a mixture with lactose [70:30 (w/w)] has been proposed using PLE at 1500 psi for 30 min. Different temperatures (from 40 to 130 °C) and ethanol:water ratios (70:30, 80:20, 90:10, 95:5, and 100:0) for the extraction solvent were assayed. The optimum extraction conditions were 40 °C and 70:30 ethanol:water. PLE extraction under the optimized conditions was also applied to purify lactulose from lactose in a mixture synthesis. Advantages of this technique over traditional methods were the short extraction time and the low solvent consumption (Ruiz-Matute *et al.* 2007).

A combination process of PLE with an in-cell packed adsorbent bed of activated charcoal was also proposed for the separation of monosaccharides in honey samples before their oligosaccharide analysis (Ruiz-Matute *et al.* 2008). Two consecutive PLE cycles using 1:99 (v/v) ethanol water for 5 min and 50:50 (v/v) ethanol/water for 10 min, respectively, both at 10 MPa and 40 °C were necessary to obtain enriched fractions of di- and trisaccharides (73% and 8% of total carbohydrate, respectively). This method was compared with other procedures (i.e., yeast treatment and activated charcoal extraction; Figure 15.5). As can be observed in the figure, all methods were useful for selective removal of monosaccharides from honey, although yeast treatment was the most effective (from 593 mg/g to 5 mg/g). However, the recovery of di- and trisaccharides was higher when the PLE procedure was used (74% and 79%, respectively). Moreover, PLE reduced extraction time and solvent volumes.

15.4.5 Microwave-assisted extraction (MWAE)

Microwave-assisted extraction is based on heating solvents in contact with samples using microwave energy with the aim of improving the partitioning of the target compounds from the matrix into the solvent. It is carried out in closed vessels and also combines the effect of pressure and temperature. Until now, applications to separate bioactive carbohydrates are scarce and mainly focused on the fractionation of high molecular weight carbohydrates such as galactoglucomannans (Lundqvist *et al.* 2003).

MWAE treatments have recently been used to fractionate carbohydrates from the coffee residue matrix remaining after preparing the beverage (Passos *et al.* 2011). Fractionation of water-soluble polysaccharides (galactomannans, type II arabinogalactans, and cellulose), oligosaccharides (mannooligosaccharides) and monosaccharides (mannose) was achieved

at 900 W, 200 °C for 5 min and 15 bar. Yields of 29.0% of water soluble material were obtained when these conditions were applied to a ratio of 1 g of dried coffee residue to 30 mL of water extraction. The relative amount of polysaccharides, oligosaccharides, and monosaccharides extracted accounted for 83.4, 12.8, and 3.4%, respectively. The increase of the ratio of coffee residue mass to volume of water (r:w) resulted in a decrease of the amount of polysaccharides while increasing the oligosaccharides and monosaccharides content. Different percentages of galactomannans and arabinogalactans were obtained depending on the r:w ratio.

Water-soluble hemicelluloses were extracted by microwave treatment from spruce chips and fractionated on the basis of their DP and composition (Lundqvist *et al.* 2002, 2003). The spruce chips were milled and impregnated (soaked) with water and NaOH. After impregnation, wood material was heat treated at a predetermined temperature (180, 190, or 200 °C) and residence time (2 and 5 min) in a microwave oven. Fractionation depended on the temperature used and, more importantly on the degree of impregnation of spruce chips in NaOH solutions of different concentrations. As an example, the highest mannan yield was obtained from water impregnated spruce chips at 190 °C for 5 min (molecular weight of 3800), whereas the highest DP carbohydrates (molecular weight of 14 000) were obtained at these conditions when impregnated with 2% NaOH (Lundqvist *et al.* 2003).

Although other works focus on the extraction of carbohydrates assisted by microwave, optimization of extraction conditions results in a fractionation that depends on the solubility of carbohydrates in the chosen solvent at the assayed temperatures. For example, MWAE was applied to produce arabinoxylans from corn pericarp, which is an industrial waste of corn starch production. Xylans were separated from cellulose by heating at 176.5 °C for 16 min using a 1/20 (g/mL) solid to liquid ratio. Under these conditions, the maximal yield attained 70.8% of carbohydrates with predominant production of xylo oligosaccharides (Yoshida *et al.* 2010).

15.5 Microbiological and enzymatic treatments

Microbiological treatments can be considered a selective method for the fractionation of carbohydrates based on enzymatic activities.

Saccharomyces cerevisiae has been used to remove monosaccharides (glucose and galactose) from GOS mixtures producing ethanol and CO_2 (Goulas *et al.* 2007). Fermentations took place at 30 °C under stirring, using 1 g of freeze-dried yeast (2.9×10^{10} cfu/g) per 100 mL of solution of carbohydrate mixtures (450 mg/mL). Although, the mixtures were successfully purified of glucose (92% of glucose removed) by fermenting with this yeast, with no losses in the oligosaccharide content, only a small decrease in galactose was observed (Figure 15.6). However, previous works described the removal of individual galactose by this yeast (Yoon *et al.* 2003). This behavior could be due to the high amounts of glucose present in the mixture, which led the yeast to metabolize it first, while leaving galactose initially unaffected (Goulas *et al.* 2007). Once glucose was removed, galactose assimilation should start, but these authors justify the presence of this monosaccharide by the high levels of the ethanol produced (7.4% v/v), which inhibited the action of yeast. Later, Hernández *et al.* (2009) also applied this treatment (*S. cerevisiae*) to purify GOS, achieving complete removal of both glucose and galactose after only 10 h of incubation. These positive results were justified considering that the concentration of GOS used in their work was lower (200 mg/mL) than that used by Goulas *et al.* (2007), and, consequently the level of ethanol produced could be lower and would not be toxic to yeast.

Li *et al.* (2008) also used *S. cerevisiae* L1 and *Kluyveromyces lactis* L3 to selectively remove monosaccharides from a GOS mixture produced by β-galactosidases, resulting in an increase of GOS purity from 28.7% to 39.4% and 97.5%, respectively.

Apart from the high efficiency of yeast treatment in the removal of monosaccharides, this procedure can also be performed directly on synthesis mixtures without the need of significant dilutions (as required by other techniques such as nanofiltration) and it is a low-cost and easily scalable process for industrial uses (Goulas *et al.* 2007). However, the main disadvantages of yeast treatment for the fractionation of GOS is the incapacity of the yeast cells to remove disaccharides such as lactose (digestible carbohydrate) and also the production of some metabolic products during fermentation, such as ethanol and trehalose (Hernández *et al.* 2009).

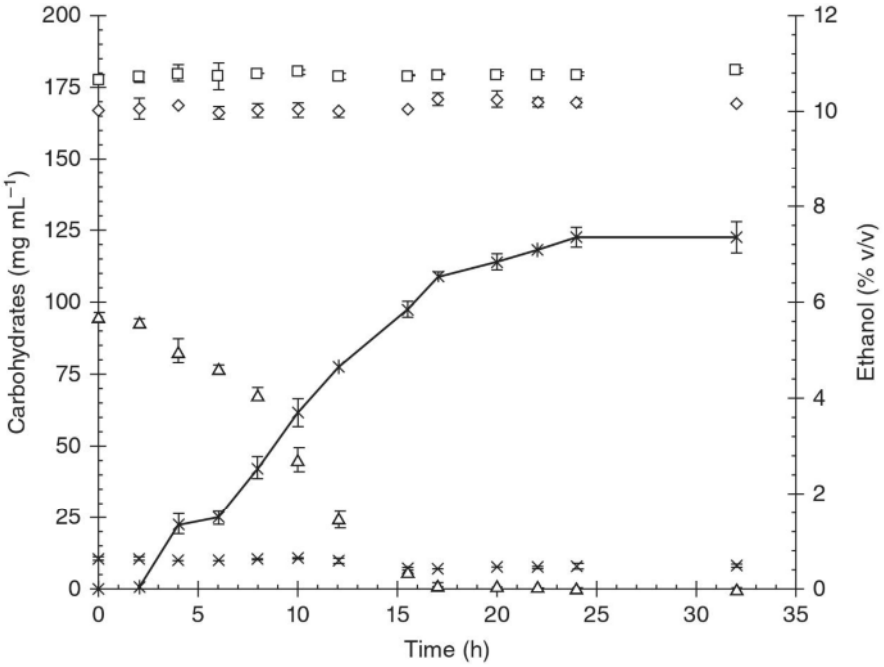

Figure 15.6 Purification of commercial GOS mixture by fermentation with *S. cerevisiae*. GOS with DP ≥ 3 (◇); disaccharides (□); glucose (Δ); galactose (×) and ethanol (*). (Goulas, Tzortzis and Gibson 2007. Reproduced with permission of Elsevier.)

S. cerevisiae has also been successfully used for the enrichment of legume extracts in bioactive inositols (free inositols, methyl-inositols and glycosyl-inositols). The selective removal of interfering carbohydrates was achieved; incubation time (3–40 h) was highly dependent on the composition of the legume considered (Ruiz-Aceituno *et al.* 2012).

Other treatments based on the use of different enzymes have been also proposed for the fractionation of carbohydrates and the purification of bioactive oligosaccharides. In this sense, Splechtna *et al.* (2001) assayed a selective enzymatic oxidation for GOS purification using fungal cellobiose dehydrogenase which is approximately 100 times more likely to react with lactose rather than GOS. Oxidation of lactose was coupled to reduction of 2,6-dichloro-indophenol which was added in catalytic concentrations. The oxidized redox mediator was continuously regenerated by fungal laccase-catalyzed reduction of molecular oxygen into water. Ion-exchange chromatography was employed to remove lactobionic acid, other ions and monosaccharides (Maischberger *et al.* 2008)

Finally, immobilized cells of the bacterium *Zymomonas mobilis* have been used to remove glucose, fructose and sucrose from different oligosaccharide mixtures (fructo-, malto-, isomalto-, gentio-, and inulin-oligosaccharides). These mono- and disaccharides were completely fermented within 12 h of incubation with no pH control or added nutrients (Crittenden and Playne 2002).

