UNIVERSIDAD AUTÓNOMA DE MADRID

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Departamento de Química-Física Aplicada

Nuevos métodos de obtención y análisis de extractos vegetales enriquecidos en iminoazúcares bioactivos

SONIA RODRÍGUEZ-SÁNCHEZ

Tesis Doctoral
2015

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Memoria presentada por:

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Para optar al grado de
Doctor en Ciencia y Tecnología de Alimentos.

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CERTIFICAN:

Que el presente trabajo titulado "**Nuevos métodos de obtención y análisis de extractos vegetales enriquecidos en iminoazúcares bioactivos**", y que constituye la Memoria que presenta Dª. Sonia Rodríguez-Sánchez para optar al grado de Doctor en Ciencia y Tecnología de Alimentos, ha sido realizado en el Departamento de Análisis Instrumental y Química Ambiental del Instituto de Química Orgánica General del C.S.I.C, bajo su dirección.

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

Fdo. Dª. María Luz Sanz Murias **Fdo. Dª. Ana Cristina Soria Monzón**

INDICE GENERAL

INDICE

ABREVIATURAS

I. Abreviaturas

^{13}C-NMR	Resonancia magnética nuclear de carbono
^{1}H-NMR	Resonancia magnética nuclear de protón
AEC	Cromatografía de intercambio aniónico
APCI	Ionización química a presión atmosférica
A_s	Factor de asimetría de pico
BEH	Fase estacionaria basada en puentes híbridos de etileno con amida enlazada trifuncionalmente
BSTFA	*N,O*-bis-(trimetilsilil)-trifluoroacetamida
CEC	Cromatografía de intercambio catiónico
CID	Disociación inducida por colisión
DAB-1	(2R,3S)-2-hydroxymethyl-3-hydroxypyrrolidine, 1,4-dideoxy-1,4-imino-D-arabinitol
d_c	Diámetro de la columna
d_f	Diámetro de fase
DFAs	Dianhídridos de fructosa
DFJ	1-deoxyfuconojirimycin
DGJ	1-deoxygalactonojirimycin
DIJ	Deoxy-L-idonojirimycin
DMDP	2,5-didesoxi-2,5-imino-*D*-manitol
DMJ	1-desoximanojirimicina
DNJ	1-desoxinojirimicina
DP	Grado de polimerización
EI	Impacto electrónico
ESI	Ionización por electrospray
F	Factor de Fisher
FABMS	Espectrometría de masas de bombardeo atómico rápido
FID	Ionización de llama
Gal	Galactosa
GC	Cromatografía de gases
GC-MS	Cromatografía de gases acoplada a espectrometría de masas
H	Altura equivalente de plato teórico

HILIC	Cromatografía de interacción hidrofílica
HMDS	Hexametildisilazano
HMJ	Homomanojirimicina
HNJ	Homonojirimicina
HPAEC	Cromatografía líquida de intercambio aniónico de alta resolución
HPLC	Cromatografía líquida de alta resolución
I	Índice de retención
i.d.	Diámetro interno
IEC	Cromatografía de intercambio iónico
ILs	Líquidos iónicos
IR	Espectroscopía infrarroja
I^T	Índice de retención lineal
L	Longitud de la columna
LC	Cromatografía líquidos
LMWC	Carbohidratos de bajo peso molecular
LOD	Límite de detección
LOQ	Límite de cuantificación
m/z	Relación masa/carga
MLR	Regresión lineal múltiple
MRM	Modo monitorización de reacciones múltiples
MS	Espectrometría de masas
MS^n	Espectrometría de masas en tándem
MSTFA	*N*-metil-*N*-(trimetilsilil) trifluoroacetamida
n_c	Capacidad de pico
N_{max}	Número de platos máximo
NMR	Resonancia magnetic nuclear
P	Polaridad
PAAN	Aldononitrilos peracetilados
PAD	Amperometría de pulsos
PCA	Análisis de componentes principales
PHEA	Fase estacionaria tipo polihidroxietil aspartamida
PLE	Extracción con líquidos presurizados
Q-TOF	Cuadrupolo acoplado a tiempo de vuelo
R_{adj}	coeficiente de correlación ajustado a los grados de libertad

RF	Factor de respuesta
RPLC	Cromatografía de fase inversa
R_s	Resolución
RSD	Desviación estándard relativa
S/N	Relación señal/ruido
SCAN	Modo barrido de masas
SIM	Modo selectivo de iones
SIR	Modo de registro selectivo de iones
SP	Fase estacionaria
SPM	Modelo de los parámetros de solvatación
SWE	Extracción con agua subcrítica
S_y	Error standard de la estimación
t_a	Tiempo de análisis total
TF	Factor de cola
TFA	Ácido trifluoroacético
t_M	Tiempo muerto
TMCS	Trimetilclorosilano
TMS	Trimetilsilil derivados
TMSI	Trimetilsililimidazol
TMSO	Trimetilsilil oximas
t_R	Tiempo de retención
TZ	Número de separación media
UTE	Utilización de eficiencia teórica
w_b	Anchura de pico en la base
w_h	Anchura de pico medida a la mitad de altura
ZIC	Fase estacionaria tipo HILIC basada en una sulfobetaína zwiterionica
σ	Desviación estándar del ruido

RESUMEN

II. RESUMEN

Los iminoazúcares (también llamados polihidroxialcaloides) son reconocidos inhibidores de glicosidasas. Esta actividad inhibitoria les confiere gran utilidad como candidatos para el tratamiento de numerosas enfermedades relacionadas con la absorción de carbohidratos, tales como diabetes y obesidad, enfermedades de gran repercusión actual. Existe, por tanto, un gran interés desde distintos ámbitos por la obtención de estos compuestos, principalmente a partir de fuentes naturales, para poder ser empleados como complementos bioactivos o ingredientes de alimentos funcionales. Sin embargo, las relativamente bajas concentraciones en las que se encuentran estos iminoazúcares en matrices vegetales, los tediosos procesos de extracción aplicados hasta el momento, la co-extracción de otros carbohidratos tales como glucosa o fructosa presentes en mayores concentraciones y que pueden interferir en la actividad de los iminoazúcares, y la gran variedad de estos compuestos con estructura similar, hacen que sea necesario abordar estudios en los que se evalué su potencial de aplicación como ingredientes con carácter funcional en alimentos.

Así, el objetivo principal de esta Tesis Doctoral se ha centrado en el **desarrollo de métodos de obtención y análisis de extractos enriquecidos en iminoazúcares bioactivos a partir de muestras de origen vegetal**. Las muestras consideradas han sido hojas, ramas y frutos de morera, bulbos de jacinto, hojas y raíces de Aglaonema y granos de trigo sarraceno.

En cuanto a los métodos analíticos, se ha evaluado el uso tanto de la cromatografía de gases acoplada a espectrometría de masas (GC) como de la de líquidos acoplada a masas tándem (LC-MS^2), para el análisis simultáneo de iminoazúcares y otros carbohidratos de bajo peso molecular presentes en los extractos vegetales en estudio. En GC, ha resultado especialmente relevante la selección del tipo de derivado y agentes derivatizantes para conseguir derivados estables y evitar reacción parciales, la optimización de condiciones cromatográficas tales como temperatura del inyector, gradiente de temperatura del horno, etc. para la elución de todos los carbohidratos presentes en los extractos, y la evaluación del uso de detectores de ionización de llama (FID) o de MS (modo registro selectivo de iones o modo barrido de iones). Se ha

llevado a cabo también un estudio exhaustivo de los datos proporcionados mediante GC-MS (índices de retención lineales y abundancia de relaciones *m/z* de iones fragmento característicos) para patrones de iminoazúcares, pudiendo relacionarlos con su estructura química y permitiendo así la caracterización/identificación tentativa de iminoazúcares desconocidos. Asimismo, se ha evaluado por primera vez el empleo de nuevas fases estacionarias comerciales basadas en líquidos iónicos para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular, previa caracterización mediante el Modelo de los Parámetros de Solvatación de las propiedades de retención de dichas columnas.

En cuanto a la LC, se ha seleccionado la cromatografía de interacción hidrofílica (HILIC) como modo de operación por sus ventajas para el análisis de moléculas polares. La evaluación de distintas fases estacionarias y condiciones cromatográficas (gradientes, flujos, modificadores orgánicos y aditivos, temperaturas del horno, etc.), así como el empleo de MS^2 permitió la detección e identificación de nuevos iminoazúcares en las muestras estudiadas.

Todos los métodos optimizados, tanto de GC como de LC, han sido validados en términos de linealidad, recuperación, precisión y sensibilidad.

Una vez desarrollados los requeridos métodos de análisis, se ha procedido a la optimización de los métodos de extracción y fraccionamientos. La extracción con disolventes presurizados (PLE) ha resultado ser una técnica apropiada para la extracción de iminoazúcares, obteniéndose rendimientos similares a los de la extracción sólido líquido convencional pero en un menor tiempo y empleando menores volúmenes de disolvente. Por otra parte, el fraccionamiento de iminoazúcares y la consiguiente eliminación selectiva de otros carbohidratos de bajo peso molecular co-existentes en extractos vegetales mediante el empleo de levaduras (*Saccharomyces cerevisiae*) ha probado ser ventajosa sobre el empleo de resinas de intercambio catiónico por su rapidez y por el carácter “limpio” de este procedimiento para su aplicación al desarrollo de alimentos funcionales.

Por último, como primera aproximación al empleo de estos extractos como ingredientes funcionales o suplementos bioactivos, y centrándonos en la muestra de Aglaonema por su complejidad y mayor contenido en

iminoazúcares, se ha comprobado su estabilidad en condiciones de almacenamiento acelerado (50 ºC, 1 mes), su alta bioactividad (ensayos *in vitro* de actividad inhibidora de *α*-glucosidasas) y su seguridad (ensayos de viabilidad celular de células Caco-2) en concentraciones menores de 125 µg mL^{-1}

Los resultados de carácter multidisciplinar descritos en esta Tesis suponen una contribución destacable en el campo del desarrollo de nuevos ingredientes y complementos bioactivos, tema éste de gran repercusión tanto científica como económica.

INTRODUCCIÓN GENERAL

1. Introducción general

1.1. Carbohidratos bioactivos en los alimentos

Los carbohidratos se definen como polihidroxi-aldehídos o polihidroxi-cetonas con fórmula general $(CH_2O)_n$, donde *n* es igual o mayor de 3. Dentro de esta categoría se incluyen también otros compuestos con distinta fórmula empírica tales comodesoxi azúcares, aminoazúcares, iminoazúcares, etc. En función de su grado de polimerización (DP), los carbohidratos se dividen en monosacáridos (DP1), disacáridos (DP2), oligosacáridos (DP3-20) y polisacáridos (DP>20).

Los carbohidratos son uno de los constituyentes más importantes de los alimentos ya que pertenecen al grupo de nutrientes básicos implicados en la alimentación y en el metabolismo. Pueden encontrarse presentes de manera natural en el alimento o ser adicionados para la mejora de diversas propiedades (sensoriales, funcionales, tecnológicas, etc.)

Así, entre otras, los carbohidratos presentan toda una serie de funciones relevantes en la industria alimentaria como edulcorantes, gelificantes, espesantes, estabilizadores y precursores de compuestos con aroma o color que se forman a partir de ellos por medio de diversas reacciones [1]. Además, se conocen en la actualidad determinados carbohidratos que, además de sus propiedades nutricionales, presentan un efecto beneficioso para la salud del consumidor. Son los denominados carbohidratos bioactivos, entre los que se incluyen prebióticos [2], inositoles [3] e iminoazúcares [4], siendo el estudio de estos últimos el objeto de esta Tesis.

1.2. Iminoazúcares

1.2.1. Definición y clasificación

Los iminoazúcares, también llamados azazúcares y polihidroxialcaloides, son carbohidratos bioactivos de bajo peso molecular con estructura similar a la de los monosacáridos, en los que el átomo de oxígeno del anillo se ha reemplazado por un átomo de nitrógeno.

Su estructura puede ser mono- o bicíclica, incluyendo un mínimo de dos grupos hidroxilo y un átomo de nitrógeno heterocíclico, con grupos estructurales basados en anillos de cinco y seis átomos de carbono. Dependiendo de su estructura química se

clasifican en cinco grupos: piperidinas (e.g. 1,5-didesoxi-1,5-imino-*D*-glucitol o 1-desoxinojirimicina (DNJ) y 2,6-didesoxi-2,6-epiimino-*D*-glicero-*L*-gulo-heptitol o *α*-homonojirimicina (*α*-HNJ)), pirrolidinas (e.g. 2,5-didesoxi-2,5-imino-*D*-manitol (DMDP)), indolicidinas (e.g. swainsonina), pirrolicidinas (e.g. australina) y nortropanos (e.g. calisteginas). En la **Figura 1.1** se puede ver un ejemplo de cada una de estas estructuras.

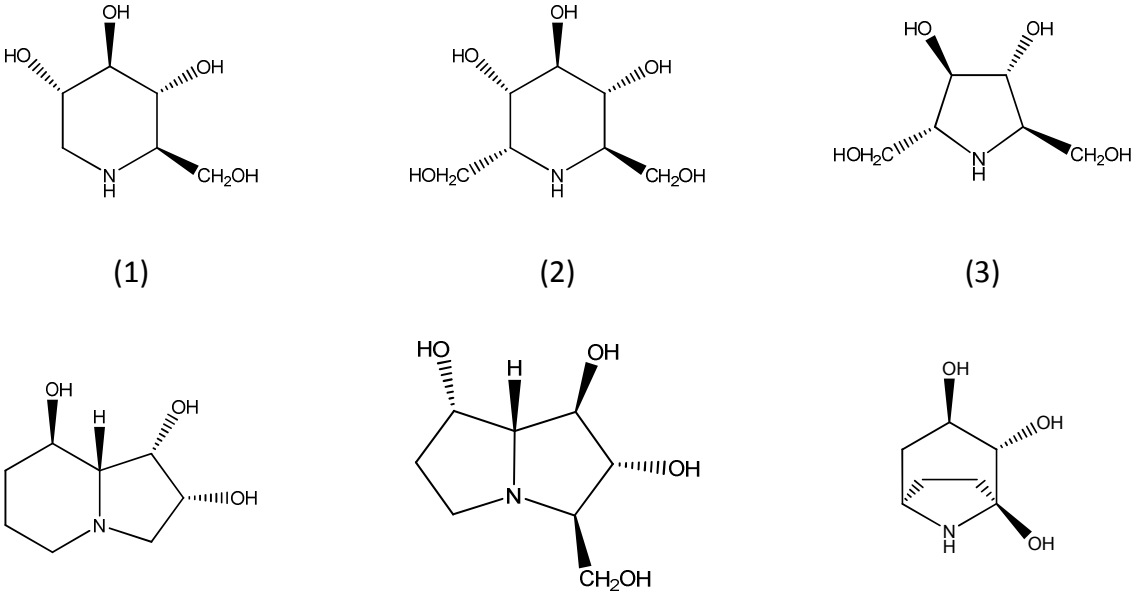


Figura 1.1. Estructuras químicas de DNJ (1), *α*-HNJ (2), DMDP (3), swainsonina (4), australina (5) y calistegina A_3 (6).

1.2.2. Presencia de iminoazúcares en fuentes naturales

Su potencial como compuestos bioactivos ha hecho que en la actualidad exista un creciente interés por la obtención de iminoazúcares, ya sea mediante síntesis química [5-7], o mediante su extracción a partir de fuentes naturales.

En la naturaleza, los iminoazúcares se encuentran presentes en numerosas familias de plantas (Moraceae, Leguminosae, Hyacinthaceae, Araceae, etc.) [8-11] y en diversos géneros de microorganismos (*Streptomyces, Bacillus*, etc.) [10, 12-14]. Entre las fuentes de origen vegetal, cabe destacar las siguientes:

1.2.2.1. Morera (*Morus* sp.)

El género *Morus* engloba entre 10 y 16 especies de árboles caducifolios, en su mayoría procedentes de Asia, principalmente de países como China, Corea, Japón y Tailandia. De entre ellas destacan la morera blanca o morera propiamente dicha (*Morus alba*) y la morera negra o moral común (*Morus nigra*). En Asia, se comercializan distintos productos de grado alimentario basados en extractos de morera [15], y los pacientes con diabetes mellitus consumen infusiones de sus hojas por sus propiedades antihiperglicémicas [16]. Dichas propiedades, como se detalla en la **sección 1.2.3**, son atribuidas al rico contenido en polihidroxialcaloides de sus hojas, raíces, ramas y frutos [17]. Las cantidades y el tipo de polihidroxialcaloides varían dependiendo tanto de la especie como de la época del año de su recogida, tamaño de las hojas, etc., siendo, en general, la DNJ y la fagomina (1,2-didesoxinojirimicina) los más abundantes (0.1-0.21 % en peso seco) [15].

Figura 1.2. *Morus alba*

1.2.2.2. Aglaonema (*Aglaonema* sp.)

El género *Aglaonema* pertenece a la familia Araceae y comprende 21 especies (*A. commutatum, A. crispum, A. nitidum), con numerosos cultivares (A. commutatum "*Treubii"*, A. commutatum "*Emerald beauty", etc.), todas ellas herbáceas de hoja perenne. Proceden fundamentalmente del sureste de Asia (noreste de India, zona sur de China, Indonesia y Nueva Guinea), donde habitan en bosques tropicales con alto grado de humedad y poca luz [18, 19]. Debido a la atractiva variedad de sus hojas y a su baja tolerancia a la luz, las especies de aglaonema se han cultivado

Figura 1.3. *Aglaonema commutatum* "Treubii"

ampliamente en China y otros países asiáticos durante siglos como plantas ornamentales de interior [20]. Es una de las fuentes naturales que posee una mayor variedad de iminoazúcares, incluyendo: DMDP, α- y β-HNJ, α- y β-homomanojirimicinas (α- y β-HMJ) y α-homo-*allo*-nojirimicina [11, 21, 22]. Los compuestos más abundantes suelen ser el DMDP y la α-HNJ, aunque los estudios sobre la composición en iminoazúcares de esta planta son todavía escasos [11].

1.2.2.3. Jacinto (*Hyacinthus* sp.)

El jacinto es una planta bulbosa herbácea ornamental de las más cultivadas, ya sea interiormente como en exterior. Pertenece a la familia de las liliáceas, subfamilia escilóideas y subclase monocotiledónea [23]. Es una planta de unos 25-30 cm de altura, con un grueso bulbo provisto de túnica (película que tiene como finalidad proteger las escamas carnosas). Se cultiva a lo largo de todo el mundo, aunque su origen se centra en el Mediterráneo y Sudáfrica [24]. En la actualidad, se comercializan unas 50 especies procedentes de las plantaciones de Holanda, Francia, Inglaterra y Alemania [23]; una de las más conocidas es el *Hyacinthus orientalis*, en cuyos bulbos pueden encontrarse un gran número de polihidroxialcaloides [25], incluida la α-HNJ, que es el iminoazúcar que se encuentra en mayor cantidad en esta especie [26]. Al igual que para la Aglaonema son escasos los trabajos en los que se estudia su contenido en iminoazúcares.

Figura 1.4. *Hyacinthus orientalis*

1.2.2.4. Trigo sarraceno o alforfón (*Fagopyrum esculentum*)

Se trata de una planta anual herbácea cultivada por sus granos para consumo humano y animal. Se considera popularmente un cereal, aunque realmente no lo sea; ya que, aunque posee características similares, no pertenece a la familia de las gramíneas, sino a las poligonáceas. Es originario del Asia Central, aunque se ha cultivado también tradicionalmente en muchos otros países. Hoy en día los principales países productores son también los mayores consumidores: China, seguida por Rusia, Ucrania y Polonia. En la actualidad, la harina de alforfón se utiliza para la elaboración de diferentes productos alimenticios como fideos chinos, farinetes en Cataluña, cerveza, galletas, pan, etc. [27]. Es la fuente principal de *D*-fagomina (6.7-44 mg kg^{-1}) y de diferentes isómeros de la misma [28, 29].

Figura 1.5. *Fagopyrum esculentum*

1.2.3. Actividad antiglicosidasa de los iminoazúcares

La actividad antiglicosidasa de los iminoazúcares se basa en su propiedad de mimetizar la estructura de los azúcares sencillos, lo que permite que puedan ocupar el lugar de éstos en reacciones enzimáticas catalizadas por las enzimas que digieren dichos azúcares [10, 30], las glicosidasas.

Las glicosidasas (también llamadas glicosil-hidrolasas) son enzimas ampliamente distribuidas en todos los sistemas vivos, que catalizan la ruptura de enlaces glicosídicos de oligosacáridos y glicoconjugados, y su actividad es fundamental para varios procesos biológicos tales como:

i) Degradación de polisacáridos dietéticos, dando lugar a azúcares más sencillos, que son después metabólicamente absorbidos para su uso dentro del organismo.

ii) Catabolismo lisosomal de glicoconjugados y procesado de glicoproteínas.

iii) Biosíntesis de unidades de oligosacáridos en glicoproteínas y glicolípidos.

Algunas de estas enzimas son específicas dependiendo del número, posición o configuración de los grupos hidroxilo de la molécula de azúcar. Así por ejemplo, las *α*- y β-glucosidasas rompen los enlaces glicosídicos de glucosa terminal unida al carbono anomérico mediante enlace *α*- y β-, respectivamente [31]. Su peso molecular varía considerablemente entre 20-100 kDa por monómero, aunque la mayoría de ellas se encuentran en el rango de 40-70 kDa y suelen actuar en un rango óptimo de pH neutro [32].

La inhibición competitiva de estas enzimas por parte de los iminoazúcares se basa fundamentalmente, tanto en la influencia de su estructura (número, posición y configuración de los grupos hidroxilo) como de su carga electrostática [31]. En este sentido, Stütz y Wrodnigg [32], en un trabajo orientado a establecer la posible relación estructura-actividad inhibitoria de los iminoazúcares, aportaron valiosos datos sobre las enzimas inhibidas (*D*-galactosidasa, *D*-manosidasa, *D*-glucosidasa, α-*L*-fucosidasa, etc.) por distintos iminoazúcares.

Entre los iminoazúcares naturales con mayor actividad antiglicosidasa se encuentran DNJ, fagomina, *α*-HNJ y el DMDP [33]. Esta actividad inhibitoria les confiere gran utilidad como candidatos para el tratamiento de numerosas enfermedades relacionadas con la absorción de carbohidratos, tales como cáncer, infecciones virales, etc., así como contra obesidad y diabetes [8, 34-36]. Estas enfermedades suponen una gran problemática para la sociedad actual, sobre todo en países desarrollados donde se consideran epidemias modernas, ya que su incidencia es particularmente alarmante entre niños y adolescentes [37, 38].

1.2.3.1. 1-Desoxinojirimicina (DNJ)

La DNJ (**Figura 1.1**) es un derivado polihidroxilado de la piperidina que se considera un análogo estructural de la *D*-glucosa, en la que el átomo de oxígeno del anillo de piranosa ha sido sustituido por un grupo -NH [39, 40]. Es un iminoazúcar muy estable, tanto química- como metabólicamente [41].

Es uno de los inhibidores más potentes de *α*-glucosidasas, con un IC_{50} en torno a 30 μM [15, 42-45], así como de sacarasas y maltasas [46, 47], presentando así un gran

potencial terapéutico frente a diversas enfermedades tales como diabetes tipo II [10, 30, 39], cáncer, infecciones víricas u obesidad [48].

La DNJ fue sintetizada por primera vez en 1967 por Paulsen y Todt [49]. Desde entonces se han descubierto nuevas rutas sintéticas basadas en procedimientos quimioenzimáticos [7] e incluso se ha aplicado la síntesis asimétrica para la obtención de sus diferentes diastereoisómeros [5].

Este iminoazúcar, aislado por primera vez a partir de hojas de morera en 1976 por Yagi *y col*. [50], se ha descrito también en distintas especies de morera [33, 51, 52], donde es el iminoazúcar más abundante [51], así como en otras especies vegetales tales como *Lonchocarpus* sp. [53], *Hyacinthus orientalis* [26], etc. Además, la DNJ se encuentra también en microorganismos de los géneros *Streptomyces* y *Bacillus* [54, 55].

1.2.3.2. 1,2-didesoxinojirimicina (fagomina)

La fagomina es un iminociclitol con anillo de seis átomos de carbono que se aisló por primera vez a partir de semillas de trigo sarraceno o alforfón (*Fagopyrum esculentum* Moench) [56]. La fagomina se encuentra además en diversas plantas de las familias Moraceae (*Morus* sp.), Leguminosae (*Castanospermum australe*, *Xanthocersis zambesiaca*), Solanaceae (*Lyciumchinese*) [6, 38, 52, 57], etc. Al igual que la DNJ, es un iminociclitol química- y metabólicamente muy estable [41] y se ha sintetizado mediante diversos procedimientos, consiguiéndose incluso su síntesis enantioespecífica (enantiómeros *D-* y *L-*) [6]. Recientemente, Clapés *y col*., [7] han patentado una estrategia quimioenzimática para su obtención con excelentes rendimientos y con potencial aplicabilidad industrial por su sencillez con respecto a los antecedentes previos. Entre sus propiedades biológicas, destaca su actividad frente a α-glucosidasas intestinales de mamíferos [46], además de ser un potenciador de la secreción de insulina [58]. Recientemente, Gómez *y col*., [38] la describen como un agente para la disminución tanto de la concentración de glucosa en sangre, como de la aglutinación de enterobacterias de la mucosa intestinal. Así pues, recomiendan su uso como ingrediente dietético o componente de alimentos funcionales indicados para

reducir el riesgo para la salud asociado con la ingesta excesiva de carbohidratos de rápida absorción, o la adhesión de un exceso de bacterias potencialmente patógenas.

1.2.3.3. *α*-Homonojirimicina (*α*-HNJ)

La *α*-HNJ se aisló por primera vez en 1988 a partir de *Omphalea diandra* [59], junto con 1,5-didesoxi-1,5-imino-*D*-manitol o 1-desoximanojirimicina (DMJ), siendo el primer iminoazúcar descrito como derivado de origen natural de la DNJ sustituido en el carbono 1 [30]. Otras fuentes vegetales que contienen *α*-HNJ son la *Aglaonema commutatum* "Treubii" [11, 21] y los bulbos de *Hyacinthus orientalis* [26]. Además se ha encontrado en adultos, pupas y huevos de la polilla neotropical *Urania fulgens*, cuyas larvas se alimentan de la *Omphalea diandra* [60]. Se sintetizó por primera vez en 1990 por Aoyagi *y col*. [61].

Es un excelente inhibidor de glicosidasas con una especificidad superior a la de la DNJ [40] y se utiliza, entre otros, como precursor de agentes con alta efectividad frente al tratamiento de la enfermedad de Fabry (enfermedad hereditaria de almacenamiento lisosómico relacionada con mutaciones en el gen que codifica la enzima *α*-galactosidasa) [54].

1.2.3.4. 2,5-didesoxi-2,5-imino-*D*-manitol (DMDP)

El DMDP es un iminoazúcar que se describió por primera vez en las hojas de *Derris elliptica* (Fabaceae) [62], encontrándose con posterioridad en otras especies vegetales tales como semillas de *Lonchocarpus sericeus* [33], *Aglaonema commutayum* "Treubii" [11], hojas de *Omphalea diandra* L., en diversos microorganismos [59, 63], así como en la polilla neotropical (*Urania fulgens*) [60], lo que indica que es un metabolito bastante común [30]. Debido al gran número de derivados biológicamente activos, su síntesis es de gran interés [64]. Fue sintetizado por primera vez por Fleet y Smith [65], en 1985 a partir de *D*-glucosa. Desde entonces dicho proceso ha evolucionado mucho, hasta conseguir su síntesis a partir de *L*-xylosa y *L*-sorbosa [66, 67] e incluso su síntesis estereoselectiva [68].

Es un inhibidor de numerosas glicosidasas [64] y posee efecto anti-hiperglicémico, reduciendo significativamente la glucosa en sangre 2 horas después de su administración intraperitoneal [30].

Se han sintetizado además otros inhibidores, incluso con una mayor capacidad de inhibición, a partir de los iminoazúcares anteriormente mencionados, incluyendo la isofagomina (afesostat), la desoxigalactonojirimicina y los derivados alquilados de los mismos [30, 31, 40]. En este sentido, el miglitol (1,5-didesoxi-1,5-((2-hidroxietil)imino)-*D*-glucitol o *N*-hidroximetil-DNJ) y el miglustat (1,5-(butilimino)-1,5-didesoxi-*D*-glucitol o *N*-butil-DNJ), inhibidores de glucosidasas derivados de la DNJ, son utilizados clínicamente. El miglitol está probado para el tratamiento de la diabetes tipo II. Disminuye moderadamente los niveles de glucosa postprandial y de insulina en sangre y está indicado como suplemento de la dieta en el tratamiento de esta enfermedad en los casos en los que el tratamiento dietético resulta insuficiente. El miglustat es un inhibidor de α-glucosidasas que se ha desarrollado como fármaco anti-sida y para el tratamiento de la enfermedad de Gaucher tipo 1 [69].

1.3. Extracción de iminoazúcares

Dada la tendencia actual por la obtención de compuestos bioactivos a partir de fuentes naturales, la extracción de iminoazúcares a partir de muestras vegetales ha cobrado un gran interés. En este sentido, y previamente optimizadas las condiciones de extracción, los extractos obtenidos, enriquecidos en dichos compuestos, pueden ser utilizados como complementos alimenticios y/o ser incorporados a los alimentos como ingredientes bioactivos. Para alcanzar este fin se han desarrollado diversos métodos principalmente basados en extracciones sólido-líquido.

1.3.1. Extracción sólido-líquido convencional

La extracción de iminoazúcares a partir de fuentes naturales se lleva a cabo a partir de la muestra molida y previamente secada, ya sea al aire o mediante liofilización, empleando distintos disolventes y condiciones. Debido a la polaridad y bajo peso molecular de los iminoazúcares, el uso de disoluciones acuosas de metanol o

etanol (25-70 % v/v) resulta apropiado, debido a la gran solubilidad de estos compuestos en disolventes polares [27, 35, 36, 60, 70]. En ocasiones también se emplea sólo agua fría o caliente [71, 72]. Sin embargo, todos estos disolventes presentan como desventaja la co-extracción de otros muchos compuestos polares como carbohidratos de bajo peso molecular, alguno de los cuales pueden interferir en la bioactividad de dichos extractos.

Se ha descrito también la extracción de iminoazúcares con agua acidulada, en agitación y seguida de centrifugación [16, 73, 74]. De este modo se evita la descomposición de ciertas pirrolidinas que se degradan a pHs básicos [35, 75]. Otro procedimiento alternativo consiste en la obtención de extractos en acetonitrilo-agua (50:50 v/v con acetato amónico 6,5 mM a pH 5,5) por sonicación, que permite extraer DNJ y fagomina en el sobrenadante obtenido tras la centrifugación de dichos extractos [39].

Dependiendo de la matriz puede resultar necesaria una extracción Soxhlet previa con hexano o con éter de petróleo/diclorometano, que permita la separación de grasas y ceras, ya que estos compuestos pueden encapsular a los iminoazúcares y disminuir su solubilidad. Sin embargo, el empleo de estos disolventes no sería adecuado si la finalidad última es la obtención de un ingrediente de grado alimentario, excepto si se realiza un paso previo de eliminación del disolvente. Posteriormente, los compuestos de interés se extraerían con metanol, ya sea mediante extracción sólido-líquido [53],o bien mediante extracción Soxhlet con metanol [76].

Aunque los iminoazúcares no son muy abundantes en las diferentes matrices vegetales en las que se encuentran, no excediendo el 0,20 % en peso seco de muestra en la mayoría de los casos [10, 15, 35], los rendimientos de extracción de los métodos descritos son, en general, bastante elevados, obteniéndose unos porcentajes de recuperación entre un 86-99 % del iminoazúcar [16]. Sin embargo, son procesos lentos, que normalmente implican el uso de grandes cantidades de disolvente, poco automatizados y que requieren un tedioso manejo de la muestra, lo que puede ocasionar pérdidas importantes. Por tanto, y para satisfacer la creciente demanda de extractos ricos en iminoazúcares de aplicación en la industria alimentaria, es necesario el desarrollo de métodos avanzados que permitan la extracción más rápida, eficaz y

limpia de dichos compuestos, tras la cuidadosa optimización de las diferentes condiciones de operación para cada muestra en estudio [77]. Entre las técnicas de extracción a considerar por sus ventajas para este tipo de estudios destaca la extracción con líquidos presurizados (PLE).

1.3.2. Extracción con líquidos presurizados (PLE)

La extracción con líquidos presurizados se basa en el empleo de disolventes a elevadas presiones (4-20 MPa) y temperaturas (50-200 ºC), de manera que estos se mantienen en estado líquido durante todo el proceso. Bajo estas condiciones de presión, la constante dieléctrica del disolvente, y con ella su polaridad, disminuyen bruscamente conforme aumenta la temperatura. Además, por acción de la presión, la tensión superficial del disolvente disminuye, haciéndolo también su viscosidad, lo que mejora la solubilidad de los analitos en el disolvente [78, 79].

La **Figura 1.6** muestra el esquema de un equipo de PLE. Este equipo consta de un depósito de disolvente conectado a una bomba de alta presión, que es la encargada de introducir dicho disolvente en la celda de extracción situada en el interior de un horno. Existen también varias válvulas que permiten controlar la presión en todos los puntos del sistema. A la salida del equipo se dispone de un vial colector al que llega el extracto resultante. Adicionalmente, el sistema puede incluir un dispositivo refrigerante con el fin de evitar la evaporación del extracto por la caída de presión a la salida de la celda de extracción, o un circuito de nitrógeno que sirve para purgar las vías una vez finalizada la extracción [80].

Para el desarrollo de una metodología de extracción con PLE es necesario tener en cuenta una serie de parámetros, tales como polaridad, tanto de los compuestos de interés como del disolvente a emplear, volumen del mismo, cantidad de muestra, temperatura, tiempo y número de ciclos de extracción, entre otros. De entre ellos, la naturaleza del disolvente y la temperatura de extracción suelen resultar los parámetros más críticos en el proceso de extracción. Así, por un lado, el disolvente elegido va a influir en la selectividad de la extracción y, por otro, no hay que olvidar que a las presiones de trabajo del PLE, la variación de temperatura puede cambiar las

características del disolvente, sobre todo si éste es agua (SWE, extracción con agua subcrítica). Además, el empleo de disolventes extractantes "verdes" como el agua es también preferido desde el punto de vista de la potencial explotación de estos extractos por la industria alimentaria.

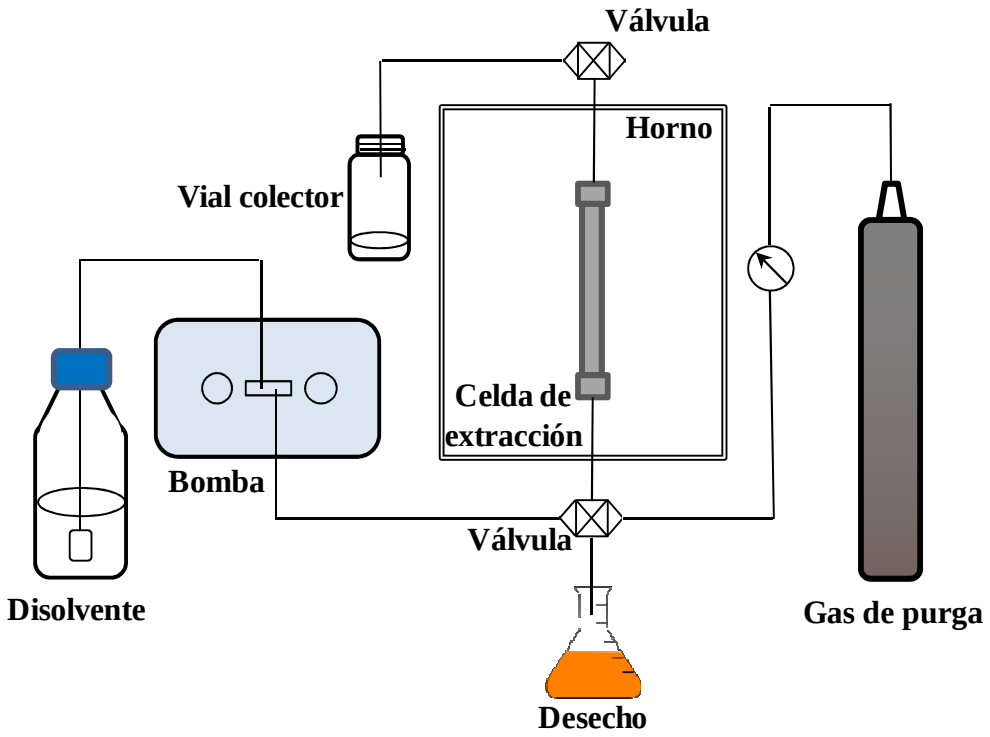


Figura 1.6. Esquema de un equipo de extracción con líquidos presurizados (PLE).

En la actualidad, la PLE presenta numerosas aplicaciones para la extracción de un gran número de compuestos, la mayor parte de ellos apolares, provenientes de distintas matrices (alimentos, plantas, etc.) [78, 81-83]. Sin embargo, las aplicaciones para compuestos polares y, más concretamente, en el campo de los carbohidratos son más bien escasas. Ruiz-Matute *y col*. [84] emplearon con éxito la PLE para la separación de lactosa y lactulosa. A su vez, Alañón *y col*. [85] utilizaron la PLE para la extracción de monosacáridos y polialcoholes presentes en maderas utilizadas para el envejecimiento del vino. En 2010, Guan *y col*. [86] optimizaron mediante un diseño de experimentos la extracción por PLE de carbohidratos de bajo peso molecular y polisacáridos del hongo *Cordyceps*. Más recientemente, Ruiz-Aceituno *y col.* [87] han empleado esta técnica para la extracción de inositoles a partir de piñones. Sin

embargo, hasta el momento esta técnica no se ha aplicado a la extracción de iminoazúcares.

De entre las ventajas que presenta la PLE, podemos destacar que es una técnica respetuosa con el medio ambiente, ya que requiere un bajo consumo de disolvente y proporciona una reducción considerable del tiempo de extracción con respecto a las técnicas convencionales [77, 88].

Esta técnica muestra también limitaciones comunes a las técnicas de extracción convencionales como la falta de selectividad que da lugar a la co-extracción de compuestos que pueden actuar como interferentes [89, 90], así como la dilución de los analitos, especialmente si se realiza un gran número de ciclos de extracción. Para solventar estas limitaciones puede recurrirse a etapas adicionales de limpieza de la muestra, de pre-concentración o de fraccionamiento, previas o posteriores a la obtención del extracto [88, 91].

1.4. Fraccionamiento de carbohidratos

La mayoría de los procedimientos de extracción de iminoazúcares previamente descritos dan lugar a la co-extracción de compuestos presentes en la matriz y en principio no deseados tales como carbohidratos de bajo peso molecular (glucosa, fructosa, sacarosa, etc). Dichos carbohidratos interferirían en las propiedades bioactivas (actividad antiglicosidasa) de los extractos obtenidos, por lo que se hace imprescindible su eliminación.

El fraccionamiento de carbohidratos no es una tarea sencilla considerando la similitud de sus estructuras y las generalmente diferentes concentraciones en que es estos se encuentran presentes en extractos naturales. En la bibliografía se han descrito para tal fin muchas técnicas tales como cromatografía de exclusión molecular, nanofiltración, tratamiento con carbón activo, etc. El empleo de una u otra depende del objetivo a alcanzar: fraccionamiento por grado de polimerización, por enlace glicosídico, etc. y a menudo es necesario el complementarlas con otros procedimientos.

La técnica más empleada hasta el momento para el fraccionamiento de iminoazúcares y otros carbohidratos de bajo peso molecular es la cromatografía de intercambio iónico [9, 35, 72].

1.4.1. Cromatografía de intercambio iónico (IEC)

La cromatografía de intercambio iónico está basada en la interacción electrostática de carácter reversible de moléculas cargadas con una fase sólida (resina) funcionalizada con grupos de carga contraria. En la actualidad, existen en el mercado numerosos tipos de resinas con diferentes tamaños de poro que permiten distintas capacidades y flujos.

En la práctica, el proceso comprende una serie de etapas:

a) Equilibrado de la superficie de intercambio iónico con un contraión (anión o catión) a unas condiciones de pH adecuadas.

b) Carga de la muestra: desplazamiento del contraión de la resina por los componentes de la muestra debido a la interacción más favorable de éstos con los grupos funcionales de la resina (las moléculas que poseen la misma carga que la superficie no se retienen).

c) Elución de los compuestos retenidos por incremento de la concentración del contraión o por cambio del pH del sistema, lo que reduce la fuerza de la interacción con la fase sólida. Así, en esta etapa las moléculas van eluyendo más o menos rápidamente en base a su carga y al pH del tampón utilizado para esta elución.

d) Regeneración de la superficie de la resina y equilibrado de la misma para su empleo con la siguiente muestra.

Según el grupo funcional de la resina, podemos distinguir dentro de esta técnica dos categorías: cromatografía de intercambio aniónico (AEC), que emplea resinas funcionalizadas con grupos dietilaminoetil, amonio cuaternario, etc., y cromatografía de intercambio catiónico (CEC), en la que las resinas contienen grupos funcionales tales como carboximetil, metilsulfonato, etc. Dentro de estas categorías, existen intercambios débiles (protón intercambiable con pK_a dentro del rango de pH normal

de los compuestos de interés) e intercambios fuertes (iones con carga formal fija o con un pk_a extremo, fuera de los rangos normales de pH de los compuestos a separar) [92].