15.6 Conclusions

Fractionation of carbohydrates is a required task in the production of bioactive oligosaccharides and the analysis of complex carbohydrate mixtures. Although several advanced techniques have emerged for this purpose, traditional methods are still effective and widely used. The main disadvantages are still the time consuming methods and lower yields of purified carbohydrates.

Most of the techniques are suitable for the fractionation of oligosaccharide mixtures with different molecular weight (see Table 15.2); however, the fractionation of carbohydrates with the same DP but different glycosidic linkages or monosaccharide composition is still a challenging task. Some attempts have been described for specific carbohydrates

Table 15.2 Comparison of fractionation techniques to obtain food bioactive oligosaccharides based on their molecular weight or glycosidic linkages and/or monosaccharide composition.

	Fractionation based on	
Techniques / treatments	Molecular weight	Glycosidic linkages and/or monomeric composition
Activated charcoal	XXX	–
Size-exclusion chromatography	XXX	–
Ion-exchange chromatography	XX	XX
Membrane-based	XX	–
Yeast	X	XX
Supercritical fluid extraction[a]	X	XX
Pressurized liquid extraction[a]	X	XX

Notes: XXX = Efficient fractionation. XX = Moderate fractionation. X = Limited fractionation. – = No fractionation. [a]= Scarce data.

(e.g., fractionation of lactulose and lactose by PLE (Ruiz-Matute *et al.* 2007)), but further development is required to extend their use to general applications. Noteworthy progress in procedures based on chromatographic and related techniques has been seen mainly at the analytical scale, including the development of new column packing and/or support materials, advances in the orthogonality of two-dimensional chromatographic separations and/or hyphenated techniques, which have improved the separation of structurally related carbohydrates. However, advances in carbohydrate fractionation at the semi- or preparative scale have primarily been focused on reducing the treatment time, while the selectivity between oligosaccharides having the same DP still needs to be improved. This is especially important in the case of bioactive oligosaccharides, as it is mandatory to establish a clear relationship between the claimed beneficial function and the chemical structure in order to comply with regulatory requirements.

Acknowledgments

This work has been financed by Projects AGL2009-11909, AGL2011-27884 and Consolider Ingenio 2010 FUN-C-FOOD CSD2007-00063 from Ministerio de Ciencia e Innovación, Project S2009/AGR-1464, ANALISYC-II from Comunidad Autónoma of Madrid (Spain) and European funding from FEDER program, project POII10-0178-4685 from Junta de Comunidades de Castilla-La Mancha and European Regional Development Fund (ERDF), and project AGR-7626 from Junta de Andalucía.

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Anexo 2

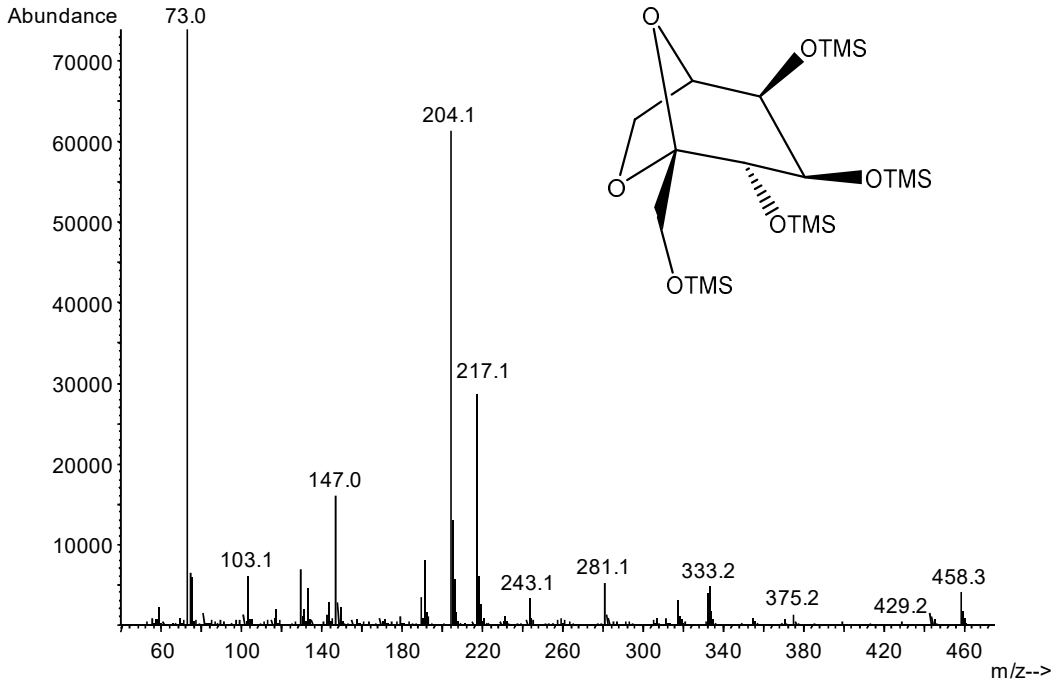
Gas chromatographic-based techniques for the characterization of low molecular weight carbohydrates and phenylakanoid glycosides of *Rhodiola rosea* food supplements

C. Carrero-Carraleroa, S. Rodríguez-Sáncheza,, I. Calvillo, I. Martínez-Castroa, A.C. Soriaa, L. Ramos, M.L. Sanz

Supplementary Material

A)

2,7-Anhydro-β-D-*altro*-heptulopyranose



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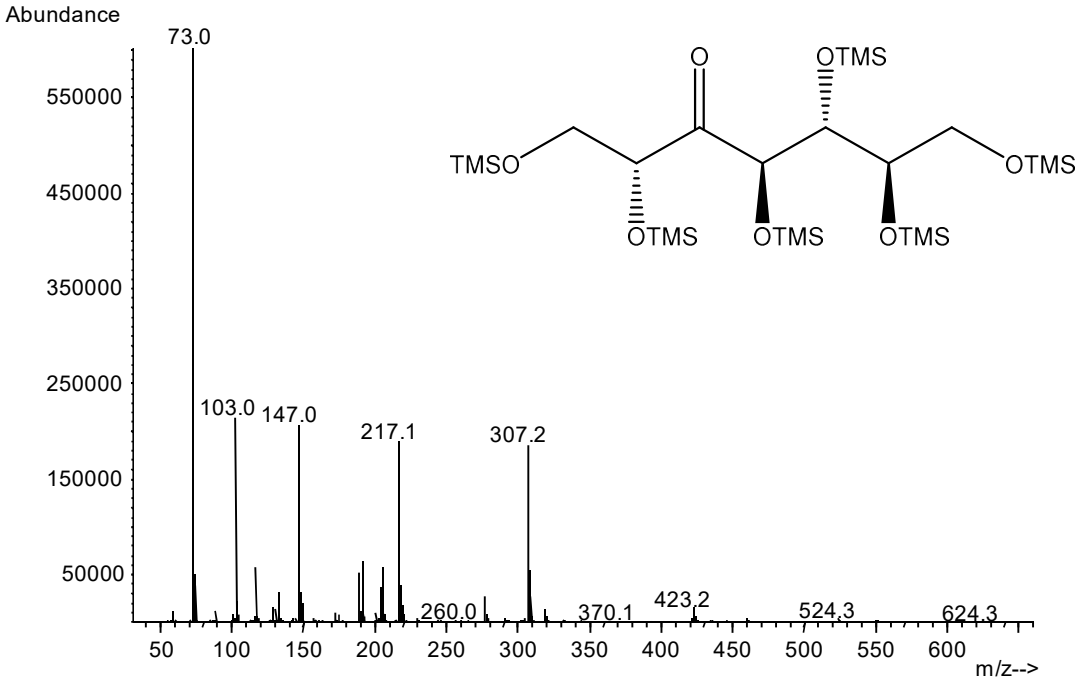

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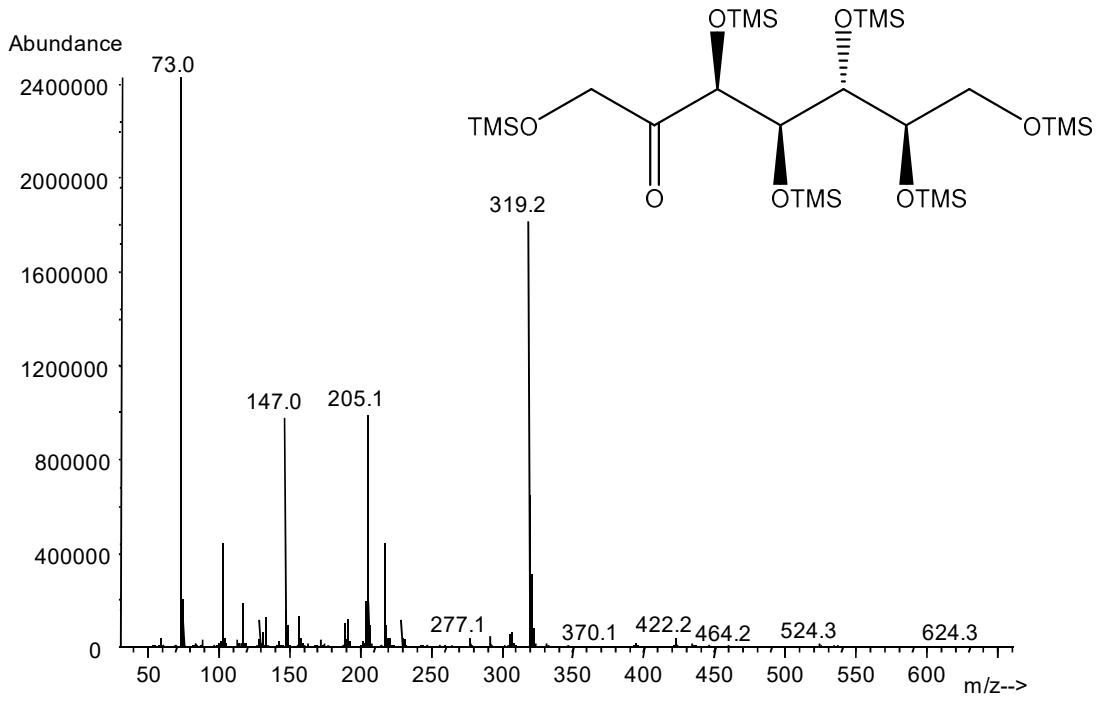

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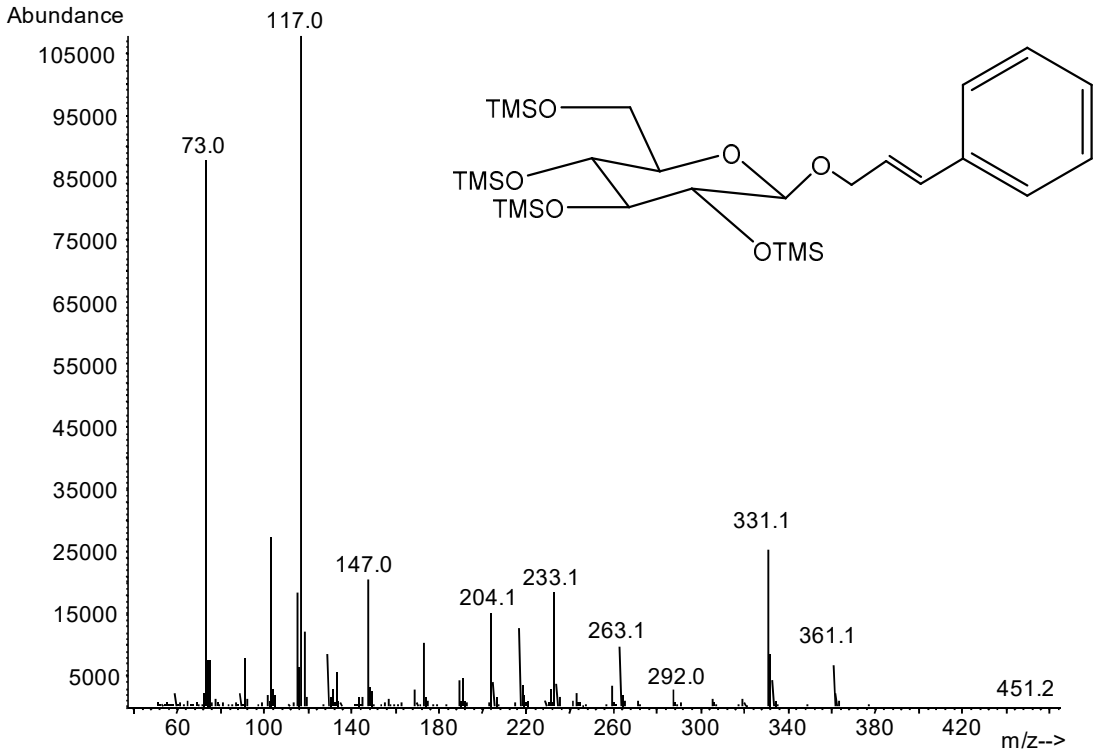

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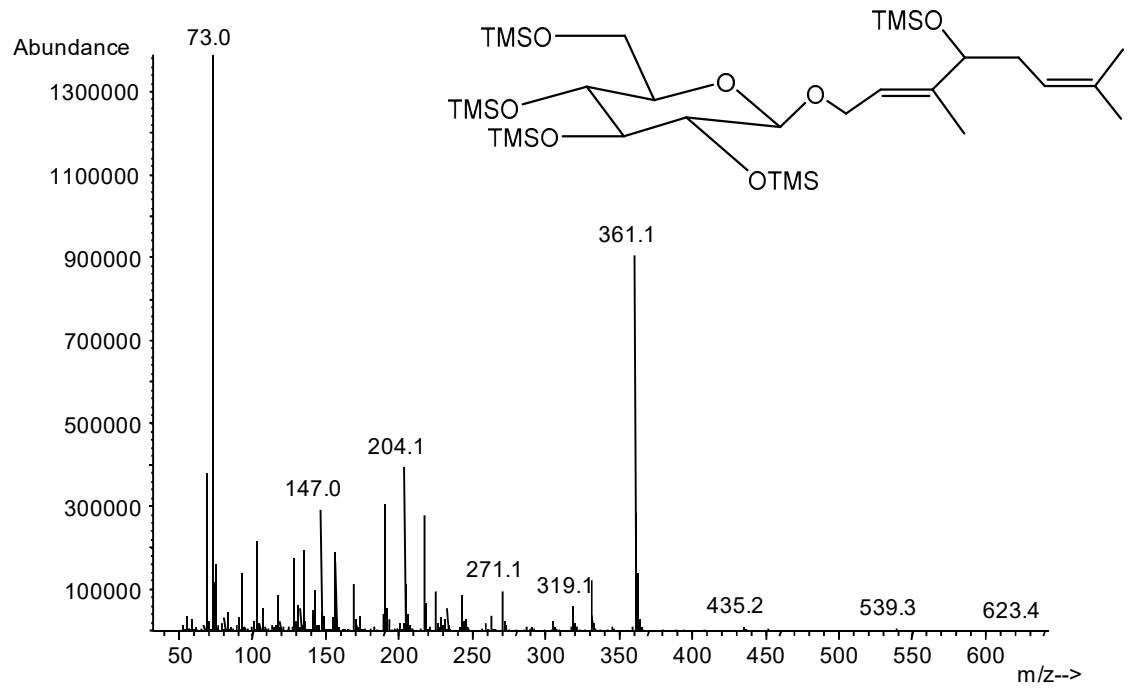

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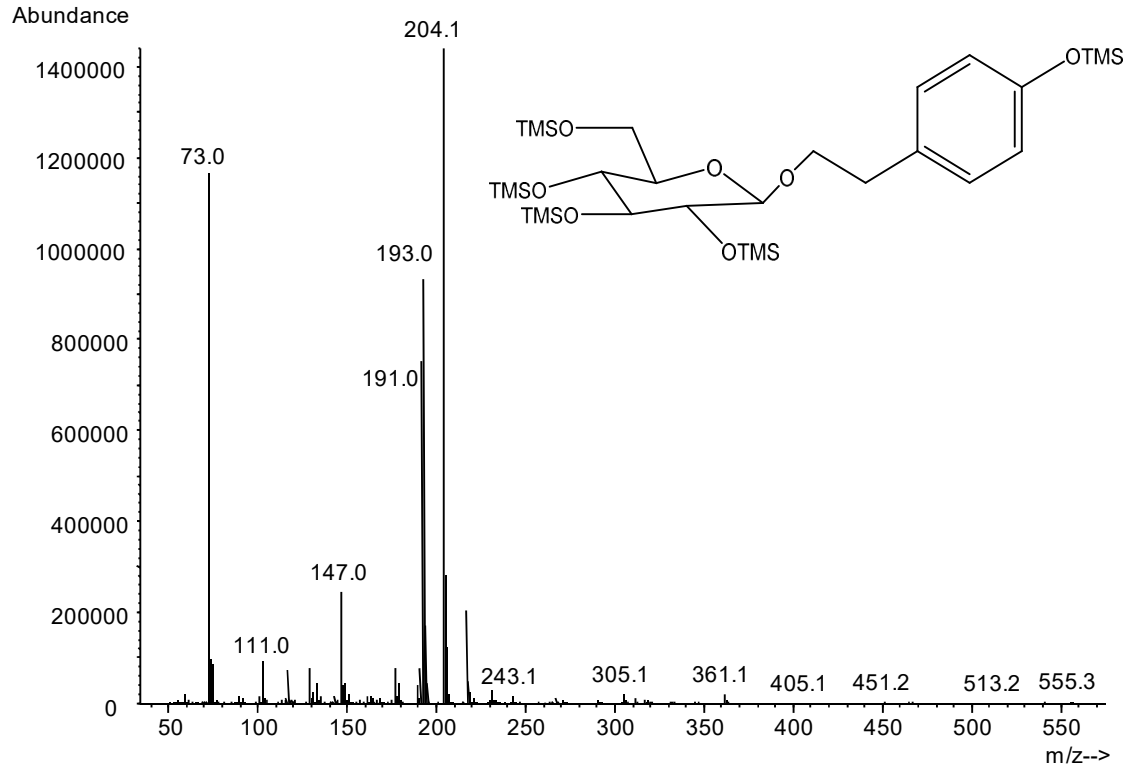

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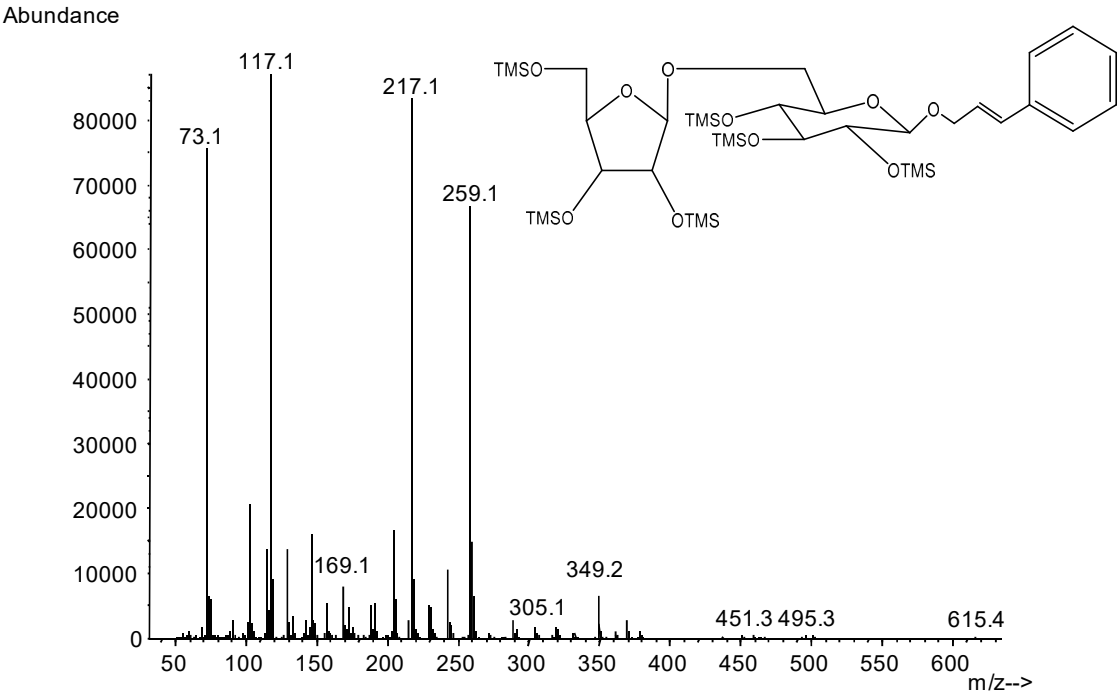