La cromatografía de intercambio iónico es comúnmente utilizada para el fraccionamiento de polihidroxialcaloides y, más concretamente, de iminoazúcares, empleándose diversas resinas aniónicas y catiónicas (Dowex 50 o Amberlite GC120 en sus formas iónicas NH_4^+ o H^+ son de las más empleadas), tanto en columnas como en sistemas de agitación [93-95]. Por otra parte, las muestras de interés pueden también someterse a varios tratamientos consecutivos con diferentes resinas de intercambio iónico en función del grado de enriquecimiento o fraccionamiento deseado [9, 96-98]. La desventaja de esta técnica es que implica procesos tediosos y, aunque la pureza obtenida es alta, los rendimientos suelen ser bajos, en torno al 0,1 % en peso seco de muestra [9, 39].

1.4.2. Tratamientos microbiológicos

Los tratamientos microbiológicos y, más concretamente con levaduras, permiten la eliminación selectiva de ciertos carbohidratos que pueden actuar como posibles interferentes. Así, con objeto de analizar ciclitoles, Baumgartner *y col*. [99] utilizaron *Saccharomyces bayanus* para eliminar sacarosa, fructosa y glucosa presentes en polvo de algarroba. Tras el tratamiento con levaduras, los carbohidratos que aún permanecen en la muestra, son eliminados mediante cromatografía de intercambio aniónico.

También se ha descrito el uso de *Saccharomyces cerevisiae* para la separación selectiva de algunos mono- y disacáridos. Carbohidratos como fructosa, galactosa, glucosa, manosa y disacáridos con uniones α- se eliminarían del medio, mientras que otros azúcares como ramnosa, sorbosa, disacáridos con uniones β- y oligosacáridos de tres o más unidades, no se verían afectados por la acción de dichas levaduras [100].

Este tratamiento se ha utilizado también para el enriquecimiento en di- y trisacáridos de muestras de miel, por eliminación de las grandes cantidades de monosacáridos presentes en este tipo de muestras [101], así como para el

enriquecimiento en inositoles bioactivos de extractos de legumbres mediante la eliminación de carbohidratos de bajo peso molecular (mono- y disacáridos) [102].

Por otra parte, la eliminación selectiva de los carbohidratos mayoritarios en miel (principalmente glucosa y fructosa), ha permitido el análisis de compuestos minoritarios en esta compleja muestra. Así, Ruiz-Matute *et al.* [103] consiguieron detectar adulteraciones de mieles con jarabes de alto contenido en fructosa y de azúcar invertido, basadas en la detección de dianhídridos de fructosa (DFAs), componentes minoritarios de estos jarabes [103].

Asimismo, se ha descrito la inmovilización de *Zymomonas mobilis* en bolas de alginato para el fraccionamiento de fructosa, glucosa y sacarosa en mezclas de oligosacáridos de grado alimentario [104]. Dicho fraccionamiento se logró tras 12 horas de incubación (sin control de pH ni adición de nutrientes).

Además de la alta eficacia del tratamiento con levaduras en la eliminación de monosacáridos, este proceso puede llevarse a cabo fácilmente en mezclas sintéticas, sin la necesidad de las diluciones requeridas por otras técnicas tales como la nanofiltración. Es además una técnica económica y fácilmente escalable para usos industriales [105].

En consecuencia, dicha técnica puede ser de gran interés para la industria alimentaria por ser una técnica limpia que no se ha aplicado hasta el momento para el enriquecimiento de iminoazúcares.

1.5. Análisis de iminoazúcares

Se han utilizado una gran variedad de técnicas para el análisis de iminoazúcares, tales como la resonancia magnética nuclear de carbono (^{13}C-RMN) y de protón (^{1}H-RMN) [51, 98], la espectroscopía infrarroja (IR) [56], etc. Sin embargo, dado que los iminoazúcares y sus correspondientes isómeros suelen formar parte de muestras complejas y encontrarse generalmente en baja concentración, son las técnicas cromatográficas de alta resolución tales como la cromatografía de gases (GC) o la cromatografía de líquidos (HPLC) y sus acoplamientos a espectrometría de masas (MS) las más empleadas para tal fin por su resolución y sensibilidad.

1.5.1. Cromatografía de líquidos (LC)

La LC ha sido la técnica de elección en la mayor parte de los estudios previos para el análisis de iminoazúcares [17, 35, 106]. Los **modos de operación** de LC más comúnmente empleados para el análisis de iminoazúcares son la cromatografía de fase inversa (RPLC), cromatografía de alta eficacia de intercambio aniónico (HPAEC), cromatografía de intercambio catiónico (CEC) y la cromatografía de interacción hidrofílica (HILIC).

1.5.1.1. Cromatografía de fase inversa (RPLC)

La RPLC es uno de los modos de operación más comunes debido a sus numerosas ventajas que incluyen la fácil utilización de diferentes gradientes de elución, la compatibilidad con muestras de carácter acuoso, las posibles diferencias en las separaciones variando el pH, el uso de distintos modificadores orgánicos o aditivos, etc. [107].

Emplea fases estacionarias no polares tipo alquilsilice (C_8, C_{18}), siendo la fase móvil más polar (mezclas de agua con modificadores orgánicos). El mecanismo de retención se basa en interacciones hidrofóbicas de los analitos con la fase estacionaria de la columna, lo que va a depender tanto de la naturaleza de los analitos, como de las fases móviles empleadas. Sin embargo, su aplicabilidad a iminoazúcares y carbohidratos en general es limitada debido a la débil interacción de estos compuestos polares con la fase estacionaria de la columna, lo que se traduce en la poca o nula retención de los mismos [70]. La solución a este problema es someter los compuestos a un paso previo de derivatización que permita aumentar la retención de los mismos en estas fases, como describen algunos autores [16, 106] para la determinación de DNJ en hojas de *Morus alba*.

1.5.1.2. Cromatografía de alta eficacia de intercambio aniónico (HPAEC)

La HPAEC es una de las técnicas más utilizadas para el análisis de carbohidratos [108, 109] debido a su alta sensibilidad y selectividad. Este tipo de cromatografía está basado en la ionización de los carbohidratos en condiciones alcalinas (pH 9-13) y en su

separación en columnas con resinas de intercambio aniónico. El eluyente utilizado normalmente es el hidróxido sódico, incluyéndose acetato de sodio para aumentar la fuerza iónica. La detección se lleva a cabo utilizando amperometría de pulsos (PAD), que consiste en la medida de la corriente eléctrica generada por la oxidación de los carbohidratos en la superficie de un electrodo de platino u oro y su posterior reducción para limpieza del electrodo.

Existen muy pocas referencias que utilicen HPAEC para el análisis de polihidroxialcaloides, ya que mientras que el análisis de patrones resulta adecuado, la resolución alcanzada para muestras reales (e.g. extracto de *Castanospermum australe*) donde se encuentran presentes otros carbohidratos de bajo peso molecular es baja [110].

Recientemente, se ha desarrollado un método para el análisis de iminoazúcares mediante HPAEC, que se ha aplicado al análisis de DNJ en ingredientes alimentarios derivados de hoja de morera [111]. Sin embargo, este método presenta varios inconvenientes, tales como la dificultad para su acoplamiento a la espectrometría de masas debido al uso de fases móviles con un alto contenido en sales y a la frecuente presencia de isómeros, que no son siempre resueltos en su totalidad, lo que suele dificultar la identificación inequívoca en su aplicación a muestras reales. Estos problemas hacen imprescindible el uso de patrones, disponibles comercialmente sólo para un limitado número de iminoazúcares, para la inequívoca identificación de los mismos.

1.5.1.3. Cromatografía de intercambio catiónico (CEC)

La CEC, además de emplearse con fines preparativos para el fraccionamiento de carbohidratos, es otro de los modos de HPLC utilizados para el análisis de un amplio rango de moléculas que van desde aminoácidos y nucleótidos, hasta proteínas y carbohidratos. La cromatografía de intercambio catiónico separa los iones y moléculas polares basándose en su carga. La fase estacionaria contiene grupos funcionales cargados negativamente a los que están unidos los denominados contraiones con los que compiten en interacción electrostática los compuestos de interés. Las típicas fases estacionarias utilizadas para el análisis de oligosacáridos son las de

poliestirenosulfonado con contraiones Ca^{2+} y Ag^{+}, mientras que para los monosacáridos se emplean los contraiones Pb^{2+} [54]. Existe también en estas fases un mecanismo adicional de separación por exclusión molecular por lo que el orden de elución es inverso a su peso molecular [112].

Dado que el tipo de interacción es iónica, la unión del analito con la fase estacionaria o resina, debe tener lugar bajo condiciones de baja fuerza iónica. Así, la elución se consigue, o bien aumentando la fuerza iónica de la fase móvil, o mediante un cambio de pH de la fase móvil que altere el estado de ionización del analito [113].

Es una técnica comúnmente utilizada para el análisis de iminoazúcares. Así, y a modo de ejemplo, se ha descrito su empleo para la determinación de iminioazúcares *N*-alquilados en muestras biológicas [113], así como para el análisis de *D*-fagomina en trigo y morera [28].

1.5.1.4. Cromatografía de interacción hidrofílica (HILIC)

Es una técnica muy útil para el análisis de compuestos polares e hidrofílicos que no se separan bien en fase inversa por su baja o nula retención, como es el caso de los carbohidratos. Utiliza fases estacionarias altamente polares e hidrofílicas como la sílice o funcionalizadas con grupos amina, amida o zwitteriónicos, entre otros [114]. La fase móvil más común está compuesta por acetonitrilo o metanol en una alta proporción (50-95 %) y otra fase tipo acuosa con aditivos de muy diversos tipos (ácidos, bases, sales, etc.).

Su mecanismo de retención aún en la actualidad no está claro, aunque parece que la teoría más aceptada lo explica como el reparto del analito entre la fase móvil orgánica y una película acuosa parcialmente inmovilizada en la fase estacionaria [114-116] (**Figura 1.7**). Dependiendo del tipo de fase estacionaria pueden existir otros tipos de mecanismos adicionales como ocurre, por ejemplo, en las fases zwiteriónicas, donde pueden tener lugar algunas interacciones electroestáticas entre los analitos y la propia fase estacionaria en función del pH de la fase móvil [116].

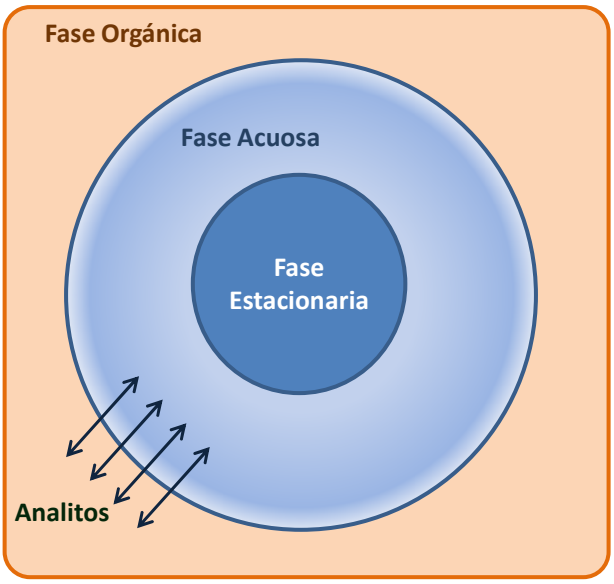


Figura 1.7. Mecanismo de retención para la separación en modo HILIC

Existen algunas aplicaciones para el análisis de iminoazúcares con distintas fases estacionarias que operan en modo HILIC [39, 45]. Las más comúnmente utilizadas son del tipo amida [17, 27, 39, 70], polihidroxietil aspartamida (PHEA) [117], y tipo silícea [118]. Sin embargo, la comparación entre estas fases o con otras distintas es todavía un estudio pendiente de realizar, que puede resultar de gran utilidad para la caracterización de mezclas complejas donde coexisten iminoazúcares y otros carbohidratos de bajo peso molecular.

En cuanto a los **detectores** empleados en HPLC, el índice de refracción es uno de los más comunes ya que no necesita derivatización previa de los carbohidratos. Sin embargo, sólo se puede emplear cuando la elución es en modo isocrático, por lo que su aplicación al análisis de mezclas complejas de iminoazúcares es limitada [119]. Los detectores de absorción ultravioleta-visible y de fluorescencia son también ampliamente utilizados, pero en estos casos se debe llevar a cabo una derivatización pre- o post-columna previa debido a la ausencia de grupos cromóforos o fluoróforos en los iminoazúcares [120]. El principal inconveniente de estos métodos es la mayor manipulación de la muestra que conlleva una mayor complejidad de análisis [35, 47]. El detector de pulsos amperométricos, acoplado comúnmente a HPAEC, donde no se necesita derivatización previa posee una alta sensibilidad y permite la detección de carbohidratos en cantidades del orden de picomoles [121].

Actualmente, el acoplamiento de espectrómetros de masas a equipos de HPLC, particularmente con ionización por electrospray (ESI) o ionización química a presión atmosférica (APCI), es una técnica muy prometedora para el análisis de iminoazúcares sin derivatización previa. Así, se ha llevado a cabo el análisis de alcaloides tipo pirrolicidina en miel por HPLC-APCI-MS, utilizando una columna C_8 de fase inversa [122], y el de DNJ en hojas de morera mediante HILIC-ESI-MS [45].

Como se ha comentado previamente, la coelución de los iminoazúcares con otros carbohidratos presentes en la muestra, es una de las principales dificultades del análisis de estos compuestos. Este problema puede solucionarse en la mayoría de los casos cuando el espectro de masas se adquiere en modo monitorización selectiva de iones (SIM), adquiriéndose únicamente la señal correspondiente a ciertas relaciones masa/carga (*m/z)* específicas de los iminoazúcares [17]. Como contrapartida, la información proporcionada por el espectro de masas es más limitada que en modo barrido de masas (SCAN), lo que dificulta la identificación/caracterización tanto de iminoazúcares como de otros compuestos presentes en la muestra.

Los acoplamientos de HPLC a sistemas de MS tándem (HPLC-MS^n) con analizadores de cuadrupolo-tiempo de vuelo (Q-TOF) han experimentado un gran desarrollo, siendo recientemente aplicados al análisis de iminoazúcares. Encontramos algunos ejemplos para el análisis de derivados de isofagomina [123], determinación estructural de iminoazúcares a partir de extractos de *Suregada glomerulata* [98], etc. Si bien, Nuengchamnong *y col.* [70] fueron los primeros en describir la aplicación de esta técnica en modo monitorización de reacciones múltiples (MRM) y usando un triple cuadrupolo, para la identificación inequívoca de DNJ, posteriormente ha sido utilizada por otros autores como Nakagawa *y col*. [17] para el análisis de este iminoazúcar en hojas de morera. Aún así, los trabajos existentes en la bibliografía son todavía escasos y, además, la mayoría de ellos se centran en el análisis de iminoazúcares aislados, no en el de mezclas complejas de estos compuestos bioactivos.

1.5.2. Cromatografía de gases acoplada a espectrometría de masas (GC-MS)

La GC es una técnica muy adecuada para el análisis de mezclas complejas de compuestos volátiles o semivolátiles presentes en bajas concentraciones, dada su elevada sensibilidad y resolución. El acoplamiento de la cromatografía de gases a la espectrometría de masas (GC-MS) proporciona una información cualitativa adicional para la identificación de compuestos desconocidos. Sin embargo, su aplicación para el análisis de iminoazúcares hasta el momento es limitada [53, 120, 124]. El principal motivo radica en la necesidad de transformar estos compuestos en sus derivados volátiles (derivatización) previo a su inyección en el sistema cromatográfico. Otros aspectos como la frecuente coelución de estos compuestos con otros carbohidratos de bajo peso molecular en mezclas complejas como los extractos naturales, y la similitud de sus estructuras que dificulta la identificación, hacen necesario el profundizar en el desarrollo de métodos por GC-MS para el análisis de estos compuestos.

1.5.2.1. Derivatización

Del mismo modo que para los azúcares, para estos alcaloides se requiere llevar a cabo una reacción de derivatización previa que les confiera la volatilidad y estabilidad tèrmica necesarias para su posterior análisis por GC. En este paso previo de derivatización se sustituyen los átomos de hidrógeno activos de la molécula de iminoazúcar por otros grupos más apolares.

Dado que los iminoazúcares se encuentran normalmente en presencia de mezclas complejas de otros carbohidratos de bajo peso molecular, los procedimientos de derivatización, así como las condiciones cromatográficas deben elegirse cuidadosamente para conseguir derivados estables y evitar las coeluciones en el análisis por GC. Mientras que la sustitución de los hidrógenos activos correspondientes a los grupos hidroxilo no presenta problemas, la presencia del grupo imino en su estructura hace que puedan sufrir derivatizaciones parciales o poco reproducibles, problema que ha sido objeto de estudio por diferentes autores [35, 125, 126]. Los grupos hidroxilo se derivatizan muy fácilmente, sin embargo, la reacción del grupo imino depende del reactivo y de las condiciones de derivatización, resultando uno o dos derivados para cada iminoazúcar [35].

Uno de los procedimientos de derivatización más empleados para estos compuestos es la formación de trimetilsilil derivados (TMS). Existen diferentes reactivos (trimetilsililimidazol (TMSI); trimetilclorosilano (TMCS); hexametildisilazano (HMDS); *N*-metil-*N*-(trimetilsilil)trifluoroacetamida (MSTFA); *N,O*-bis-(trimetilsilil)-trifluoroacetamida (BSTFA); etc) y condiciones de sililación [35].

La sililación de diferentes clases de polihidroxialcaloides (DNJ, fagomina, DMDP, castanospermina, etc.) a partir de fuentes naturales se describió por primera vez con TMCS-HMDS-piridina (1:3:9) a 50 ºC durante 15 minutos [127]. Los derivados sililados se formaban rápidamente, siendo estables durante 24 horas a temperatura ambiente.

Posteriormente, se observó que el tratamiento con MSTFA a 60 ºC durante 1 hora de una mezcla de calisteginas, daba lugar a la derivatización total tanto de los grupos hidroxilo como del grupo imino de la molécula, aunque tiempos más cortos de tratamiento produjeron la derivatización parcial de estos compuestos [125]. Sin embargo, en trabajos posteriores con calisteginas y otros polihidroxialcaloides en extractos de *Ipomoea* sp. [128, 129], el grupo imino permanecía sin derivatizar incluso a temperaturas y tiempos mucho mayores.

En 1997, Kite *y col*. [126] realizaron un estudio de las condiciones necesarias para la derivatización tanto de los grupos hidroxilo, del grupo imino y de ambos para derivados de piperidinas, tales como ácidos hidroxipipecólicos. Todos los agentes empleados daban proporciones variables de los derivados sililados de los grupos hidroxilo y del grupo imino, excepto para el caso de muestras liofilizadas y expuestas a la humedad ambiental previamente a la derivatización con TMCS-HMDS-piridina, en el que sólo se derivatizaban los grupos hidroxilo.

Otros estudios muestran que la swainsonina (iminoazúcar tipo pirrolicidina) puede derivatizarse de forma completa empleando, tanto MSTFA como BSTFA y HMDS-TMCS, como agentes sililantes. Sin embargo con BSTFA, la castanospermina (iminoazúcar tipo indolicidina) originó tres picos cromatográficos mayoritarios asociados a una derivatización parcial, incluso después de un calentamiento prolongado [130].

Por otra parte, algunos reactivos de sililación podrían dar lugar a subproductos no volátiles que causarían obturaciones de la jeringa de inyección o de la columna capilar.

Molyneux *y col*. [35] observaron que el MSTFA en piridina es uno de los agentes sililantes más adecuados ya que los subproductos que origina son altamente volátiles cuando la reacción se mantiene a 60 ºC durante 1 hora.

Otro procedimiento de derivatización que se ha empleado previo al análisis de iminoazúcares por GC consiste en la acetilación con anhídrido acético en piridina a 20 ºC durante 20 horas. Este método, descrito por primera vez por Magalhães *y col*. [53], permitió la caracterización/identificación de cinco polihidroxialcaloides acetilados en base a sus espectros de masas en extractos de *Lonchocarpus* sp. Además, se propusieron los correspondientes mecanismos de fragmentación en espectrometría de masas para dichos compuestos.

Considerando los limitados estudios previos dirigidos únicamente a la derivatización de iminoazúcares específicos, surge la necesidad de llevar a cabo estudios exhaustivos que permitan encontrar los reactivos y condiciones de sililación más adecuadas para mezclas complejas de distintos iminoazúcares y otros carbohidratos de bajo peso molecular, comparando los distintos derivados posibles, así como las ventajas y limitaciones en su aplicación a muestras reales.

1.5.2.2. Fases estacionarias

La selección de la **fase estacionaria** resulta determinante en cualquier separación por GC. La **selectividad** de una fase estacionaria viene dada por las interacciones fisicoquímicas de las moléculas de soluto con dicha fase. Se establece como la capacidad para dar un tipo concreto de interacción y sus diferencias van a ser las responsables de la separación de compuestos con valores de polaridad similares.

Una de las propiedades más importantes de las fases estacionarias es la **polaridad**. Por polaridad se entiende la selectividad de la fase hacia compuestos polares y puede evaluarse en función de la suma de todas las interacciones que es capaz de dar dicha fase estacionaria. Depende del número y tipo de grupos funcionales polares que presenta la fase, y podría estimarse a partir de la combinación de una serie de parámetros moleculares debidamente sopesados. Sin embargo, como muchas de las fases empleadas son polímeros (o mezclas de ellos) cuyo peso molecular sólo se

conoce aproximadamente, y el número de grupos funcionales no se puede medir con facilidad, se han desarrollado una serie de escalas arbitrarias. Muchas de ellas comparan las retenciones de distintos solutos modelo que se analizan en unas condiciones determinadas y dan lugar a series de datos de retención, que en algunos casos se modifican matemáticamente. Los más conocidos son las constantes de McReynolds [131]. En este método se utilizan los índices de retención a 120 °C de cinco solutos (benceno, 1-butanol, 2-pentanona, 1-nitropropano y piridina) que representan interacciones diferentes con la fase estacionaria en estudio y en otra fase apolar tomada como referencia, el escualano. Así, las diferencias entre ambos índices para cada compuesto constituyen las llamadas constantes de McReynolds, que dan una idea de la selectividad si se consideran individualmente y de la polaridad si se consideran en conjunto.

Sin embargo, el método de McReynolds no es capaz de diferenciar completamente las interacciones individuales molécula-fase estacionaria, ya que en realidad la retención de cada uno de estos solutos es debida a la acción simultánea de distintas fuerzas, como son fuerzas de dispersión, capacidad para donar y/o aceptar protones, fuerzas dipolares, etc. En cambio, el modelo de los parámetros de solvatación (SPM) [132, 133] sí que permite evaluar dichas interacciones intermoleculares y ha sido ampliamente empleado para evaluar las características de retención de una variedad de fases estacionarias [134, 135].

Las fases estacionarias que más comúnmente se han empleado para el análisis de iminoazúcares son las compuestas por polisiloxanos (metil-, fenil- y cianopropil-derivadas). Sin embargo, aunque resultan apropiadas debido a su baja polaridad, su selectividad no es, en general, destacable. Durante la última década, han surgido columnas basadas en el empleo de líquidos iónicos (ILs) como fases estacionarias y que han suscitado un gran interés, ya que poseen propiedades físico-químicas únicas como se describe a continuación, lo que potencialmente les confieren una selectividad diferente a la de las fases estacionarias convencionales, pudiendo ser de gran versatilidad en numerosas aplicaciones.

- **Líquidos iónicos (ILs) como fases estacionarias**

Los ILs son sales orgánicas que poseen puntos de fusión por debajo de los 100 ºC. Poseen una serie de propiedades, tales como presión de vapor despreciable, baja volatilidad, elevada viscosidad, conductividad electrolítica, miscibilidad variable, baja inflamabilidad, alta estabilidad térmica, etc. [136, 137], lo que les hace buenos candidatos como alternativa "verde" a los disolventes orgánicos volátiles y como fases estacionarias en cromatografía de gases [138].

La mayoría de los líquidos iónicos están compuestos por cationes orgánicos (e.g. piridinio, pirrolidinio, imidazolio, amonio, fosfonio, etc.) (**Figura 1.8**), mientras que los aniones suelen ser tanto inorgánicos (e.g. Cl^-, PF_6^-, BF_4^-, etc.) como orgánicos (trifluorometilsulfonato $[CF_3SO_3]^-$, bis[(trifluorometil)sulfonil]imida $[(CF_3SO_2)_2N]^-$, trifluoroacetato $[CF_3CO_2]^-$, etc.) [136] (**Figura 1.9**). Estos cationes y aniones determinan las propiedades físicas y químicas del IL, por lo que su combinación ofrece un gran número de posibilidades.

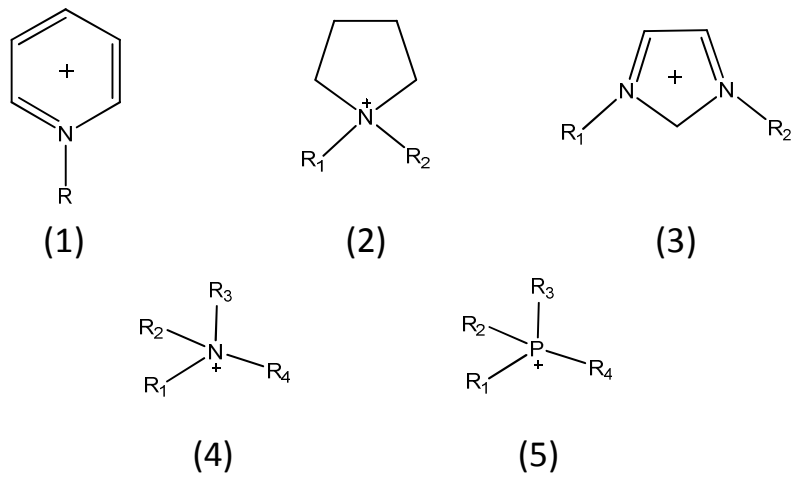


Figura 1.8. Estructuras químicas de algunos de los cationes más comúnmente utilizados en los líquidos iónicos: piridinio (1), pirrolidinio (2), imidazolio (3), tetraalquilamonio (4) y tetraalquilfosfonio (5).

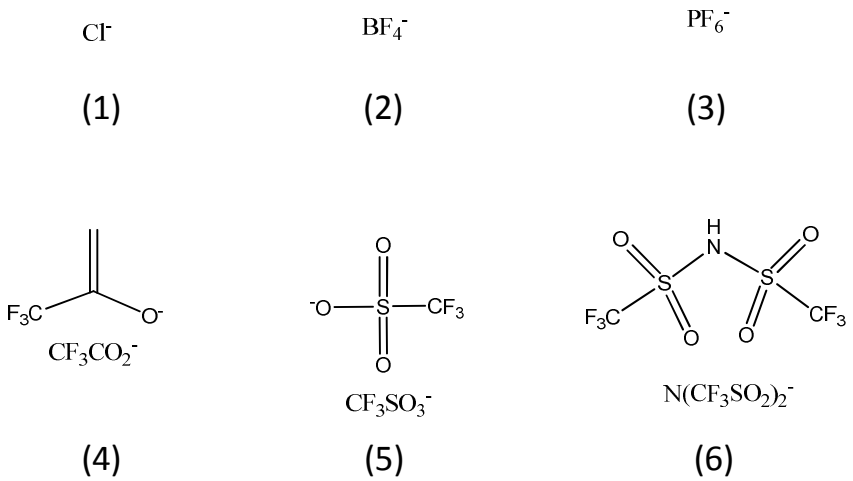


Figura 1.9. Estructuras químicas de algunos de los aniones más comúnmente utilizados en los líquidos iónicos

Como consecuencia de la potencial utilidad de estos compuestos se han sintetizado diversas fases estacionarias de cromatografía de gases basadas en ILs con iones fosfonio, guanidinio, imidazolio, etc. [139-142], existiendo también fases estacionarias comerciales (Supelco®). Hasta el momento, se han comercializado siete fases estacionarias compuestas por líquidos iónicos (SLB-IL59, SLB-IL60, SLB-IL61, SLB-IL76, SLB-IL82, SLB-IL100 y SLB-IL111) con distintas polaridades. Dichos valores de polaridad (P), calculados a partir de las constantes de McReynolds y posteriormente normalizados a la fase SLB-IL100 (P = 100), se muestran en la (**Figura 1.10)** [143] junto a otras fases estacionarias comúnmente empleadas en GC.

Hasta el momento los líquidos iónicos se han aplicado con exito principalmente para el análisis de ácidos grasos[144, 145], aceites esenciales [146], metabolitos de cafeína [147], compuestos volátiles de plantas [148], etc. Sin embargo, la utilidad de estas fases estacionarias para el análisis de iminoazúcares y de otros carbohidratos de bajo peso molecular en general no ha sido abordada.

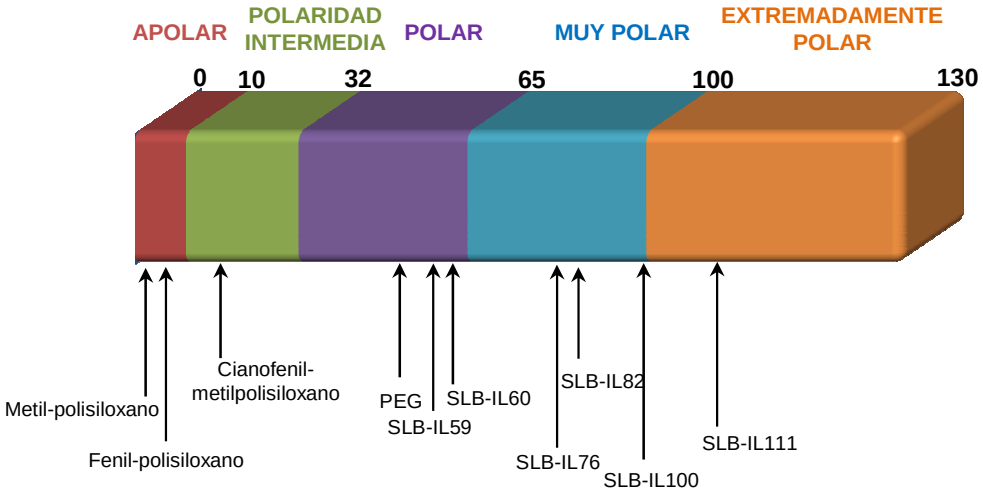


Figura 1.10. Escala de polaridad de algunas fases estacionarias para uso en cromatografía de gases.

1.5.2.3. Acoplamientos GC-MS

Los espectros de masas obtenidos por ionización por impacto electrónico de los carbohidratos en general, y los iminoazúcares en particular, presentan perfiles de fragmentación muy similares y se distinguen básicamente por las diferencias de intensidad relativa de sus fragmentos [149]. La combinación de los datos de retención obtenidos por GC y los espectros de masas sería, por tanto, de utilidad para la identificación de la mayoría de estos compuestos, lo que convierte a la GC-MS en una de las técnicas más apropiadas para el análisis de distintos iminoazúcares presentes en mezclas complejas, aunque los trabajos existentes en la bibliografía son hasta el momento relativamente escasos [29, 36, 150].

JUSTIFICACIÓN Y OBJETIVOS

2. Justificación y objetivos

Esta Tesis Doctoral tiene como punto de partida el proyecto del Plan Nacional (AGL2009-11909), financiado por el Ministerio de Ciencia e Innovación, y que tiene como principal objetivo el desarrollo de métodos de extracción, enriquecimiento y análisis de carbohidratos bioactivos, tales como los iminoazúcares, a partir de matrices vegetales.

La sociedad actual está sufriendo con gran incidencia y a edades cada vez menores afecciones que, si bien pueden estar condicionadas por factores genéticos, también pueden estar relacionadas o ser consecuencia del estilo de vida y los hábitos alimenticios. Dichas enfermedades, como pueden ser la obesidad y la diabetes, están relacionadas con la absorción de carbohidratos de bajo peso molecular, tales como glucosa, sacarosa, etc. Estos y otros azúcares simples son digeridos en el intestino gracias a enzimas específicos para cada uno de ellos (glucosidasas, sacarasas, etc.), de manera que si se logra su inhibición, será posible regular la absorción de dichos carbohidratos y, por tanto, la mejora o el control de las enfermedades anteriormente mencionadas.

En este sentido, existe un gran interés por la búsqueda de compuestos que sean capaces de inhibir a los enzimas que digieren los carbohidratos de bajo peso molecular presentes en la gran mayoría de los alimentos, con el fin de regular su absorción. Además, si estos compuestos bioactivos son de origen natural, su empleo supone un valor añadido frente a los sintéticos, ya que el consumidor busca, por lo general y cada vez con mayor frecuencia, alimentos funcionales, suplementos alimenticios, etc. compuestos en su mayoría por ingredientes naturales.

Los iminoazúcares son conocidos inhibidores de glicosidasas y se encuentran en relativamente bajas concentraciones en diversas fuentes vegetales. Por tanto, su extracción mediante métodos rápidos, eficaces y respetuosos con el Medio Ambiente resulta de gran interés con vistas a su potencial explotación por parte de la industria alimentaria, entre otras. Un paso posterior de enriquecimiento de dichos extractos, que permita eliminar los azúcares de bajo peso molecular inevitablemente presente en los mismos y que pueden interferir en la bioactividad de los iminoazúcares, es también obligado. El seguimiento de ambos procesos debe ir siempre acompañado del

desarrollo de metodologías analíticas avanzadas y ventajosas en cuanto a sensibilidad, resolución, etc., que permitan la caracterización exhaustiva de la composición en iminoazúcares de dichos extractos, con el fin de relacionar ésta con su bioactividad.

Dado que el objetivo final de estos extractos es su incorporación como ingredientes bioactivos de alimentos funcionales o su empleo como suplementos alimenticios, es necesario también evaluar la estabilidad de dichos compuestos en condiciones de almacenamiento controladas, así como comprobar mediante estudios *in vitro* (como paso previo a los requeridos estudios *in vivo)*, que no son tóxicos a las dosis requeridas para que se produzca la actividad deseada, principalmente para extractos procedentes de fuentes no alimentarias.

De esta manera, el objetivo general de esta Tesis Doctoral ha sido **el desarrollo de métodos de obtención y análisis de extractos enriquecidos en iminoazúcares bioactivos a partir de muestras de origen vegetal (Morera, Jacinto, Aglaonema y Trigo sarraceno).**

Partiendo de este objetivo general, se plantearon los siguientes **objetivos parciales**:

- Desarrollar métodos de extracción sólido-líquido convencional y de extracción con líquidos presurizados (PLE), así como de fraccionamiento, para la obtención a partir de fuentes naturales de extractos enriquecidos en iminoazúcares.

- Optimizar la requerida preparación de muestra (derivatización), así como las condiciones cromatográficas para la caracterización (datos de retención cromatográfica y espectros de masas) por cromatografía de gases acoplada a espectrometría de masas (GC-MS) de iminoazúcares bioactivos y otros carbohidratos de bajo peso molecular presentes en extractos vegetales.

- Evaluar la selectividad de nuevas fases estacionarias comerciales para GC basadas en líquidos iónicos y su aplicación al análisis de iminoazúcares y otros

carbohidratos de bajo peso molecular (monosacáridos, disacáridos, inositoles, etc.).

- Desarrollar metodologías basadas en cromatografía de líquidos en modo HILIC acopladas a espectrometría de masas (LC-MS) y a espectrometría de masas tándem (LC-MS^2) para la caracterización cuali- y cuantitativa de mezclas de iminoazúcares bioactivos y otros carbohidratos de bajo peso molecular presentes en extractos vegetales.

- Evaluar la bioactividad, estabilidad y potencial toxicidad de extractos enriquecidos en iminoazúcares.

METODOLOGÍA Y PLAN DE TRABAJO

3. Metodología y Plan de trabajo

El desarrollo de métodos de obtención de extractos enriquecidos en iminoazúcares requiere de la puesta a punto y optimización previa de métodos cromatográficos que permitan, tanto la caracterización exhaustiva de estos extractos, como el seguimiento de los cambios en su composición durante los procesos de extracción y fraccionamiento.

Para alcanzar estos objetivos, el **plan de trabajo** seguido (**Figura 3.1**) se ha estructurado en dos grandes bloques, "Análisis" y "Extracción", que engloban las distintas secciones en las que se han subdividido los resultados incluidos en la presente Memoria. Es de reseñar también que para el análisis de extractos vegetales ricos en iminoazúcares bioactivos y otros carbohidratos interferentes de bajo peso molecular, se han seguido dos estrategias diferentes basadas en el empleo de la GC-MS (sección 4) y de la LC-MS (sección 5), con el fin de seleccionar aquella técnica que proporcionara los mejores resultados o evaluar el uso complementario de ambas. Por otra parte, la sección 6 aborda la optimización de la extracción, tanto sólido-líquido convencional como haciendo uso de líquidos presurizados, de iminoazúcares a partir de fuentes naturales, así como el fraccionamiento de los extractos obtenidos con el fin de eliminar compuestos interferentes con la actividad antiglicosidasa de los iminoazúcares.

Todos estos estudios se han llevado a cabo empleando como fuente natural de iminoazúcares distintas matrices de origen vegetal (hojas, frutos y ramas de diversas especies de morera, bulbos de jacinto, hojas y raíces de Aglaonema y granos de trigo sarraceno).

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

Sección 4. Desarrollo de métodos mediante GC-MS para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular.

Sección 4.1. Desarrollo de un procedimiento de derivatización para el análisis simultáneo por GC-MS de iminoazúcares (DNJ y fagomina) y otros carbohidratos de bajo peso molecular en extractos de morera (*Morus* sp.).

- Evaluación y selección del tipo de derivado y agentes derivatizantes óptimos.
- Validación del método (intervalo de linealidad, recuperación, precisión y sensibilidad).
- Aplicación del método al análisis de extractos obtenidos por extracción sólido-líquido convencional a partir de hojas, frutos y ramas de diversas especies de morera (*Morus alba*, *Morus nigra*).

Sección 4.2. Mejora de un método por GC-MS para el análisis de iminoazúcares y otros carbohidratos bioactivos.

- Optimización de condiciones cromatográficas (temperatura del inyector, rampa de temperatura del horno, etc.).
- Evaluación del efecto sobre la reproducibilidad y sensibilidad de detección de iminoazúcares bioactivos del uso de detectores de ionización de llama (FID) o de MS, operando éste último en modo registro selectivo de iones (SIM) o en modo barrido de iones (SCAN).
- Aplicación del método mejorado a la caracterización cuali- y cuantitativa de extractos sólido-líquido de trigo sarraceno, jacinto y morera.

Sección 4.3. Caracterización por GC-MS de los trimetilsilil derivados de iminoazúcares.

- Análisis por GC-MS de patrones de iminoazúcares previamente derivatizados.
- Establecimiento de relaciones entre estructura química y datos cromatográficos (índices de retención lineales, I^T) y de espectrometría de

masas (abundancia de relaciones *m/z* de iones fragmento característicos) para los iminoazúcares analizados.

- Aplicación de las conclusiones obtenidas a la caracterización de iminoazúcares desconocidos en extractos de raíces de *Aglaonema treubii*.

Sección 4.4. Nuevas fases estacionarias basadas en líquidos iónicos (ILs) para el análisis por GC-MS de iminoazúcares y otros carbohidratos de bajo peso molecular.

Sección 4.4.1. Caracterización por el Modelo de los Parámetros de Solvatación (SPM) de las propiedades de retención de columnas comerciales de GC basadas en ILs.

- Evaluación de las interacciones soluto-fase estacionaria (IL) a partir de los datos obtenidos en la inyección, en un rango de temperaturas entre 80-160 ºC, de 95 compuestos de distinta funcionalidad.
- Comparación de la selectividad proporcionada por dichas columnas y por columnas de GC convencionales.

Sección 4.4.2. Evaluación de diferentes fases estacionarias basadas en ILs para el análisis por GC-MS de carbohidratos.

- Inyección de patrones de carbohidratos de bajo peso molecular como mono-, di- y trisacáridos, inositoles e iminoazúcares. Evaluación de parámetros cromatográficos (anchura, simetría, resolución, etc.) para estos compuestos, obtenidos en distintas condiciones de análisis (isotermas y rampas de temperatura).
- Aplicación de la fase estacionaria óptima al análisis de extractos de jacinto y de morera.

Sección 5: Desarrollo de métodos mediante LC-MS para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular.

Sección 5.1. Evaluación de diferentes fases estacionarias hidrofílicas para el análisis simultáneo por LC-MS^n de iminoazúcares y otros carbohidratos de bajo peso molecular en extractos vegetales.

- Análisis en modo HILIC de patrones de iminoazúcares y otros carbohidratos de bajo peso molecular en distintas fases estacionarias y condiciones cromatográficas (gradientes, flujos, modificadores orgánicos y aditivos). Obtención de parámetros cromatográficos (anchura, simetría, resolución, etc.) para los compuestos analizados.
- Determinación de precisión y sensibilidad del método seleccionado.
- Aplicación del método optimizado a la caracterización cuali- y cuantitativa de extractos de morera, jacinto y trigo sarraceno, ricos en iminoazúcares.
- Empleo de los datos de espectrometría de masas tándem (MS^2) para la caracterización estructural de iminoazúcares desconocidos en los extractos analizados.