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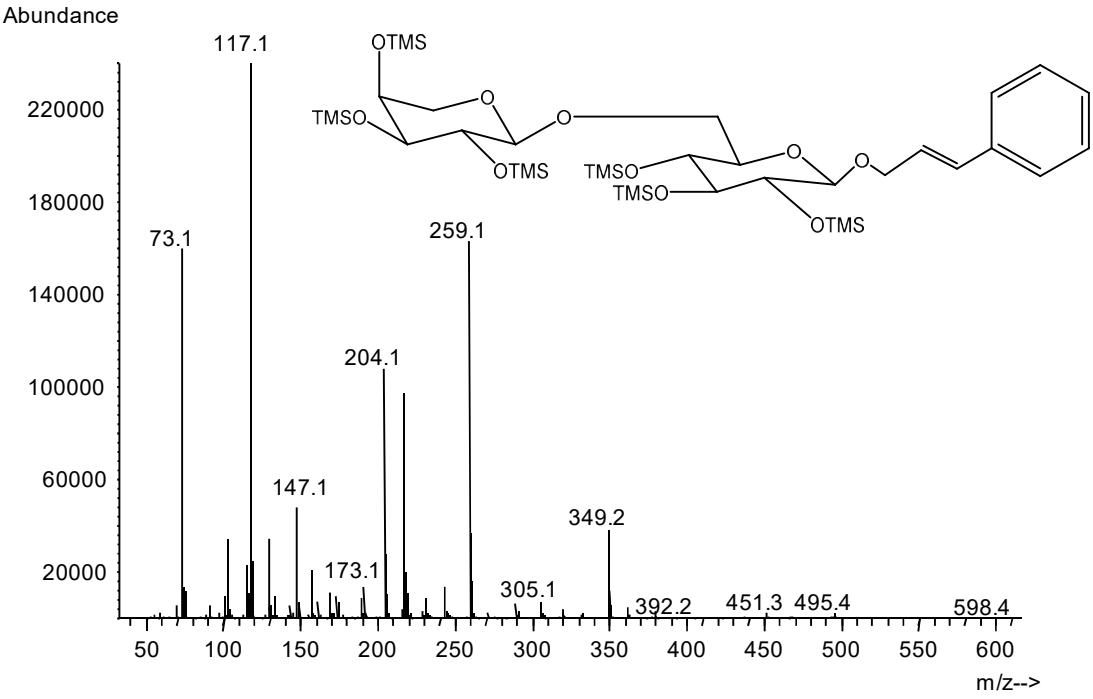