Sección 5.2. Cromatografía líquida de interacción hidrofílica (HILIC) acoplada a espectrometría de masas para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular en extractos de Aglaonema.

- Análisis de extractos de Aglaonema empleando la columna hidrofílica previamente seleccionada en la sección 5.1. y nuevas condiciones cromatográficas (eluyentes, aditivos, temperatura de la columna, etc). Evaluación del efecto sobre la retención, resolución, anchura de pico y simetría de compuestos seleccionados de dichas condiciones experimentales.
- Aplicación del método optimizado al análisis de extractos de hojas de diferentes muestras de Aglaonema. Caracterización estructural mediante datos de MS^2 de iminoazúcares desconocidos.

Sección 6: Optimización de métodos de extracción y fraccionamiento de iminoazúcares bioactivos a partir de fuentes vegetales.

Sección 6.1. Nuevas metodologías para la extracción y fraccionamiento de carbohidratos bioactivos a partir de hojas de morera (*Morus alba*).

- Optimización de condiciones experimentales (temperatura y tiempo de extracción, cantidad de muestra y número de ciclos) para la extracción por PLE de iminoazúcares y otros carbohidratos bioactivos. Comparación de resultados con los de la extracción convencional.
- Puesta a punto de métodos de fraccionamiento basados en la incubación con levaduras y la cromatografía de intercambio catiónico para el enriquecimiento en iminoazúcares e inositoles de extractos de morera. Comparación de resultados y selección de condiciones óptimas.

Sección 6.2. Extracción con líquidos presurizados de iminoazúcares a partir de muestras de *Aglaonema* sp. Estudio de bioactividad, viabilidad celular y estabilidad térmica de los extractos obtenidos.

- Caracterización por GC-MS de extractos de Aglaonema empleando las condiciones previamente optimizadas en la sección 4.2.
- Aplicación de las conclusiones obtenidas en la sección 4.3 sobre la relación estructura química-datos de GC-MS para la identificación de iminoazúcares desconocidos en extractos de Aglaonema.
- Evaluación del efecto de distintas condiciones experimentales (temperatura y tiempo de extracción, cantidad de muestra y número de ciclos) sobre la extracción por PLE de iminoazúcares a partir de muestras de Aglaonema.
- Optimización de la incubación con levaduras para el enriquecimiento en iminoazúcares de dichos extractos.
- Obtención de extractos enriquecidos en iminoazúcares a partir de distintos cultivares de *Aglaonema* sp.
- Puesta a punto del ensayo y determinación de la actividad inhibidora de α-glicosidasas de los extractos de Aglaonema anteriormente obtenidos.

- Estudio *in vitro* de la viabilidad de células Caco-2 tras el tratamiento con extractos de Aglaonema como medida de la potencial toxicidad de compuestos coextraídos con los iminoazúcares en dichos extractos.
- Evaluación de los posibles cambios en el contenido en iminoazúcares bioactivos durante el almacenamiento en condiciones de temperatura controlada como indicador de la estabilidad térmica de extractos de Aglaonema.

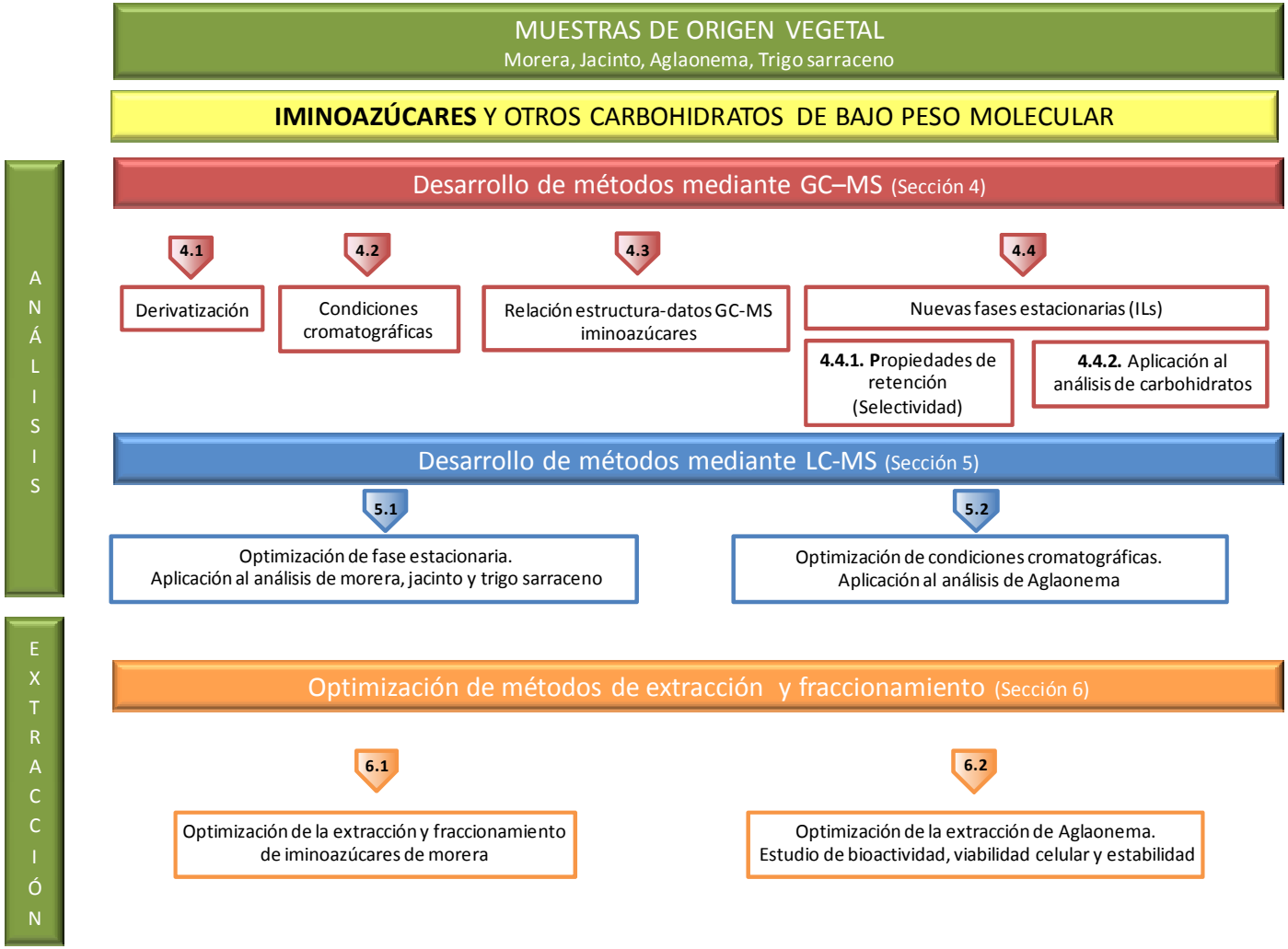


Figura 3.1. Esquema general del plan de trabajo seguido en la presente Tesis Doctoral.

ANALISIS POR GC-MS

4. Desarrollo de métodos mediante GC–MS para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular

Como se ha comentado previamente en la Introducción de esta Memoria (**sección 1.5**), el análisis de extractos vegetales ricos en iminoazúcares mediante técnicas cromatográficas presenta varios problemas, tales como coeluciones con otros carbohidratos de bajo peso molecular e interferentes con la bioactividad de los iminoazúcares, carencia de patrones comerciales que faciliten la identificación de estos compuestos bioactivos, falta de estabilidad y reproducibilidad de los derivados formados para su análisis por GC, etc.

Con objeto de desarrollar un método por GC-MS, adecuado para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular presentes en extractos vegetales, se han llevado a cabo en esta sección avances en: (i) la optimización del proceso de preparación de muestra (derivatización), (ii) la selección de las condiciones cromatográficas óptimas y (iii) el estudio de datos de retención cromatográfica (I^T, índices de retención lineales) y de fragmentaciones típicas en espectrometría de masas con ionización por impacto electrónico (EI) de iminoazúcares. Además, se ha evaluado el uso de nuevas fases estacionarias de GC basadas en líquidos iónicos para el análisis de estos compuestos, previa caracterización de su polaridad, eficacia y selectividad.

Para la optimización de los métodos, se han empleado patrones comerciales de iminoazúcares y otros carbohidratos de bajo peso molecular (mono- y disacáridos, inositoles, etc.), así como extractos acuosos de hojas de morera, hojas y raíces de Aglaonema, bulbos de jacinto y granos de trigo sarraceno, obtenidos mediante extracción sólido-líquido convencional.

La **sección 4.1.** está basada en el trabajo titulado "**A derivatization procedure for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates by GC-MS in mulberry (*Morus* sp.)**" de Rodríguez-Sánchez *y col.*, publicado en *Food Chemistry* 126 (2011) 353-359. En este trabajo se desarrolla, optimiza y valida un proceso de derivatización de iminoazúcares (DNJ y fagomina) y otros carbohidratos de bajo peso molecular, como azúcares sencillos e inositoles, para su aplicación al análisis de extractos de hojas, frutos y ramas de morera (*Morus sp.*). El proceso de derivatización optimizado permite la derivatización "completa" de los

iminoazúcares, así como su análisis conjunto con otros carbohidratos de bajo peso molecular.

Una vez seleccionadas las condiciones de derivatización óptimas, se abordó el problema de la cuantificación de iminoazúcares presentes en muy bajas concentraciones en mezclas complejas. Se optimizaron, entre otras, condiciones cromatográficas como la temperatura del inyector, aspecto poco abordado en el análisis de iminoazúcares por GC, y se evaluó el efecto sobre la sensibilidad de detección del uso de detectores FID y de MS, trabajando este último en modo registro selectivo de iones (SIM) o en modo barrido de iones (SCAN). Los resultados obtenidos se resumen en la **sección 4.2.**, en el trabajo titulado "**Improvement of a gas chromatographic method for the analysis of iminosugars and other bioactive carbohydrates**" de Rodríguez-Sánchez *y col.*, publicado en *Journal of Chromatography A* 1289 (2013) 145-148.

Por otra parte, la identificación/caracterización de iminoazúcares en extractos vegetales mediante GC-MS no es una tarea fácil, ya que se carece de antecedentes bibliográficos que relacionen datos de retención en GC y de fragmentación por MS con sus estructuras químicas, las cuales son muy similares entre sí. Como avance en este sentido, la **sección 4.3.** incluye el trabajo titulado "**Characterization of trimethylsilyl ethers of iminosugars by gas chromatography-mass spectrometry**" de Rodríguez-Sánchez *y col.*, publicado en *Journal of Chromatography A* 1372 (2014) 221–227. En dicho trabajo se lleva a cabo un estudio exhaustivo de los datos de retención (índices de retención lineales (I^T) en dos columnas con distinta fase estacionaria) y de los espectros de masas (abundancia de relaciones *m/z* de iones fragmentos característicos) de doce patrones de iminoazúcares (previamente derivatizados), estableciéndose relaciones entre estos datos y sus estructuras químicas. Dicho estudio resulta de gran utilidad para la posterior identificación/caracterización de iminoazúcares presentes en extractos vegetales.

Otra cuestión de especial relevancia en la optimización de un método cromatográfico es la selección de la fase estacionaria. La reciente comercialización de nuevas fases estacionarias basadas en ILs, y a las que se atribuye una selectividad diferente a la de las fases convencionales, hizo de interés el evaluar su posible

aplicación al análisis de mezclas complejas de carbohidratos de bajo peso molecular, incluyendo entre otros iminoazúcares, aplicación no contemplada hasta el momento en la bibliografía.

La **sección 4.4.** incluye el estudio y aplicación de cuatro fases estacionarias basadas en ILs (SLB-IL59, SLB-IL76, SLB-IL82 y SLB-IL100), proporcionadas por la empresa Supelco gracias a un convenio de colaboración. Previo a la evaluación de su utilidad para el análisis de carbohidratos, fue necesario llevar a cabo una caracterización de dichas fases basada en estudios de selectividad. Por tanto, esta sección está dividida a su vez en dos subsecciones:

La **sección 4.4.1** incluye el trabajo titulado "**Characterization by the solvation parameter model of the retention properties of comercial ionic liquid columns for gas chromatography**" de Rodríguez-Sánchez *y col.*, publicado en *Journal of Chromatography A* 1326 (2014) 96-102. En este trabajo se han caracterizado los mecanismos de retención de las fases estacionarias basadas en ILs previamente mencionadas. Para tal fin, y considerando las limitaciones del método de McReynolds relacionadas con la falta de diferenciación de las interacciones individuales molécula-fase estacionaria, previamente mencionadas en la Introducción de esta Memoria (**sección 1.5.2.2**), se empleó el Modelo de los Parámetros de Solvatación (SPM) en un rango de temperaturas entre 80-160 ºC. La evaluación de las interacciones existentes para las distintas fases en estudio puso de manifiesto que dichas fases ocupan un espacio, en cuanto a selectividad se refiere, no cubierto hasta el momento por las columnas convencionales, lo que puede contribuir a aumentar la capacidad de separación de esta técnica.

Una vez caracterizadas, dichas fases estacionarias se emplearon por primera vez para el análisis de carbohidratos de bajo peso molecular (iminoazúcares, inositoles, mono-, di-, y trisacáridos). En este estudio, además de las cuatro columnas indicadas previamente, se incluyó la columna SLB-IL61 de reciente comercialización. La selección de fase estacionaria y condiciones cromatográficas óptimas, basada en parámetros cromatográficos como anchura de pico, simetría y resolución, se llevó a cabo a partir de los resultados obtenidos en el análisis de patrones comerciales. Dichas condiciones experimentales fueron las elegidas para el análisis de extractos de jacinto y morera

ricos en iminoazúcares. Estos resultados se recogen en el artículo titulado "**Evaluation of different ionic liquid stationary phases for the GC-MS analysis of carbohydrates**" de Rodríguez-Sánchez *y col.*, enviado para su publicación a la revista *Journal of Chromatography A*, y constituyen la **sección 4.4.2** de esta Memoria.

4.1. A derivatization procedure for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates by GC-MS in mulberry (*Morus* sp.)

S. Rodríguez-Sánchez, O. Hernández, A.I. Ruiz-Matute, M.L. Sanz

Food Chemistry (2011) 126: 353-359

4.1.1. Introduction

Polyhydroxyalkaloids (also known as iminosugars) are hydroxylated derivatives of piperidine, pyrrolidine, indolizidine, pyrrolizidine and nortropane [35], which have been detected in a high number of plant families (Leguminosae, Moraceae, Hyacinthaceae, Campanulaceae, etc.) and microorganism genus (*Streptomyces, Bacillus*, etc.) [10]. Different pharmacological and ecological activities have been attributed to these compounds, the inhibitory effect against glycosidases being the most studied, which makes them potential drug candidates for the treatment of several diseases (e.g. diabetes, cancer, AIDS, and viral infections) [6].

Deoxynojirimycin (1,5-dideoxy-1,5-imino-*D*-glucitol; DNJ) is a polyhydroxyalkaloid typical of the six-membered ring piperidine group. It has been shown to be a potent α-glucosidase inhibitor, although DNJ does not inhibit α-amylases [151]. Its presence has been described in leaves and roots of *Morus* sp., *Hyacinthus orientalis* bulbs and larvae of *Bombyx mori* [10] in which its content has been used as antihyperglycemic quality criterion [16].

Many reports have been published about the chemical synthesis of iminosugars [152, 153]; however, there is a high interest in obtaining these compounds from natural sources. Different extraction procedures based on the use of polar solvents such as ethanol, methanol, hot water [35] or acidulated water [16] have been carried out. However, all these procedures are not selective for the target compounds and the extraction of interferences such as other low molecular weight carbohydrates takes also place; a purification step being necessary. Therefore, the development of analytical methods which allow the simultaneous determination of iminosugars and interferences to control their extraction is of crucial importance.

HPLC has been used for the analysis of polyhydroxylated alkaloids; however, the poor resolution achieved by this technique and the extreme hydrophilicity and low solubility of these compounds in non-hydroxylic organic solvents make difficult the selection of the most suitable solvent system-column packing combination [35] and require the search for different alternatives. Moreover, the lack of chromophores in these chemical structures limits their detection. The use of HILIC systems coupled to evaporative light scattering detectors [39] or amino columns coupled to MS detectors [154, 155] have, to some extend, reduced these problems. Furthermore, different methods using direct ESI MS analysis have been proposed for rapid detection of polyhydroxyalkaloids [156].

However, GC-MS combines the good resolution of GC and the structural information obtained by MS. Both trimethylsilyl (TMS) [120] and acetylated [53] derivatives have been used to transform iminosugars into volatile compounds. Nevertheless, the appearance of multiple peaks for each reducing carbohydrate is one of their main disadvantages [120], the development of new derivatization procedures to analyse iminosugars free from interferences being necessary.

Therefore, the aim of this work was to develop a derivatization procedure for the simultaneous analysis of iminosugars (mainly DNJ and fagomine) and other carbohydrates of low molecular weight, and validate its application to extracts of mulberry leaves, fruits and branches, which are known to be a natural source of these compounds.

4.1.2. Materials and methods

4.1.2.1. Standards

Analytical standards of fructose, glucose, galactose, ribose, sucrose, *myo*-inositol, galactinol (O-*α*-*D*-galactopyranosyl-(1-1)-*L*-*myo*-inositol), pipecolic acid (2-piperidinecarboxylic acid), nipecotic acid (3-piperidinecarboxylic acid), isonipecotic acid (4-piperidinecarboxylic acid), *α*-methyl-*L*-proline (2-methylpyrrolidine-2-carboxylic acid) and phenyl-*β*-*D*-glucoside were obtained from Sigma Chemical Co. (St. Louis, US). DNJ was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Glyceryl-

β-galactoside was obtained by transglycosidation reaction of lactose treated with *β*-galactosidase [157]. This product was kindly gifted by Dr. Montilla (CSIC, Spain).

4.1.2.2. Samples

Samples of *Morus alba* leaves (M1L and M2L) were obtained from Tarancón (Cuenca, Spain) and from Madrid (Spain), respectively. Leaves (M3L), fruits (M3F) and branches (M3B) of *Morus alba* were collected from Hoyo de Pinares (Ávila, Spain). Samples of leaves (M4L), fruits (M4F) and branches (M4B) from *Morus nigra* were obtained from Madrid (Spain).

4.1.2.3. Carbohydrate extraction

The extraction of carbohydrates from mulberry samples was carried out using acidulated water, previously suggested by other authors [16], as it has been described that the presence of acid may suppress the decomposition of some pyrrolidines [35]. One gram of samples was ground before extraction into 10 mL of water acidulated with 0.1 % (v/v) HCl for 24 hours. Samples were filtrated through Whatman no. 4 paper and kept at -20 ºC until analyses.

4.1.2.4. Carbohydrate analysis

- Derivatización procedures

Except for those derivatization procedures specifically indicated, 1 mL of extracts 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 dried under vacuum at 38-40 ºC.

- *Silylation.* Trimethylsilyl (TMS) ethers were prepared according to Troyano *y col*. [158]. Hundred µL of anhydrous pyridine, 100 µL of trimethylsilylimidazole (TMSI) and 100 µL of trimethylchlorosilane (TMCS) were added to the evaporated samples. Extraction of the TMS ethers was carried out using 100 µL of hexane and 200 µL of Milli-Q water. One µL of the hexane upper layer was injected into the GC.

- *Acetylation.* Acetylation was carried out following the procedure described by Magalhães *et al*. [53]. Samples (10 mg) were treated with acetic anhydride (0.7 mL)

in pyridine (0.5 mL) for 24 h at room temperature. After reaction, samples were centrifuged at 8000 rpm for 10 min, and 1 µL of supernatants was analyzed by GC-MS.

- *Oximation+Acetylation*. Oximes were obtained by addition of 350 µL of a solution of 2.5 % hydroxylamine chloride in pyridine after 30 min at 75 ºC according to Brobst & Lott [159]. Then, they were acetylated with acetic anhydride (0.45 mL) for 5 h. After reaction, samples were centrifuged at 8000 rpm for 10 min, and 1 µL of supernatants was analyzed by GC-MS.
- *Oximation+Silylation*. Taking into account the problems found for iminosugar derivatization, different procedures were followed for the formation of TMSO. Extracts were dried by two different ways:

(i) under vacuum, with and without acetone addition to help water removal and

(ii) freeze-drying: Freeze dried samples were also treated by two different procedures: exposing them to atmospheric moisture prior to derivatization according to Kite & Hughes [126], and in closed vials without moisture exposition.

Oximes were formed as indicated above for acetylated oximes using a solution of 2.5 % hydroxylamine chloride in pyridine, whereas TMS derivatives were obtained by four different procedures:

(i) 300 µL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) and 3 µL of trimethylchlorosilane (TMCS) at 60 ºC for 1 h [35];

(ii) 90 µL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and 10 µL of trimethylchlorosilane (TMCS) at room temperature for 1 h [160];

(iii) 100 µL of trimethylsilylimidazole (TMSI) and 100 µL of trimethylchlorosilane (TMCS) at room temperature for 30 min [158];

(iv) 350 µL of hexamethyldisilazane (HMDS) and 35 µL of trifluoroacetic acid (TFA) at 45 ºC for 30 min [159].

After reaction, samples were centrifuged at 8000 rpm for 10 min and kept at 4 ºC for 24 h before analysis. One µL of supernatants was injected into the GC injection port.

- GC-MS analysis

GC-MS analyses of derivatised samples were carried out in a Hewlett-Packard 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using He at ~1 mL min^{-1} as carrier gas. A 30 m x 0.25 mm i.d. x 0.25 µm film thickness TRB-1 (crosslinked methyl silicone) column from Teknokroma (Barcelona, Spain) was used. Oven temperature program was optimised, the best conditions being: from 100 ºC to 200 ºC at 15 ºC min^{-1}, kept at this temperature for 15 min and finally programmed to 300 ºC at 15 ºC min^{-1} and kept at this temperature for 10 min. Injector temperature was 300 ºC and injections were made in the split mode with a split ratio 1:20. Mass spectrometer was operating in electron impact (EI) mode at 70 eV, scanning the 35-650 *m/z* range. Interface and source temperature were 280 ºC and 230 ºC, respectively. Acquisition was done using a HPChemStation software (Hewlett-Packard, Palo Alto, CA, USA).

Linear retention indices (I^T) were calculated from the retention times of TMSO derivatives and suitable *n*-alkanes.

Quantitative values were calculated using the internal standard method. Response factors were determined by injecting standards of different concentration containing internal standard (phenyl-*β*-*D*-glucoside) in triplicate. Limit of detection (*LOD*) was calculated as three times the *S/N* (signal-to-noise) ratio while limit of quantitation (*LOQ*) was considered ten times this ratio according to Foley & Dorsey [161]. Accuracy was estimated by carrying out recovery assays by the addition of known amounts of glucose, DNJ and *myo*-inositol standards to a *Morus alba* extract; the three carbohydrates were measured in both unspiked and spiked samples and recoveries were calculated. Relative standard deviation (*RSD* %) was calculated for the results obtained of a standard mixture and a *Morus alba* extract previously derivatised and analysed in 5 different days.

4.1.3. Results and discussion

4.1.3.1. Derivatization assays

Figures 4.1A and **4.1B** show the GC-MS profiles of acetyl and TMS derivatives of mulberry M1L extract. Derivatised sugars (ribose, glucose, fructose and sucrose), *myo*-inositol, galactinol and DNJ were identified by comparison of their linear retention indices and mass spectra with those of corresponding derivatised standards. Fagomine was identified by its characteristic *m/z* fragment ions for acetylated derivatives according to Magalhães *et al.* [53] (*m/z*: 43 (100), 98 (29), 140 (92), 182 (57), 196 (11), 242 (3)), and according to the fragmentation suggested by Molyneux *et al.* [35] for TMS derivatives of alkaloids (**Table 4.1**). However, up to five peaks can appear per reducing sugar (both methods keep the sugar ring intact and can provide a peak for each tautomer), leading to overlapped peaks. Although TMS derivatization has been the most common procedure used to analyse iminosugars [120], and DNJ could easily be quantified using this procedure in mulberry extract, fagomine coeluted with one of the five tautomeric peaks of ribose. In the acetylated sample, DNJ had the same retention time as fructose, making also its quantification difficult.

Therefore, a different derivatization procedure for the simultaneous analysis of sugars, iminosugars and inositols was assayed.

Figure 4.1C shows the GC-MS profile of carbohydrates in mulberry M1L, previously subjected to the oximation + acetylation derivatization as indicated in Materials and Methods. By using this derivatization method, reducing aldoses are converted into per-acetylated aldononitriles (PAAN), giving rise to a single peak. Non-reducing sugars, inositols and iminosugars gave rise to a single derivative corresponding to the per-acetylated compound. Reducing ketoses should form the per-acetylated oximes (with *syn* (*E*) and *anti* (*Z*) isomers) giving rise to two different peaks. However, derivatization of these carbohydrates was not complete, and up to five different peaks could appear for each ketose.

The effect of acetylation time of glucose, fructose, *myo*-inositol and DNJ, previously to the oximation step, was evaluated by sampling the reaction mixture every hour by triplicate. **Figure 4.2** shows the behavior of these standards during acetylation. Although previous studies suggested 24 h at 20 ºC for carrying the

acetylation of iminosugars out [53], incubation for 5 h was enough for the complete derivatization of glucose, *myo*-inositol and DNJ. Nevertheless, acetylation of fructose was variable during the 24 h period studied. Therefore, this derivatization procedure was discarded for mulberry samples, where fructose is one of the main sugars.

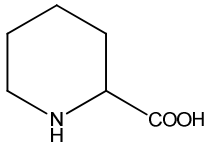
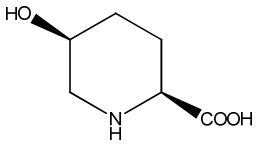
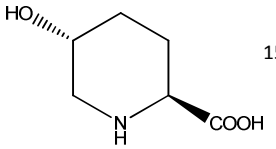
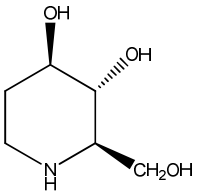
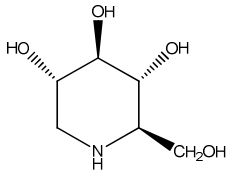
Figure 4.1D shows the GC-MS profile of carbohydrates in mulberry M1 extract previously to the oximation + trimethylsilylation procedure. Both reducing aldoses and ketoses showed two peaks from the *syn* (*E*) and *anti* (*Z*) isomers whereas non-reducing sugars and inositols gave rise to one peak. However, partial derivatization of iminosugars was the major drawback of this reaction: while the hydroxyl groups were readily silylated, the reaction for the amino group depended on the compound and on the derivatization conditions, resulting in one or two peaks for each iminosugar.

Therefore, different conditions were assayed trying to achieve a complete derivatization of iminosugars. Residual moisture and derivatization reagents appear to be important in controlling the extent of trimethylsilylation of these alkaloids.

Concerning residual moisture, previous studies have shown that a complete silylation of the hydroxyl groups of hydroxypipecolic acids occurs without silylation of the amino group when samples are first freeze-dried and then exposed to atmospheric moisture before derivatization [126]. When this procedure was assayed in a triplicate analysis of pipecolic acid and DNJ, with and without exposing samples to atmospheric moisture after freeze-drying, no differences were found for both treatments. Moreover, pipecolic acid and DNJ solutions were also evaporated under vacuum before derivatization treatment to compare the effect and no notable differences were observed. Similar results were also obtained for in those samples treated with and without acetone before the complete evaporation under vacuum.

Regarding derivatization, four different approaches (MSTFA/TMCS, BSTFA/TMCS, TMSI/TMCS and HMDS/TFA) were followed after treatment of pipecolic acid (**Figure 4.3A**) and DNJ (**Figure 4.3B**) with 2.5% hydroxylamine chloride in pyridine:

Table 4.1. Linear retention indices (I^T) and MS data of TMS derivatives of polyhydroxy alkaloids found in mulberry.

Polyhydroxyalkaloid	Structure	I^T	Mass spectrum
Pipecolic acid		1250[a] 1371[b]	56 (7)*, 75 (7), 84 (100), 103 (4), 186 (6) [a] 73 (24), 156 (100), 147 (4), 230 (4), 258 (1)[b]
Methylpyrrolidinecarboxylic acid	N.C.**	1458[a] 1506[b]	75 (28), 84 (100), 103 (3), 186 (7), 201 (1)[a] 73 (56), 147 (17), 156 (100), 230 (6), 258 (6)[b]
cis-5-Hydroxypipecolic acid		1532[a]	73 (27), 82 (100), 156 (5), 172 (80), 289 (1)[a]
Hydroxypipecolic acid with a methyl group	N.C.	1565[a]	73 (100), 142 (87), 147 (29), 186 (66), 288 (9), 303 (1)[a]
trans-5-Hydroxypipecolic acid		1578[a]	73 (88), 82 (100), 156 (4), 172 (82), 289 (1)[a]
Fagomine		1698[a]	73 (91), 129 (27), 144 (100), 170 (29), 260 (67), 348 (2)[a]
DNJ		1940[a] 1854[b]	73 (100), 144 (47), 217 (82), 258 (34), 348 (52), 436 (1)[a] 73 (100), 147 (26), 216 (32), 258 (6), 348 (8), 420 (87), 508 (0.7)[b]

**m/z* values (Relative abundance in brackets)
** N.C. not confirmed
[a]Data corresponding to the compound with only hydroxyl groups derivatised.
[b] Data corresponding to the compound with both hydroxyl and imino groups derivatised.

(i) MSTFA gave rise to the formation of two silyl derivatives for both pipecolic acid and DNJ differing in the derivatization in the imino group. On the contrary, Molyneux *et al.* [35] reported the formation of single derivative for polyhydroxyalkaloids (e.g.: swainsonine, castanospermine) when using MSTFA at 60 ºC.

(ii) The use of BSTFA afforded a single peak corresponding to the non-derivatised amino group of DNJ. However, the extent of derivatization was non-reproducible for pipecolic acid giving randomly rise to one or two peaks; formation of these peaks was not possible to be controlled.

(iii) After derivatization with TMSI and TMCS, DNJ with the non-derivatised amino group was the only peak formed. However, pipecolic acid was not derivatised under these conditions (data not shown).

(iv) The best results were obtained using HMDS and TFA: all the hydroxyl groups were derivatized for both DNJ and pipecolic acid, whereas for pipecolic acid only traces of the derivatised amino group were detected. These derivatives are sufficiently volatile for analysis [35] and the formation of a single peak is more appropriate to avoid coelutions and quantification problems. Therefore, the use of HMDS and TFA was selected for further analyses.

Derivatized samples were kept at 4 ºC for at least for 24 h until analysis. This period of preservation turned out to be critical for stability of derivatives.

4.1.3.2. Qualitative analysis

Figure 4.4 shows close-up views of the GC-MS profile of the mulberry extract M1L previously subjected to the oximation + sylilation derivatization procedure. No coelution was observed for DNJ and fagomine; other alkaloids were also detected. Pipecolic acid (I^T = 1250; peak 1) was observed at trace levels. Two peaks showing an I^T= 1458 (peak 4) and an I^T = 1506 (peak 6) were also observed. Their mass spectra were similar to that of monosilylated pipecolic acid and disilylated pipecolic acid (see **Table 4.1**). Their GC retention times were compared with those of available standard compounds having a similar structure to that of pipecolic acid (methylproline, nipecotic and isonipecotic acids) with negative results. Taking into account the similarity among their mass spectra, these peaks could correspond to a methylated pyrrolidine carboxylic isomer.

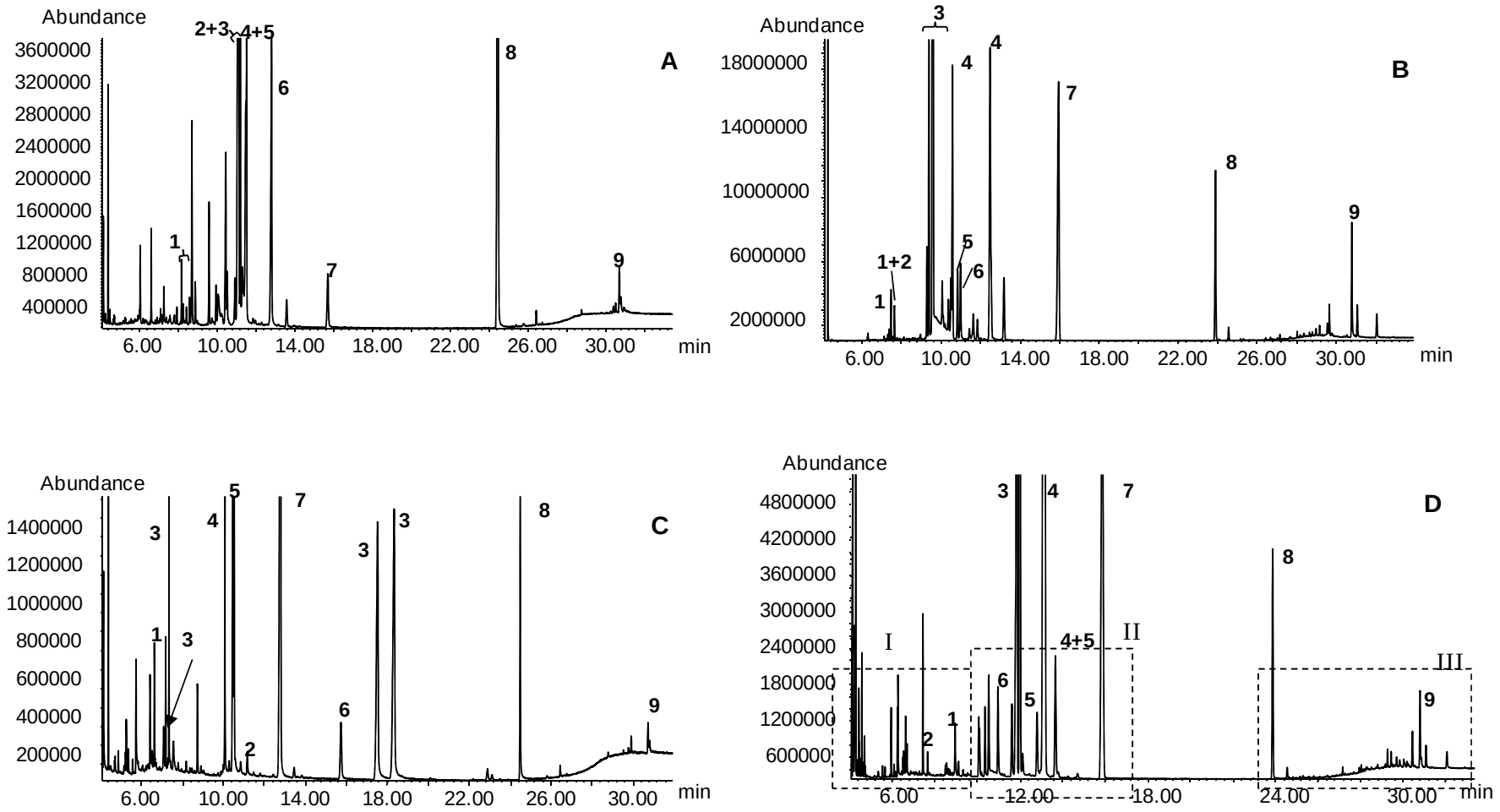


Figure 4.1. GC-MS profiles of mulberry leaves extract converted into their (A) acetylated, (B) trimethylsilyl, (C) acetylated oxime and (D) trimethylsilyloxime derivatives. (1) Ribose, (2) fagomine, (3) fructose, (4) glucose, (5) galactose, (6) *myo*-inositol, (7) DNJ, (8) phenyl-*β*-*D*-glucopyranoside (i.s.), (9) galactinol.

Two peaks with mass spectra corresponding to 5-hydroxypipecolic acid (**Table 4.1**) with I^T of 1532 (peak 7) and 1578 (peak 9) could be assigned to *cis*- and *trans*- isomers, respectively, according to Kite & Hughes [126]. This compound had previously been described in mulberry roots [162]. A compound with an I^T of 1565 (peak 8) was also identified as a derivative of pipecolic acid: although its chemical structure could not be confirmed, its mass spectrum (**Table 4.1**) is compatible with a hydroxypipecolic acid structure with an additional methyl group.

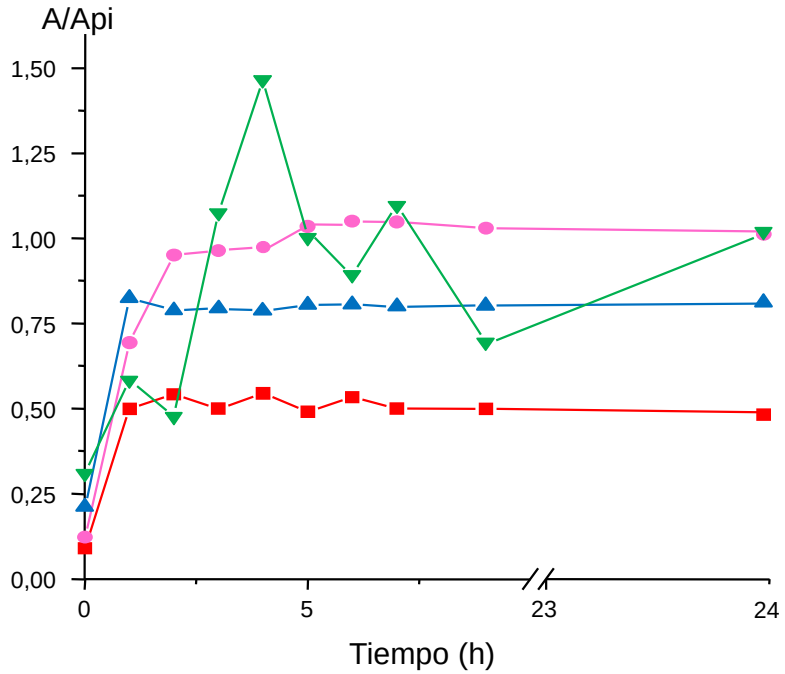


Figure 4.2. Optimization of acetylation time of glucose (■), fructose (▼), *myo*-inositol (●) and DNJ (▲).

Different acids such as glyceric (I^T = 1333, peak 3), malic (I^T = 1490, peak 5), citric (I^T = 1808, peak 12), quinic (I^T = 1877, peak 13), gluconic (I^T = 2115, peak 19) acids and the gluconic lactone (I^T = 1899, peak 14), were also detected. Moreover, a peak with I^T = 2784, and a characteristic *m/z* ion at 337 (peak 22) was identified as a glyceryl-*β*-galactoside by comparison with previous data [157].

4.1.3.3. Validation of the method

The range of linearity of MS response was checked by employing calibration curves in the range from 0.013 to 1.3 mg mL^{-1} of standards previously converted to their TMSO. Response factors relative to phenyl-*β*-D-glucoside varied from 0.75 for DNJ to 2.1 for *myo*-inositol and galactinol.

An average of *LOD* and *LOQ* of 54 ng g^{-1} of product and 180 ng g^{-1} of product were obtained, respectively. The average recovery values ranged from 91 to 94 %, whereas precision of the method ranged from 5.9 % to 7.0 % for all the target compounds which can be considered adequate for quantitative analysis. However, as indicated before, derivatization procedure and preservation of samples should be very carefully controlled to obtain these reproducibility values.

4.1.3.4. Sample analysis

Table 4.2 shows the sugar, iminosugar and inositol contents of leaves of mulberries M1L and M2L and leaves, fruit and branches of mulberries M3 and M4. The highest values of DNJ were detected in samples M3B, M1L and M4F (4.75, 2.68, and 2.08 mg g^{-1} of product, respectively). However, among all samples under study, mulberry M2L showed the highest concentration of fagomine (0.66 mg g^{-1} of product). In general, carboxylic acid derivatives were higher for leaf samples; M1L sample showed the highest value. Only minor amounts of these compounds were detected in bark samples (M3B and M4B).

Regarding to sugars, both M1L and M2L leaf samples showed the highest fructose and ribose concentrations; however, glucose and galactose were the most abundant in M4L. Galactinol and its isomers also showed the highest values in mulberry M4L.