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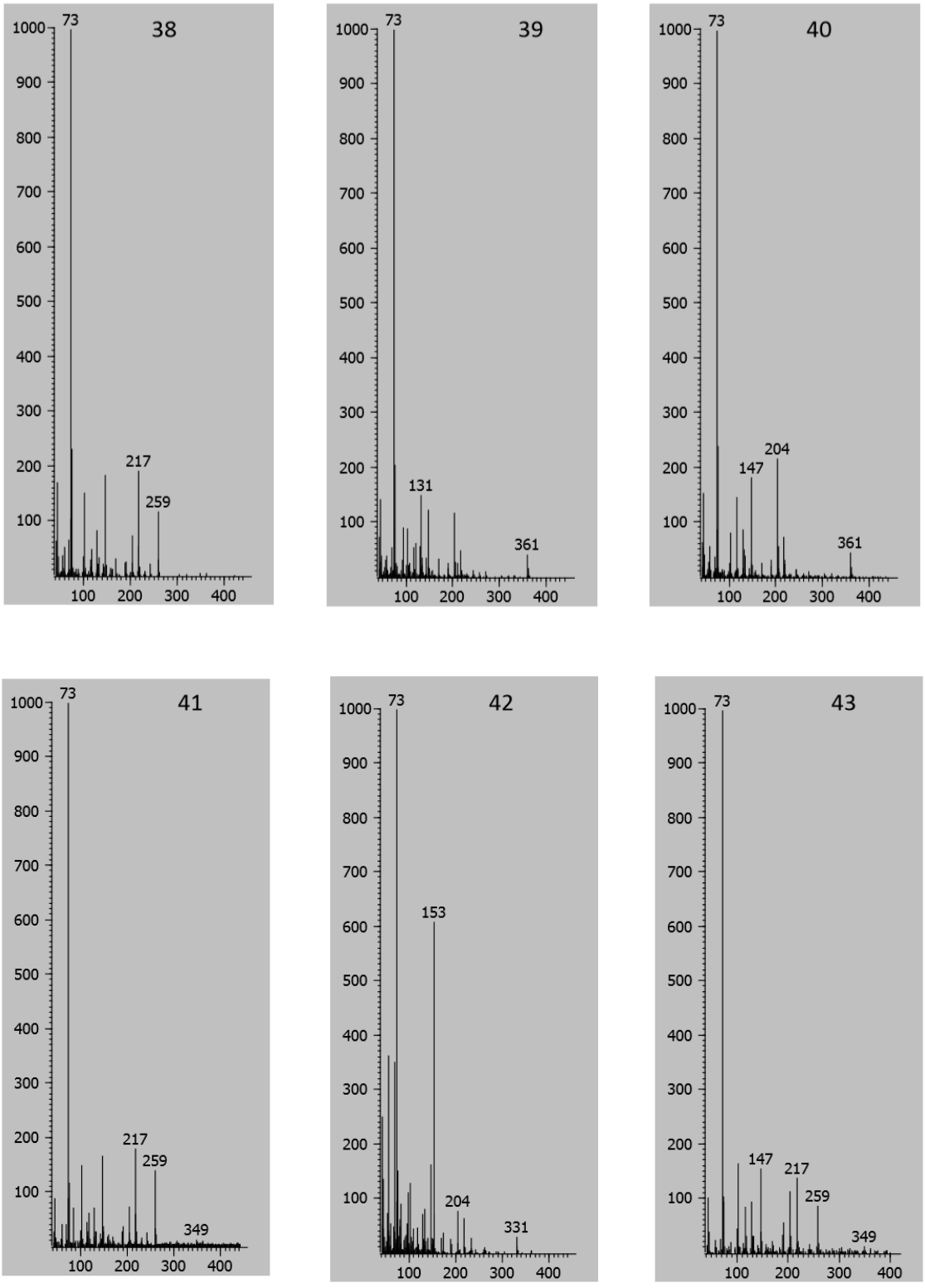
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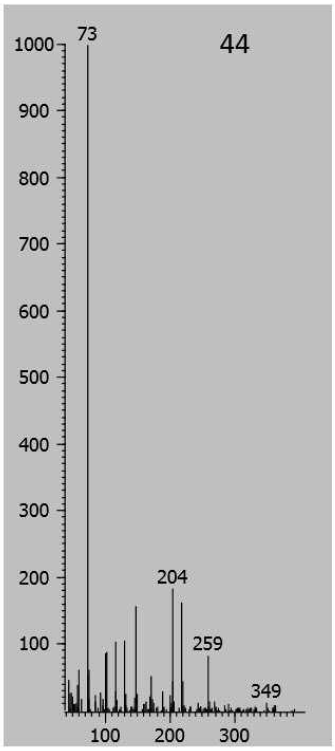
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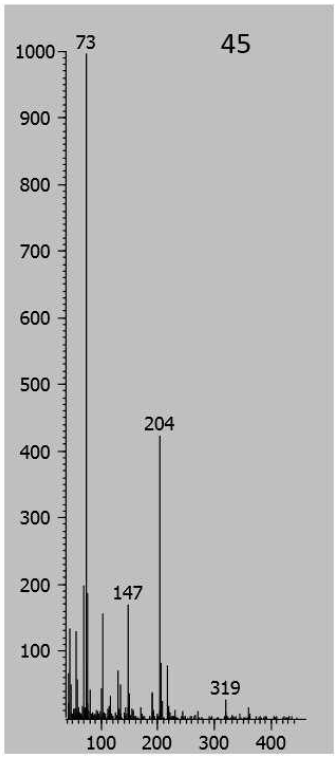
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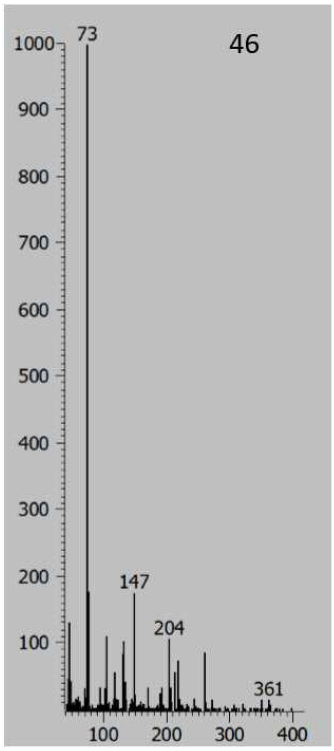
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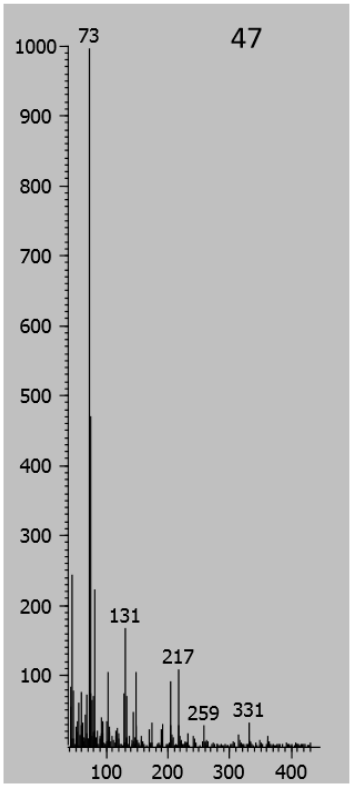
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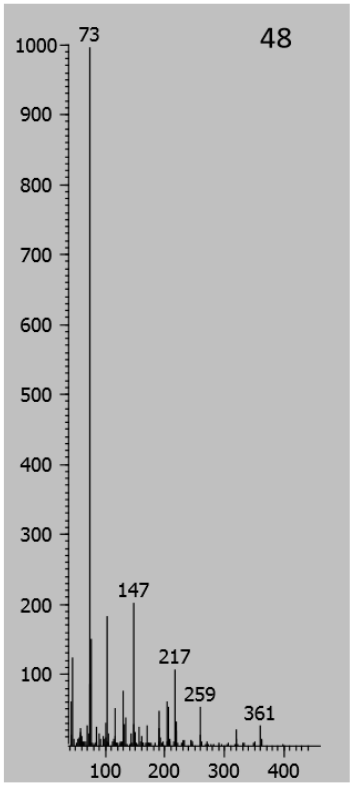
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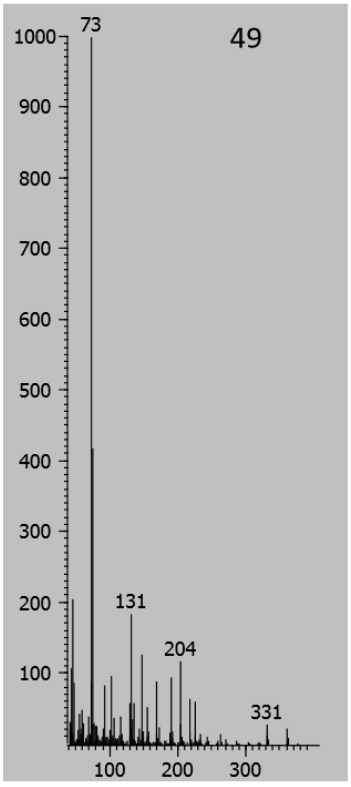
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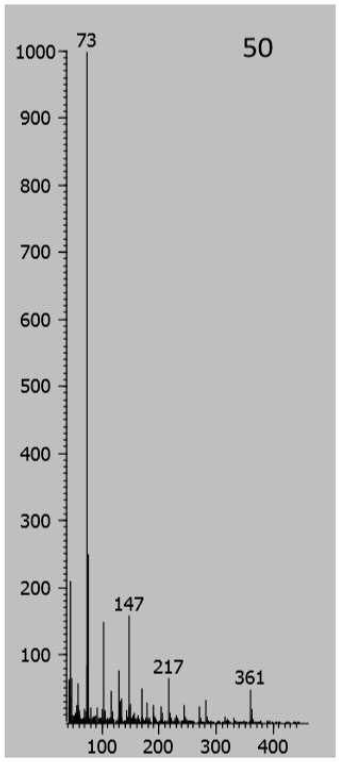
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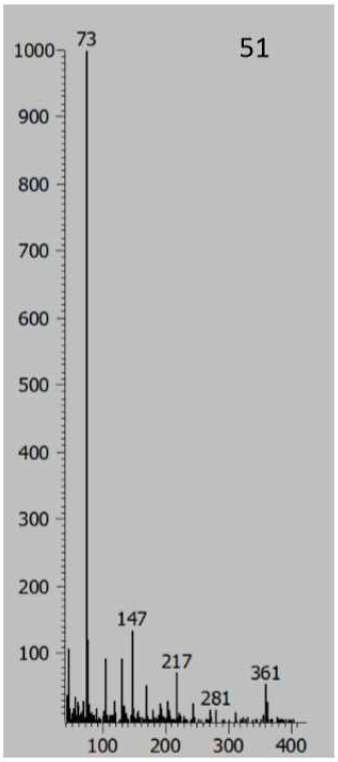
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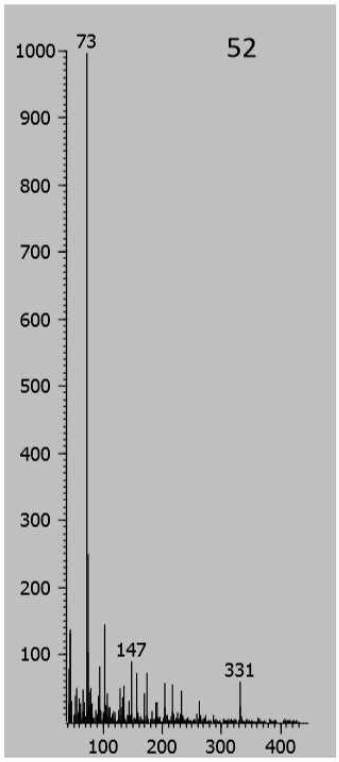
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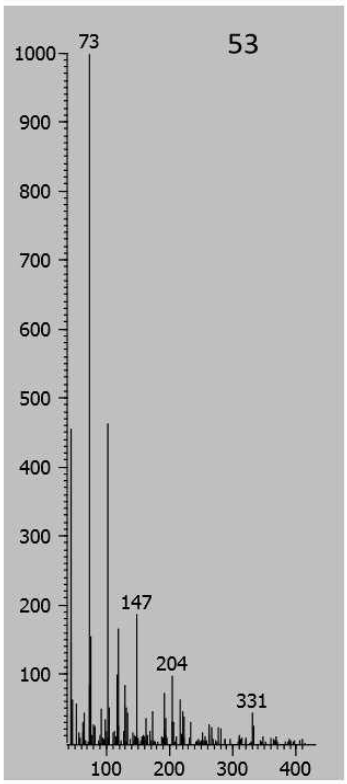
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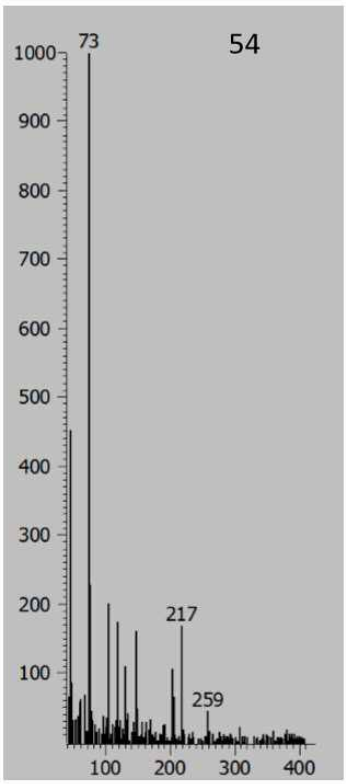
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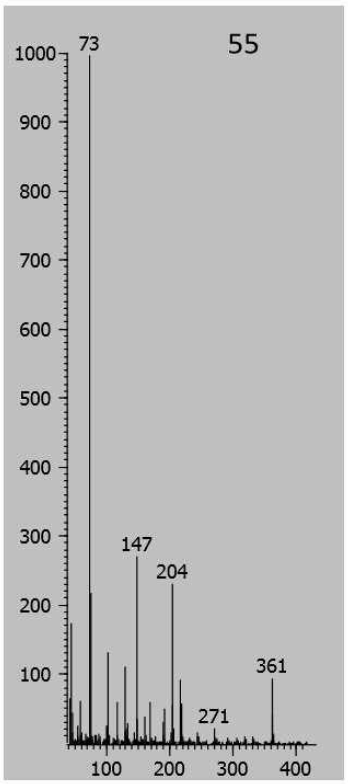
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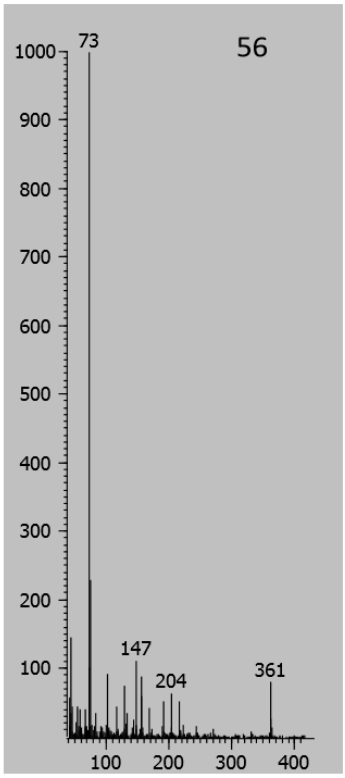
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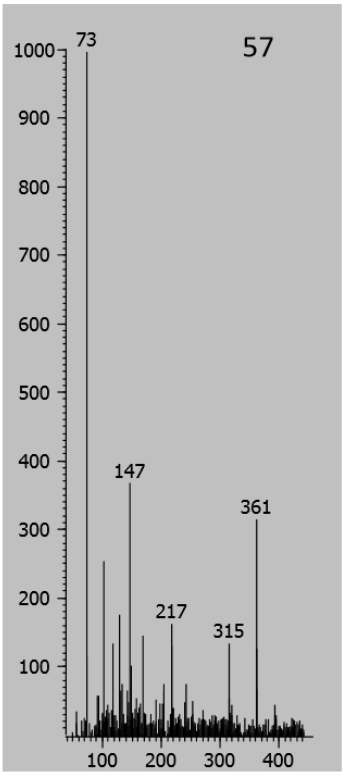
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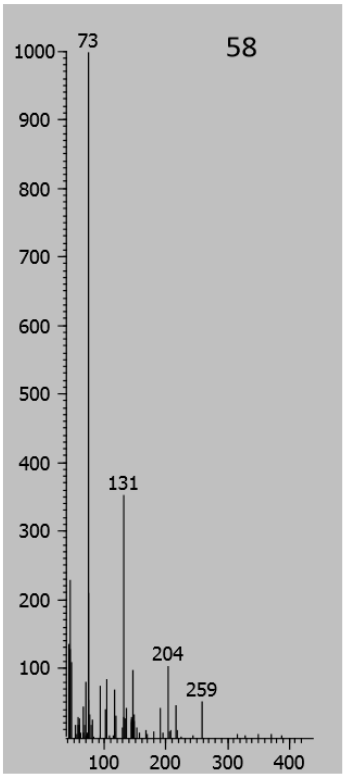
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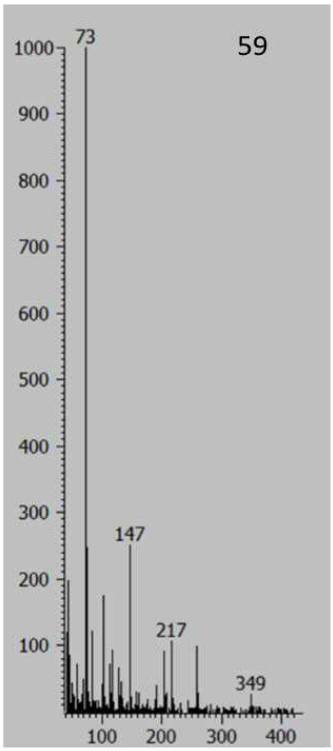
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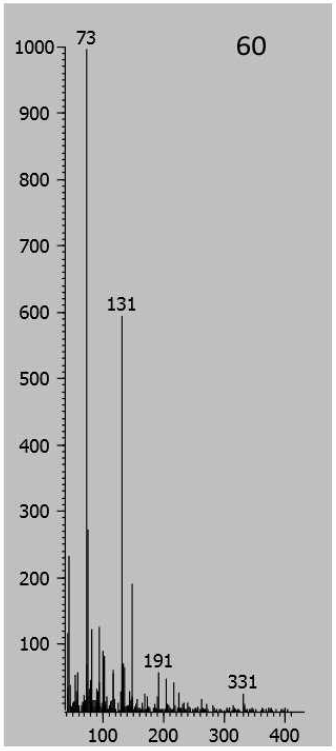
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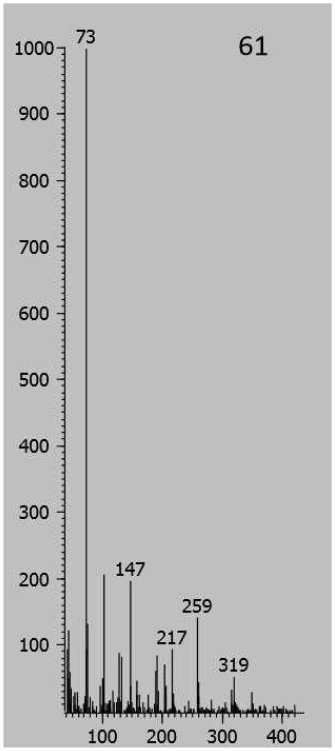
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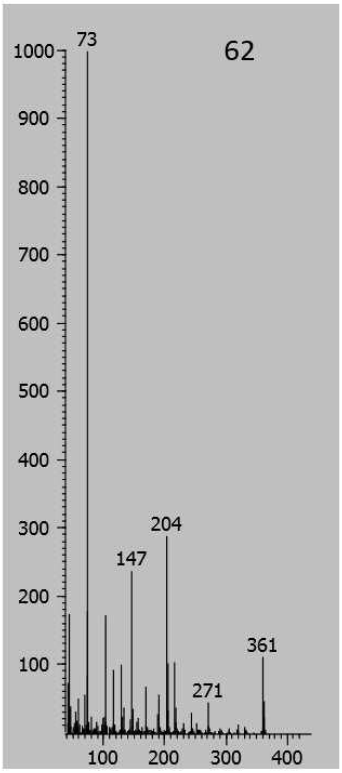
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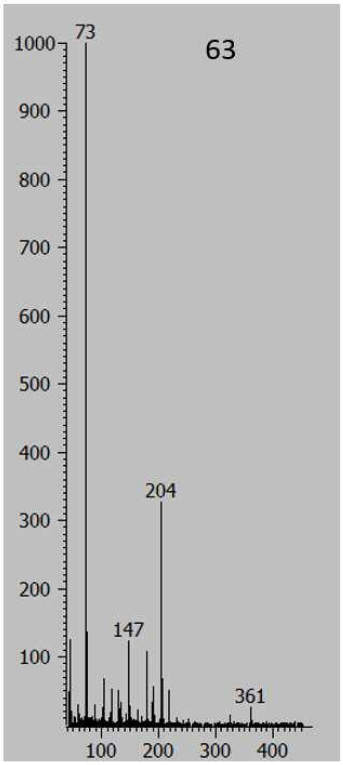
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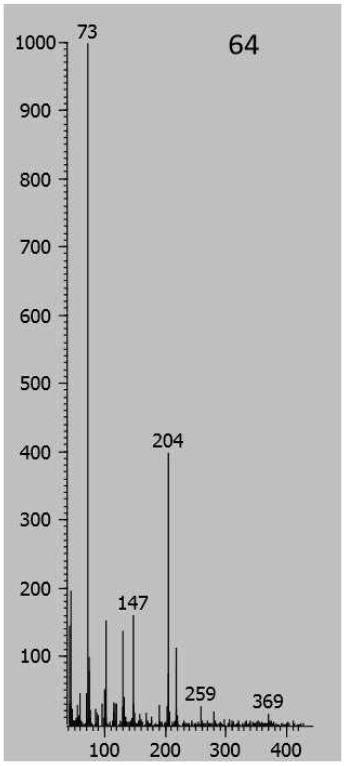
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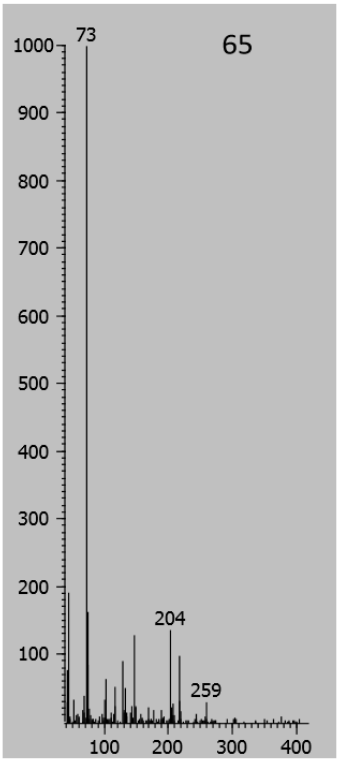
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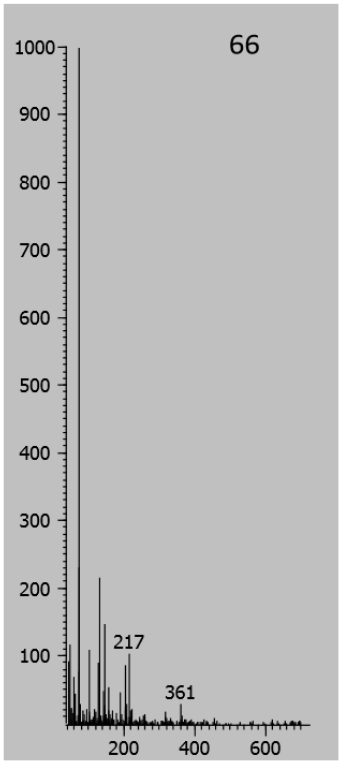
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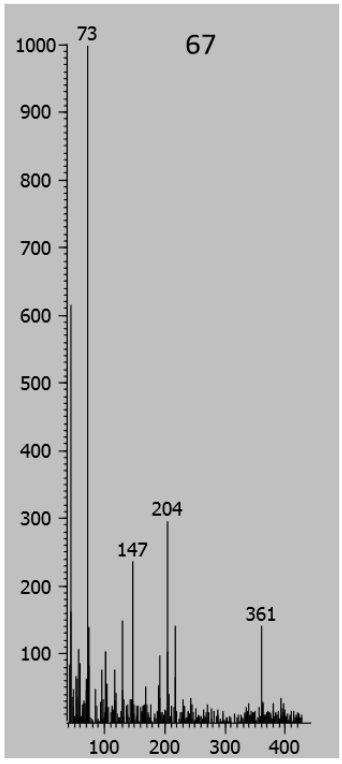
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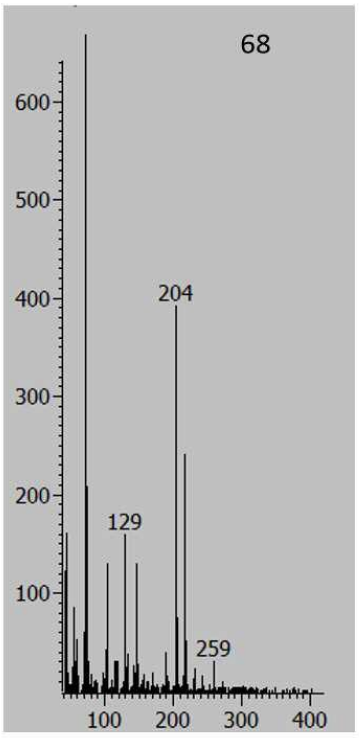
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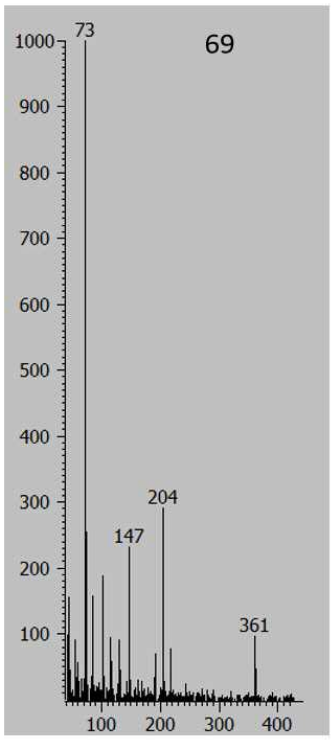
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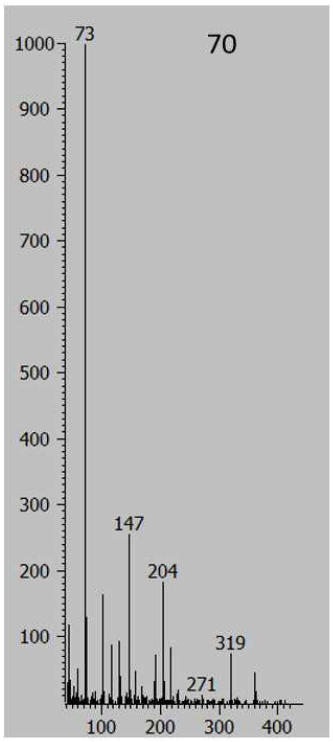
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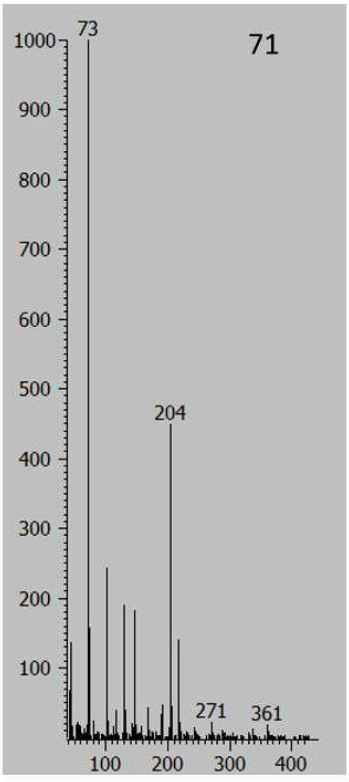