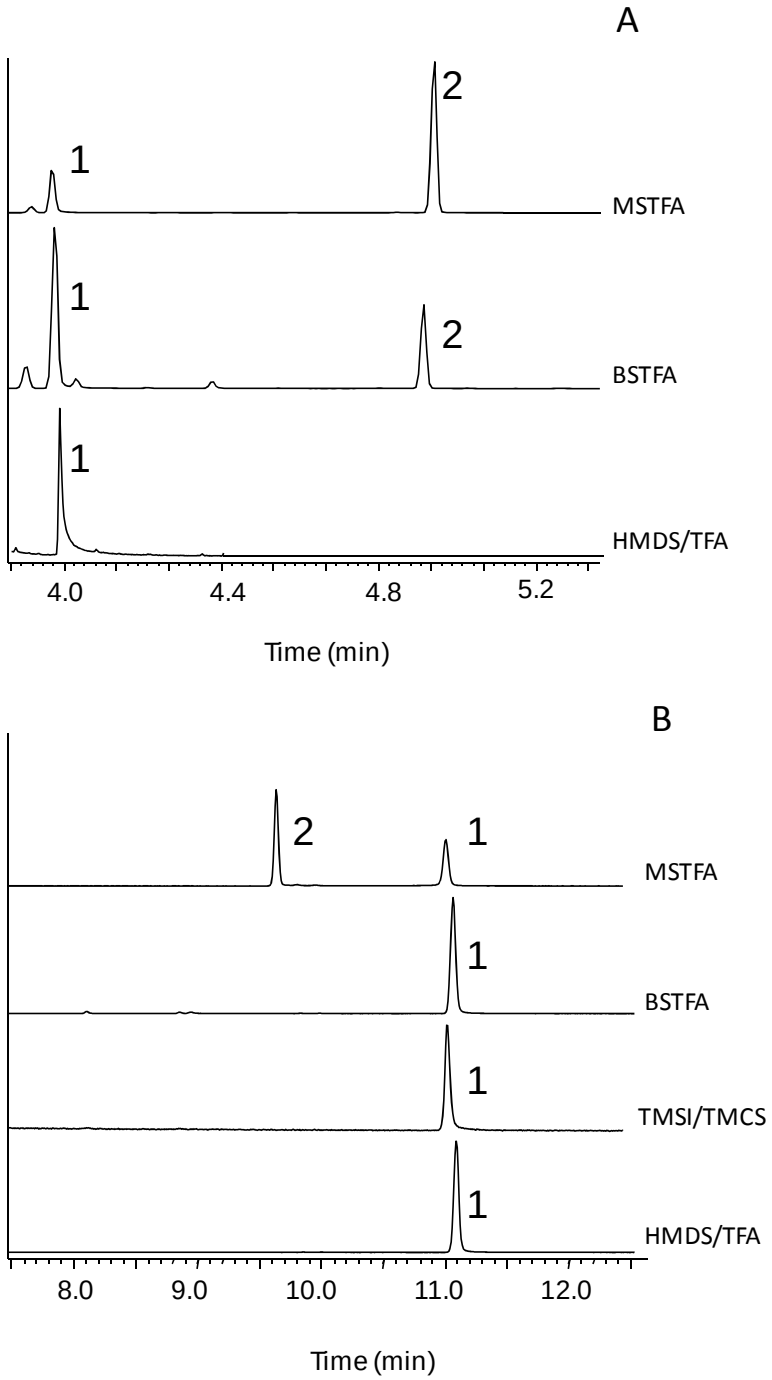


Figure 4.3. GC-MS profiles of TMSO of pipecolic acid (A) and DNJ (B) obtained using MSTFA, BSTFA, TMSI/TMCS and HMDS/TFA. (1) monosilylated derivatives, (2) disilylated derivatives.

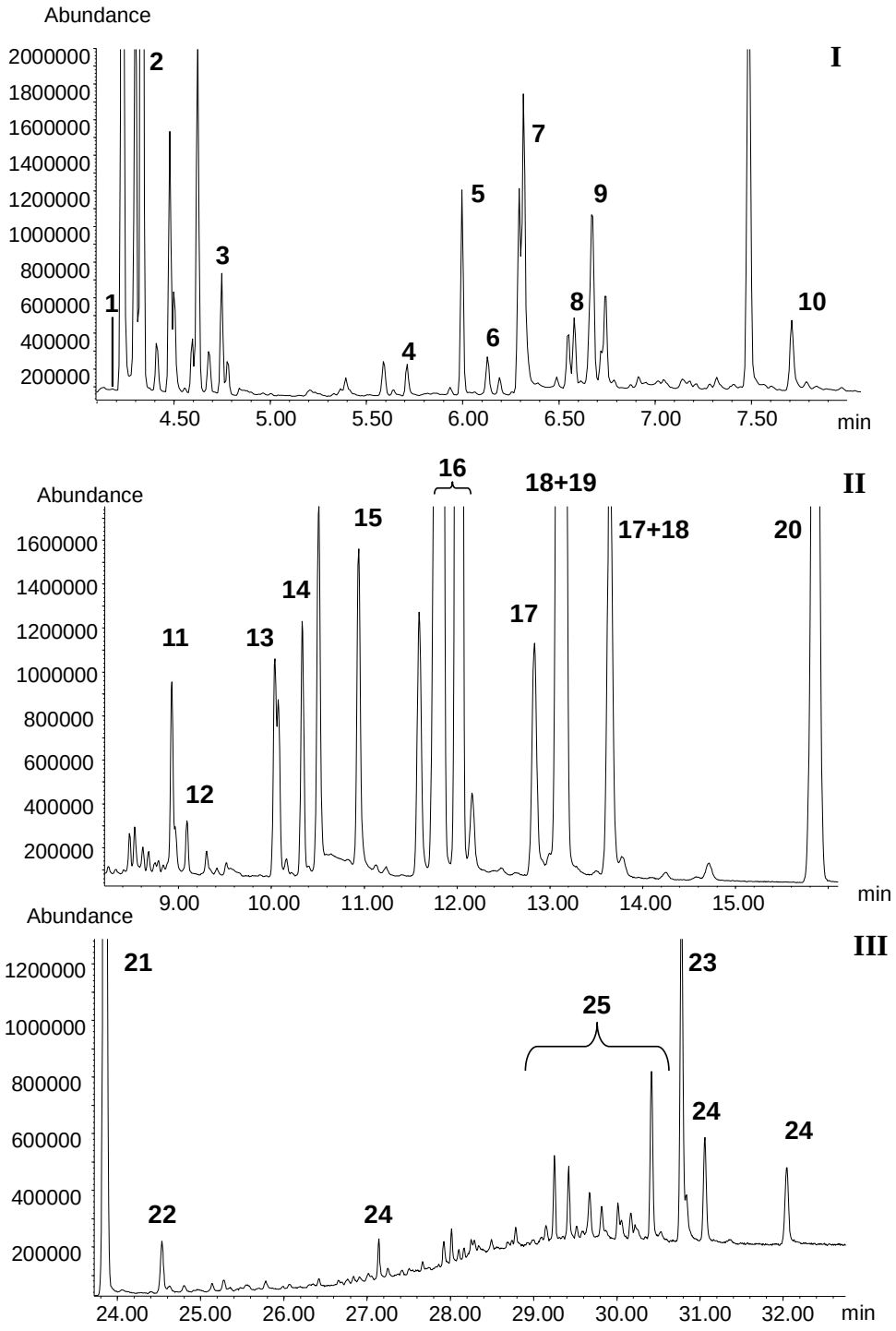


Figure 4.4. Close-up views of GC-MS profile of mulberry leaves (**Figure 4.1D**) previously converted into their trimethylsilyloximes under optimized conditions. I, II and III: regions from **Figure 4.1D**. (1) Pipecolic acid, (2) glycerol, (3) glyceric acid, (4) monosilylated methyl-pyrrolidinecarboxylic acid, (5) malic acid, (6) disilylated methyl-pyrrolidinecarboxylic acid, (7) *cis* 5-hydroxypipecolic acid, (8) hydroxypipecolic acid with a methyl group, (9) *trans* 5-hydroxypipecolic acid, (10) fagomine, (11) ribose, (12) citric acid, (13) quinic acid, (14) gluconic lactone, (15) DNJ, (16) fructose, (17) galactose, (18) glucose, (19) gluconic acid, (20) *myo*-inositol, (21)phenyl-*β*-*D*-glucopyranoside (i.s.), (22) glyceryl-galactoside, (23) galactinol, (24) isomers of galactinol, (25) disaccharides.

4.1.4. Conclusions

Iminosugars are frequently present in natural products together with other low molecular weight carbohydrates. GC-MS is a powerful tool for their analysis, but it requires a previous derivatization step, which can lead to problems in both qualitative and quantitative determination. The proposed procedure for conversion into TMSO appears to be the most appropriate method to simultaneously quantify sugars, inositols and iminosugars in mulberry extracts, although for the last compounds, control of derivatization conditions is crucial. Derivatization of complex samples containing other different iminosugars will possibly require a similar study of the effect of the silylation procedures and their conditions.

Table 4.2. Content (mg g^{-1} of product) of iminosugars, sugars and inositols in mulberry samples.

	M1L	M2L	M3L	M3F	M3B	M4L	M4F	M4B
Pipecolic acid	tr*	tr	tr	tr	tr	tr	tr	tr
Methylpyrrolidine carboxylic acid	0.68 (0.05)**	0.34 (0.02)	0.55 (0.06)	tr	0.14 (0.06)	0.05 (0.00)	0.36 (0.03)	tr
cis-5-Hydroxypipecolic acid	5.98 (0.27)	0.52 (0.10)	0.11 (0.02)	tr	tr	0.02 (0.00)	0.31 (0.04)	tr
Hydroxypipecolic acid with a methyl group	1.39 (0.09)	0.85 (0.00)	0.70 (0.01)	0.13 (0.03)	0.08 (0.00)	0.16 (0.03)	0.58 (0.02)	tr
trans-5-Hydroxypipecolic acid	1.53 (0.35)	0.31 (0.11)	0.25 (0.03)	0.11 (0.02)	tr	0.30 (0.04)	0.54 (0.06)	tr
Fagomine	0.12 (0.01)	0.66 (0.09)	0.05 (0.00)	0.10 (0.04)	tr	0.05 (0.00)	0.22 (0.10)	0.05 (0.01)
DNJ	2.68 (0.52)	1.23 (0.03)	0.04 (0.00)	0.11 (0.01)	4.75 (1.48)	0.22 (0.03)	2.08 (0.05)	0.27 (0.08)
Ribose	1.76 (0.20)	1.08 (0.04)	0.34 (0.01)	0.39 (0.01)	0.28 (0.10)	0.32 (0.10)	0.41 (0.02)	0.13 (0.06)
Fructose	72.88 (0.32)	57.40 (0.18)	12.84 (0.19)	14.39 (0.88)	8.91 (0.54)	38.08 (1.69)	31.71 (1.30)	6.24 (0.38)
Galactose	2.67 (0.16)	2.07 (0.06)	0.93 (0.00)	0.35 (0.08)	1.72 (0.66)	5.66 (0.85)	1.34 (0.43)	0.86 (0.03)
Glucose	16.50 (1.07)	20.72 (0.40)	12.16 (0.14)	25.92 (0.81)	10.44 (3.53)	40.51 (4.51)	29.40 (4.11)	1.98 (0.16)
Myo-inositol	11.17 (0.16)	6.59 (0.14)	3.09 (0.01)	4.37 (0.77)	6.91 (2.61)	7.12 (0.37)	12.34 (2.94)	0.75 (0.09)
Galactinols	2.04 (0.33)	0.41 (0.08)	0.28 (0.01)	0.03 (0.00)	0.06 (0.00)	3.37 (0.37)	0.27 (0.01)	0.01 (0.00)
Disaccharides	1.57 (0.14)	0.27 (0.07)	0.36 (0.02)	0.12 (0.00)	0.15 (0.04)	2.74 (0.13)	0.34 (0.12)	0.06 (0.03)

4.2. Improvement of a gas chromatographic method for the analysis of iminosugars and other bioactive carbohydrates

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Journal of Chromatography A (2013) 1289: 145-148

4.2.1. Introduction

Iminosugars (also called azasugars or polyhydroxyalkaloids) are monosaccharides with the endocyclic oxygen substituted by a nitrogen atom which confers them bioactive properties [8, 30]. They can be synthetized by chemical and enzymatic procedures [5-7] and are naturally present in different plants (Moraceae, Leguminosae, Hyacinthaceae, etc.) and microorganism genus (*Streptomyces*, *Bacillus*, etc.) [21, 31, 116]. Their inhibitory effect against glycosidases makes of iminosugars potential drug candidates for the treatment of several diseases such as diabetes, cancer, viral infections, etc. [8, 10, 30]. Moreover, therapeutic applications on different fields of medicine (oncology, neurodegenerative and metabolic diseases, etc.) have also been attributed to these compounds [163].

Different methods have been proposed for the analysis of iminosugars by HPLC, GC and direct ESI MS [35, 45, 111]; however, their resolution from other low molecular weight carbohydrates (LMWC) which could interfere with their biological properties is generally not achieved. We have recently developed a GC-MS method for the analysis of iminosugars and other LMWC in extracts of mulberry leaves, fruits and branches (**section 4.1**). Derivatization conditions were carefully optimized to avoid the appearance of multiple peaks for each iminosugar and to confer stability and reproducibility of analysis on derivatives. However, when this procedure was applied to the analysis of extracts such as buckwheat, in which iminosugars were present at very low levels, limitations regarding sensitivity we found, as non-gaussian peaks were occasionally obtained and even in some cases peaks could not be detected.

Therefore, and with the aim of improving the previously developed method to allow the sensitive determination of iminosugars and other bioactive LMWC in different plant sources, a study on stability with time of derivatized extracts and on

evaluation of chromatographic conditions (injection temperature and oven program) and detectors (FID and MS) was carried out. To the best of our knowledge, this is the first time that the relevance of the injection port temperature, and the determination of sensitivity provided by either GC-FID or GC-MS using SCAN and SIM acquisition modes have been evaluated for the analysis of iminosugars.

4.2.2. Materials and methods

4.2.2.1. Standards

Analytical standards of fructose, glucose, galactose, sucrose, *myo*-inositol, galactinol (O-*α*-*D*-galactopyranosyl-(1-1)-*L*-*myo*-inositol), phenyl-*β*-*D*-glucoside and deoxy-mannojirimycin (DMJ) were obtained from Sigma Chemical Co. (St. Louis, US). Deoxy-nojirimycin (DNJ) was purchased from Toronto Research Chemicals Inc. (Ontario, Canada) and *α*-homonojirimycin (*α*-HNJ) was purchased from Dextra Laboratories (Reading, UK).

4.2.2.2. Samples

Two samples of mulberry (*Morus alba*) leaves were collected in Madrid (Spain). Four samples of hyacinth (*Hyacinthus orientalis*) bulbs and two of buckwheat (*Fagopyrum esculentum*) grains were acquired in local markets in Madrid.

4.2.2.3. Carbohydrate extraction

One gram of air-dried sample was ground in a domestic mill (Moulinex) before extraction for 2 hours at ambient temperature into 10 mL of water acidified with 0.1 % (v/v) HCl under stirring. Samples were centrifuged at 3800 *g* and precipitates were re-extracted in 10 mL of the same solvent to achieve a complete extraction of iminosugars. Combined extracts were filtrated through Whatman no. 4 paper and kept at -20 ºC until analysis. Mulberry, buckwheat and hyacinth extracts were prepared in triplicate.

4.2.2.4. Carbohydrate analysis

- Derivatization procedure

A two-step derivatization procedure (oximation + silylation) (**section 4.1**) was carried out prior to GC analysis of carbohydrates. Oximation, aimed to suppress the anomeric centre of reducing carbohydrates, allows reducing the number of chromatographic peaks corresponding to the different (up to five) silylated tautomeric forms of reducing carbohydrates in solution, to only two signals: *anti*- and *syn*-isomers. Previous studies have shown that this methodology results in a better GC resolution of iminosugars and other bioactive carbohydrates from other coextractives usually taking part of the complex mixtures present in plant extracts (**section 4.1**).

The procedure was as follows: One milliliter of extract was mixed with 0.2 mL of a 70 % ethanolic solution of phenyl-*β*-*D*-glucoside (1 mg mL^{-1}) employed as internal standard and dried under vacuum at 38-40 °C. Oximes of coextracted reducing sugars were formed using 350 µL of 2.5 % hydroxylamine chloride in pyridine at 75 ºC for 30 min. Trimethylsilyl (TMS) ethers were later obtained by treatment with 350 µL of hexamethyldisylazane and 35 µL of trifluoroacetic acid at 45 ºC for 30 min [164]. After reaction, samples were centrifuged at 4400g for 10 min and kept into the fridge for 24 h before analysis. One microlitre of supernatant was injected into the injection port of the GC instrument.

- Gas chromatographic analysis

GC-MS analyses of derivatized samples were carried out in a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (both from Agilent Technologies, Palo Alto, CA, USA), using He as carrier gas at 0.91 mL min^{-1}. A 30 m x 0.25 mm i.d. x 0.25 µm film thickness ZB-1MS (crosslinked methyl silicone) column from Phenomenex (Torrance, CA) was used. Oven temperature program was optimized and the best conditions were from 100 ºC to 200 ºC (15 min) at 6 ºC min^{-1} and finally programmed to 300 ºC at 15 ºC min^{-1} and kept at this temperature for 50 min. Injector temperature was varied from 200 to 300 ºC and injections were made in split mode with a split ratio of 1:20. Mass spectrometer was operated in electron impact (EI) mode at 70 eV, under both full SCAN (35-650 *m/z* range) and SIM acquisition modes. **Table 4.3** lists the three characteristic *m/z* ions selected for

quantitation of target analytes when working under SIM acquisition mode. Interface and source temperature were 280 ºC and 230 ºC, respectively. Data acquisition was done using HP ChemStation software (Agilent Technologies).

Table 4.3. Characteristic *m/z* ions selected for acquisition in SIM mode.

Initial Time (min)	Characteristic *m/z* ions	Compound
5.0	144/260/348	Fagomine
8.5	144/258/348	DMJ and DNJ
11.2	246/270/450	*α*-HMJ
11.6	265/305/318	*Chiro*-inositol
12.3	246/270/450	*α*-HNJ
15.0	265/305/318	*Myo*-inositol
23.0	147/217/361	Phenyl-*β*-*D*-glucoside (i.s.)
28.3	204/361/365	Glycosil-inositols

GC-FID analyses were carried out under similar chromatographic conditions using a 7890A gas chromatograph (Agilent Technologies), using nitrogen at 0.71 mL min^{-1} as carrier gas.

Chromatographic parameters considered for optimization of operating conditions were peak width at half height (w_h), resolution (R_s) and peak asymmetry factor (A_s), calculated as the ratio of the tailing and leading edges of the peak measured at 10 % peak height.

Identification was done using commercial standards and/or data (linear retention indices and mass spectra) reported in **section 4.1**. Identities were considered to be tentative when only experimental GC and MS data were available and no further comparison was possible due to the lack of standards or literature data.

Quantitative values were calculated using the internal standard method. Standard solutions over the expected concentration range in mulberry/hyacinth/buckwheat were injected in triplicate to assess the linearity of response in the range evaluated and to calculate the response factor (*RF*) relative to the internal standard (phenyl-*β*-*D*-glucoside). *RF* of those compounds for which standards were not commercially

available, namely fagomine, α-homomannojirimycin (α-HMJ) and glycosyl-inositols, were assumed to be those of DNJ, α-homonojirimycin and galactinol, respectively.

According to Foley and Dorsey [161], *LOD* and *LOQ* were calculated as three and ten times standard deviation of the nose (σ), respectively. Inter-day precision (relative standard deviation, *RSD*) was calculated from the results obtained for a hyacinth extract (hyacinth 2) previously derivatized and analysed in 5 different days. Intra-day precision was determined from *n* = 10 replicates of the analysis of hyacinth 2 extract.

4.2.3. Results and discussion

Iminosugar composition of buckwheat, mulberry and hyacinth samples was firstly explored by GC-MS under SCAN acquisition mode. As previously described, fagomine was present in buckwheat [28], whereas this iminosugar and DNJ were detected in mulberry (**section 4.1**). Four peaks with a mass spectrum characteristic of iminosugars were detected in hyacinth samples (**Figure 4.5**). These peaks were assigned to DMJ (peak 1), DNJ (peak 3), and α-HNJ (peak 6) by comparison with the corresponding standards. Peak 4 was tentatively assigned to α-homomannojirimycin (α-HMJ) by comparison of its retention order and characteristic *m/z* ions with data from literature [11].

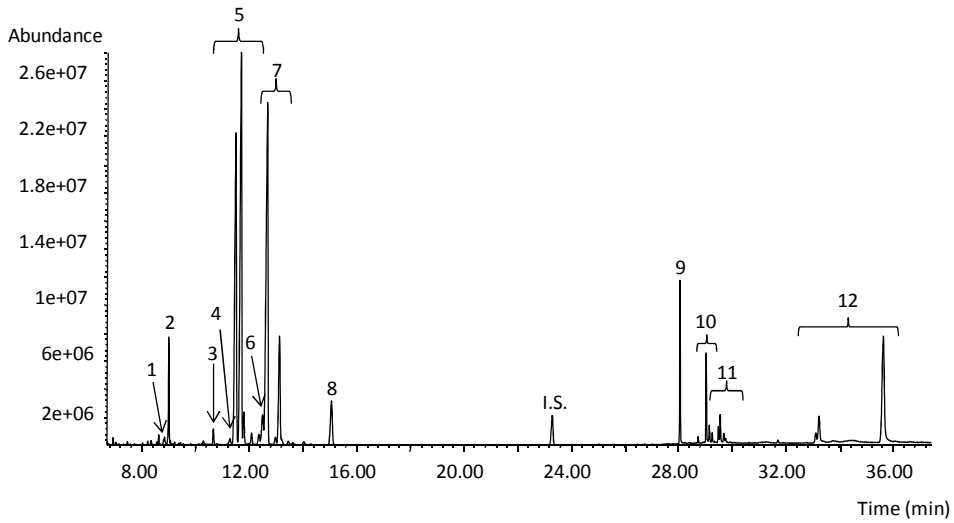

Figure 4.5. GC-MS (SCAN) profile of derivatized extract of hyacinth 4. DMJ (1), citric acid (2), DNJ (3), α-HMJ + manitol (4), fructose (5), α-HNJ (6), glucose (7), *myo*-inositol (8), phenyl-*β*-*D*-glucoside (I.S.), sucrose (9), glycosyl-inositols (10), disaccharides (11), trisaccharides (12).

Considering the low expected concentration of iminosugars and other bioactives in some of the samples under study, optimization of different chromatographic parameters was carried out with the aim of improving the reproducibility and sensitivity of their analysis.

Oven temperature program of GC-MS instrument was optimized for the resolution of iminosugars from other low molecular weight carbohydrates naturally present in the extracts (interfering mono- and disaccharides, and other bioactive carbohydrates such as inositols, glycosyl-inositols). *Rs* values higher or equal to 1 were obtained for all the target compounds except for *α*-HMJ, detected only in hyacinth samples. This compound was overlapped with the front part of the peak corresponding to manitol, present at higher levels. Coelutions of bioactive LMWC with other interferences were not detected under these optimized conditions.

For quantitative analysis, all the samples were injected in quintuplicate and occasionally iminosugar peaks were not detected or tails were observed. Derivatization procedure was first questioned; however, as previously reported by Rodríguez-Sánchez *et al*. (**section 4.1**), reproducibility among quintuplicates of well eluting peaks (e.g. fructose, glucose, inositols, etc.) was appropriate (≤ 5 % *RSD*). Moreover, a re-injection of the same derivatized sample with tailing peaks, gave later gaussian peaks. This behaviour led us to evaluate the stability and equilibration of derivatives and the reproducibility of their injection.

One extract of mulberry leaves (mulberry 1) was injected into the GC just after been derivatized, every hour up to 15 h, and every day up to five consecutive days (a total of 20 injections, **Figure 4.6**). Although precise analysis of other LMWC such as glucose, galactinol or *myo*-inositol was achieved, DNJ and fagomine randomly decreased or disappeared. However, this behaviour could not be correlated with the derivatization time. Stability of the derivatives was also evaluated up to three months and results were similar to those commented above.

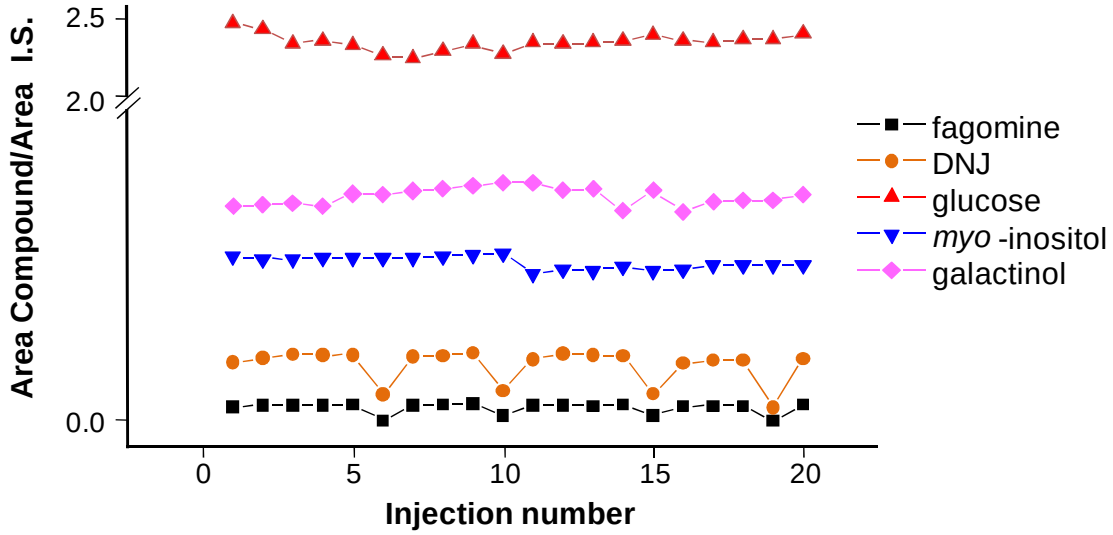

Figure 4.6. Relative areas of glucose, *myo*-inositol, galactinol, DNJ and fagomine derivatives versus internal standard in mulberry 1 injected just after been derivatized (injection no. 1), every hour up to 15 h (injection no. 2-16), and every day up to five consecutive days extract (injection no. 17-20). Injection temperature = 300 ºC.

Considering that this behaviour was not a consequence of the derivatization process, and taking into account that evaluation of this parameter has not previously been addressed in the literature, optimization of the injection port temperature for analysis of iminosugars was subsequently done. Although injection port was initially set at 300 ºC for considering this temperature appropriate for the volatilization of TMSO of carbohydrates [165], an A_s of 1.53 and a relatively high w_h value (0.088 min) were occasionally observed for DNJ; fagomine was not even detected (**Figure 4.7A** and **Table 4.4**). Therefore, lower temperatures (200 and 240 ºC) were assayed. Several injections of samples carried out at these temperatures showed no degradation of iminosugars and provided efficient and symmetric DNJ and fagomine peaks in all cases (**Figure 4.7A** and **Table 4.4**). However, other compounds highly retained on the GC column such as galactinol (I^T= 3907) were negatively affected as shown by an A_s of 3.25 and a w_h of 0.081 min at 200 ºC and an A_s of 1.33 and a w_h of 0.063 min at 240 ºC (**Table 4.4** and **Figure 4.7B**). As beneficial properties related to the metabolism of galactinol and its isomers, complementary to those of iminosugars (e.g. insulin mediators; [166]), have been attributed to these compounds, their precise and

reproducible analysis in these samples was also considered for selection of optimal conditions. Intermediate temperatures of the injection port (220 and 260 ºC) were also evaluated (data not shown). However, 240 ºC resulted to be the optimum temperature to achieve a compromise between the required stability of TMSO of iminosugars (DNJ and fagomine) and the appropriate vaporization of glycosyl-inositols (**Figure 4.7B**).

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Precision and limits of detection and quantitation were further evaluated under optimized injection conditions. Both intra-day and inter-day relative standard deviations were lower than 4.5 % for iminosugars and other LMWC. *LOD* and *LOQ*, calculated for iminosugars under full SCAN acquisition mode, were on average 54 ng g^{-1} and 180 ng g^{-1}, respectively.

In order to develop a more sensitive methodology of application to those plant extracts in which iminosugars are present in very low concentrations, the use of a FID detector and of a mass spectrometer operating under SIM acquisition mode was considered. An improvement of 60 and 270 times on average of sensitivity was found for GC-FID (*LOD*: 0.6-1 ng g^{-1} and *LOQ*: 2.1-4.1 ng g^{-1}) and GC-MS (SIM) (*LOD*: 0.1-0.3 ng g^{-1} and *LOQ*: 0.2-1.1 ng g^{-1}) methods, respectively.

Table 4.4. Peak width at half height (w_h, min) and peak asymmetry factor (A_s) for selected compounds at different temperatures of the injection port.

Temperature (ºC)	w_h			A_s		
	Fagomine	**DNJ**	**Galactinol**	**Fagomine**	**DNJ**	**Galactinol**
200	0.023	0.047	0.081	0.96	0.88	3.25
240	0.022	0.045	0.063	0.96	0.84	1.33
300	-	0.088	0.055	-	1.53	0.92

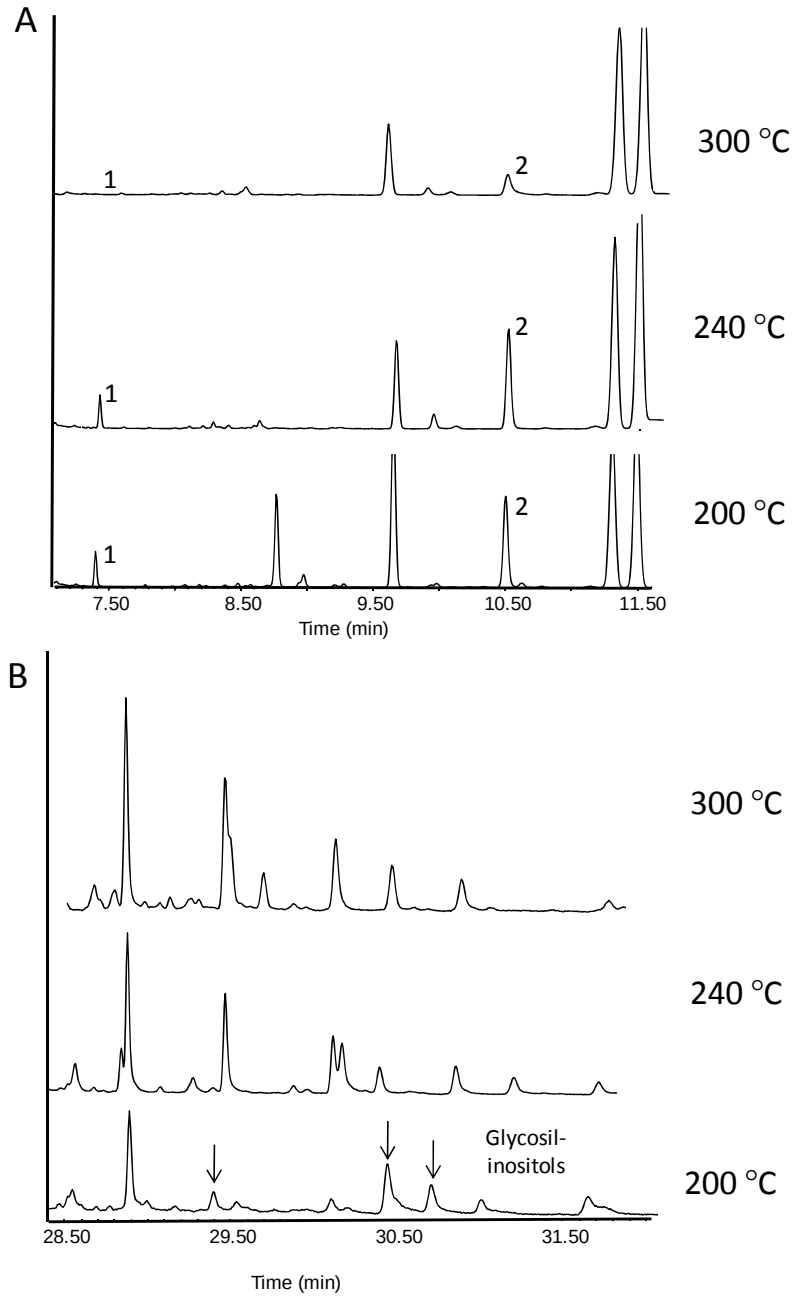

Figure 4.7. GC-MS (SCAN) profile of: (A) iminosugars (fagomine (1) and DNJ (2)) and (B) glycosyl-inositols, at different temperatures of the injector port: 200 ºC, 240 ºC and 300 ºC.

Taking into account the higher sensitivity of GC-MS (SIM) and its advantages for quantitation of coeluting compounds, this technique was selected for the analysis of target compounds in the samples under study. Hyacinth extracts showed the highest content of iminosugars (**Table 4.5**), mainly due to the noticeable contribution of α-HNJ (2.5-5.5 mg g^{-1}). Buckwheat showed the highest amounts of glycosyl-inositols (3.5-4.6

mg g^{-1}), whereas hyacinths presented the highest abundance of *myo*-inositol. Relatively high concentrations of *chiro*-inositol were only detected in buckwheat samples.

4.2.4. Conclusions

In this work, improvement in terms of reproducibility and sensitivity of a previously developed GC method for analysis of iminosugars and other bioactive carbohydrates was aimed. Once proved the stability of derivatives, the effect of different chromatographic conditions, some of them not previously studied, was determined. Setting the injection port temperature at 240 ºC was found determinant to get gaussian peaks and good reproducibility in the analysis of target compounds. Either GC-FID or GC-MS (SIM) methods provided the sensitivity required for the detection of these bioactives, with the advantage of this last technique for the quantitation of coeluting compounds. These methods could be applied for the analysis of iminosugars and inositols in other plant extracts in which these compounds are present at lower concentrations. However, in both cases the previous use of GC-MS (SCAN) to verify the presence of iminosugars and other LMWC of interest is always required.

Table 4.5. Content (mg g^{-1}) of iminosugars, inositols and glycosyl-inositols determined by GC-MS (SIM mode) in the samples under study.

Sample	Fagomine	DMJ	DNJ	*α*-HMJ	*α*-HNJ	*Chiro*-inositol	*Myo*-inositol	Sum of glycosyl-inositols
Hyacinth 1	-	0.0248 (0.0001)*	0.32 (0.02)	0.038 (0.004)	2.51 (0.09)	-	1.55 (0.03)	0.436 (0.007)
Hyacinth 2	-	0.080 (0.002)	0.78 (0.01)	0.175 (0.003)	5.5 (0.2)	-	4.17 (0.08)	0.039 (0.001)
Hyacinth 3	-	0.0044 (0.0003)	0.36 (0.06)	0.097 (0.006)	3.2 (0.1)	-	3.0 (0.1)	0.0293 (0.0009)
Hyacinth 4	-	0.064 (0.001)	0.49 (0.02)	0.197 (0.004)	2.00 (0.02)	-	2.18 (0.01)	0.077 (0.003)
Buckwheat 1	0.054 (0.001)	-	-	-	-	0.98 (0.03)	0.80 (0.03)	3.5 (0.2)
Buckwheat 2	0.046 (0.001)	-	-	-	-	0.96 (0.01)	1.13 (0.03)	4.56 (0.1)
Mulberry 1	0.34 (0.01)	-	1.54 (0.01)	-	-	-	0.70 (0.02)	0.88 (0.07)
Mulberry 2	0.22 (0.02)	-	1.86 (0.03)	-	-	-	0.25 (0.02)	1.01 (0.07)

*Standard deviation in brackets.

4.3. Characterization of trimethylsilyl ethers of iminosugars by gas chromatography-mass spectrometry

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Journal of Chromatography A (2014) 1372: 221-227

4.3.1. Introduction

Iminosugars are polyhydroxyalkaloids consisting of monocyclic or bicyclic derivatives of pyrrolidine, piperidine, pyrrolizidine, indolizidine or nortropane ring structures. Many of them are able to inhibit glycosidases and to interfere with sugar receptors [33]. They can be found in natural sources such as plants from the Araceae, Leguminosae, Moraceae, Hyacinthaceae, Campanulaceae, etc. families and microorganisms such as *Streptomyces*, *Bacillus*, etc. [10], but they can also be synthetically produced [5, 49, 167].

Simple mixtures or individual iminosugars are commonly analyzed by high performance liquid chromatography (HPLC) [27, 39, 47]. However, the analysis of complex mixtures such as those extracted from natural sources is not straightforward considering the low abundance of iminosugars and the often poor separation among the different iminosugars and among these bioactives and other coextracted low molecular weight carbohydrates (LMWC). Gas chromatography (GC) provides good resolution and sensitivity, although a previous derivatization step is mandatory (**sections 4.1** and **4.2**). Trimethylsilylation confers iminosugars and other LMWC the necessary stability and volatility for their GC analysis [168]. Although special emphasis has been paid to the optimization of silylation reagents [35 and **section 4.1**] and chromatographic conditions (**section 4.2**), there is still a lack of comprehensive studies regarding the different retention of iminosugars depending on the stationary phase. In this sense, Nash *et al*. [127] reported the separation of seven trimethylsilyl (TMS) derivatives of naturally occurring polyhydroxyalkaloids ((2*R*,3*S*)-2-hydroxymethyl-3-hydroxypyrrolidine,1,4-dideoxy-1,4-imino-*D*-arabinitol (DAB-1), fagomine, 2,5-dideoxy-2,5-imino-*D*-manitol (DMDP), swainsonine, 1-deoxynojirimycin (DNJ) and castanospermine) in two different GC columns (methyl and phenylmethylsilicone). However, retention indices of these compounds were not provided and

chromatographic data on a larger number of naturally occurring iminosugars would be useful for their further identification in real samples.

Additional qualitative information, valuable for the characterization of unidentified iminosugars, can be obtained by coupling gas chromatography to mass spectrometry (GC-MS). Characteristic electron impact (EI)MS ions such as those originated by the loss of methyl and/or CH_2OTMS groups have been provided for some trimethylsilylated iminosugars (e.g. DMDP and *α*-homonojirimycin (*α*-HNJ), [11]; australine, [125]). Moreover, EI mass spectra of DAB-1, DNJ and swainsonine [169] and of several calystegines [74, 129, 170, 171] have also been reported.

Although a combination of GC retention data and mass spectral patterns (abundance of characteristic *m/z* ratios) could provide useful information for the characterization/identification of iminosugars, these data are only available for a very few compounds (e.g.: linear retention indices (I^T) and (EI)MS fragmentation of fagomine and DNJ (**section 4.1**)). In the present work, I^T on two stationary phases and (EI)MS data of 12 trimethylsilylated piperidine and pyrrolidine iminosugars are provided. These data have been correlated with their structures in an attempt to help in the further identification of these compounds in natural sources such as *Aglaonema treubii* root extracts.

4.3.2. Material and methods

4.3.2.1. Standards and samples

Analytical standards of DNJ,1-deoxymannojirimycin (DMJ), *N*-methyl-1-deoxynojirimycin (*N*-methyl-DNJ) and *N*-nonyl-1-deoxynojirimycin (*N*-nonyl-DNJ) were obtained from Sigma Chemical Co. (St. Louis, US). Deoxy-*L*-idonojirimycin (DIJ) and 1-deoxygalactonojirimycin (DGJ) were purchased from Toronto Research Chemicals Inc. (Ontario, Canada) and *α*-homonojirimycin (*α*-HNJ), *N*-methyl-*trans*-4-hydroxy-*L*-proline, 1-deoxyfuconojirimycin (DFJ) and DMDP were purchased from Dextra Laboratories (Reading, UK). Purity of all these standards was higher than 95 %. Miglitol (1-(2-hydroxyethyl)-2-(hydroxymethyl)-piperidine-3,4,5-triol) was acquired in a local pharmacy and fagomine (1,5-imino-1,2,5-trideoxy-*D*-arabino-hexitol) was extracted

from mulberry leaves, as is indicated in **section 4.1**. Solutions (1 mg mL^{-1}) of standards in ultrapure Milli-Q (Millipore) water were prepared for analysis.

A plant of *A. treubii* was acquired in a local garden center in Madrid. LMWC were extracted from *Aglaonema* roots by conventional solid-liquid extraction with acidulated water, as previously described in **section 6.1**.

4.3.2.2. Derivatization

One mL of standard solutions or *A. treubii* root extract was mixed with 0.5 mL of internal standard (phenyl-*β*-*D*-glucoside 1 mg mL^{-1}) and evaporated under vacuum prior to carbohydrate derivatization. A two-step derivatization procedure (oximation + silylation) [149] was carried out prior to GC-MS analysis of iminosugars. Previous studies have shown that this methodology results in a better GC resolution of iminosugars and other bioactive carbohydrates from other coextractives usually taking part of the complex mixtures present in plant extracts (**section 4.1**).

Oximes were obtained by addition of 350 µL of a solution 2.5 % hydroxylamine chloride in pyridine after 30 min at 75 °C. They were then silylated with hexamethyldisilazane (350 µL) and trifluoroacetic acid (35 µL) at 45 °C for 30 min. Under these derivatization conditions only one peak is observed for each iminosugar, corresponding to the *O*-persilylated form, and *N*-silylated compounds are not obtained, as previously indicated in **section 4.1**. After reaction, samples were centrifuged at 4400g for 10 min, and 1 µL of supernatant was injected into the GC injection port.

4.3.2.3. GC-MS analysis

GC-MS analyses (*n* = 3 replicates) were carried out following the validated method in **section 4.2**, using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA). Analyses were carried out on a 30 m x 0.25 mm *i.d.*, 0.25 µm d_f, methylpolysiloxane capillary column (HP-1, Agilent Technologies, Palo Alto, CA, USA) and a 50 % phenylmethylpolysiloxane column (BPX-50, SGE Europe Ltd, UK) of the same dimensions, using helium at ~ 1 mL min^{-1} as

carrier gas. For both columns, the oven temperature was programmed from 100 °C to 200 °C (15 min) at a heating rate of 15 °C min^{-1} and finally programmed to 300 °C (10 min) at 15 °C min^{-1} (total analysis time of 38 min). Injections were carried out in split mode (1:20) at 240 °C. The transfer line and ionization source were thermostated at 280 and 230 °C, respectively. Mass spectra were recorded in EI mode at 70 eV within the mass range *m/z* 35-650. Acquisition was done using HPChem Station software (Agilent Technologies, Palo Alto, CA, USA). I^T were calculated from retention times of TMS iminosugars and suitable *n*-alkanes, as described in [172, 173].

4.3.3. Results and discussion

Table 4.6 shows I^T data on two stationary phases (methyl and phenylmethyl silicone) and abundances of characteristic ions in the EI mass spectra of the 12 *O*-persilylated iminosugars under study. Full scan mass spectra for these compounds can be found in **Figure 1S** of **Anexo I**.

4.3.3.1. Chromatographic data

No change in the elution order of iminosugars on both stationary phases (HP-1 and BPX-50) was observed (**Table 4.6**). As reported by Nash *et al*. [127], a close correlation of retention time with degree of hydroxylation was found (see **Figure 2S** of **Anexo I**).

Thus, *N*-methyl-*trans*-4-hydroxy-*L*-proline was the first compound to elute, followed by those iminosugars with three, four, etc -OTMS groups. As expected, among the compounds with identical molecular formula (e.g. DFJ and fagomine), the retention was affected by the nature of the substituents and their corresponding slight differences in polarity.

Table 4.6. Linear retention indices (I^T) on methylpolysiloxane (HP-1) and 50 % phenylmethylpolysiloxane (BPX-50) stationary phases and relative abundance for characteristic *m/z* ratios of iminosugars.

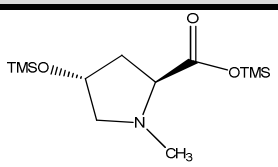
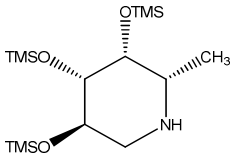
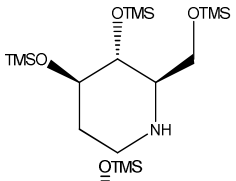
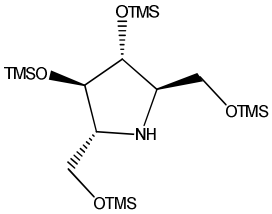
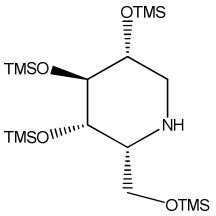
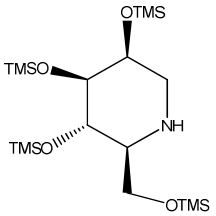
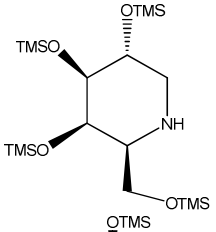
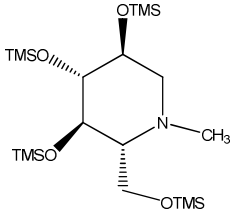
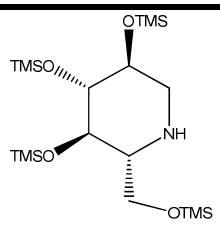
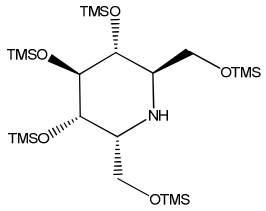
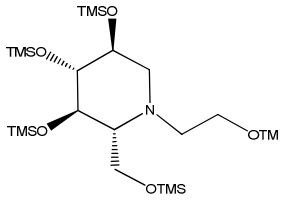
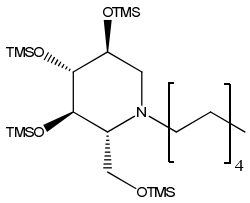
Iminosugar	Structure	Molecular weight (uma)	I^T		*m/z*
			HP-1	BPX-50	
***N*-methyl-*trans*-4-hydroxy-*L*-proline**		289	1432	1525	73 (34), 82 (98), 130 (4), 172 (100), 274 (7)
DFJ		363	1628	1629	73 (34), 147 (9), 184 (3), 217 (100), 258 (4), 273 (3), 348 (1)
Fagomine		363	1698	1740	73 (76), 144 (100), 170 (31), 260 (93), 348 (4)
DMDP		451	1804	1793	73 (100), 144 (13), 147 (20), 168 (10), 217 (16), 258 (12), 348 (83), 436 (2)
DIJ		451	1822	1800	73 (100), 144 (43), 147 (23), 217 (80), 258 (41), 348 (64), 436 (3)
DMJ		451	1832	1812	73 (100), 144 (34), 147 (22), 217 (80) 258 (22), 348 (41), 436 (1)
DGJ		451	1871	1833	73 (100), 144 (36), 147 (24), 217 (72), 258 (40), 348 (54), 436 (1)
***N*-methyl-DNJ**		465	1903	1893	73 (61), 147 (18), 158 (57), 182 (3), 272 (5), 362 (100), 450 (2)

Table 4.6. (cont.)

DNJ		451	1958	1956	73 (100), 144 (40), 147 (27), 217 (70), 258 (35), 348 (50), 436 (1)
***α*-HNJ**		553	2061	2006	73 (100), 147 (24), 217 (20), 246 (20), 270 (7), 360 (5), 450 (50), 538 (2)
Miglitol		567	2116	2066	73 (99), 147 (23), 260 (20), 374 (3), 464 (100), 552 (3)
***N*-nonyl-DNJ**		577	2415	2358	73 (46), 147 (13), 270 (13), 384 (2), 386 (3), 474 (100), 562 (3)

Regarding iminosugars with four -OTMS groups, the lowest linear retention index was observed for DMDP followed by DIJ, DMJ, DGJ and DNJ. The potential higher volatility of DMDP, a five-membered ring with two $–CH_2OTMS$ substituents, could justify its lower retention. It is also worth noting the differences in I^T values for iminosugars only differing in the orientation (axial or equatorial) of their hydroxyl groups (e.g. DIJ, DMJ, DGJ and DNJ). This behaviour is similar to that found for trimethylsilylated monosaccharides [174]. In agreement with this, Asano *et al*. [11] reported differences in chromatographic retention for various iminosugar isomers and, as an example, they found that *α*-homomannojirimycin (*α* -HMJ) eluted before *α* -HNJ using a BPX-5 capillary column.

In both columns, *N*-methyl-DNJ (with molecular formula $C_7H_{15}NO_5$) eluted before DNJ (molecular formula $C_6H_{13}NO_4$). Higher retention indices were observed for α-HNJ, miglitol and *N*-nonyl-DNJ.

4.3.3.2. Mass spectra

As expected for persilylated sugars, molecular ions were not observed in the spectrum of any of the standards under study; $[M-15]^+$ ions were, however, present in all of them (see **Table 4.6** and **Figure 1S** of **Anexo I**).

TMS *N*-methyl-*trans*-4-hydroxy-*L*-proline mass spectrum showed a characteristic *m/z* 172 ion ($[M-117]^+$, base peak) corresponding to the loss of -COOTMS from the molecular ion. This fragment loses a TMSOH group giving an abundant ion at *m/z* 82. Kite and Hughes [126] reported a similar fragmentation pattern for hydroxypipecolic acids. In these compounds, the intensity of the ratio between *m/z* 56 and *m/z* 82 ions was found to be crucial to distinguish between 4- and 5-hydroxypipecolic acids; however, the former ion was not present in the mass spectrum of *N*-methyl-*trans*-4-hydroxy-*L*-proline, where the loss of a methyl group from the molecular ion (*m/z* 274 ion) was detected at relatively high intensity.

Noticeable differences in the MS fragmentation pattern of TMS derivatives of fagomine and DFJ were found. For fagomine, characteristic ions at *m/z* 348 ($[M-15]^+$) and 260 ($[M-103]^+$) corresponded to the loss of $-CH_3$ and $-CH_2OTMS$ groups, respectively. Ion at *m/z* 144 (base peak) could be the result of a fragmentation pathway proposed for cyclic amines [175] that in the case of TMS fagomine would lead to $CH_2=N^+=CH-CH_2OTMS$ ion. The loss of TMSOH from $[M-103]^+$ (*m/z* 170 ion) was also observed. In the mass spectrum of the TMS derivative of DFJ, consecutive losses of a $-CH_3$ group (*m/z* 348 ion) and of TMSOH (*m/z* 258 ion) were detected, although these ions showed a low intensity. Base peak of DFJ mass spectrum corresponded to *m/z* 217 ion which indicates the presence of three adjacent hydroxyl groups.

Although characteristic *m/z* ions of the TMS derivatives of tetrahydroxylated piperidine (DIJ, DMJ, DGJ and DNJ) and pyrrolidine (DMDP) iminosugars were similar, remarkable differences regarding their relative abundances were observed. Ions at *m/z*

436 and 348 corresponding, respectively, to the loss of -CH_3 and -CH_2OTMS groups from the molecular ion were detected in all cases with similar abundances. However, the latter loss was noticeably more favored in the case of DMDP considering its two -CH_2OTMS substituents. Losses of two successive TMSOH from $[M-103]^+$ (ions at *m/z* 258 and 168) were also detected in this pyrrolidine iminosugar.

The *m/z* 217 ion was remarkably more abundant in piperidine iminosugars than in DMDP (70-80 vs 16 % relative abundance, respectively). A relatively abundant characteristic ion at *m/z* 144 (abundances of 34-43 % of base peak), similar to that found in fagomine, was also detected in tetrahydroxylated piperidine iminosugars. However, only slight differences in the mass spectrum of the four piperidine isomers were detected: DIJ and DNJ showed an intensity ratio of *m/z* 258 and 144 ions of 0.9, whereas this ratio was1.1 for DGJ and 0.6 for DMJ. While the three iminosugars DIJ, DNJ and DGJ, showed an intensity ratio of *m/z* 217 and 258 ions around 2.0, DMJ showed a ratio of 4.2 for these ions.

Regarding the mass spectrum of *N*-methyl-DNJ, *m/z* 362 ion ($[M-CH_2OTMS]^+$), was the base peak. Low abundant ions at *m/z* 272 and 182 corresponding to two consecutive losses of TMSOH from *m/z* 362 ion were also detected. Moreover, *m/z* 158 ion equivalent to the fragment *m/z* 144 of DNJ, showed a relatively high abundance (57 % of the base peak). A similar fragmentation pattern was observed for TMS derivatives of miglitol and *N*-nonyl-DNJ, for which $[M-103]^+$ ions (*m/z* 464 and 474, respectively) were the base peak. Ions at *m/z* 260 and 270 (corresponding to $CH_2=NR^+$-CH=CHOTMS ions, where R is CH_2CH_2OTMS for miglitol and $(CH_2)_8$-CH_3 for *N*-nonyl-DNJ) were also characteristic. These last ions could probably have the same structural origin as *m/z* 144 ion in piperidine and *m/z* 158 ion in *N*-methyl-piperidine iminosugars.

A characteristic ion at *m/z* 450 ($[M-103]^+$) was detected for TMS *α*-HNJ. Similarly to DMDP, the high abundance of this ion could be justified by the two CH_2OTMS substituents of *α*-HNJ. Consecutive losses of TMSOH were also detected (ions at *m/z* 360 and 270). Ion at *m/z* 246 corresponds to the loss of CH_2OTMS-CHOTMS-CHOTMS probably through the mechanism described for the *m/z* 144 fragment in piperidine iminosugars.

4.3.3.3. Analysis of iminosugars in *A. treubii* extracts

As an example of application, **Figure 4.8** shows the GC-MS profile on a HP-1 capillary column of an *A. treubii* root extract previously subjected to the derivatization procedure indicated in Materials and Methods. Glucose, fructose and sucrose were the most abundant carbohydrates detected. Different peaks with mass spectra compatible with iminosugar structures according to previous results were also detected. **Table 4.7** shows I^T data on two stationary phases (HP-1 and BPX-50) and (EI)MS fragmentation data of these potential iminosugars. Peaks 2 and 7 were assigned as DMDP and α-HNJ, respectively, by comparison with data (I^T and characteristic MS fragments) for corresponding standards. Two other peaks with mass spectra similar to that of α –HNJ and I^T of 1987 and 2040 on HP-1 and of 1923 and 1958 on BPX-50 were also observed (peaks 3 and 5). These compounds were tentatively identified as α-HMJ and α-*allo*-HNJ according to data previously reported by Asano *et al*. [9, 11], who isolated them by different ion exchange resins and identified their structures by fast atom bombardment mass spectrometry (FABMS) and nuclear magnetic resonance (NMR). Peak 1 showed I^T (HP-1) = 1783 and I^T (BPX-50) = 1826, and characteristic *m/z* ions at 436, 348, 320, 258 and 191. Similarly to the MS pattern of DMDP and other tetrahydroxylated piperidine iminosugars, *m/z* fragments at 436 and 348 could correspond to the loss of -CH_3 and -CH_2OTMS groups from the molecular ion (*m/z* 451). However, in contrast to these iminosugar standards, relative abundances of characteristic fragments were quite different (as compared to DMDP, *m/z* 436 (2) vs (36); *m/z* 348 (84) vs (4), *m/z* 258 (10) vs (1), see **Tables 4.6 and 4.7**. Moreover, a relatively abundant *m/z* fragment at 320 was detected in mass spectrum of peak 1. This ion could correspond to the loss of a CH_2-CH_2-CH_2OTMS group from the molecular ion. A *m/z* fragment at 191, not present neither in DMDP nor in DIJ, DMJ, DGJ and DNJ, was also observed. According to DeJong *et al*. [176], this fragment obtained via rearrangement is composed of two OTMS groups and one –CH, and it might be indicative of the presence of three adjacent OTMS groups in a piperidine iminosugar of molecular weight $C_6NO_4H_{13}$. Therefore, this compound was tentatively assigned as 2,3,4-trihydroxy-5-hydroxymethyl piperidine.

Peak 6 showed a MS profile similar to that of miglitol. However, its I^T on both HP-1 and BPX-50 columns was lower than that of the commercial standard; therefore this peak could only be tentatively assigned as a miglitol isomer. To the best of our knowledge, these last two iminosugars (peaks 1 and 6) have not been previously detected in *A. treubii* root extracts. Finally, peak 10 showed *m/z* fragments characteristic of HNJ (*m/z* 450) and a glycoside (*m/z* 361), and therefore it could be assigned as a glycosyl-HNJ.

4.3.4. Conclusions

A comprehensive study of TMS derivatives of 12 iminosugars analyzed by GC–(EI) MS has been carried out. A combination of GC retention data (I^T) on two different stationary phases and relative abundances for characteristic *m/z* ratios has been reported for the first time for most of compounds. These data have been correlated with iminosugar structures in an attempt to provide insight in the characterization/identification of unknown iminosugars. As an example of application, the iminosugar composition of an *Aglaonema treubii* root extract has been fully determined. A total of seven iminosugars were detected, two of these compounds, tentatively identified as 2,3,4-trihydroxy-5-hydroxymethylpiperidine and a miglitol isomer, were described for the first time in this source.

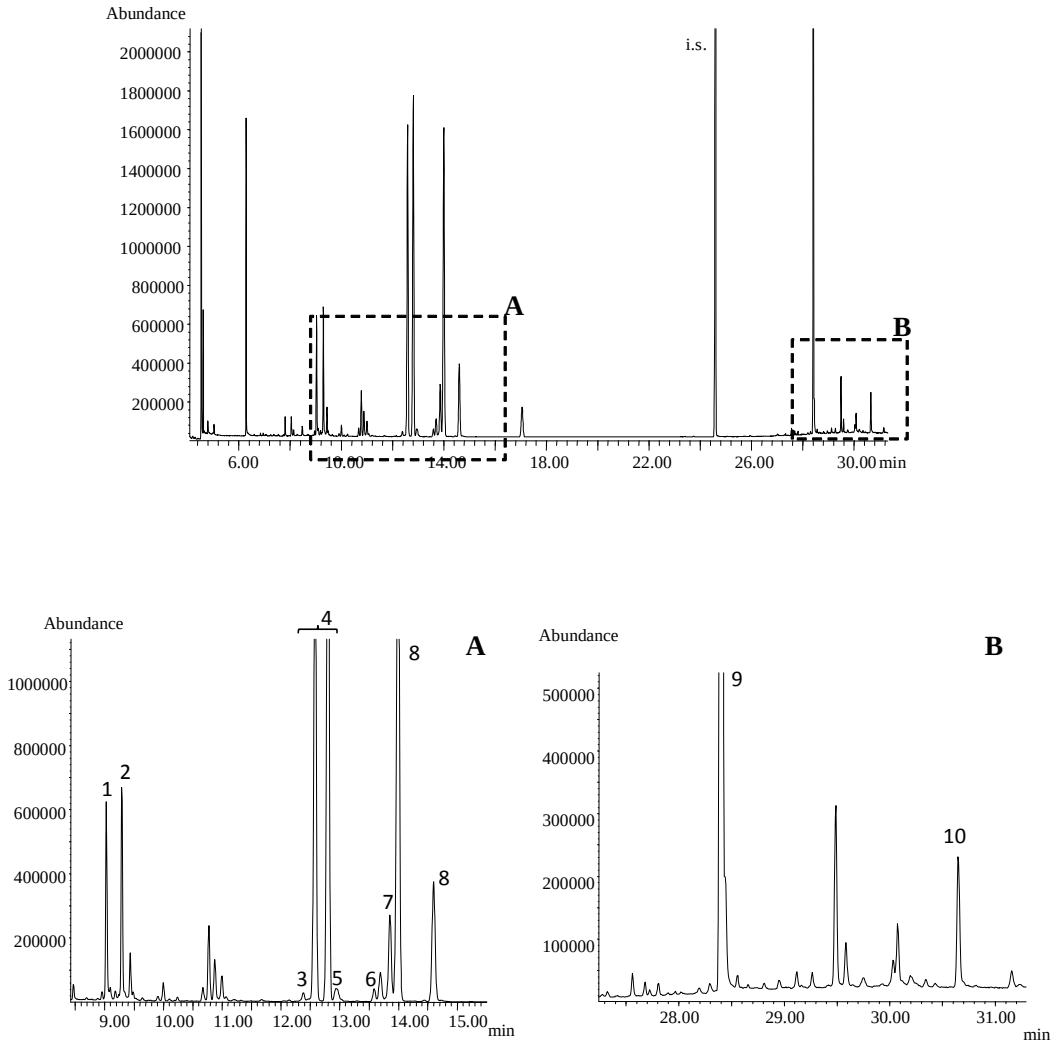


Figure 4.8. GC-MS profile of a derivatised extract of *Aglaonema treubii* root analysed on a HP-1 capillary column. A and B are close-up views of the elution zones of iminosugars analysed: (1) 2,3,4-trihydroxy-5-hydroxymethylpiperidine, (2) DMDP, (3) *α*-HMJ, (4) fructose, (5) *α*-*allo*-HNJ, (6) miglitol isomer, (7) *α*-HNJ, (8) glucose, (9) sucrose, (10) glycosyl-HNJ, (i.s.) internal standard.

Table 4.7. Linear retention indices (I^T) on methylpolysiloxane (HP-1) and 50 % phenylmethylpolysiloxane (BPX-50) stationary phases and relative abundances for characteristic *m/z* ratios of TMS iminosugars detected in *Aglaonema treubii* root extract.

Iminosugar	Molecular weight of TMS derivatives (*u*)	I^T		*m/z*
		HP-1	BPX-50	
2,3,4-trihydroxy-5-hydroxymethylpiperidine	451	1783	1826	73 (100), 144 (1), 147 (23), 168 (1), 217 (13), 258 (1), 191 (12), 320 (24), 348 (4), 436 (36)
DMDP	451	1806	1793	73 (100), 144 (14), 147 (20), 168 (10), 217 (18), 258 (10), 348 (84), 436 (2)
α-HMJ	553	1987	1923	73 (100), 147 (24), 217 (20), 246 (18), 270 (7), 360 (6),450 (47), 538 (2)
allo-HNJ	553	2040	1958	73 (100), 147 (23), 217 (22), 246 (21), 270 (7), 360 (7), 450 (52), 538 (2)
Miglitol isomer	567	2056	1984	73 (100), 147 (15), 260 (13), 374 (9), 464 (43), 552 (2)
α-HNJ	553	2063	2005	73 (100), 147 (26), 217 (18), 246 (20), 270 (8), 360 (6), 450 (52), 538 (2)
Glycosyl-HNJ	931	3036	2940	73 (100), 147 (29), 217 (33), 244 (18), 361 (16), 450 (21), 492 (4), 624 (0.5)

4.4. Nuevas fases estacionarias para GC-MS

Esta sección se compone de dos trabajos que pretenden evaluar y caracterizar cuatro nuevas fases estacionarias compuestas por líquidos iónicos (**sección 4.4.1**), así como su aplicación de las mismas al análisis de carbohidratos de bajo peso molecular (mono-, di, trisacáridos, inositoles e iminoazúcares) para seleccionar, finalmente, aquella más apropiada para el análisis de dichos compuestos (**sección 4.4.2**)

4.4.1. Characterization by the solvation parameter model of the retention properties of commercial ionic liquid columns for gas chromatography

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Journal of Chromatography A (2014) 1326: 96-102

4.4.1.1. Introduction

Over the past decade, ionic liquids (ILs) have generated great interest because of their unique and tunable physicochemical properties and their versatility for various applications [136, 177]. They are organic salts with melting points below 100 °C that typically possess negligible vapor pressure, wide liquid ranges, high viscosity and good thermal stability. All these properties make them ideal candidates as stationary phases (SPs) for gas chromatography (GC) [135, 137]. From the first papers published about this application of ILs [178, 179] to the present, a great deal of development has been aimed at preparing columns that have higher thermal stability and different selectivity than commercial columns.

Polycationic [138, 180] and polymerized [142] ILs, as well as IL bonded polysiloxanes [181, 182], have shown the best characteristics as GC stationary phases.

To date, only seven different ILs have been used to prepare commercial capillary columns (SLB IL59, SLB-IL60, SLB-IL61, SLB-IL76, SLB-IL82, SLB-IL100 and SLB-IL111). Their main advantages are that they offer different separation properties than columns prepared with polysiloxanes and poly(ethylene glycols), as well as lower

column bleed, longer life time, higher thermal stability and greater resistance to damage from moisture and oxygen. Thus, they have been successfully used in the analysis of complex mixtures, as fatty acid methyl esters [183], essential oils [146], caffeine metabolites [147], pollutants [184] and haloanisoles [185], among others. Such a large number of applications suggests a wide variety of interactions, which are not completely explained by the high polarity of these commercial columns.

Polarity estimation in GC is based on the McReynolds method [131], which uses the retention indices (*I*) of five probes to represent a distinct interaction, and the combination of their retention indices is used to determine the polarity of the SP. However, this approach cannot fully differentiate individual interactions since the retention of probes is not driven by a single force but is most often due to several simultaneous interactions. In contrast, the solvation parameter model (SPM) [132, 133] can quantitatively evaluate the individual intermolecular interactions between a SP and a substance. For that reason it has been extensively used to evaluate the retention characteristics of a variety of SPs, including ILs [135, 186].

The aim of this work was to characterize by means of the solvation parameter model the retention properties of four commercial ionic liquid columns (SLB-IL59, SLB-IL76, SLB-IL82 and SLB-IL100), studying the effect of temperature on selectivity, and comparing their separation characteristics to those of conventional SPs in capillary GC.

4.4.1.2. Experimental

- *Materials and equipment*

Three HP-5890A and HP-5890 Series II (Agilent, Palo Alto, CA, USA) gas chromatographs with split/splitless injection systems and flame ionization detectors (FID) were used. Data acquisition and processing were carried out using Clarity Lite chromatographic software (Data Apex Ltd., Prague, Czech Republic).

The results presented here were obtained on four fused-silica capillary columns coated with ionic liquids as stationary phases (Supelco Sigma Aldrich, Bellefonte, PA, USA) and whose characteristics are summarized in **Table 4.7**. In order to simplify the

notation throughout the manuscript, the columns will be referred to hereafter without the SLB- prefix.

All solutes were purchased from Merck (Darmstadt, Germany), Carlo Erba Reagenti (Rodano, Milano, Italy) and Sigma-Aldrich Co. (St. Louis, MO, USA), except for methane, which was obtained from natural gas.

- *Methods*

For all experiments, the injection port and detector temperatures were 230 and 250 °C, respectively, with the exception of the IL100 column for which 180 and 200 °C were used instead.

A solution of naphthalene in undecane (10 μg μL^{-1}) was used to evaluate column efficiency at 110 °C by the maximum plate number per meter (N_{max}/m). The quality of the column coating was expressed by the utilization of theoretical efficiency (UTE).

The Grob test was used to evaluate the overall chromatographic properties of the capillary columns, and the test mixture was prepared in house from the pure standards [187]. The average separation number (*TZ*) was calculated as the average of the two values obtained from the methyl esters of the Grob test.

Mixtures of the McReynolds probes (benzene, 1-butanol, 2-pentanone, 1-nitropropane and pyridine) and *n*-alkanes were injected at 120 °C. In all cases, the *n*-alkanes present in the mixtures were chosen in order to bracket the McReynolds probes in the chromatograms. Hold-up times (t_M) and retention indices (*I*) were estimated by the LQG method [188, 189], which provides reliable and reproducible t_M values and very accurate *I* data. McReynolds constants were obtained by subtracting the retention indices of the five McReynolds probes on the SP under study, and those in the reference non-polar SP (squalane). The polarity of the SP was calculated as the sum of the five McReynolds constants [131].

In order to cover the broad range of possible solute-stationary phase interactions, 95 solutes with varied functional groups were selected for the characterization of the IL columns by the SPM. This model is described for GC [190] by the equation:

$$\log k = c + eE + sS + aA + bB + lL \quad \text{Eq. (1)}$$

where *k* is the retention factor of a solute on the SP at a specific temperature; c is the model intercept; the capital letters (*E*, *S*, *A*, *B*, and *L*) represent the solute descriptors that are probe specific parameters determined for many substances; and the lowercase letters (*e*, *s*, *a*, *b*, and *l*) are referred to as the system constants, in which all information concerning the solvation properties of the SP is represented. Specifically, *e* defines the capability of the SP for *π*-*π* and *n*-*π* interactions and *s* for dipole-type interactions; while *a* and *b*, are the hydrogen-bond basicity and acidity of the SP, respectively; and *l* describes the overall dispersive-type interactions. The solutes selected were grouped into different mixtures and chromatographed isothermally from 80 to 160 °C at increments of 20 °C to obtain their *k* values. System constants, for each IL column and temperature, were calculated by multiple linear regression (MLR) analysis from experimental *k* values of the probes selected and their descriptor values. **Table S1** in the **Anexo I** section shows the solutes selected, as well as their solute descriptors.

Radar plots for SPM data were created normalizing the total length of axes (radii) to unity. Each radius represents a system constant (*e*, *s*, *a*, *b* and *l*) at 120 °C and each radar plot a chromatographic column. Four radar graphs were drawn, each with data corresponding to one of the IL columns under study and to two additional columns (HP-INNOwax and SP-2340) for comparison. For these last two columns, the system constants were obtained from Poole and Poole [186].

Microsoft Office Excel (Microsoft Corp. Redmond. WA, USA) and Statgraphics Centurion XV (StatPoint Technologies, Warrenton, VA, USA) were used for data handling, basic calculations, radar plots and, non-linear and multiple linear regressions. Unscrambler X (CAMO Software AS, Oslo, Norway) was used for principal component analysis.

4.4.1.3. Results and discussion

➢ *Evaluation of chromatographic columns*

Results showed that columns efficiency was up to about 4700 plates per meter ($k_{naphthalene}$> 4.9) at 110 °C (**Table 4.8**). The high *UTE %* values (> 96 %, except for the IL76 column) indicate that the columns have a very uniform film thickness, similar to those typically obtained for non-polar stationary phases [191]. The *TZ* values, between 27 and 38, agree with this behavior.

However, according to the Grob test analysis (**Figure 4.9**), the columns showed low inertness to compounds with hydrogen bonding capabilities. Moreover, in agreement with the McReynolds constants and the polarities determined (**Table 4.8)**, these IL columns are highly polar with an important acid-base behavior.

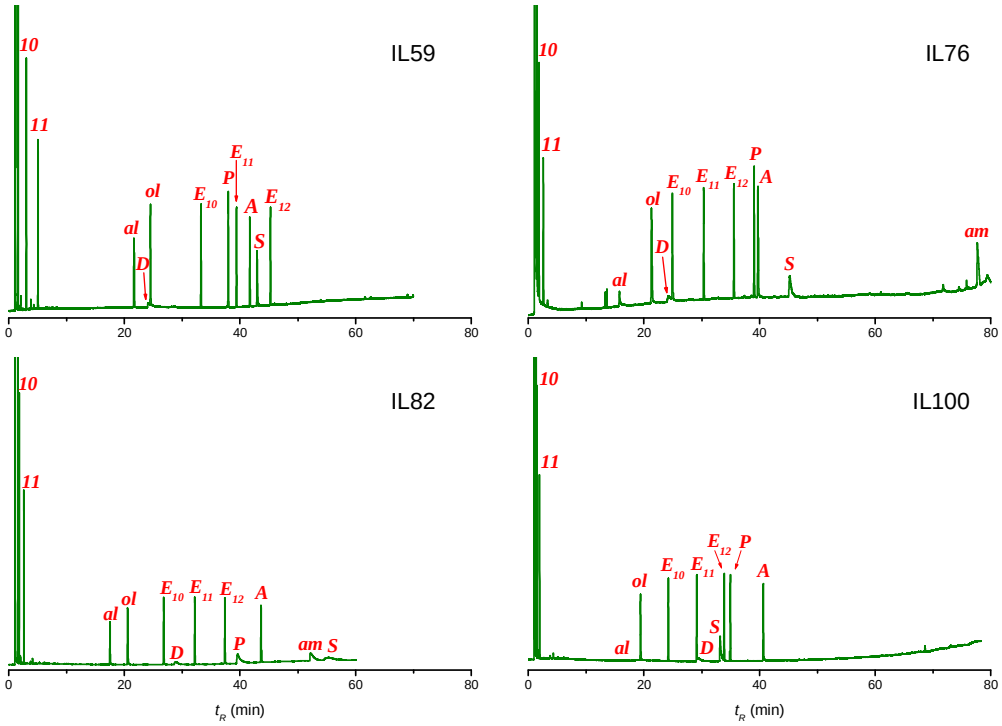


Figure 4.9. Chromatograms of the Grob test mixture on the IL columns studied. For peak identification and experimental conditions, see [187].

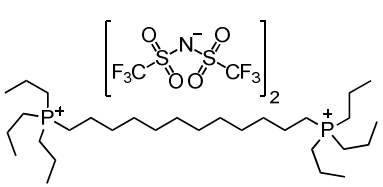
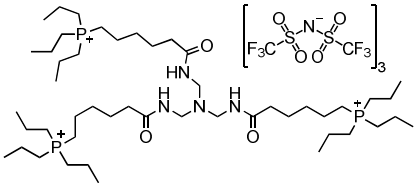
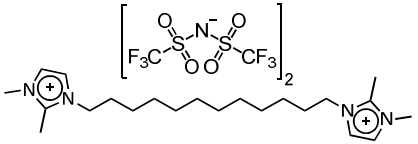
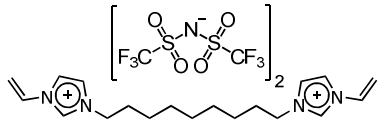
➢ *Solvation parameter model*

To further understand the interactions that occur in the commercial IL columns studied, the SPM was used. **Table 4.9** lists the system constants with their standard deviations and the goodness-of-fit statistics for each case. Very good regression

models, without any significant trend in the residual plots reinforce the statistical reliability of the built models.

All the ILs studied possess the bis(trifluoromethylsulfonyl)imide anion, so differences in the system constants will mainly be attributed to the cations. Thus, dipolar-type interactions (*s*) are one of the most predominant forces in these columns. The *s* values ranged from 1.51 to 1.78 at 120 °C, falling well within the interval observed for poly(cyanopropylmethylsiloxanes) with 50-75 % substitution percentages of cyanopropyl group [192]. The IL82 and IL100 columns showed higher *s* values than the IL59 and IL76 columns because of the polarizability of imidazolium moiety. Regarding the *a* system constant, a high capability for H-bond base interactions with solutes was observed. It has been described that the basicity of ILs is dominated by anion but modified by cation [193], hence, the small differences in *a* values must be related to the different chemical structures of cations. Imidazolium ring could act more as an H-bond acceptor than the phosphonium cation, so it could be expected that the IL59 and IL76 columns would have lower values than the IL82 and IL100 columns. However, this was only observed for the IL59 column. The higher value obtained for the IL76 column could be due to the amide groups that contribute positively as H-bond accepting groups, increasing its *a* value. The central core nitrogen in this IL could also contribute, but it was sterically hindered for effective H-bonding [194].

Table 4.8. Characteristics of the commercial IL columns studied.

Parameter	Column			
	IL59	**IL76**	**IL82**	**IL100**
L (m)	30	30	30	30
d_c (mm)	0.25	0.25	0.25	0.25
d_f (µm)	0.2	0.2	0.2	0.2
Stationary phase	1,12-di(tripropylphosphonium) dodecane bis(trifluoromethyl sulfonyl)imide	Tri(tripropylphosphoniumhexanamido) triethylamine bis(trifluoromethyl sulfonyl)imide	1,12-Di(2,3-dimethylimidazolium) dodecane bis(trifluoromethyl sulfonyl)imide	1,9-di(3-vinylimidazolium) nonane bis(trifluoromethyl sulfonyl)imide
				
TZ	38	27	33	31
N_{max}/m	4448	3730	4373	4714
UTE %	98	81	96	96
$k_{naphthalene}$	7.8	6.1	7.7	4.9
$u_{optimum}$(cm s^{-1})	13.5	14.0	12.8	15.7
McReynolds constants				
Benzene	352	472	529	587
1-Butanol	544	712	725	845
2-Pentanone	554	673	721	849
1-Nitropropane	683	858	928	1050
Pyridine	627	793	840	1026
Polarity	2760	3509	3741	4357

The *l* system constant accounts for the opposing contributions of solute-SP dispersion interactions that favor retention, and cavity formation that reduces retention. The ease of cavity formation depends on the size of the solute and on the cohesive energy of the SP, the latter determined by the strength of solvent-solvent interactions. At least in a qualitative sense, the *l* system constant can be interpreted in gas chromatography as an indication of the peak spacing between homologs [135]. Typically, non-polar columns held the highest *l* values (e.g., 0.58 at 100 °C for poly(phenylmethylsiloxanes) with a 5 % of phenyl content), although some authors have found similar values for polar ILs [195-198]. This fact triggered the concept of the "dual nature" selectivity of ILs [199], because they are capable of separating polar molecules as if they were polar SPs and non-polar molecules like non polar SPs. However, this concept is not applicable to all ILs, and the commercial columns studied in this work are good examples: their *l* system constants showed low values, similar to the non-ionic, highly polar SPs (i.e., poly[*bis*(3-cyanopropyl)siloxanes]); low retention for non-polar compounds; and poor separation of members of homologous series. This was more evident as the SP polarity increased (from IL59 to IL100), since the SP became more cohesive and it was more difficult to create the necessary cavity.

With respect to the H-bond acidity (*b*) of these ILs, it has been reported that the hydrogen bond acidity of unfunctionalized ILs largely depends on the nature of the cation and can be regulated by the anion [200]. By examining the IL structures (**Table 4.8**), it can be seen that the imidazolium cations possess the ability to act as a hydrogen bond acid, while phosphonium cations do not. That explains why the IL59 and IL76 columns have the lowest *b* values. However, although the IL82 column has imidazolium cations, its H-bond donating capability was lower than expected. The substitution of the hydrogen in position 2 (the most acidic in the imidazolium ring) by a methyl group, substantially decreases its acidity. The non-zero value for the *b* system constant is the most remarkable feature of these ILs columns, in contrast with the polymers commonly used as SPs in capillary GC, which lack H bond acidity [186]. As the majority of the substances that are analyzed by GC are basic, that is, they have heteroatoms with lone electron pairs, the acidic characteristics of these commercial ILs

columns would give them a greater flexibility and a supplementary ability to resolve complex mixtures.

Finally, the lowest contribution to global retention was given by the *e* system constant. Results show that π-π and n-π interactions with solutes are low, attractive, and non-significant for the IL59 column. Since the *e* value is dominated by the cation [197], the presence of π-electron-rich imidazolium cations and amide groups in the other three columns seems to accentuate their interactions with solutes containing non-bonding and π-electron systems and, therefore, provides significant *e* values. Although the differences among columns are very low, a slightly higher *e* value for the IL82 column was observed. The methyl group in position 2 of the imidazolium cation can release electrons toward the aromatic π-system by positive inductive effect and hyperconjugation, increasing the electron density on the aromatic ring and explaining the greater capability for π-type interactions observed in the IL82 column (higher *e* value).

Table 4.9. System constants for the IL columns studied.

Column name	Temperature (°C)	System constant[a]						Statistics[b]			
		e	***s***	***a***	***b***	***l***	***c***	S_y	***F***	R_{adj}	***n***
IL59	100	-0.058 (0.027)	1.594 (0.031)	1.406 (0.040)	0.307 (0.041)	0.469 (0.005)	-2.950 (0.031)	0.043	1748	0.996	70
	120	0	1.515 (0.024)	1.278 (0.030)	0.284 (0.034)	0.420 (0.004)	-2.993 (0.026)	0.042	3017	0.997	80
	140	0	1.460 (0.023)	1.161 (0.029)	0.242 (0.032)	0.375 (0.004)	-3.015 (0.024)	0.041	2985	0.996	85
	160	0	1.373 (0.024)	1.016 (0.029)	0.227 (0.031)	0.328 (0.004)	-2.976 (0.027)	0.037	2059	0.995	76
IL76	100	0.073 (0.021)	1.711 (0.027)	1.593 (0.044)	0.382 (0.035)	0.422 (0.003)	-3.121 (0.023)	0.034	3569	0.998	73
	120	0.095 (0.020)	1.628 (0.025)	1.455 (0.041)	0.320 (0.033)	0.374 (0.003)	-3.131 (0.021)	0.033	3554	0.998	78
	140	0.116 (0.022)	1.555 (0.029)	1.288 (0.046)	0.307 (0.046)	0.329 (0.003)	-3.159 (0.025)	0.036	2128	0.997	74
	160	0.119 (0.023)	1.441 (0.037)	1.140 (0.054)	0.315 (0.040)	0.286 (0.005)	-3.113 (0.039)	0.036	842	0.994	56
IL82	100	0.087 (0.024)	1.818 (0.031)	1.475 (0.050)	0.345 (0.040)	0.418 (0.004)	-3.064 (0.026)	0.040	2728	0.997	76
	120	0.155 (0.028)	1.783 (0.036)	1.386 (0.059)	0.348 (0.047)	0.351 (0.004)	-3.086 (0.030)	0.048	1788	0.996	79
	140	0.137 (0.020)	1.649 (0.026)	1.231 (0.041)	0.273 (0.033)	0.324 (0.003)	-3.101 (0.021)	0.033	2964	0.997	79
	160	0.144 (0.022)	1.538 (0.032)	1.108 (0.048)	0.280 (0.037)	0.282 (0.004)	-3.075 (0.033)	0.034	1164	0.995	61
IL100	80	0	1.980 (0.026)	1.742 (0.042)	0.606 (0.038)	0.442 (0.004)	-3.088 (0.025)	0.044	4317	0.998	78
	100	0.081 (0.023)	1.858 (0.026)	1.556 (0.031)	0.551 (0.034)	0.390 (0.003)	-3.128 (0.021)	0.040	4331	0.998	86
	120	0.117 (0.021)	1.758 (0.024)	1.377 (0.029)	0.510 (0.032)	0.343 (0.003)	-3.160 (0.021)	0.037	3912	0.998	85
	140	0.139 (0.022)	1.661 (0.025)	1.230 (0.030)	0.470 (0.033)	0.298 (0.003)	-3.168 (0.021)	0.038	2908	0.997	86

[a] Values in parentheses are the standard deviations of the system constants.

[b] S_y: standard error of the estimate; *F*: Fisher's factor; R_{adj}: correlation coefficient adjusted for degrees of freedom; *n*: number of solutes included in the model.

On the other hand, as deduced from **Table 4.9**, the dependence of system constants on temperature is approximately linear. The *s*, *a*, *b* and *l* values decrease with increasing temperature, leading to the reduction of solute retention typically observed in GC. The dipole-dipole (*s*) and H-bond (*a* and *b*) interactions are highly dependent on the orientation of SPs and solutes molecules participating in the interaction. Therefore, it should be expected that these forces will be weakened as temperature increases, due to the translational and rotational energy increase of the molecules [201]. Nevertheless, dispersion forces have no temperature term, so it might be expected that the *l* system constant (cavity formation/dispersion forces) will increase with temperature because cavity formation should require less energy at higher temperatures due to the weakening of solvent-solvent interactions. However, because dispersion interactions are strongly distance-dependent, the weakening of solvent-solvent interactions that makes cavity formation easier at higher temperatures also diminishes dispersion interactions by increasing the average distance between solute and solvent ions, leading to lower retention and *l* values. Moreover, the behavior found for the *e* system constant is especially noteworthy: as temperature increases, its value rises slightly and the relative importance of π-type interactions becomes more significant. It is assumed that electrons are less tightly bound to atoms at higher temperatures, thereby favoring these interactions and leading to an increase in the *e* system constant.

Lastly, temperature does not have the same effect on all system constants. The most substantial decrease was observed for the *a* system constant (H-bond basicity), while the rest exhibited a weak temperature dependence suggesting that these interactions may persist well beyond the temperature range studied in this work. This fact could be very useful for optimizing separation in programmed temperature.