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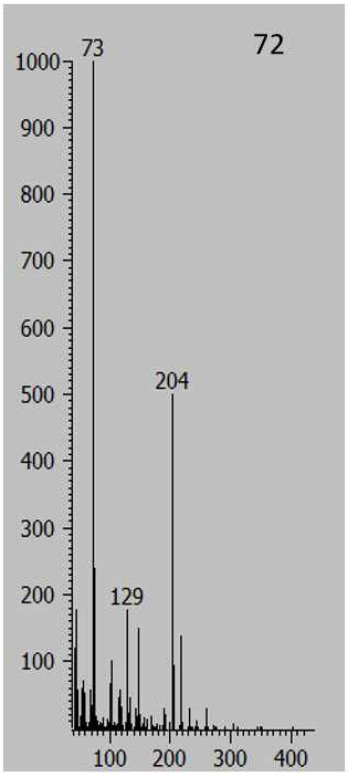
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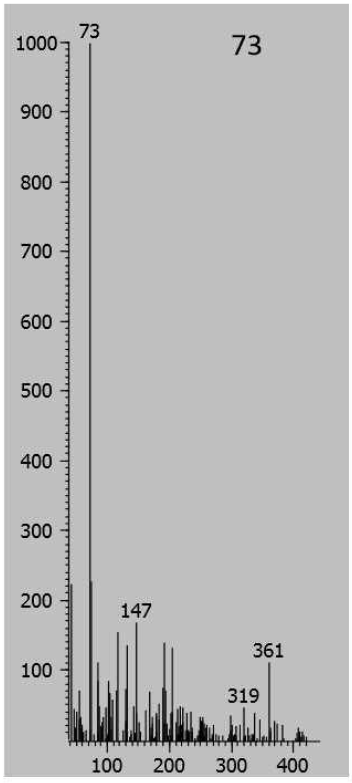
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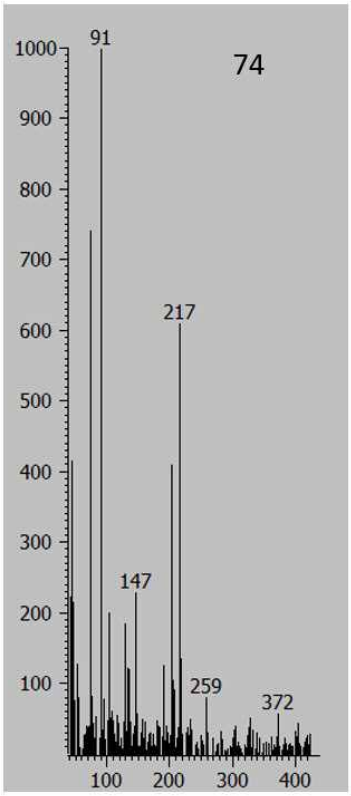
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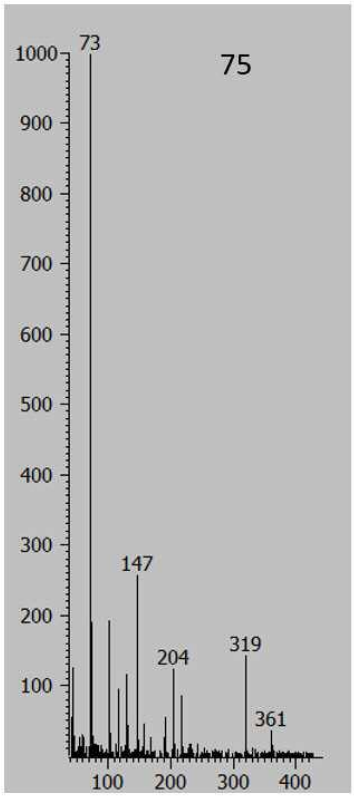
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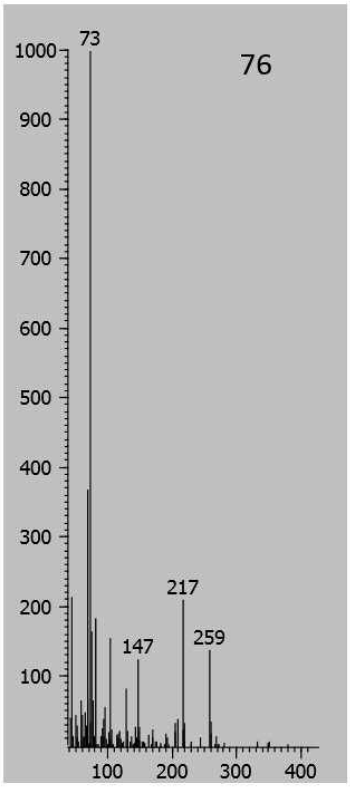
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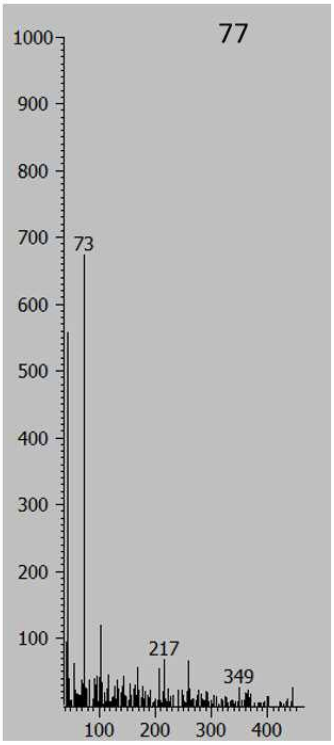
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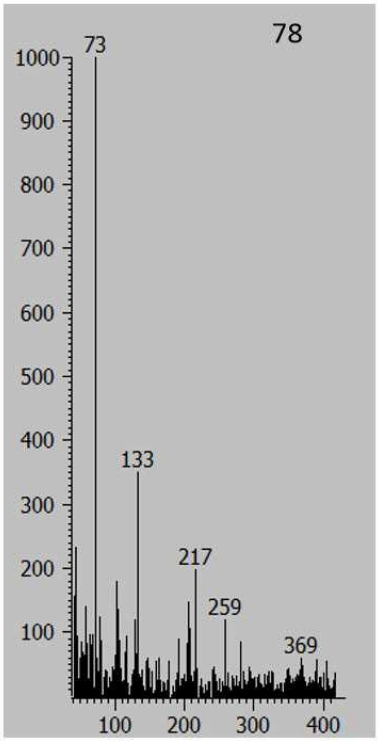
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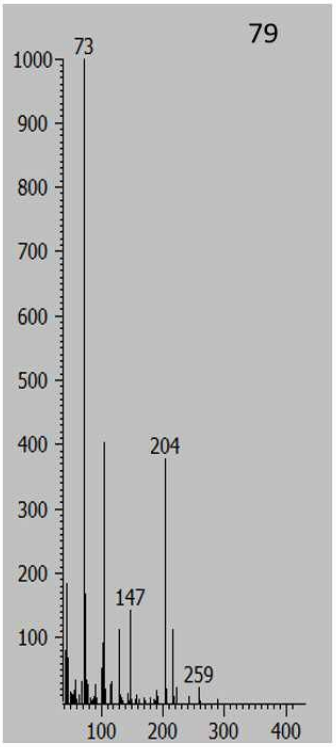
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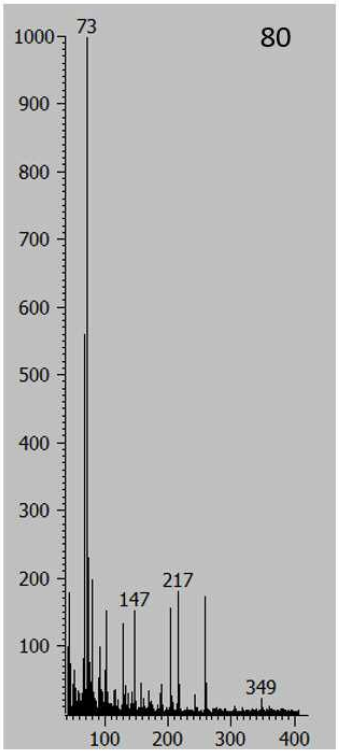
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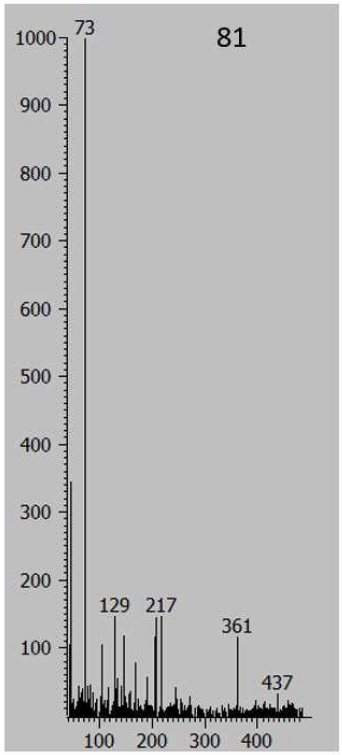
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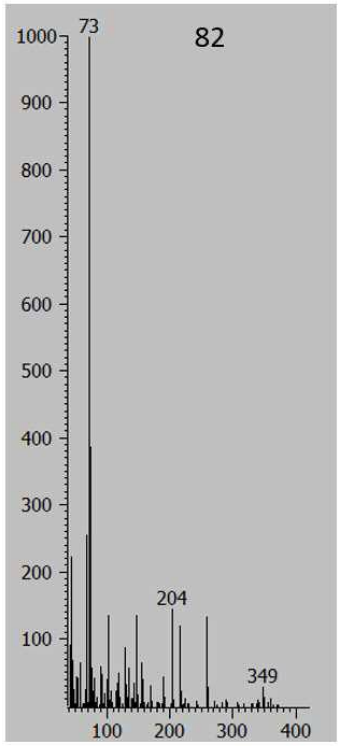
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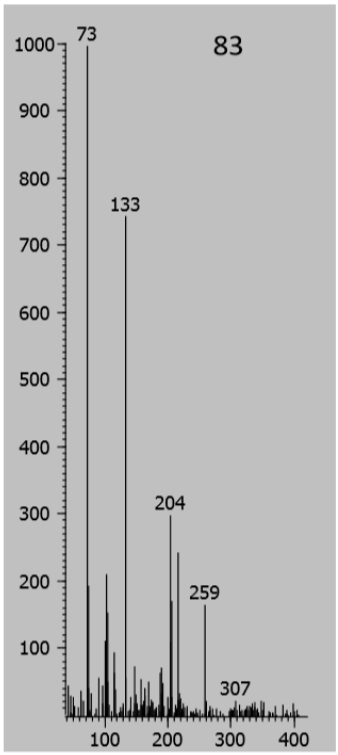
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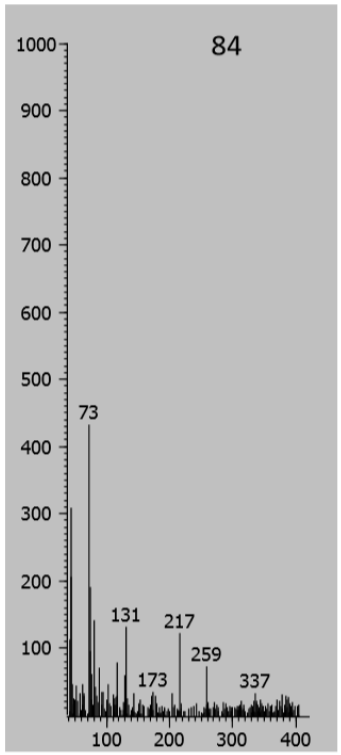
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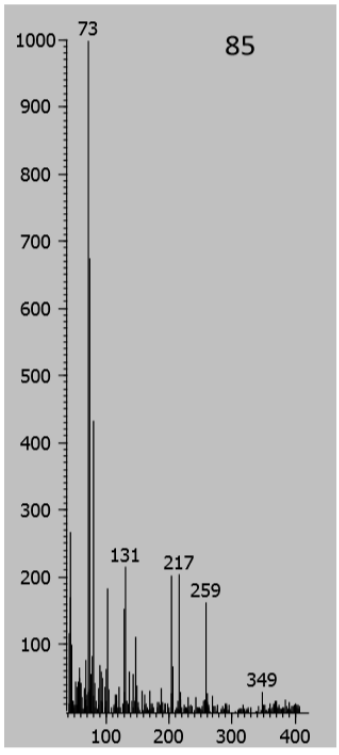
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Anexo 3