- Comparison with other stationary phases for capillary GC columns

As discussed in **section 4.4.1.1**, IL columns from Supelco have been used to analyze complex mixtures of substances that were traditionally carried out on poly(ethylene glycol) and poly[*bis*(3-cyanopropyl)siloxane] columns, searching for a different selectivity in separations. In order to go in depth into the reasons for this different selectivity, the system constants of these conventional SPs were compared

with those of the ILs studied in this work. Radar plots were shown to be a useful tool to display multivariate observations in a two-dimensional chart. **Figure 4.10** shows four radar graphs, one for each IL column, along with HP-INNOwax and SP 2340 radar plots for comparison. The former was selected as a representative example of poly(ethylene glycol) stationary phases, and the latter of poly[*bis*(3-cyanopropyl)siloxane] phases. The different shapes observed in the radar plots for both SPs prove their different selectivity. Specifically, the higher capability of the HP-INNOwax column for n-π interactions (*e*), along with its lower capability for dipolar-type interactions (*s*), implies changes in the chromatographic behavior of compounds with multiple bonds with respect to the SP-2340 column.

If only IL columns are considered, their radar plot shapes are very similar, except for the IL59 column, on which π-type interactions (*e*) are non-significant. When compared with the above two non-ionic polar columns, more regular polygons are obtained. This indicates a more homogeneous contribution to all possible solute-SP interactions. Therefore, these IL columns could have a more general applicability than poly(ethylene glycol) and poly[*bis*(3-cyanopropyl)siloxane] phases, while maintaining their polar character. Divergences are especially significant for the *b* system constant, i.e., on the capability for H-bond donor interactions. In the HP-INNOwax and SP-2340 columns this acidic capability is non-existent, and in the ILs it is moderate, increasing from the IL59 to the IL100 columns. Consequently, the additional retention of basic compounds such as esters, ketones, ethers, etc. in these IL columns could be used to separate them by a different mechanism than on the HP-INNOwax and SP-2340 columns. Moreover, the apices corresponding to the *l*, *e* and *s* system constants for the IL plots are generally between those of the two conventional columns considered, whereas the *a* system constant apex is always below them. This is very interesting, as all conventional SPs that are strong H-bond bases are strongly dipolar/polarizable as well [186]. The higher *s/a* ratio in these IL columns together with the non-null *b* system constant are promising tools for selectivity optimization.

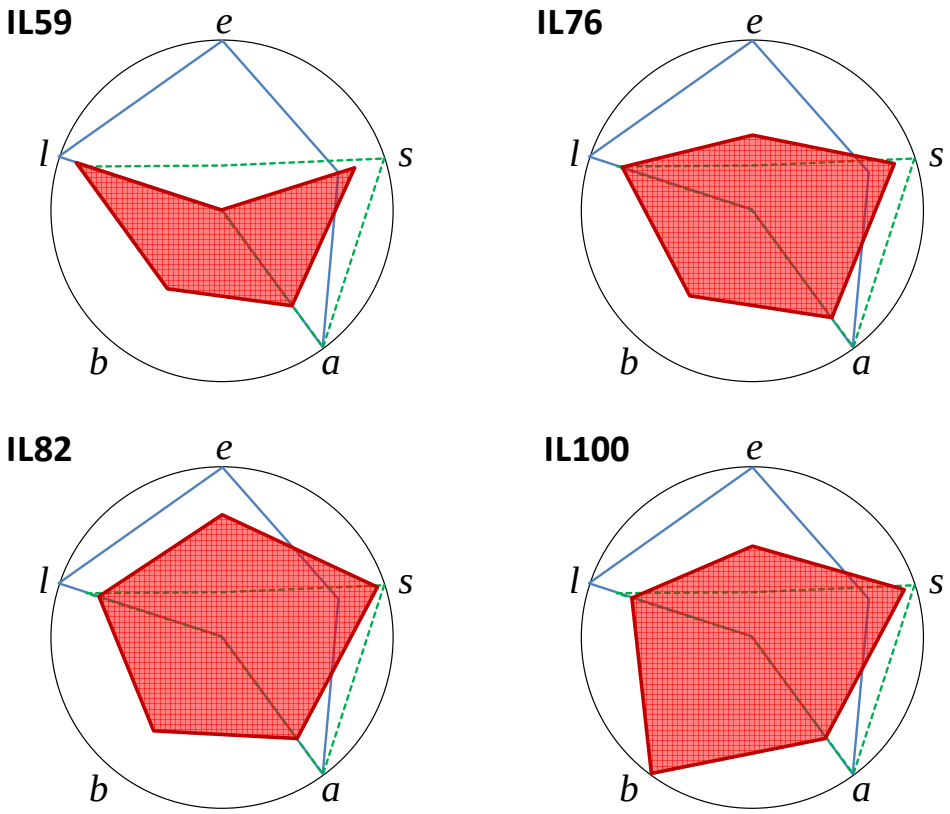


Figure 4.10. Radar plots at 120 °C for the commercial IL columns studied (filled polygons) along with those of the HP-INNOwax (blue, solid line) and SP-2340 (green, dashed line) columns.

Finally, in order to obtain an overview of the selectivity of these IL columns compared to those typically used in capillary GC, a principal component analysis (PCA) was carried out. System constants at 120 °C of 45 different capillary columns [186, 192, 202] together with the four ILs studied were compiled and subjected to PCA. The first three principal components explained 99.2 % of the variance, representing the whole column selectivity space quite well. Principal components 1 and 2 took into account contributions from the *a* and *s* system constants, while principal component 3 was mainly related to the *e* and *b* system constants. **Figure 4.11** shows the 3D score plot obtained. It can be seen that the siloxane-type SPs are arranged in three variability lines corresponding to trifluoropropyl, phenyl and cyanopropyl substituents (dashed lines in **Figure 4.11** labeled as 1, 2 and 3, respectively). Furthermore, there are two other clearly differentiated groups of SPs, corresponding to the poly(ethylene glycol) (cluster I) and IL (cluster II) phases. The PCA score plot reveals that the ILs employed to prepare these commercial columns possess separation characteristics that could not

be replicated by non-ionic SPs, because they are the only ones that simultaneously have high values of PC1 and PC3. That is, among all the SPs here collected, none are capable of simultaneously providing intense H-acceptor plus dipolar interactions (high *s*+*a* value, PC1), and H-donor plus *π*-*π* interactions (high *e*+*b* value, PC3).

4.4.1.4. Conclusions

In this work, four commercial capillary columns (IL59, IL76, IL82 and IL100) with ILs as SPs have been thoroughly studied in order to better understand their retention mechanisms. According to the SPM results, dipolar-type (*s*) and hydrogen-bond base interactions (*a*) were the dominant contributions to retention, while *π*-*π* and n-*π* interactions (*e*) were barely significant. Moreover, they were moderately hydrogen-bond acid SPs (*b*) with a low separation of members of homologous series (*l*). All these interactions decreased with temperature except for the *π*-type ones, though the variation was only noticeable for hydrogen-bond basicity. As a result of the different retention mechanisms involved, the IL columns studied are highly versatile and have a noteworthy capacity to resolve complex mixtures.

One of the most outstanding characteristics found for these ILs when compared with other non-ionic SPs, was the non-zero value for the *b* system constant. This acidity along with their high polarity confer them a distinct selectivity, as it has been clearly shown by principal component analysis. On the contrary, it should be emphasized that they present a low inertness to compounds with a high capability to interact through H-bonding, which makesthe use of these columns impractical to quantify low levels of such compounds.

Finally, we consider that our contribution would be an important step forward in understanding the type and strength of the different interactions involved in the retention process of an analyte on these columns, which could be essential to plan future separations of complex mixtures (e.g. plants extracts) and to broaden the scope of these commercial IL columns.

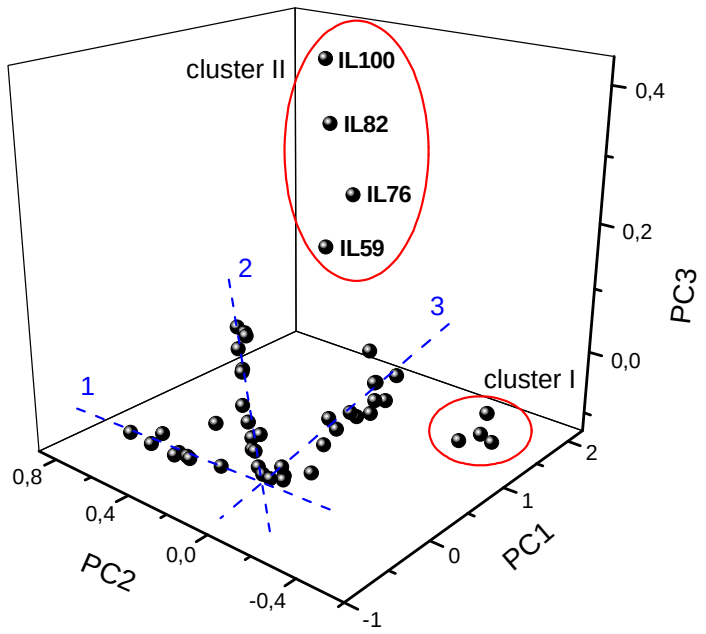


Figure 4.11. PCA scores for the system constants of 45 non-ionic capillary columns together with the IL columns studied at 120 °C. Dashed lines indicate the trend of distribution of siloxane-type SPs substituted with trifluoropropyl (1), phenyl (2) and cyanopropyl (3) groups. Clusters of poly(ethylene glycol) (cluster I) and IL (cluster II) phases are highlighted by circles.

4.4.2. Evaluation of different ionic liquid stationary phases for GC-MS analysis of carbohydrates

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Journal of Chromatography A (submitted)

4.4.2.1. Introduction

Carbohydrates are one of the most abundant organic substances in Nature and they usually occur as complex mixtures, with large isomer diversity and great variety of functional groups suited to their different biological roles in the living organisms. Thus, high-resolution analytical techniques such as high performance liquid chromatography (HPLC) or gas chromatography (GC) are required for their determination. GC coupled to mass spectrometry (GC-MS) has been widely applied to the analysis of low molecular weight carbohydrates (LMWC) [164, 168] due to the high resolution, sensitivity and structural information that this technique provides. Derivatization of carbohydrates prior to their GC analysis is a mandatory task. Formation of trimethylsilyl (TMS) derivatives is one of the most common procedures; however, because tautomeric forms of reducing sugars can produce multiple peaks, formation of oximes to suppress the anomeric center before silylation is usually carried out.

Carbohydrates are usually analyzed by GC using poly(dimethylsiloxane) stationary phases since they have good thermal stability and high permeability towards solutes [149]. However, data regarding retention on more polar stationary phases such as those based on different percentages of phenyl-, trifluoropropyl-, vinyl- or cyanopropyl groups in the polysiloxane chain are also found in the literature [165, 174, 203-205]. High-temperature stationary phases based on a carborane-modified siloxane phases have also been used for the determination of oligosaccharides with high degree of polymerization [102, 206]. On the contrary, poly(ethylene glycol)stationary phases have been scarcely used for LMWC analysis due to their limited temperature range and stability [165, 204, 207]. Considering that selectivity is one of the most important parameters on GC separations and it is governed by the interaction mechanisms of the compounds with the stationary phase and the relative strengths of these mechanisms

to each other [186], the evaluation of new stationary phases with different selectivity is of great interest for improving and modifying carbohydrate resolution.

Ionic liquids(ILs) are defined as organic salts with melting points below 100 °C, consisting of organic cations associated with organic/inorganic anions [208]. Since their first application by Barber et al. in 1959 [178], ILs have been used as GC stationary phases, due to their specific properties such as low volatility, high thermal stability, low column bleed, high viscosity, variable polarities, versatility and different separation selectivity [209]. IL stationary phases have been shown to have complex retention mechanisms that depend on multiple types of intermolecular forces, leading to unique retention properties [210 and **section 4.4.1**].

Recently, several IL-based GC capillary columns with different stationary phases and polarities have been introduced in the market (SLB^{TM}-IL59, SLB^{TM}-IL60, SLB^{TM}-IL61, SLB^{TM}-IL76, SLB^{TM}-IL82, SLB^{TM}-IL100 and SLB^{TM}-IL111). They have been applied to the analysis of different compounds such as fatty acids, biodiesel blends, plasticizers and synthetic fragrances [144, 211-213]. However, to the best of our knowledge, these columns have not been previously used for carbohydrate analysis and therefore, no chromatographic data (retention, peak width or symmetry) of LMWC using ILs as GC stationary phases have been reported.

Thus, in this work, retention indices of different TMS oximes of LMWC, including mono-, di- and trisaccharides, several inositols (cyclic polyalcohols) and iminosugars (monosaccharides where the endocyclic oxygen atom has been replaced with nitrogen) on different commercial IL capillary columns are reported. Two columns with the classical GC stationary phases poly(dimetilsiloxane) and poly(ethylene glycol)are included in this study for comparative purposes. The effect of temperature operation on retention, peak width and tailing factor of LMWC eluted on the most appropriate IL column has also been evaluated. Finally, the selected chromatographic conditions were applied to the GC-MS analysis of LMWC in real samples.

4.4.2.2. Materials and methods

➢ *Standards*

- Mono-, di- and trisaccharides

Analytical standards of α,α-, α,β- and β,β-trehalose, arabinose, erlose, fructose, galactose, glucose, isomaltose, kestose, kojibiose, lactulose, lactose, laminaribiose, maltose, maltotriose, melezitose, phenyl-*β*-*D*-glucoside, raffinose, ribose, sucrose and turanose were obtained from Sigma Chemical Co.(St. Louis, US).

- Inositols

chiro-inositol, galactinol (1-*O*-*α*-*D*-galactopyranosyl-*L*-*myo*-inositol), *myo*-inositol, pinitol and *scyllo*-inositol were purchased from Sigma Chemical Co.

- Iminosugars

Deoxy-mannojirimycin (DMJ) and 1-deoxynojirimycin (DNJ) were obtained from Sigma Chemical Co. *α*-homonojirimycin (*α*-HNJ) and 2,5-dideoxy-2,5-imino-*D*-mannitol (DMDP) were acquired from Dextra Laboratories (Reading, UK) while miglitol (Distabol, Sanofi-Aventis) ((2*R*,3*R*,4*R*,5*S*)-1-(2-hydroxyethyl)-2-(hydroxymethyl) piperidine-3,4,5-triol) was purchased in a local pharmacy.

➢ *Samples*

Hyacinth (*Hyacinthus orientalis*) bulbs and mulberry (*Morus alba*) leaves were acquired in local markets and collected, respectively, in Madrid (Spain). Carbohydrates were extracted from these matrices with Milli-Q water as described in **section 6.1**. In brief, one gram of air-dried sample was ground in a domestic mill (Moulinex) before extraction for 2 hours at ambient temperature into 10 mL of water under stirring, filtrated through Whatman no. 4 paper and kept at -20 ºC until analysis. Extracts were prepared in triplicate.

➢ *Derivatization*

A derivatization procedure was carried out prior to GC analysis of carbohydrates as indicated by Sanz et al. [149]. One mL of carbohydrate standards (1 mg mL^{-1} in methanol:water (30:70 v/v) or 1 mL of extract was evaporated under vacuum and treated with 350 µL of a 2.5% solution of hydroxylamine hydrochloride in pyridine and

then heated for 30 min at 75 °C. Compounds were then silylated with hexamethyldisilazane (350 µL) and trifluoroacetic acid (35 µL) at 45 °C for 30 min. After reaction, samples were centrifuged at 4400 g for 10 min. In order to remove the excess of silylation reagents to avoid the stationary phase damage, 50 µL of supernatant were treated with 50 µL of heptane and 100 µL of water. Organic phase containing the derivatized carbohydrates were recovered and 1 µL was injected on the injection port. The derivatization procedure previously described gives rise to a single chromatographic peak for non-reducing sugars, corresponding to their TMS ethers, whereas two peaks are detected for reducing sugars, corresponding to their *syn* (*E)* and *anti (Z)* oxime isomers.

- *GC-MS analysis*

Five IL columns (SLB^{TM}-IL59, SLB^{TM}-IL60, SLB^{TM}-IL76, SLB^{TM}-IL82 and SLB^{TM}-IL100), kindly provided by Supelco Sigma-Aldrich (Bellefonte, PA), were evaluated. As reference columns, a poly(dimethylsiloxane) column (HP-1, Agilent Technologies, Palo Alto, CA, USA) and a bonded poly(ethyleneglycol) capillary column (SUPELCOWAX$^{®}$ 10, Supelco, Bellefonte, PA) were also included in this study. **Table 4.10** summarizes the specific characteristics of the different columns.

GC-MS analyses were carried out using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA) using helium at ~ 1 mL min^{-1} as carrier gas. Different oven temperature programs were assayed for each of the columns tested. Apart from the oven temperature program selected as optimal for SLB^{TM}-IL82, a linear gradient (from 80ºC to 250 ºC at 10 ºC min^{-1} and held 10 min; ramp 1) and different isothermal conditions (140ºC, 160ºC, 180ºC, 230ºC and 250ºC) were also assayed for the elution of target compounds in this column.

Samples were injected in split mode (1:20) at 240 ºC. 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 HPChem Station software (Agilent Technologies, Palo Alto, CA, USA).

Table 4.10. Main characteristics of the columns and temperature programs used in GC-MS analysis.

Column	Stationary phase	Dimensions	GC Temperature program
SLB^{TM}-IL100	1,9-Di(3-vinylimidazolium) nonane bis(trifluoromethylsulfonyl) imide	30 m × 0.25 mm i.d.× 0.2 µm df	80ºC (5 min)10ºCmin-1 220ºC (60 min)
SLB^{TM}-IL82	1,12-Di(2,3-dimethylimidazolium) dodecane bis(trifluoromethylsulfonyl) imide	30 m × 0.25 mm i.d.× 0.2 µm df	
SLB^{TM}-IL76	Tri(tripropylphosphoniumhexanamide)triethyl amine bis(trifluoromethylsulfonyl) imide	30 m × 0.25 mm i.d.× 0.2 µm df	80ºC (5 min)10ºCmin-1 220ºC (10 min) 10ºCmin^{-1} 250ºC (50 min)
SLB^{TM}-IL60	1,12-Di(tripropylphosphonium) dodecane bis(trifluoromethylsulfonyl) imide	30 m × 0.25 mm i.d.× 0.2 µm df	
SLB^{TM}-IL59	1,12-Di(tripropylphosphonium) dodecane bis(trifluoromethylsulfonyl)imide	30 m × 0.25 mm i.d.× 0.2 µm df	
SUPELCOWAX®	Poly(ethyleneglycol)	30 m× 0.25 mm i.d. × 0.25 µm df	100ºC 10ºC min-1 220ºC (10 min) 10ºCmin-1 260ºC (10 min)
HP-1	Poly(dimethylsiloxane)	25 m× 0.25 mm i.d. × 0.25 µm df	100ºC 15ºC min-1 200ºC (15 min) 15ºC min-1 300ºC (20 min)

Linear retention indices (I^T) were calculated from retention times of derivatized carbohydrates and suitable *n*-alkanes. Resolution (R_s) was calculated as follows:

$$R_s = 2*(t_{R2} - t_{R1})/(w_{b1} + w_{b2}) \quad \text{Eq. (2)}$$

Where *1* and *2* refer to two consecutive eluting peaks and w_b is the peak width at base. Peak width (w_h) was measured at half height of the peak and tailing factor (*TF*) was calculated using the equation:

$$TF = (A05_i + B05_i)/(2 * A05_i) \quad \text{Eq. (3)}$$

where $A05_i$ is the left half-width of the peak at 5% height and $B05_i$ is the right half-width at 5% height. Peak capacity (n_c), defined as the maximum number of non-overlapping peaks in a given interval [214], was calculated as follows:

$$n_c = 1 + (t_g/\overline{\omega}_b) \quad \text{Eq. (4)}$$

considering three different intervals: (i) monosaccharides, inositols (except galactinol) and iminosugars, (ii) disaccharides and galactinol and (iii) trisaccharides, where $\overline{\omega}_b$ is the mean peak width value at base and t_g is the difference between t_R in the given interval.

4.4.2.3. Results and discussion

- *Effect of stationary phase composition on LMWC retention*

To evaluate the effect of IL stationary phases on the retention of LMWC different commercial standards, previously derivatized as indicated in **materials and methods section**, were analyzed on five capillary columns (SLB™-IL59, SLB™-IL60, SLB™-IL76, SLB™-IL82, SLB™-IL100). HP-1 and SUPELCOWAX® were also included in this study as reference of conventional non-polar and polar columns, respectively, for comparative purposes. Compounds were selected to include monosaccharides, disaccharides with different glycosidic linkages and monomeric composition, reducing and non-reducing trisaccharides, inositols and iminosugars. Considering the different boiling points of these compounds and the maximum temperatures of the columns, several oven temperature programs were assayed; the optimal conditions for each column are shown in **Table 4.10**.

Taking into account IL columns, only SLB^{TM}-IL82 allowed the elution of all the target carbohydrates. SLB^{TM}-IL59, SLB^{TM}-IL60 and SLB^{TM}-IL76 columns provided the elution of mono- di-, trisaccharides and inositols; however, iminosugars could not be determined in any of them, except for DNJ and DMJ which were only eluted in SLB^{TM}-IL76. None of the studied carbohydrates were eluted in SLB^{TM}-IL100. The low inertness of these columns to compounds with H-bonding capabilities (**section 4.4.1**) can undergo the adsorption or degradation of the iminosugars and related compounds. Other authors have reported similar problems in these columns for some organochlorine pesticides [213, 215], polybrominated diphenyl ethers [215] and volatile suspected allergens [213]. On the other hand, both HP-1 and SUPELCOWAX® columns allowed the elution of all the target carbohydrates. However, a noticeable increase of the baseline signal was observed in SUPELCOWAX® column when the oven temperature increased and a low response factor was obtained for the most retained compounds (e.g. galactinol, trisaccharides) which restrict its use for the analysis of these compounds.

I^T obtained for the derivatized LMWC are summarized in **Table 4.11**. As previously observed by other authors [216], I^T were noticeably lower in the SUPELCOWAX® column (between 1331 and 2996), whereas, I^T values in IL columns increased when polarity of the stationary phase increased (SLB^{TM}-IL82 provided the highest values and SLB^{TM}-IL59 the lowest). In general, monosaccharides, inositols (except for galactinol) and iminosugars eluted first, followed by disaccharides and galactinol. As expected, trisaccharides were the most retained compounds. It is worth noting that available alkanes were scarcely retained in SLB^{TM}-IL76 and SLB^{TM}-IL82 columns (e.g. C_{40} 20.4 and 20.9 min), in agreement with the high cohesive energy and low capability for dispersive interactions of their stationary phases (**section 4.1.1**).Thus, I^T values for highly retained carbohydrates (t_R trisaccharides 23.6-24.0 min and 28.5-33.67 min, respectively) could not be determined and they have been indicated as I^T >4000 in **Table 4.11**.

Table 4.11. Linear retention indices (I^T) obtained for the TMSO derivatives of carbohydrate standards analyzed in the different columns assayed.

	Standard	I^T					
		SLB™-IL82	SLB™-IL76	SLB™-IL60	SLB™-IL59	SUPELCOWAX®	HP-1
Monosaccharides	Arabinose 1	1879	-*	1727	1684	-	1795
	Arabinose 2	1891	-	1731	1693	1331	1806
	Ribose 1	1905	1836	1762	1714	1335	1821
	Ribose 2	1924	1840	1767	1720	1343	-
	Fructose 1	2076	1995	1883	1840	1402	2003
	Fructose 2	2083	2000	1894	1844	1404	2012
	Galactose *E*	2140	2043	1950	1899	1430	2047
	Galactose *Z*	2159	2068	1993	1943	1438	2078
	Glucose *E*	2155	2060	1966	1914	1438	2058
	Glucose *Z*	2155	2069	1990	1938	-	2078
Disaccharides	Sucrose	2995	2828	2644	2530	1841	2681
	α,α-Trehalose	3072	2917	2739	2596	1936	2722
	β,β-Trehalose	3278	3092	2891	2735	2184	2743
	α,β-Trehalose	3316	3143	2895	2925	2065	2741
	Lactulose 1	3202	-	-	-	2029	2744
	Lactulose 2	3221	-	-	-	2057	2748
	Lactose *E*	3217	3039	2808	2649	2039	2749
	Lactose *Z*	3217	3042	2816	2657	2039	2755
	Laminaribiose *E*	3284	3108	2870	2706	2200	2768
	Laminaribiose *Z*	3301	3127	2905	2745	2223	2785
	Turanose 1	3264	3098	2872	2709	2118	2782
	Turanose 2	3264	3098	2872	2709	2126	2786
	Maltose *E*	3248	3079	2863	2701	2115	2784
	Maltose *Z*	3257	3087	2880	2722	2115	2793
	Kojibiose *E*	3277	3099	2869	2704	2173	2789
	Kojibiose *Z*	3297	3122	2907	2744	2216	2812
	Isomaltose *E*	3484	3254	3004	2857	2553	2835
	Isomaltose *Z*	3508	3278	3042	2906	2594	2857

Table 4.11. (cont.)

Trisaccharides	Raffinose	>4000**	>4000	3546	3372	2967	2857
	Kestose	>4000	>4000	3540	3365	2955	3084
	Erlose	>4000	-	3612	3497	3014	3100
	Melezitose	>4000	>4000	3571	3401	2954	3122
	Maltotriose *E*	>4000	-	>4000	-	2996	3212
	Maltotriose *Z*	>4000	-	>4000	-	-	3226
Inositols	Pinitol	1895	1851	1779	1734	1334	1882
	chiro-inositol	2047	1981	1914	1859	1380	2019
	scyllo-inositol	2142	2081	2027	1971	1422	2085
	myo-inositol	2207	2169	2109	2052	1451	2146
	Galactinol	3375	3209	3012	2868	2337	2851
Iminosugars	DMDP	2170	-	-	-	1377	1808
	DMJ	2173	2227	-	-	1368	1831
	DNJ	2253	2300	-	-	1465	1959
	α-HNJ	2557	-	-	-	1463	2051
	Miglitol	2521	-	-	-	1503	2119

* Peaks were not detected

** >4000:retention times higher than available alkanes (C_{40})

Elution order of monosaccharide was similar for all the stationary phases; pentoses (arabinose and ribose) eluted before hexoses (fructose, galactose and glucose). Only slight differences on their separation were observed (e.g. galactose *Z* and glucose *Z* showed R_S <1 on HP-1 (I^T 2078) and SLB^{TM}-IL76 (I^T 2068) and were separated (R_S > 1) on SLB^{TM}-IL59 (I^T 1943 and 1938, respectively)).

No differences were found in the elution order of inositols (pinitol, a methyl-inositol eluted first followed by *chiro*-, *scyllo*- and *myo*-inositol and finally galactinol, a glycosyl-inositol) among the different columns; however, iminosugars were highly affected by the selectivity of the different stationary phases. As an example, **Figure 4.12** shows the GC-MS profiles of monosaccharides (labelled as ■), iminosugars (labelled as ▲) and inositols (labelled as ★) eluted on SLB^{TM}-IL82, HP-1 and SUPELCOWAX® columns. In the HP-1 column, elution is almost independent of the

functional groups, as the substances are separated mainly by dispersive interactions. Therefore, solutes tend to elute as the number of carbon atoms in the molecule and the size of the ring increase. Consequently, clusters of compounds belonging to the three studied families were not observed in the chromatograms. When a polar stationary phase was used, as that on the SUPELCOWAX® column, new directional and more specific polar interactions were involved in the separation process. Poly(ethylene glycols) are dipolar/polarizable stationary phases with H-bond acceptor characteristics [186], so iminosugar retention was increased with respect to oligosaccharides and inositols by the additional H-bonding through the hydrogen atom of the amine group. An interesting fact is the high retention of DNJ in comparison with its isomer DMJ. The higher number of substituents in equatorial positions of the former provides a greater surface area and a lower steric hindrance to polar interactions. In the SLB™-IL82 column, in general, iminosugars were more retained than monosaccharides and inositols. This column is less H-bond basic and more dipolar/polarizable than poly(ethylene glycols), but it is also moderately H-bond acid. This last characteristic confers to all the IL-based columns studied in this work a distinct selectivity. In particular, a high retention for basic compounds, that is, compounds with heteroatoms with lone electron pairs (such as the studied carbohydrates) is expected. Moreover, the stronger localization of the unshared electron pairs around the oxygen atom than around the nitrogen atom makes this type of interaction easier for the iminosugars. Therefore, an increase in their retention was observed because they are separated by an additional mechanism than on the SUPELCOWAX® column.

In terms of resolution, several differences were observed for the different columns. As an example, DNJ and HNJ could not be resolved in SUPELCOWAX® column (peaks 12 and 14 in **Figure 4.12**, R_S: 0.8), however these peaks were exceptionally separated in SLB™-IL82 (R_S: 51.6) and in HP-1 (R_S: 22.9). However, in this last column a poor separation of HNJ with galactose *E* was observed (peaks 6 and 14; R_S:0.9). Similarly, arabinose 2 and DMDP coeluted in HP-1 (peaks 1 and 9 in **Figure 4.12**, R_S: 0.1), whereas these peaks were clearly separated in SLB™-IL82 (R_S: 37.0) and in SUPELCOWAX® (R_S: 18.5). Taking into account that resolution is difficult to be objectively measured in complex mixtures of variable composition, peak capacity was

considered in this study. Similar n_c for monosaccharide, inositol and iminosugar interval was achieved by using HP-1 and SLB™-IL82 (n_c: 89 and 81, respectively), whereas this value was remarkably lower for SUPELCOWAX® (n_c: 68), SLB™-IL59 (n_c: 68), SLB™-IL60 (n_c: 69) and SLB™-IL76 (n_c: 53).

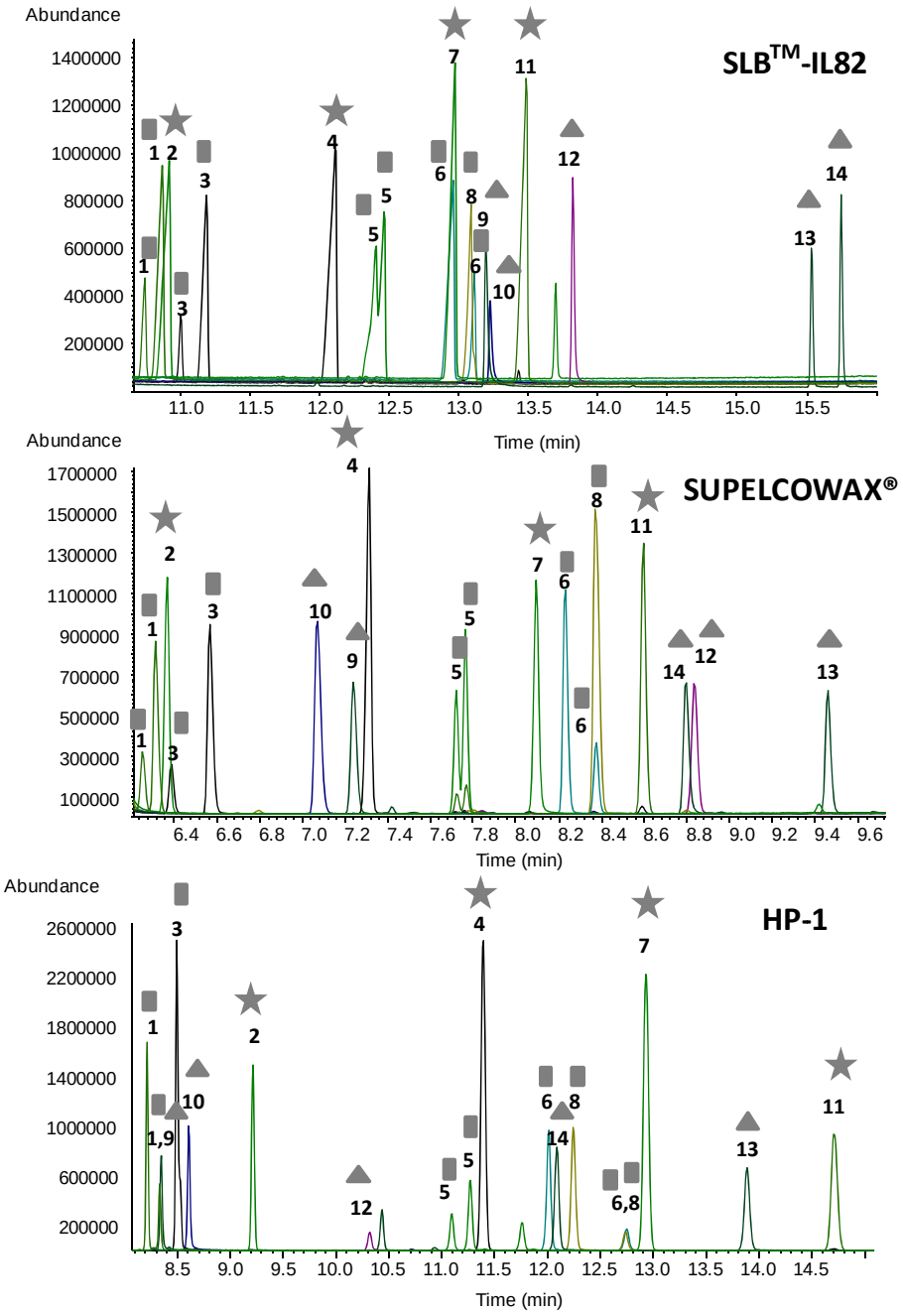

Figure 4.12. GC-MS chromatographic profile obtained for carbohydrate standards analyzed in SLB™-IL82, SUPELCOWAX® and HP-1 columns: 1) arabinose,2) pinitol,3) ribose, 4) *chiro*-inositol, 5) fructose, 6) galactose, 7) *scyllo*-inositol, 8) glucose, 9) DMDP, 10) DMJ, 11) *myo*-inositol, 12) DNJ, 13) miglitol and 14) HNJ. ■: monosaccharides; ★: inositols; ▲: iminosugars

Regarding disaccharides, sucrose was the first to elute in all the evaluated columns and, in general, isomaltose (with 1→6 glycosidic linkage) the last. This chromatographic behavior has been previously observed by other authors in dimethyl- and phenyl-silicone stationary phases [216]. However, as a result of the different selectivity of these stationary phases, some variations in retention order of these carbohydrates were also observed. **Figure 4.13** shows the GC profiles of disaccharides eluted in SLB^{TM}-IL82, SUPELCOWAX$^{®}$ and HP-1 as an example. Changes in retention order were clearly observed for α,α-, α,β- and β,β-trehaloses. In SUPELCOWAX$^{®}$, α,α-trehalose (peak 2) was the first to elute followed by α,β- and β,β-trehaloses (peaks 10 and 8, respectively); this chromatographic behavior was also observed to a lesser extent in HP-1 (**Table 4.11**). However, this retention order was changed in IL columns in which α,β-trehalose eluted latter, although both α,β- and β,β-trehaloses almost coeluted in SLB^{TM}-IL60. Differences in retention of these carbohydrates among the studied columns could be attributed to the additional capability of these IL stationary phases to act as H-bond acids (**section 4.4.1**). They can interact with the lone electron pairs of oxygen atoms of disaccharides, increasing their retention. However, the directional nature of hydrogen bonding would promote a greater retention of α,β-trehalose due to its higher number of equatorial substituents in one of the rings, which mean less steric hindrance between this oligosaccharide and the stationary phase.

With respect to resolution, it is worth to note that isomers *E* and *Z* of reducing glucosyl-glucose disaccharides (e.g. maltose, laminaribiose, kojibiose and isomaltose) were highly separated in HP-1 (R_s:2.6-6.0), SLB^{TM}-IL59 (R_s:1.4-3.5) and SLB^{TM}-IL60 (R_s:1.8-4.8), whereas resolution values for these isomers were lower in SLB^{TM}-IL76 (R_s:0.7-1.7) and SLB^{TM}-IL82 (R_s:0.6-1.4). Worse resolution was achieved for those disaccharides having fructose as the reducing moiety (R_s:0.0-1.4) and for lactose (R_s:0.0-0.8) in all the ILs columns. Considering peak capacity for disaccharides, best results were obtained for HP-1 column (n_c: 53), whereas the smallest values were achieved when using SUPELCOWAX$^{®}$ and SLB^{TM}-IL59 columns (n_c: 29 and 30, respectively).

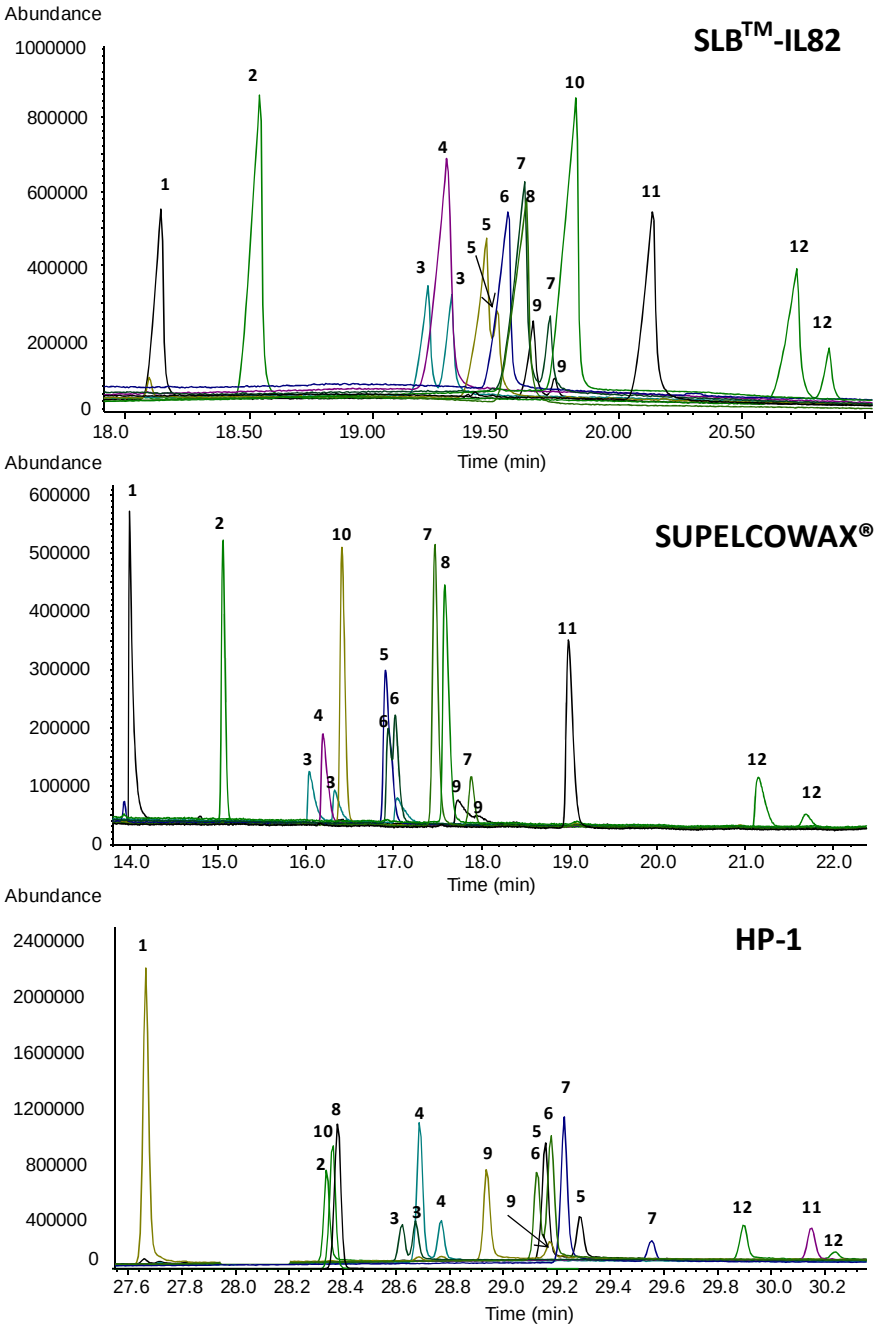

Figure 4.13. GC-MS chromatographic profile obtained for disaccharide standards analyzed in SLB™-IL82, SUPELCOWAX® and HP-1 columns: 1) sucrose, 2) *α*,*α*-trehalose, 3) lactulose, 4) lactose, 5) maltose, 6) turanose, 7) kojibiose, 8) *β*,*β*-trehalose, 9) laminaribiose, 10) *α*,*β*-trehalose, 11) galactinol, 12) isomaltose.

Non-reducing trisaccharides eluted first in all the columns under study, followed by maltotriose (reducing trisaccharide), except for SUPELCOWAX® for which maltotriose eluted before erlose (**Table 4.11**). However, differences in the elution order of non-reducing trisaccharides were also observed among the columns tested. **Figure 4.14** shows the GC profiles of trisaccharides eluting in SLB™-IL82 and HP-1

columns. Melezitose was the first eluting trisaccharide in SLB^{TM}-IL82 (t_R: 28.5 min), SLB^{TM}-IL76 (t_R: 23.6 min) and SUPELCOWAX$^{®}$ (t_R: 29.4 min), followed by kestose (t_R: 29.4, 23.7 and 29.5 min, respectively) and raffinose (t_R: 29.9,24.0 and 29.8 min, respectively). However, kestose eluted first in SLB^{TM}-IL59 and SLB^{TM}-IL60 followed by raffinose and melezitose, whereas raffinose was the first trisaccharide to elute in HP-1 followed by kestose, erlose and melezitose (**Table 4.11**). In the HP-1 column, trisaccharides were found to elute according to the number and size of rings (as dispersion interactions with the stationary phase rise accordingly), with an increase in the retention when the fructose unit were in a intermediate position. However, the opposite effect was found when the polar stationary phases were involved. Trisaccharides with a fructose in the central position have globular structures with more methyl groups bound outwards, hindering polar interactions. Therefore, a decrease of retention of melezitose and kestose as polarity of stationary phase increases was found, and hence a very different elution pattern to HP-1 column.