Characterization of (semi-)volatile compounds migrating into food simulants from polypropylene food containers by GC×GC–ToF MS

C. Carrero-Carralero, J. Muñoz-Arnanz, M. Ros, S. Jiménez-Falcao, M.L. Sanz, L. Ramos

Supplementary Material

Standards

PAH Mix 9 (US EPA 16), containing the 16 EPA priority PAHs in acetonitrile, and PAH mix 183, containing the 15+1 EU PAHs in isooctane, were acquired form Dr. Ehrenstorfer Laboratories (Augsburg, Germany). Two stock standard solutions containing environmentally or chromatographically relevant PCBs (PCB # 18, 28, 31, 33, 45, 47, 52, 55, 66, 74, 77, 80, 81, 84, 88, 91, 95, 99, 101, 105, 110, 114, 118, 119, 122, 123, 124, 126, 128, 129, 131,132, 135, 136, 138, 139, 141, 144, 149, 153, 155, 156, 157, 164, 167, 169, 170, 171, 173, 174, 175, 176, 178, 180, 183, 187, 189, 190, 194, 196, 197, 200, 201, 202, 206, 209) and PBDEs (PBDEs # 17, 28, 47, 66, 85, 99, 100,153 y 154) were prepared in isooctane from individual standards also purchased from Dr. Ehrenstorfer Laboratories. Syn- and anti-isomers of dechlorane plus (DP) were purchased from Wellington Laboratories Inc. (Guelph, ON, Canada). A number of phthalates and organophosphorus flame retardant standards were also available in the working group from previous studies (Escobar-Arnanz et al., 2018). Standard solutions were prepared to contain ca. 10 ng μL^{-1} of each of the corresponding compound.

Reference

Escobar-Arnanz, J., Mekni, S., Blanco, G., Eljarrat, E., Barceló, D., & Ramos, L. (2018). Characterization of organic aromatic compounds in soils affected byan uncontrolled tire landfill fire through the use of comprehensive two-dimensional gas chromatography–time-of-flight mass spectrometry. *J. Chromatogr. A, 1536*, 163-175.

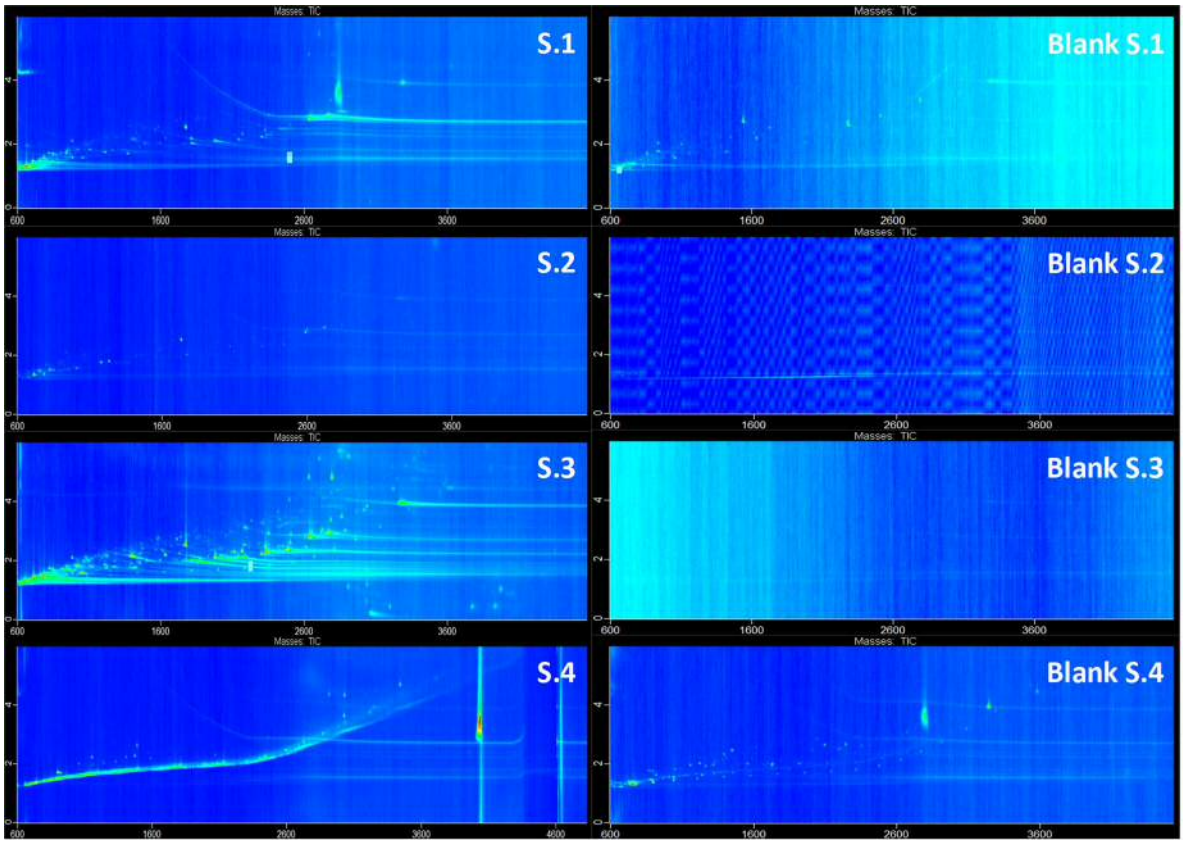

Figure. S.1. Overview of the two-dimensional chromatograms obtained for simulants obtained after treatment of food container A (left column), and corresponding reagent blanks (right column). Simulant identification: (S.1) water, (S.2) 3% acetic acid, (S.3) 10% ethanol, and (S.4) isooctane.