The highest peak capacity was obtained in HP-1 column (n_c: 69), whereas extremely small values were obtained for SLB^{TM}-IL76 (n_c: 3) and SLB^{TM}-IL59 (n_c: 9), indicating a high coelution of these compounds in these stationary phases.

At the sight of the above exposed, SLB^{TM}-IL82 is a useful phase for the analysis of carbohydrates, mainly monosaccharides, inositols and iminosugars and it was selected for further studies.

- *Influence of temperature on peak width and symmetry (tailing factor)*

The effect of temperature conditions (programmed and isothermal) on peak width and tailing factor (TF) of the different target LMWC was evaluated for SLB^{TM}-IL82. Isothermal conditions were chosen on the basis of the optimal temperature for the elution of each type of carbohydrates on this column: 160 and 180ºC for monosaccharides, inositols and iminosugars, 180, 230 and 250ºC for disaccharides and 230 and 250ºC for trisaccharides. **Table 4.12** shows peak widths of monosaccharides, inositols and iminosugarsat 140ºC, 160 ºC as well as in the optimal gradient for carbohydrate elution in this column (ramp 2) and in a linear gradient (ramp 1) as indicated in **section 4.4.2.2**.

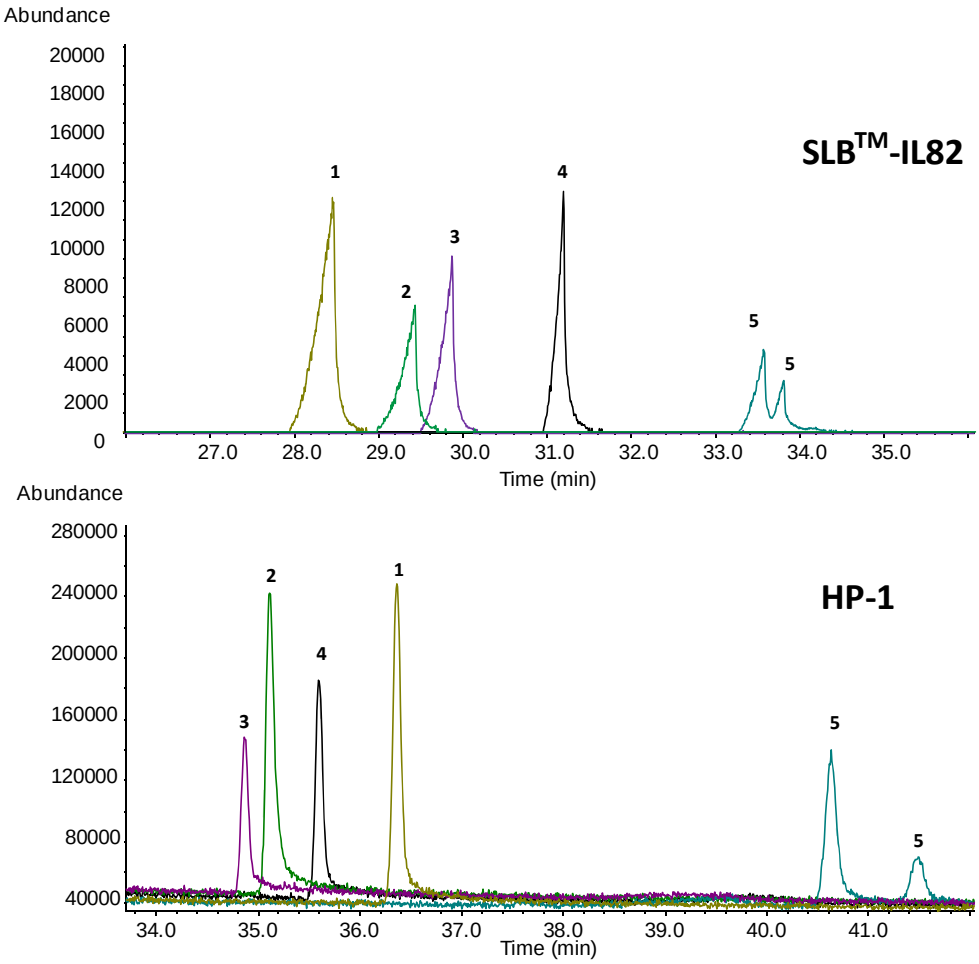

Figure 4.14. GC-MS chromatographic profile obtained for trisaccharide standards analyzed in SLB™-IL82 and HP-1 columns: 1) melezitose, 2) kestose, 3) raffinose, 4) erlose and 5) maltotriose

Under isothermal conditions peak widths (w_h) varied from 0.03 to 0.15 min, with the exception of HNJ at 140 ºC which was a highly retained and broad peak (w_h: 0.26 min). Slightly narrower peaks were obtained in programmed temperatures, mainly for carbohydrates eluting in the optimal ramp (ramp 2; w_h: 0.03-0.07 min). Regarding tailing factor, values higher and lower than 1 were obtained depending on the type of carbohydrates. In general, good TF values were obtained for monosaccharides, with the exception of galactose eluted in ramp 2 and fructose 1 and glucose *E* eluted at 160ºC which presented TF values lower than 1. TF values lower than 1 indicates that solute-stationary phase interactions are relatively weak compared with solute-solute or stationary phase-stationary phase interactions or that column is overload as a result of large sample sizes [217].

Table 4.12. Retention times (t_R), peaks widths (w_h) and tailing factor (TF) obtained for the TMSO derivatives of monosaccharide, iminosugar and inositol standards analyzed at different conditions in SLB^{TM}-IL82 column.

Sample	Isothermal						Programmed					
	140°C			160°C			Ramp 1			Ramp 2		
	t_R (min)	Width (min)	TF	t_R (min)	Width (min)	TF	t_R (min)	Width (min)	TF	t_R (min)	Width (min)	TF
Arabinose	-	-	-	-	-	-	7.41	0.03	1.00	10.75	0.04	1.00
	2.67	0.10	1.00	2.15	0.07	0.93	7.47	0.03	1.10	10.87	0.04	1.50
Ribose	-	-	-				6.21	0.04	1.00	11.01	0.03	1.00
	2.87	0.07	0.80	2.18	0.07	0.88	6.40	0.04	1.80	11.19	0.04	1.50
Fructose	3.84	0.05	0.81	2.52	0.04	0.63	7.52	0.03	1.20	12.40	0.03	0.87
	3.92	0.06	1.4	2.54	0.03	1.50	7.57	0.03	1.30	12.46	0.03	1.30
Galactose	4.53	0.07	1.06	2.73	0.05	0.81	7.97	0.03	1.10	12.96	0.07	0.68
	4.78	0.07	0.94	2.82	0.04	0.86	8.11	0.03	1.00	13.11	0.04	0.71
Glucose	4.68	0.06	1.00	2.77	0.06	0.64	8.08	0.03	1.00	13.08	0.04	0.94
	4.72	0.04	0.88	2.80	0.03	1.50	-	0.03	1.15	-	-	-
Pinitol	2.82	0.06	1.06	2.18	0.05	1.00	6.22	0.03	1.25	10.91	0.03	1.50
chiro-Inositol	3.58	0.06	1.06	2.44	0.10	1.40	7.24	0.03	1.25	12.12	0.03	1.50
scyllo-Inositol	4.55	0.06	1.10	2.74	0.10	1.30	8.01	0.04	1.50	12.97	0.04	1.60
myo-Inositol	5.56	0.07	1.08	3.10	0.11	1.30	8.51	0.04	1.30	13.49	0.03	1.50
DMDP	5.12	0.06	1.29	2.95	0.05	1.50	8.50	0.05	1.30	13.20	0.03	1.33
DMJ	5.29	0.06	1.29	3.05	0.07	1.25	8.62	0.10	1.90	13.22	0.03	1.83
DNJ	6.67	0.07	1.11	3.58	0.08	0.94	9.14	0.06	1.50	13.82	0.03	1.33
α-HNJ	18.68	0.26	2.75	6.49	0.10	2.08	10.96	0.04	1.33	15.75	0.03	1.17
Miglitol	18.07	0.15	2.04	5.89	0.07	1.43	10.75	0.04	1.50	15.54	0.03	1.17
MAX		**0.26**	**2.75**		**0.10**	**2.50**		**0.10**	**1.90**		**0.07**	**1.83**
MIN		**0.03**	**0.56**		**0.03**	**0.63**		**0.03**	**0.59**		**0.03**	**0.57**
AVERAGE		**0.09**	**0.98**		**0.06**	**1.12**		**0.06**	**0.97**		**0.05**	**0.90**

Considering that in this work the injected amount of sample has been carefully controlled to avoid overloading, this behavior could be attributed to the first fact. On the contrary, iminosugars and inositols gave TF values higher than 1, which indicates peak tailing, as a result of the existence of adsorption phenomena in the column. A similar behavior was observed for amino-compounds in SLB^{TM}-IL82 columns (**section 4.4.1**) which could be justified by the low inertness of this stationary phase to compounds with hydrogen bonding capabilities.

As expected, peak widths for di- and trisaccharides (**Table 4.13**) were higher at isothermal elution conditions, mainly at 180 and 230 ºC, (w_h: 0.05-0.30 min) than at programmed temperature (0.02-0.11 min and 0.02-0.24 min for ramp 1 and 2, respectively). Whereas peak widths of disaccharides were appropriate using both temperature programs (w_h0.03-0.04 min), trisaccharides, the most retained compounds, eluted as broader peaks in ramp 2 (w_h:0.05-0.24 min).

Peak fronting (TF >1) was observed for disaccharides eluted at 180 ºC, however, peak tailing was, in general, detected at higher temperatures (230 and 250 ºC) while in programmed temperature most of the TF values were close to 1. A similar behavior was observed for trisaccharides. Peak fronting was found for trisaccharides eluted at 230 ºC, except for kestose and melezitose, whereas peak tailing was, in general, detected at 250 ºC. As commented before for inositols and iminosugars, peak tailing could be attributed to the existence of adsorption phenomena in the column. Appropriate tailing factor values were obtained for trisaccharides at both programmed temperature conditions (mean TF values of 0.97 and 0.83for ramp 1 and 2, respectively).

Therefore, the described chromatographic behavior in SLB^{TM}-IL82 column indicates that it could be a useful phase for the analysis of LMWC with different structural characteristics (mono-, di- and trisaccharides, inositols and iminosugars) choosing the most appropriate temperature for each type of carbohydrates.

- *Analysis of real samples*

The analysis of carbohydrates present in real samples was carried out using SLB^{TM}-IL82 column under optimal temperature programmed conditions (ramp 2).

Hyacinth (*Hyacinthus orientalis*) bulbs and mulberry (*Morus alba*) leaf extracts were chosen as an example of real samples considering their previously reported content on the different kinds of carbohydrates (mono- and disaccharides, iminosugars and inositols) (**sections 4.1** and **4.2**). Identification of the peaks was done by means of comparison of their retention times with those of standard compounds and by the information obtained from their mass spectrum.In those cases in which no standards were available the identification was performed by comparing with data reported in the bibliography.

GC-MS analyses of hyacinth extract using SLB^{TM}-IL82 column allowed the separation of all the LMWC present in the sample (**Figure 4.15A)**. Different iminosugars (DMJ, DNJ, HMJ and HNJ, peaks 3, 5, 6 and 7, respectively) as well as monosaccharides (fructose and glucose, peaks 1 and 2, respectively) and sucrose (peak 9) were identified. *Myo*-inositol (peak 4) and different unknown glycosyl-inositols and disaccharides (labeled as peak 10) were also detected. As expected, some differences in the peak elution order were observed when comparing the obtained GC profile with that reported in a HP-1 column (**section 6.1)**. Iminosugars were more retained than monosaccharides (fructose and glucose), the most abundant carbohydrates in hyacinth, in SLB^{TM}-IL82, contrarily to retention order observed in HP-1 column, where iminosugars eluted before or in between fructose and glucose.

A similar behavior was observed for mulberry leaf extract analysis. **Figure 4.15B** shows the GC profile of this extract analyzed using SLB^{TM}-IL82column. Fructose, galactose, glucose, and sucrose (peaks 2, 3, 4 and 9, respectively), *myo*-inositol (peak 5) and minor amounts of iminosugars: fagomine (peak 1) and DNJ (peak 6) were detected. Iminosugars were more retained than in HP-1 column, however, fagomine (1,5-imino-1,2,5-trideoxy-D-arabino-hexitol) eluted before fructose.

These differences in retention can be an advantage when carbohydrates of different structural characteristics are present in the analyzed samples and SLB^{TM}-IL82 column can offer complementary information to conventional HP-1 columns.

Table 4.13. Retention times (t_R), peaks widths (w_h) and tailing factor (TF) obtained for the TMSO derivatives of di- and trisaccharide standards analyzed at different conditions in SLB^{TM}-IL82 column.

Sample	Isothermal									Programmed					
	180°C			230°C			250°C			Ramp 1			Ramp 2		
	t_R (min)	Width (min)	TF	t_R (min)	Width (min)	TF	t_R (min)	Width (min)	TF	t_R (min)	Width (min)	TF	t_R (min)	Width (min)	TF
Sucrose	7.05	0.19	0.55	2.15	0.06	0.75	-			13.10	0.02	1.00	18.14	0.03	1.00
α,α-Trehalose	8.22	0.11	1.50	2.27	0.05	1.13	1.93		1.13	13.53	0.04	1.10	18.54	0.03	1.10
α,β-Trehalose	17.65	0.11	0.89	2.59	0.07	0.94	2.03	0.05	1.14	14.79	0.03	1.6	19.82	0.04	1.20
β,β-Trehalose	13.80	0.11	0.87	2.52	0.05	1.17	2.00	0.08	1.40	14.60	0.04	1.10	19.62	0.03	1.10
Lactulose	11.83	0.12	0.72							14.17	0.03	1.10	19.22	0.03	0.86
	12.42	0.12	0.71	2.47	0.08	0.62	2.00	0.20	1.24	14.27	0.03	1.00	19.32	0.03	1.10
Lactose	12.05	0.09	0.81	2.46	0.10	1.78	2.00	0.10	2.00	14.24	0.04	1.10	19.30	0.04	1.20
Laminaribiose	14.27	0.11	0.70							14.51	0.03	1.10	19.65	0.04	1.1
	14.68	0.08	1.19	2.54	0.06	1.32	2.02	0.09	3.47	14.57	0.03	1.00	19.74	0.02	1.00
Turanose	12.40	0.10	1.55	2.55	0.10	1.25	2.03	0.06	1.33	14.48	0.03	1.00	19.55	0.03	1.20
Maltose	12.89	0.30	0.56							14.40	0.02	0.88	19.46	0.04	1.1
	13.14	0.15	0.66	2.51	0.09	1.30	2.04	0.06	1.42	14.44	0.03	1.00	19.51	0.04	1.1
Galactinol	18.07	0.18	0.59	2.77	0.08	1.80	2.14	0.10	1.65	15.02	0.04	1.00	20.13	0.04	1.1
Kojibiose	14.06	0.18	0.58	2.57	0.11	1.00				14.54	0.04	0.93	19.61	0.04	0.87
	14.60	0.11	0.75	2.59	0.12	2.10	2.03	0.07	1.25	14.63	0.04	1.00	19.72	0.03	1.00
Isomaltose				3.05	0.07	0.63	2.22	0.06	0.69	15.42	0.04	1.10	20.72	0.03	1.00
				3.11	0.05	0.90	2.25	0.04	1.50	15.51	0.03	1.00	20.85	0.04	1.00
Raffinose				7.92	0.27	0.61	3.88	0.15	1.08	18.56	0.04	1.00	29.87	0.07	1.05
Kestose				7.46	0.21	1.13	3.73	0.07	1.23	18.42	0.09	0.82	29.40	0.16	0.69
Erlose				9.26	0.17	0.88	4.24	0.09	1.96	19.11	0.07	1.24	31.20	0.10	0.90
Melezitose				6.97	0.17	1.53	3.62	0.11	1.50	18.25	0.08	0.82	28.48	0.24	0.77
Maltotriose				13.35	0.30	0.77	5.62	0.13	0.83	20.65	0.11	0.76	33.44	0.05	1.1
				13.91	0.20	0.90	5.79	0.19	1.69	20.85	0.08	1.20	33.67	0.07	1.04
MAX		**0.41**	**0.69**		**0.30**	**3.70**		**0.19**	**2.00**		**0.11**	**1.24**		**0.24**	**2.33**
MIN		**0.06**	**0.53**		**0.05**	**0.61**		**0.04**	**0.69**		**0.03**	**0.58**		**0.04**	**0.65**
AVERAGE		**0.23**	**0.61**		**0.12**	**1.24**		**0.09**	**1.36**		**0.07**	**0.79**		**0.08**	**0.89**

4.4.2.4. Conclussions

Several commercial IL capillary columns have been explored for the first time for the analysis of mono- di-, trisaccharides, inositols and iminosugars. SLB^{TM}-IL82 resulted to be the most useful IL column for these analyses. Although poly(dimethylsiloxane) is a more appropriate stationary phase for LMWC separation, carbohydrates from natural products can be very complex mixture and can require the use of GC columns with different selectivity. Therefore, SLB^{TM}-IL82 can be an interesting alternative to classical GC stationary phases and can provide useful additional information for the analysis of complex mixtures with different purposes.

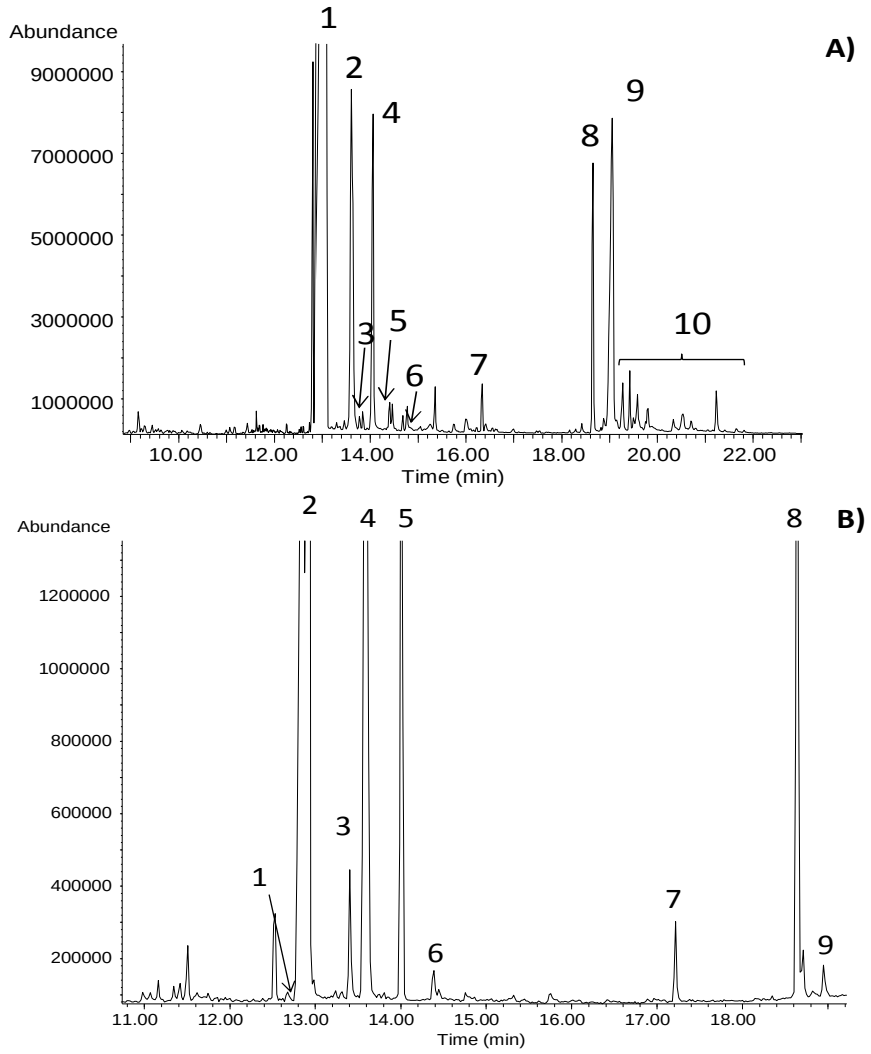

Figure 4.15. GC-MS chromatographic profile of **A)** hyacinth extract. 1) fructose, 2) glucose,3) DMJ, 4) *myo*-inositol, 5) DNJ, 6) HMJ, 7) HNJ, 8) phenyl-*β*-*D*-glucoside, 9) sucrose, 10) disaccharides + glycosyl-inositols and **B)** mulberry extract. 1) fagomine, 2) fructose, 3) galactose,4) glucose, 5) *myo*-inositol, 6) DNJ, 7) glycosyl-glycerol, 8) phenyl-*β*-*D*-glucoside and 9) sucrose.

ANALISIS POR LC-MS

5. Desarrollo de métodos mediante LC-MS para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular

Como se muestra en la **sección 4**, la GC-MS ha demostrado ser una técnica adecuada para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular presentes en extractos vegetales. En esta sección se pretende evaluar también la utilidad de la LC-MS para el análisis de estos compuestos, con el fin de seleccionar la técnica que resulte más adecuada o evaluar el uso complementario de ambas. De entre los distintos modos de operación en HPLC, se ha seleccionado la HILIC, ya que como se indica en la Introducción (**sección 1.5.1**), es una técnica apropiada para la separación de compuestos polares e hidrofílicos. La detección de los compuestos se realizó mediante MS y MS^2 con el fin de obtener información estructural de dichos carbohidratos.

Para conseguir el objetivo planteado fue necesario optimizar y validar métodos de análisis mediante HILIC-MS, seleccionándose: (i) fase estacionaria, (ii) modificador orgánico, (iii) aditivo y gradiente de la fase móvil y (iv) temperatura de la fase estacionaria. Los métodos optimizados se aplicaron al análisis simultáneo de iminoazúcares y otros carbohidratos de bajo peso molecular presentes en los extractos mencionados en la **sección 4** (hojas de morera, granos de trigo sarraceno, bulbos de jacinto y hojas de Aglaonema).

La **sección 5.1.** está basada en los resultados del trabajo titulado **"Evaluation of different hydrophilic stationary phases for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates in vegetable extracts by liquid chromatography tandem mass spectrometry"** de Rodríguez-Sánchez *y col.,* publicado en *Journal of Chromatography A* (1372) 81-90. En este trabajo se ha evaluado la utilidad de tres fases estacionarias hidrofílicas diferentes, empleando distintas condiciones cromatográficas (gradientes, flujos, modificadores orgánicos y aditivos), para el análisis de patrones de iminoazúcares y otros carbohidratos de bajo peso molecular. Los criterios empleados para la selección de la fase estacionaria más apropiada fueron resolución, eficacia, simetría y anchura de pico. El método. El método optimizado se aplicó a la caracterización cuali- y cuantitativa mediante LC-MS^2 de extractos de hojas de morera, bulbos de jacinto y granos de trigo sarraceno,

permitiendo la identificación de iminoazúcares ya descritos en estos extractos y previamente detectados por GC-MS, así como la caracterización estructural de iminoazúcares desconocidos hasta el momento.

Una vez seleccionada la fase estacionaria más adecuada para el análisis de estos compuestos por HILIC-MS, se amplió el estudio previo con la evaluación del efecto de nuevas condiciones cromatográficas (eluyentes, aditivos, temperatura de la columna, etc.) sobre la retención, resolución, simetría y anchura de pico de algunos iminoazúcares, mono- y disacáridos seleccionados en extractos de hojas de Aglaonema. El método optimizado se aplicó al análisis mediante HILIC-MS^2 de extractos procedentes de hojas de distintos cultivares, con el fin de contribuir a ampliar el conocimiento sobre la composición en iminoazúcares de dichos extractos, mediante la detección de nuevos iminoazúcares no descritos previamente en la bibliografía. Los resultados obtenidos se recogen en la **sección 5.2.** que incluye el trabajo titulado "**Analysis of iminosugars and other low molecular weight carbohydrates in *Aglaonema* sp. extracts by hydrophilic interaction liquid chromatography coupled to mass spectrometry**" de Rodríguez-Sánchez *y col.*, enviado al *Journal of Agricultural and Food Chemistry* (2015) y pendiente de revisión.

5.1. Evaluation of different hydrophilic stationary phases for the simultaneous analysis of iminosugars and other low molecular weight carbohydrates in vegetable extracts by liquid chromatography tandem mass spectrometry

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Journal of Chromatography A (2014) 1372: 81-90

5.1.1. Introduction

Iminosugars or iminocyclitols are low molecular weight carbohydrate (LMWC) analogues in which the endocyclic oxygen has been replaced by a nitrogen atom. They can be obtained by chemical synthesis [5-7, 218], but can also be isolated from different plants (Leguminosae [64], Araceae [22], Moraceae [219], Campanulaceae [220], Polygonaceae [27], Hyacinthaceae [9]) and microorganisms such as *Streptomyces* and *Bacillus* [10, 13]. Iminosugars have the capability of competitively inhibiting glycosidases because they are able to mimic the transition state of pyranosidic or furanosidic units of natural glycosidase substrates [31]. Due to the important role that glycosidases play in many essential life processes, iminosugars might have different potential therapeutic applications (antiviral, anticancer, antibiotic, etc.) [10]. Among them, those aimed to the reduction of the risk of developing insulin resistance and for overweight control have been the most reported [15, 221, 222].

Nojirimycin was the first iminosugar discovered in 1966 by Inouye *et al*. [223]. It was found to be a good inhibitor of both *α*- and *β*-glucosidases of different origins. Since then, several works have been focused on the study of other iminosugars mainly deoxynojirimycin (DNJ) and fagomine. DNJ is a highly potent intestinal *α*-glucosidase inhibitor present, among others, in mulberry (*Morus* sp.) leaves and in silkworms [17]. Fagomine was first isolated from buckwheat (*Fagopyrum esculentum*) seeds and later found in different natural sources such as *Xanthocercis Zambesiaca* (Leguminosae) seeds, *Morus bombycis* and *Morus alba* (Moraceae) leaves and roots, respectively, *Lycium chinese* (Solanaceae) roots, etc. [63]. Recent studies have attributed a double

action to fagomine: lowering the postprandial blood glucose and modulating bacterial adhesion [38]

Analysis of iminosugars has been addressed by different techniques [36, 51, 56], and high performance liquid chromatography (HPLC) has become one of the most commonly applied for this purpose [35]. Several liquid chromatographic separation modes such as anion [111] and cation [28] exchange, hydrophilic interaction [17, 118] and reversed-phase [39] have been applied for the analysis of iminosugars. Among them, the reported advantages of hydrophilic interaction liquid chromatography (HILIC) methods for the analysis of polar compounds and the possibility of direct coupling to mass spectrometry (MS) have contributed to extend the use of this HPLC mode for the analysis of complex mixtures of carbohydrates [224-226].

Regarding iminosugar detection, the lack of a chromophore or fluorophore group in their structures prevents the direct use of spectrophotometric detectors unless derivatization procedures are carried out [16, 70, 111]. Alternatively, other detectors such as evaporative light scattering and pulsed amperometric detectors have been used [39]. However, the structural information provided by MS makes this technique the most powerful tool for the characterization and elucidation of new or unidentified compounds.

On the other hand, most of the analytical methods developed so far have been focussed on the determination of DNJ [16, 17, 39, 111] and/or fagomine [27, 28] in different matrices, and the number of manuscripts devoted to the analysis of other iminosugars is more limited [21, 219, 227]. Moreover, iminosugars are generally present at low concentrations in natural sources together with other LMWC (monosaccharides, disaccharides, inositols, etc.), which negatively contribute to the use of these extracts as bioactives (**section 4.1**). The simultaneous analysis of all these compounds, scarcely considered in most previous papers, is therefore required, but it is not straightforward, considering the complexity of the mixtures and the similarity of carbohydrate structures and corresponding MS data.

A gas chromatography-mass spectrometry (GC-MS) method recently optimised in our laboratories has provided a good resolution among the variety of iminosugars and LMWC present in different plant extracts (**sections 4.1** and **4.2** of this Thesis).

However, due to the low volatility and high polarity of carbohydrates, a previous derivatization step was required. Efficiency of the derivatization process and stability of the resulting derivatives were also crucial factors to be carefully controlled (**section 4.1**).

Hence, it would be of great interest the development of a sensitive and high resolution HILIC-MS method, which allows the simultaneous determination of the complex mixtures of iminosugars and LMWC present in extracts from natural sources without a prior derivatization process. To achieve that aim, three hydrophilic stationary phases (amide, aspartamide and zwitterionic) have been evaluated in this paper in terms of resolution, time of analysis, peak width and peak symmetry. As an example of application, selected phase and conditions have been used for the analysis of these compounds in three different vegetable (*Morus alba* leaves, *Fagopyrum esculentum* seeds and *Hyacinthus orientalis* bulbs) extracts. Tandem MS data have also been used to confirm the identity of iminosugas or for elucidation of new structures.

5.1.2. Materials and methods

5.1.2.1. Standards

2,5-dideoxy-2,5-imino-*D*-mannitol (DMDP) and α-homonojirimycin (*α*-HNJ) were purchased from Dextra Laboratories (Reading, UK), whereas fructose, glucose, *myo*-inositol, *chiro*-inositol, galactinol (O-*α*-*D*-galactopyranosil-(1→1)-*D*-*myo*-inositol), sucrose, 1-deoxynojirimycin hydrochloride (DNJ), 1-deoxymannojirimycin hydrochloride (DMJ), *N*-(2-hydroxyethyl)-1-deoxynojirimycin (miglitol), and *N*-methyl-1-deoxynojirimycin (*N*-methyl-DNJ) were obtained from Sigma (St. Louis, USA).

Standard solutions (0.01-0.02 mg mL^{-1}) in CH_3CN:water (80:20, v:v) were filtered through nylon FH membranes (0.22 μm) (Millipore, Bedford, MA) before injection.

5.1.2.2. Samples

Mulberry (*Morus alba*) leaves were collected in Madrid (Spain). Samples of hyacinth (*Hyacinthus orientalis*) bulbs and buckwheat (*Fagopyrum esculentum*) seeds

were acquired in local markets in Madrid. All the samples were air-dried, ground in a domestic mill (Moulinex) and sieved (< 500 µm) before carbohydrate extraction.

5.1.2.3. Carbohydrate extraction

Samples (0.05 g) were extracted with 0.5 mL of acidulated water at room temperature 50 ºC for 2 h. Extracts were diluted with acetonitrile to achieve a final 80:20 (v:v) CH_3CN:water ratio.

5.1.2.4. LC-MS analysis

Two LC-MS instruments (both from Agilent Technologies, Santa Clara, CA, USA) were used in this study. The first one was a 1200 Series HPLC system, provided with a binary pump, a Rheodyne 7125 injection valve and an oven (Kariba Instruments) to control column temperature, coupled via an electrospray ionization (ESI) interface working under positive polarity to a single quadrupole MSD 1100 mass spectrometer. The electrospray source parameters were adjusted as follows: spray voltage, 4 kV; drying gas (N_2, 99.5% purity) temperature, 300 °C; drying gas flow, 12 L min^{-1}; nebulizer (N_2, 99.5% purity) pressure, 276 kPa; and fragmentor voltage, 80-100 V. Optimization of ion transmission into the analyzer was performed by infusing the default test mixture. Molecular ion adducts for sugars and iminosugars were recorded in the selected ion monitoring (SIM) mode. Data acquisition and processing were performed using HP Chemstation Rev. A.07.01 software.

The second instrument was an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler, and a column oven) coupled to a 6520 quadrupole-time of flight (QTOF) mass spectrometer, using an ESI interface working in the positive-ion mode. The electrospray voltage was set at 4.5 kV, the fragmentor voltage at 150 V and the drying gas temperature at 300 °C. Nitrogen (99.5% purity) was used as nebulizer (207 kPa) and drying gas (6 L min^{-1}), while nitrogen of higher purity (99.999%) was used as the collision gas.

Optimization of ion transmission into the analyzer was performed by infusing the default test mixture. Full scan mass spectra were recorded in the 130–2000 *m/z* range.

Tandem mass spectra were obtained by collision induced dissociation (CID), applying collision energies between 10 and 37 eV to the selected precursor ions ($[M+H]^+$ for iminosugars and $[M+NH_4]^+$ for other LMWC). These MS^2 spectrum data were used to confirm the identity of iminosugars present in natural extracts and for characterization of unknowns. Data acquisition and processing were performed using Agilent Mass Hunter Workstation Acquisition Rev. B.02.00 software.

The LC experiments were carried out on three different columns: i) PolyHydroxyethyl-aspartamide stationary phase (PolyHydroxyethyl-A column (PHEA); 100 mm x 2.1 mm, 3 µm, 300 Å pore size; The Nest Group, Inc., Southborough, MA, USA); ii) sulfoalkylbetaine zwitterionic stationary phase (ZIC®-HILIC column (ZIC); 150 mm x 2.1 mm, 3.5 µm, 200 Å pore size; SeQuant™, Umea, Sweden) and iii) ethylene bridge hybrid with trifunctionally bonded amide phase (XBridge column (BEH); 150 mm x 4.6 mm, 3.5 µm, 135 Å pore size, Waters, Hertfordshire, UK). Injection volume was 5 µL, and the column temperature was maintained at 25 °C.

Different binary (CH_3CN: water) gradients and additives (0.1% acetic acid or 0.1% ammonium hydroxide) were assayed for each of the columns tested.

Mixtures of standards (see **section 5.1.2**) were injected in order to optimize the flow rate (in the range of optimal theoretical flows and compatible with MS detection) and to evaluate the performance of the HILIC method, as measured by different chromatographic parameters, namely retention time (t_R), peak width at half height (w_h) and peak asymmetry factor (A_s), calculated as the ratio of the back half to front half widths at 10% of the peak height. Resolution (R_s) and height equivalent to theoretical plate (H), calculated according to equations 5 and 6, respectively, where 1 and 2 refer to two consecutive eluting compounds, w_b is the peak width at base and L is the column length, were also evaluated from these data.

$$R_S = 2\frac{(t_{R2}-t_{R1})}{(w_{b1}+w_{b2})} \quad \text{Eq. (5)}$$

$$H = \frac{L \times w_h^2}{5{,}545\ x\ t_r^2} \quad \text{Eq. (6)}$$

Quantitative analysis of vegetable extracts by LC-MS^2 was performed in triplicate using external standard calibration curves of glucose, sucrose, DNJ and α-HNJ within the range 0.0125-100 µg mL^{-1}. Goodness of fitting for these calibration curves was evaluated using their correlation coefficients. Prior to quantitation, the matrix effect was evaluated by quantifying target analytes in solutions of the original sample extracts diluted in water at different ratios (1:1-1:500, v/v).

Reproducibility of the method was measured on the basis of the intra-day precision, calculated as the relative standard deviation (*RSD*, %) of concentrations of iminosugar and LMWC standards in n = 5 independent measurements. Limits of detection (*LOD*) and quantitation (*LOQ*) were calculated as three and ten times the standard deviation of the noise (σ), respectively.

5.1.2.5. ${}^{S}_{W}pH$ measurement

The determination of the pH of the hydro-organic solutions (${}^{S}_{W}pH$) used as mobile phase was carried out using a 780 model pH meter equipped with a 6.0258.010 model KCl electrode (Metrohm Ltd., Switzerland), after calibration with 4.01, 7.00 and 9.00 aqueous buffer solutions.

5.1.2.6. Data treatment and statistical analysis

Microsoft Office Excel (Microsoft Corp. Redmond. WA, USA) was used for data handling and basic calculations. Unscrambler X (CAMO Software AS, Oslo, Norway) was used for principal component analysis.

5.1.3. Results and discussion

5.1.3.1. Evaluation of the hydrophilic stationary phases

The performance of the three hydrophilic stationary phases under study for the simultaneous analysis of iminosugars and LMWC was evaluated by using commercial standards. Compounds were selected to include iminosugars (DNJ, α-HNJ, DMJ, miglitol, *N*-methyl-DNJ and DMDP) and LMWC (fructose, glucose, *myo*-inositol, sucrose

and galactinol) usually present in vegetable sources. **Table 5.1.** summarizes the best assayed chromatographic working conditions for each of the additives (0.1% acetic acid or 0.1% ammonium hydroxide) and columns evaluated. In all cases, after analysis, the mobile phase ramped to the initial conditions in 0.1 min and equilibration lasted for 10 min.

Table 5.1. Optimal gradients and flows for the three studied hydrophilic stationary phases using both 0.1% acetic acid and 0.1% ammonium hydroxide as mobile phases additives.

Stationary phase	Mobile phase				
	Additive	$^{S}_{W}pH$ range	Flow (mL min^{-1})	Elution program	
				Time (min)	CH_3CN (%)
PHEA				0	90
				15.0	50
	CH_3COOH (0.1%)	5.29-3.79	0.4	20.0	50
				20.1	90
				30.1	90
				0	80
				13.0	65
	NH_4OH (0.1%)	9.76-9.58	0.2	23.0	65
				23.1	80
				33.1	80
BEH				0	80
				31.0	20
	CH_3COOH (0.1%)	4.46-3.43	0.4	41.0	20
				41.1	80
				51.1	80
				0	80
				46.0	65
	NH_4OH (0.1%)	9.76-9.50	0.4	56.0	65
				56.1	80
				66.1	80
ZIC				0	80
				46.0	50
	CH_3COOH (0.1%)	4.46-3.79	0.2	56.0	50
				56.1	80
				66.1	80
				0	95
				20.0	70
	NH_4OH (0.1%)	9.90-9.62	0.2	30.0	70
				30.1	95
				40.1	95

- *Effect of acetic acid*

Table 5.2. lists the retention time, peak width, asymmetry, resolution and height equivalent to theoretical plate data of carbohydrate standards eluted from PHEA, BEH and ZIC hydrophilic columns, using acetic acid as mobile phase additive. As it can be observed, carbohydrates showed the same elution order in both PHEA and BEH columns, whereas they were differently eluted in the ZIC column. A different selectivity of the aspartamide and amide columns as compared with the sulfobetaine phase has also been observed in the separation of other compounds such as nucleic acids and nucleosides [228].

In the PHEA and BEH columns, sugars eluted first, followed by inositols (*myo*-inositol and galactinol) and iminosugars. However, remarkable differences in terms of retention times and resolution were observed between them. In the PHEA column, all the compounds were poorly retained, whereas in the BEH column two eluting zones were clearly distinguished: (i) sugars and inositols (13.5-19.4 min) and (ii) iminosugars (31.2-32.5 min). In general, good resolution values were achieved for sugars and inositols in both columns, while poorer values were generally obtained for iminosugars. Although the specific retention mechanism in HILIC is not clearly known, it has mainly been attributed to the interaction of analytes with the polar stationary phase through partitioning into a water-rich layer, which is generated through chemisorption phenomena at the surface of the stationary phase [115, 116, 229]. Nevertheless, other secondary mechanisms such as electrostatic interactions and hydrogen bonding [116] may also be involved in the HILIC retention process, and could cause the observed behaviour.

Both BEH and PHEA are considered neutral phases. However, ionization of residual surface silanol groups in BEH at pH above 4 could impart negative charges to the column, whereas in the PHEA column, the polypeptide coating can be either positively charged at pH <4.4 or negatively charged at a pH >4.4, presumably due to the presence of free *N*- and *C*-termini [229]. Considering the ${}^{S}_{w}pH$ range of the mobile phases (see **Table 5.1**), iminosugars (pK_b: 7.5-8) are positively charged under these conditions, which could justify their higher retention by electrostatic interactions with the negatively charged stationary phase. Both sugars and inositols are neutral compounds and elute earlier. Moreover, different authors have also reported that other basic

compounds such as amines or amides are also highly retained on hydrophilic phases [230].

On the other hand, the longest analysis time (47.4 min) was observed for the zwitterionic column. In this column, most of the carbohydrates were well resolved (R_s> 1) under these conditions, although some coelutions were detected (e.g. glucose and fructose, α-HNJ and DNJ, see **Table 5.2**). Mono- and disaccharides eluted first followed by *N*-methyl-DNJ, DMDP, *myo*-inositol and galactinol. The remaining iminosugar standards with remarkably higher retention times (43.6 - 47.4 min) followed a similar elution pattern as compared to PHEA and BEH columns. In the ZIC column, the sulfonate groups on the outside of the sulfobetaine ligands give the column cation-exchange properties despite the overall zwitterionic nature of the ligand [228]. This property could justify the higher retention of miglitol, DMJ, α-HNJ and DNJ which are positively charged under these conditions; however, it does not support the lower retention of *N*-methyl-DNJ and DMDP.

Poor symmetry was found for LMWC eluted using PHEA column (0.4-0.7) and for both sugars and iminosugars in the zwitterionic column (up to 4). Asymmetry factors ranging 1.0-1.5 were obtained for standards other than glucose and fructose analysed in the BEH column. Regarding efficacy, and with the exception of fructose (w_h = 0.87 min, H = 0.113 mm), BEH column provided the best results for the different standards (w_h = 0.18-0.58 min, H = 0.003-0.018 mm), whereas the broadest peaks were obtained in the ZIC column (w_h = 0.46-1.92 min; H = 0.01-2.11 mm).

Table 5.2. Chromatographic parameters for the three studied hydrophilic stationary phases using 0.1 % CH_3COOH as additive.

Compound	PHEA					BEH					Compound	ZIC				
	t_R (min)	w_h (min)	A_S	R_S	H (mm)	t_R (min)	w_h (min)	A_S	R_S	H (mm)		t_R (min)	w_h (min)	A_S	R_S	H (mm)
Fructose	4.52	0.88	0.43	0.36	0.68	13.49	0.87	0.50	0.35	0.11	Glucose	6.88	1.92	3.97	0.02	2.11
Glucose	4.99	0.63	0.60	1.90	0.29	13.86	0.36	0.72	3.15	0.02	Fructose	6.93	0.82	1.46	2.26	0.38
Sucrose	6.63	0.39	0.66	2.39	0.06	15.62	0.30	1.41	3.41	0.01	Sucrose	10.10	0.83	0.98	0.69	0.18
Myo-inositol	8.07	0.32	0.66	3.17	0.03	17.01	0.18	1.57	6.46	0.003	*N*-methyl-DNJ	10.94	0.60	1.81	3.41	0.08
Galactinol	9.96	0.38	0.66	0.25	0.03	19.38	0.25	1.47	20.94	0.01	DMDP	14.69	0.69	1.32	3.32	0.06
DMDP	10.17	0.64	1.05	0.13	0.07	31.17	0.41	1.05	0.30	0.01	*Myo*-inositol	18.02	0.49	1.54	4.86	0.02
N-methyl-DNJ	10.32	0.65	1.04	0.81	0.07	31.39	0.43	1.20	0.49	0.01	Galactinol	21.95	0.46	1.67	18.07	0.01
Miglitol	11.19	0.63	1.09	0.50	0.06	31.74	0.43	1.46	0.28	0.01	Miglitol	43.65	0.95	1.62	0.44	0.01
DMJ	11.75	0.67	0.99	1.47	0.06	31.95	0.42	1.28	0.13	0.01	DMJ	44.50	1.36	2.11	1.12	0.02
DNJ	13.23	0.52	1.09	0.26	0.03	32.06	0.58	1.34	0.48	0.01	α-HNJ	46.77	1.03	1.76	0.34	0.01
α-HNJ	13.46	0.54	0.49	-	0.03	32.47	0.44	1.24	-	0.01	DNJ	47.36	1.02	1.36	-	0.01

- *Effect of ammonium hydroxide*

Chromatographic behaviour of carbohydrate standards eluted under basic conditions in each of the three hydrophilic columns under study was also evaluated; data are shown in **Table 5.3**. Similarly to acidic conditions, the shortest retention times were obtained in the PHEA column (3.2-9.9 min), whereas the longest retentions were observed in the BEH column (28.9-49.0 min) in spite of the higher flow used (0.4 mL min^{-1}) for this last column. In general, a similar elution order of LMWC and iminosugars was observed in the three columns, with the exception of DMDP and DMJ which noticeably changed depending on the stationary phase used, and no differential elution zones could be distinguished in either of them. Tertiary *N*-substituted iminosugars (*N*-methyl-DNJ and miglitol) and fructose eluted first in the three columns, whereas *myo*-inositol and galactinol were the most retained standards. Under these basic conditions (see working $^{S}_{W}pH$ in **Table 5.1**), all stationary phases were negatively charged [231], whereas iminosugars were not charged and, therefore, their elution behaviour could be similar to that of sugars and inositols. The higher retention of sucrose, *myo*-inositol and galactinol could be due to the higher hydrophilic character of these molecules.

Concerning resolution, most of the compounds were well resolved in the BEH column, with the exception of *N*-methyl-DNJ/fructose, DMDP/glucose and DNJ/α-HNJ. In general, poorer resolution values were obtained for both the ZIC and PHEA columns, in which several coelutions were observed and only highly retained compounds (e.g. *myo*-inositol and galactinol) were baseline resolved.

BEH column provided the best efficacies (H = 0.01-0.02 mm), although peaks were broader (w_h= 0.55-0.83 min) than those obtained in the PHEA column (w_h = 0.42-0.54 min). Regarding peak shape, the worst results were obtained for the zwitterionic column (many peaks showed A_s >1.5), whereas PHEA and BEH columns provided more Gaussian peaks.

Table 5.3. Chromatographic parameters for the three studied hydrophilic stationary phases using 0.1 % NH_4OH as additive.

Compound	PHEA					Compound	BEH					Compound	ZIC				
	t_R (min)	w_h (min)	A_S	R_S	H (mm)		t_R (min)	w_h (min)	A_S	R_S	H (mm)		t_R (min)	w_h (min)	A_S	R_S	H (mm)
***N*-methyl-DNJ**	3.24	0.42	1.25	0.20	0.30	***N*-methyl-DNJ**	28.86	0.70	1.40	0.27	0.02	***N*-methyl-DNJ**	10.94	0.58	1.72	0.09	0.08
Fructose	3.40	0.54	1.21	0.26	0.45	**Fructose**	29.17	0.68	1.38	1.33	0.02	**Fructose**	11.04	0.65	1.59	0.68	0.09
Miglitol	3.61	0.42	0.81	0.47	0.24	**Miglitol**	30.64	0.60	1.42	1.28	0.01	**Miglitol**	11.83	0.72	2.02	1.12	0.10
Glucose	3.96	0.46	0.84	0.63	0.24	**DMDP**	32.15	0.78	1.90	0.12	0.02	**Glucose**	13.32	0.85	1.05	0.90	0.11
DNJ	4.49	0.52	0.75	0.10	0.24	**Glucose**	32.31	0.76	0.82	0.83	0.01	**DNJ**	14.30	0.43	1.21	0.40	0.02
α-HNJ	4.58	0.52	0.74	0.25	0.23	**DNJ**	33.24	0.55	0.85	0.51	0.01	**DMDP**	14.69	0.58	1.40	0.03	0.04
DMDP	4.79	0.47	0.93	0.68	0.17	**α-HNJ**	33.81	0.75	1.51	1.53	0.01	**α-HNJ**	14.73	0.56	2.02	1.41	0.04
Sucrose	5.33	0.46	0.67	0.53	0.13	**DMJ**	35.65	0.67	1.45	1.74	0.01	**DMJ**	16.30	0.76	2.12	0.20	0.06
DMJ	5.75	0.47	0.64	1.46	0.12	**Sucrose**	37.51	0.59	1.40	3.61	0.01	**Sucrose**	16.53	0.60	2.09	1.47	0.04
***Myo*-inositol**	6.98	0.52	0.72	3.40	0.10	***Myo*-inositol**	41.28	0.64	1.40	6.22	0.01	***Myo*-inositol**	18.02	0.59	1.87	4.23	0.03
Galactinol	9.95	0.50	0.53	-	0.05	**Galactinol**	49.02	0.83	0.76	-	0.01	**Galactinol**	21.95	0.51	1.95	-	0.02

5.1.3.2. Selection of hydrophilic column

Considering the results discussed above for the analysis of standards, each stationary phase working under acidic or basic conditions showed different advantages and disadvantages depending on the type of compound considered. The selection of the stationary phase to be used in the development of a HILIC method should therefore take into account the aim and preferences set for the analysis.

In order to provide an overview of the behavior of the three columns under study against the different analyzed compounds, a principal component analysis (PCA) of chromatographic data was carried out. The total time of analysis (t_a) and the average value for peak width ($\overline{w_h}$), asymmetry ($\overline{A_S}$) and resolution ($\overline{R_S}$) calculated from data in **Tables 5.2 and 5.3** were the variables compiled and subjected to PCA (**Table 5.4**). These data were mean centered and standardized prior to PCA.

Table 5.4. Values of the variables subjected to PCA.

	t_a (min)	$\overline{w_h}$ (min)	$\overline{A_S}$	$\overline{R_S}$
PHEA$_{acidic}$	13.46	0.568	0.204	1.124
BEH$_{acidic}$	32.47	0.425	0.204	3.599
ZIC$_{acidic}$	47.36	0.925	0.782	3.453
PHEA$_{basic}$	9.95	0.482	0.174	0.798
BEH$_{basic}$	49.02	0.686	0.299	1.744
ZIC$_{basic}$	21.95	0.621	0.731	1.053

Figure 5.1. depicts a PCA biplot where variable loadings are represented as vectors, and scores (chromatographic columns) as dots. Several interesting facts can be observed. First, some degree of correlation between the variables used for column evaluation was found, as indicated by the low angle values between $\overline{R_S}$ and t_a vectors on one hand, and $\overline{w_h}$ and $\overline{A_S}$ vectors on the other hand. Thus, under the working conditions used, a higher resolution implies a higher time of analysis, and also broader peaks are associated to more asymmetry.

As far as columns are concerned, the first remarkable fact is that PHEA$_{acidic}$ and PHEA$_{basic}$ are very close in the biplot, indicating that its behavior is rather similar under

acidic and basic conditions. In contrast, the behavior of both BEH and ZIC columns is very different when the eluent is acidic or basic. Therefore, it seems that the retention mechanism in BEH and ZIC columns is noticeably affected by the pH values.

Finally, the relationships between columns and variables can be easily obtained, just projecting a perpendicular line from every column to the vector variables included in the biplot. The choice of the best column for the intended separation will be based on these relationships. Considering the low average resolution and the overall non-adequate symmetry provided, respectively, by the PHEA and the ZIC columns, the BEH hydrophilic phase was selected as optimal for further analysis of vegetable extracts. For this selected column, appropriate resolution between target carbohydrates with intermediate analysis times and acceptable peak widths was achieved under both acidic and basic conditions.

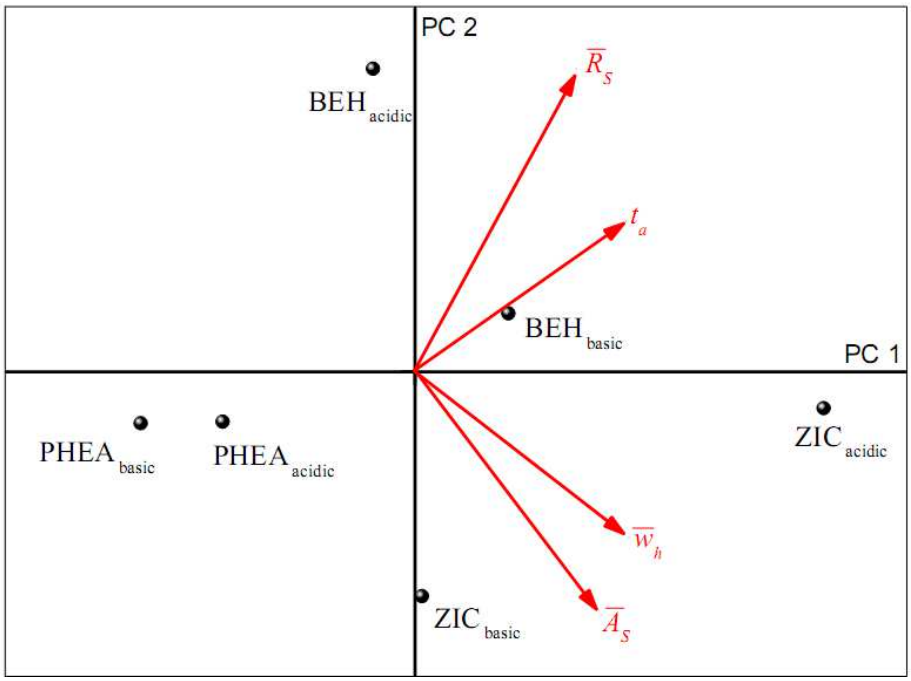

Figure 5.1. PCA biplot of chromatographic data obtained for the three HILIC columns studied working under basic (0.1% ammonium hydroxide) and acidic (0.1% acetic acid) conditions.

5.1.3.3. Analytical characterization

Once the most suitable hydrophilic column was selected, the study of different analytical parameters was addressed in order to evaluate the influence of the additive on both the sensitivity and precision of the method. As the optimized conditions were intended to be applied to the characterization of different vegetable extracts, and taking into account the advantages of MS^2 data for both the confirmation of identifications previously reported [117, 227] and the structural characterization of unknowns, evaluation of quantitative response obtained using a hybrid QTOF mass analyser was done. *LOD* and *LOQ* and precision (*RSD*, %) data calculated for a standard mixture including DNJ, *α*-HNJ, glucose and sucrose, analysed under acidic and basic conditions using the BEH column, are shown in **Table 5.5**.

Similar *LOD* and *LOQ* values were obtained for both iminosugars and LMWC under acidic conditions; sensitivity of the method was higher for sucrose as compared to the remaining standards. However, noticeable differences were observed under basic conditions, for which *LOD* and *LOQ* of iminosugars were about 10 times lower than those of LMWC. Good precision values were obtained for all standards using both additives (*RSD* ranging between 1.5 and 7.6%).

Since the highest sensitivity of HILIC-QTOF MS analysis of iminosugars was achieved using 0.1% ammonium hydroxide, and taking into account the generally low concentration of these bioactives in natural sources, this additive was selected for subsequent analyses of vegetable extracts using the BEH column.

Table 5.5. Limits of detection (*LOD*) and quantitation (*LOQ*) and precision (*RSD*) data for a standard mixture analysed by using the BEH column and either acetic acid or ammonium hydroxide as mobile phase additives.

Compound	Mobile phase additive					
	Acetic Acid			Ammonium Hydroxide		
	LOD (ng mL^{-1})	*LOQ* (ng mL^{-1})	Precision (*RSD*,%)	*LOD* (ng mL^{-1})	*LOQ* (ng mL^{-1})	Precision (*RSD*,%)
DNJ	0.14	0.46	5.21	0.025	0.08	4.01
α-HNJ	0.15	0.51	5.50	0.030	0.10	3.54
Glucose	0.10	0.33	5.42	0.28	0.92	4.93
Sucrose	0.050	0.168	7.59	0.14	0.45	1.47

5.1.3.4. Aplication to vegetable extracts

- *Qualitative analysis*

Retention times of the carbohydrates detected in the HILIC-QTOF MS analysis of hyacinth, buckwheat and mulberry extracts are shown in **Table 5.6**. MS^2 data of these compounds are also provided as **Figure 3S** (**Anexo II**). In hyacinth extract (**Figure 5.2**), DNJ, HNJ and DMJ (peaks 4, 5 and 7, respectively) were first identified by comparison of their retention times with those of corresponding standards. MS^2 data of standards were further used for confirmation purposes. Fragmentation patterns for these standards (see **Figure 3S**, **Anexo II**) were characterized by three successive losses of water molecules ($[(M+H)-18]^+$, $[(M+H)-36]^+$ and $[(M+H)-54]^+$) and a subsequent loss of 30 *m/z* units which could correspond to a CHOH group. The same array of product ions but with different intensities have also been reported by Egan *et al*. [227] when each of these standards were analysed by using a quadrupole ion trap mass spectrometer. Peak 6 of **Figure 5.2** showed a $[M+H]^+$ ion at *m/z* 194.1 and a MS^2 fragmentation pattern similar to that of HNJ. This peak was tentatively assigned as HMJ according to data previously reported in the GC-MS analysis of *Hyacinthus orientalis* extracts [21 and **section 4.2**]. Three peaks with $[M+H]^+$ ions at *m/z* 356.1 and similar MS^2 fragmentation patterns were also detected. Tandem mass spectra (see **Figure 3S**, **Anexo II**) for these compounds showed a base peak at *m/z* 194.1 corresponding to the loss of a glycosyl unit $[M+H-162]^+$ and the three characteristic successive losses of water from this ion, as previously described for *α*-HNJ. Therefore, these peaks were tentatively assigned as glycosyl-HNJ isomers (peaks 10). Asano *et al*. [21] reported the presence of 7-*O*-*β*-*D*-glucopyranosyl-*α*-HNJ in *Hyacinthus orientalis* extracts after purification of this compound by ion-exchange chromatography and further characterization by optical rotation, fast atom bombardment mass spectrometry (FABMS) and nuclear magnetic resonance (NMR). Therefore, the most abundant of these peaks eluting at 42.8 min (**Figure 5.2**) was assigned as 7-*O*-*β*-*D*-glucopyranosyl-*α*-HNJ, whereas peaks with retention times of 39.9 and 40.6 min could correspond to two other isomers of this compound with empiric formula $C_{13}H_{25}NO_{10}$ present at lower concentrations in this extract. On the other hand, three peaks with quasimolecular ions at *m/z* 178.1 and similar MS^2 spectra, compatible with those of

imino-trideoxy-heptitol structures, were also detected (peaks 2) (See MS^2 spectra in Supplementary Data). Asano *et al*. [21] described the presence of 2,5-imino-2,5,6-trideoxy-*D*-*manno*-heptitol and 2,5-imino-2,5,6-trideoxy-*D*-*gulo*-heptitol in *Hyacinthus orientalis* extract. These compounds could be two of the peaks here detected; however, the identity of the third of these isomers could not be confirmed. Other LMWC such as fructose (peak 1), glucose (peak 3), *myo*-inositol (peak 11), sucrose (peak 8) and glycosyl-inositols (peaks 9) were also detected.

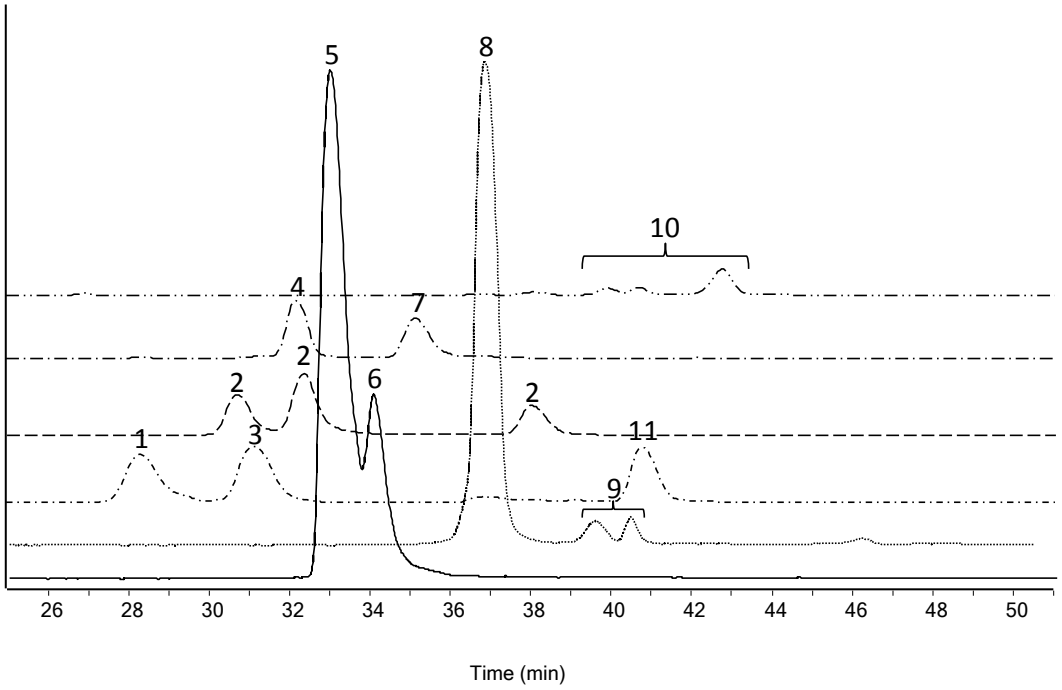

Figure 5.2. Extracted ion chromatographic profile of hyacinth extract obtained using the BEH column and 0.1% ammonium hydroxide as mobile phase additive: fructose, 1; imino-trideoxy-heptitol, 2; glucose, 3; DNJ, 4; HNJ, 5; HMJ, 6; DMJ, 7; sucrose, 8; glycosyl-inositols, 9; 7-O-*β*-D-glucopyranosyl-*α*-HNJ and isomers, 10 and *myo*-inositol, 11.

Concerning mulberry extract, the HILIC-QTOF MS profile (**Figure 5.3**) revealed the presence of *N*-methyl-DNJ and DNJ (peaks 1 and 4, respectively) which was confirmed by using both retention and MS^2 data for standards (**Table 5.6** and **Figure 3S** in **Anexo II**, respectively). Fagomine (peak 6) was identified by comparing its MS^2 spectra with data previously reported by Nakagawa *et al*. [17] for this iminosugar analyzed using a triple quadrupole mass analyzer. The same array of product ions was obtained in both configurations, although different ion intensities were observed. Ions corresponding to three consecutive losses of water molecules (*m/z* 130.1, 112.1 and 94.1) were

detected. At least four peaks with quasimolecular ions at *m/z* 326.1 and tandem mass spectral data (*m/z* 164.1 and 146.1; see **Figure 3S**, **Anexo II**) compatible with a glycosyl-DNJ isomer structure were detected (peaks 7). Nakawaga *et al*. [17] previously detected using a triple quadrupole a compound in mulberry leaves with this *m/z* ratio and similar MS^2 fragmentation pattern which was assigned as galactosyl-DNJ (Gal-DNJ). Nevertheless, the presence of other isomers was not previously described. On the other hand, Asano *et al*. [51] isolated by ion-exchange resins and identified by NMR nine glycosyl-iminosugars (galactosyl- and glucosyl-) in *Morus alba* roots extracts. Therefore, peaks 7 here detected were assigned to glycosyl-DNJ isomers.

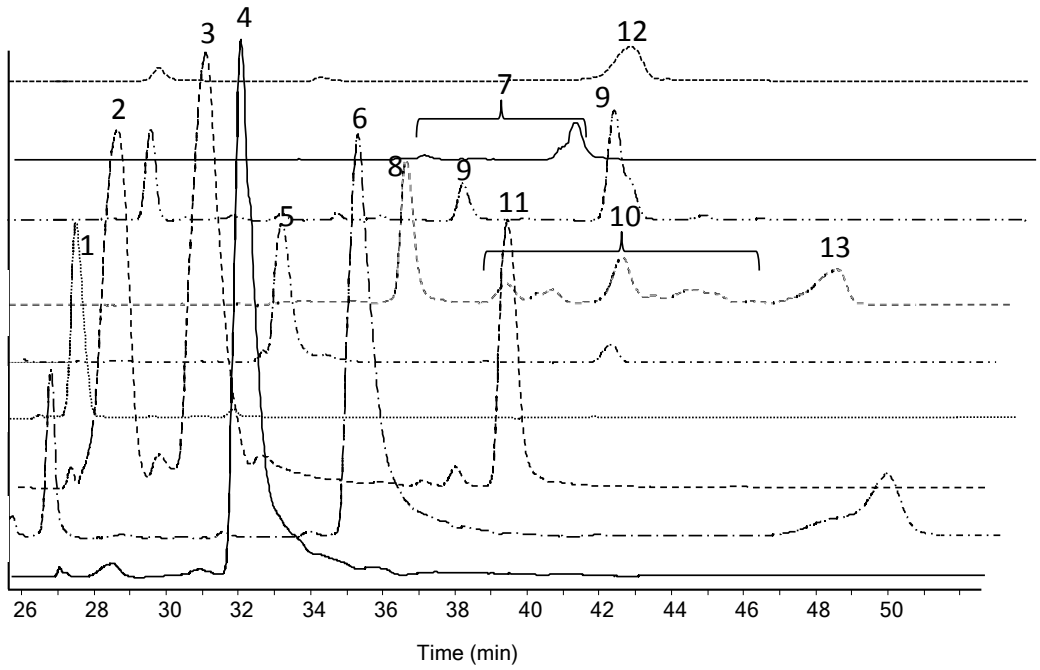

Figure 5.3. Extracted ion chromatographic profile of mulberry extract obtained using the BEH column and 0.1% ammonium hydroxide as mobile phase additive: *N*-methyl-DNJ, 1; fructose, 2; glucose, 3; DNJ, 4; pentosyl-iminosugar, 5; fagomine, 6; glycosyl-DNJ isomers, 7; sucrose, 8; glycosyl-fagomine isomers, 9; glycosyl-inositols, 10; *myo*-inositol, 11; 1,4-dideoxy-1,4-imino-(2-O-*β*-*D*-glucopyranosyl)-*D*-arabinitol, 12 and galactinol, 13.

An iminosugar with quasimolecular ion at *m/z* 296.1 was also detected at a retention time of 43.3 min (peak 12). Its $[M+H]^+$ adduct ion and MS^2 fragmentation pattern (*m/z* 134.1, 116.1 and 85.1) agree with data of 1,4-dideoxy-1,4-imino-(2-*O*-*β*-*D*-glucopyranosyl)-*D*-arabinitol, previously found by Asano *et al*. [46] in *Morus alba* root extracts. Two peaks (34.7 and 36.7 min) with $[M+H]^+$ ion at *m/z* 134.1 were also

detected and assigned, according to the same authors, to 1,4-dideoxy-1,4-imino-*D*-ribitol and 1,4-dideoxy-1,4-imino-*D*-arabinitol.

Different ions (*m/z*: 176.1, 192.1, 256.1 and 310.1) with an even mass to charge ratio compatible with the $[M+H]^+$ adduct of an iminosugar were also detected and evaluated as potential non previously identified iminosugars. Two peaks with quasimolecular ions at *m/z* 176.1 and one peak with $[M+H]^+$ at *m/z* 192.1, all of them showing characteristic consecutive losses of water molecules in their MS^2 fragmentation profiles, were detected. However, only elemental formulas of $C_7H_{13}NO_4$ and $C_7H_{13}NO_5$ could be respectively proposed for these compounds.

Peak 5, with $[M+H]^+$ 256.1 *m/z* ion showed a MS^2 spectrum characterized by a *m/z* 124.0 ion corresponding to the loss of 132 *m/z* units from $[M+H]^+$ corresponding to a pentosyl group. This compound could be tentatively assigned to a pentosyl-iminosugar. Finally, two peaks with retention times 38.0 and 42.9 and $[M+H]^+$ 310.1 *m/z* were detected (peaks 9). These compounds showed a MS^2 fragmentation profile characterized by the loss of a glycosyl group ($[(M+H)-162]^+$; *m/z* 148.1 ion, base peak) and the consecutive losses of three water molecules similarly to fagomine. Therefore, these compounds could be tentatively assigned to two glycosyl-fagomine isomers. To the best of our knowledge, these glycosyl-iminosugars have not been previously detected in mulberry leaves. Other carbohydrates such as fructose (peak 2), glucose (peak 3), sucrose (peak 8), *myo*-inositol (peak 11), galactinol (peak 13) and other glycosyl-inositols (peaks 10) were also identified.

Regarding the buckwheat HILIC-QTOF MS profile (**Figure 5.4**), fagomine (peak 3) was the only iminosugar identified by the characteristic daughter ions present in its MS^2 spectrum (**Figure 3S**, **Anexo II**). Fructose, glucose, sucrose, *chiro*-inositol, *myo*-inositol and two fagopyritols corresponding to fagopyritol A1 and B1 (peaks 1, 2, 4, 5, 6 and 7 respectively), which have been previously described by Ruiz-Aceituno *et al*. [21, 102] were also detected.

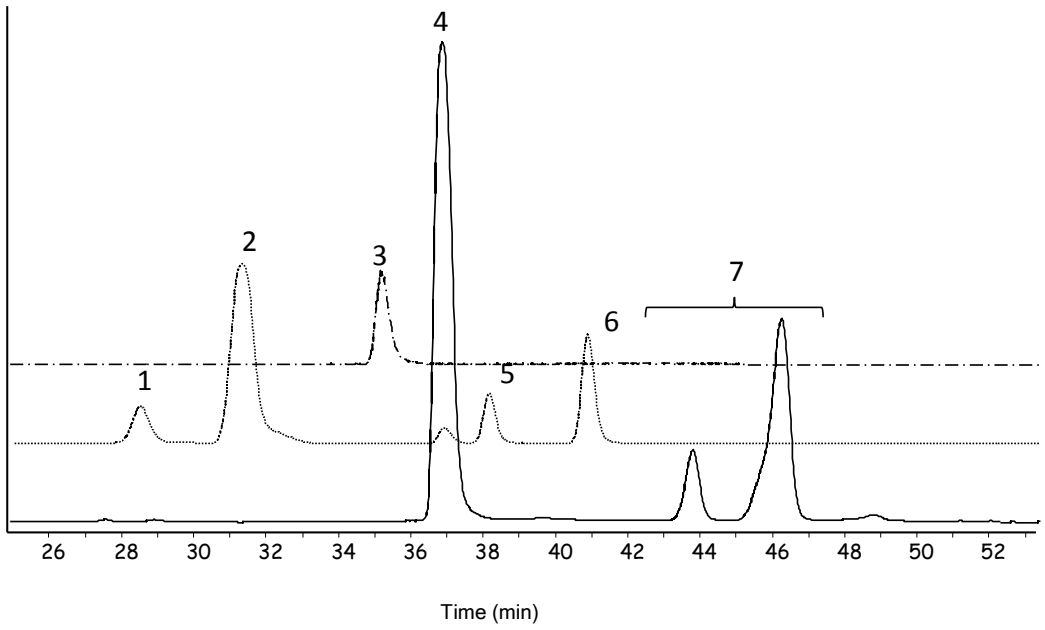

Figure 5.4. Extracted ion chromatographic profile of buckwheat extract obtained using the BEH column and 0.1% ammonium hydroxide as mobile phase additive: fructose, 1; glucose, 2; fagomine, 3; sucrose, 4; *chiro*-inositol, 5; *myo*-inositol, 6; fagopyritols, 7.

- *Quantitative analysis*

Once the iminosugar composition of the vegetable extracts under study was identified, the possible matrix effect on quantitation was ruled out by analyzing different dilutions (1:1-1:500, v/v) of each extract. Quantitative data (mg g^{-1}) for both iminosugars and other LMWC determined in hyacinth, mulberry and buckwheat extracts are listed in **Table 5.6**.

Mulberry showed the widest diversity of iminosugars and the highest amounts of other LMWC. Relatively high amounts of fagomine (0.37 mg g^{-1}) were detected in mulberry, whereas only small concentrations of this iminosugar were found in buckwheat extract (0.011 mg g^{-1}). The two isomers tentatively assigned as glycosyl-fagomine were detected at low concentrations (0.0035 and 0.016 mg g^{-1}) in mulberry extract. HNJ (1.5 mg g^{-1}) was the most abundant iminosugar present in hyacinth extract, followed by DNJ (0.175 mg g^{-1}); DMJ and HMJ were also detected at minor concentrations (0.056 mg g^{-1} and 0.85 mg g^{-1}, respectively). Buckwheat was the only extract that presented fagopyritols (2.06 mg g^{-1}) and *chiro*-inositol (0.24 mg g^{-1}).

Table 5.6. Content of iminosugars and other LMWC in hyacinth, mulberry and buckwheat extracts analyzed using the BEH stationary phase with 0.1% ammonium hydroxide as mobile phase additive.

Compound	t_R (min)	Parent ion** (*m/z*)	Mean content (mg g^{-1} sample)		
			Hyacinth	Mulberry	Buckwheat
***N*-methyl-DNJ**	27.4	178	-	0.064 (0.001) *	-
Fructose	28.8	198	4.82 (0.32)	7.0324 (0.0007)	0.44 (0.02)
Imino-trideoxy-heptitol	30.7	178	0.082 (0.002)	-	-
Glucose	31.2	198	2.48 (0.20)	7.918 (0.068)	3.84 (0.02)
DNJ	32.1	164	0.175 (0.007)	1.15(0.06)	-
Imino-trideoxy-heptitol	32.3	178	0.16 (0.01)	-	-
HNJ	33.0	194	1.50 (0.67)	-	-
Pentosyl-iminosugar	33.6	256	-	0.070 (0.002)	-
HMJ	34.0	194	0.085 (0.003)	-	-
1,4-dideoxy-1,4-imino-*D*-ribitol	34.7	134	-	0.005 (0.001)	-
Fagomine	35.1	148	-	0.37 (0.01)	0.011 (0.001)
DMJ	35.1	164	0.056 (0.005)	-	-
Glycosyl-DNJ	36.7	326	-	0.0047 (0.0003)	-
1,4-dideoxy-1,4-imino-*D*-arabinitol	36.7	134	-	0.014 (0.001)	-
Sucrose	36.8	360	3.21 (0.14)	4.49 (0.02)	2.16 (0.17)
Glycosyl-DNJ	37.4	326	-	0.0048 (0.0003)	-
Imino-trideoxy-heptitol	37.8	178	0.060 (0.002)	-	-
Glycosyl-fagomine	38.0	310	-	0.0035 (0.0007)	-
***Chiro*-inositol**	38.2	198	-	-	0.24 (0.02)
Glycosyl-HNJ	39.9	356	0.0363 (0.0001)	-	-
Glycosyl-inositols	39.5-46.0	360	0.185 (0.005)	0.22 (0.01)	-
Glycosyl-HNJ	40.6	356	0.005 (0.002)	-	-
***Myo*-inositol**	40.8	198	1.80 (0.06)	1.35 (0.05)	0.98 (0.05)
Glycosyl-DNJ	41.5	326	-	0.0264 (0.0008)	-
Glycosyl-DNJ	42.0	326	-	0.211 (0.001)	-
7-*O*-*β*-*D*-glucopyranosyl-*α*-HNJ	42.8	356	0.35 (0.01)	-	-
Glycosyl-fagomine	42.9	310	-	0.016 (0.003)	-
1,4-dideoxy-1,4-imino-(2-*O*-*β*-*D*-glucopyranosyl)-*D*-arabinitol	43.3	296	-	0.0310 (0.002)	-
Fagopyritols	43.7-46.2	360	-	-	2.06 (0.03)
Galactinol	48.5	360	-	0.42 (0.03)	-

*Standard deviation in brackets (n = 5)

**Adducts: $[M+H]^+$ for iminosugars and $[M+NH_4]^+$ for LMWC

5.1.4. Conclusions

As conclusions, a HILIC-QTOF MS^2 method using a BEH hydrophilic column and acetonitrile:water with 0.1 % ammonium hydroxide as mobile phase has been developed for the determination of bioactive iminosugars in vegetable extracts. This method has allowed for the first time the simultaneous and efficient analysis, without a prior derivatization process, of the complex mixtures of target iminosugars and interfering LMWC usually present in this kind of extracts. The method here optimized has also allowed the sensitive quantitation of these bioactives, which is an important task considering their low concentrations as compared to other co-extracted LMWC. Tandem mass spectra have been used to confirm the presence of several previously detected iminosugars and to tentatively identify for the first time other iminosugars (e.g. glycosyl-fagomine isomers) in mulberry, buckwheat and hyacinth extracts.

5.2. Hydrophilic interaction liquid chromatography (HILIC) coupled to mass spectrometry for the analysis of iminosugars and other low molecular weight carbohydrates of Aglaonema extracts

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Journal of agricultural and Food Chemistry (submitted)

5.2.1. Introduction

Iminosugars, also called azasugars, are polyhydroxyalkaloids with a potent *α*-glycosidase inhibition activity [21, 232], which have been described of usefulness for treating metabolic disorders such as diabetes and obesity [9, 38, 69, 233]. Several works have been focused on the chemical synthesis of these carbohydrates [61, 218, 234]; however, their extraction from natural sources is preferred by the food industry with the aim of incorporating these bioactive compounds as ingredients of functional foods.

Aglaonema is a genus of ornamental plants of the Araceae family native from tropical and subtropical regions of Asia. There are about 21 species described and most of the commercially grown plants are hybrids. Leaf extracts from *Aglaonema commutatum* 'Treubii' have been found to strongly inhibit *α*-glucosidases. These bioactive properties are attributed to its polyhydroxylated pyrrolidine and piperidine alkaloid composition, including *α*- and *β*-homonojirimycin (*α*- and *β*-HNJ), *α*- and *β*-homomannojirimycin (*α*- and *β*-HMJ) and 7-O-*β*-*D*-glucopyranosyl-*α*-HNJ (Glc-HNJ), among others [11].

Different methods based on gas chromatography (GC) or liquid chromatography (LC) have been proposed for the analysis of iminosugars such as 1-deoxynojirimycin (DNJ) or fagomine, present in mulberry extracts [17, 70]. However, scarce attention has been paid to the characterization of other vegetable extracts such as *Aglaonema commutatum* 'Treubii' extracts with a more complex iminosugar composition [11, 21]. Moreover, iminosugars are generally present at low concentrations in natural sources together with other low molecular weight carbohydrates (LMWC) which may interfere

in their bioactivity. References regarding the simultaneous analysis of all these carbohydrates are limited in the literature (**sections 4.1**, **4.2** and **5.1** of this Thesis).

We have recently developed a hydrophilic interaction liquid chromatographic coupled to tandem mass spectrometry (HILIC-MS^2) method for the analysis of iminosugars and other LMWC present in hyacinth, mulberry and buckwheat extracts (**section 5.1**). In that work, the performance of three different stationary phases on the basis of peak symmetry, retention, efficacy, resolution and peak width of target compounds was evaluated. The best results were obtained using an ethylene bridge hybrid (BEH) with trifunctionally bonded amide phase.

Mobile phase strength is also an important parameter in HILIC, as its main separation mechanism seems to be based on the partitioning between a water-enriched layer on the surface of the polar stationary phase and the relatively hydrophobic eluent [229]. In the present work we have extended the optimization of the previously developed HILIC method, by evaluating different conditions such as eluting solvents, mobile phase additives and column temperature. This new method has been applied to the analysis of iminosugars and other LMWC present in different Aglaonema extracts. The use of a tandem MS detector has also allowed the elucidation of new iminosugar structures in these extracts.

5.2.2. Materials and methods

5.2.2.1. Standards

2,5-dideoxy-2,5-imino-*D*-mannitol (DMDP) and *α*-HNJ were purchased from Dextra Laboratories (Reading, UK), whereas fructose, glucose, *myo*-inositol, and sucrose were obtained from Sigma (St. Louis, USA). Miglitol (1-(2-hydroxyethyl)-2-(hydroxymethyl)-piperidine-3,4,5-triol) (Distabol, Sanofi-Aventis) was acquired in a local pharmacy.

5.2.2.2. *Aglaonema* sp. Extracts

Two plants of *A. commutatum* 'Treubii' (ACT1 and ACT2), two of *A. commutatum* 'Crete' (ACC1 and ACC2), one of *A. commutatum* 'Emmerald beauty' (ACEB) and one of *A.* hybrid 'Black lance' (AHBL) were acquired in local garden centres in Madrid.

Aglaonema extracts were obtained by conventional solid-liquid extraction, as previously described in **section 5.1**.

5.2.2.3. LC-MS analyses

The two LC-MS instruments (both from Agilent Technologies, Santa Clara, CA, USA) and the operating conditions (spray voltage, drying gas temperature and flow, nebulizer pressure, fragmentor voltage, etc.) previously described in **section 5.1.2** of this Thesis were used in this study for analysis of iminosugars and other LMWC in Aglaonema extracts. As previously mentioned, MS^2 spectra carried out on both standards and compounds present in Aglaonema extracts were used to confirm the identity of some iminosugars and for characterization of unknowns. Selected precursor ions in tandem mass spectra experiments were $([M+H]^+$ for iminosugars and $[M+NH4]^+$ for other LMWC. Data acquisition and processing were performed using Agilent Mass Hunter Workstation Acquisition Rev. B.02.00 software.

LC experiments were carried out on an ethylene bridge hybrid with trifunctionally bonded amide phase (XBridge column (BEH); 150 mm x 4.6 mm, 3.5 µm, 135 Å pore size; Waters, Hertfordshire, UK). Different binary (acetonitrile:water; methanol:water) gradients, additives (0.1% acetic acid, 0.1% formic acid, 0.1% ammonium hydroxide or different concentrations of ammonium acetate) and column temperatures (25-65°C) were assayed. Injection volume was 5 µL and flow rate was 0.4 mL min^{-1} (value in the range of optimal theoretical flows for BEH column and compatible with MS detection).

Different chromatographic parameters, previously defined in **section 5.1.2**, were considered for optimization of HILIC methods: retention time (t_R), peak width at half height (w_h), asymmetry factor (A_S) and resolution (R_S).

Quantitative analysis of iminosugars and interfering LMWC in Aglaonema extracts by $LC\text{-}MS^2$ was performed in triplicate using external standard calibration curves of glucose, sucrose, DMDP and α-HNJ within the range 0.0125-100 µg mL^{-1}. Goodness of fitting for these calibration curves was evaluated from their correlation coefficients. Prior to quantitation, matrix effect was evaluated by quantifying target analytes in ACT1 extract diluted in water at different ratios (1:1-1:500, v/v).

Reproducibility of the method was measured on the basis of the intra-day precision, calculated as the relative standard deviation (*RSD*, %) in *n* = 5 independent measurements of concentrations of iminosugar and LMWC standards. Limits of detection (*LOD*) and quantitation (*LOQ*) were calculated as three and ten times the standard deviation of noise (σ), respectively.

5.2.2.4. $_{w}^{S}pH$ measurement

Determination of the pH of the hydro-organic solutions ($_{w}^{S}pH$) used as mobile phase was carried out as previously describes in **section 5.1.2** of this Thesis.

5.2.3. Results and discussion

5.2.3.1. Mobile phase strength

In order to study the effect of the strength of the mobile phase on carbohydrate separation, different isocratic mixtures of acetonitrile (one of the weakest solvents in HILIC) and water (one of the strongest solvents) were assayed for analysis of ACT1 leaf extract. As previously suggested in **section 5.1**, 0.1% ammonium hydroxide was used as additive in both eluents.

Fructose, glucose, sucrose, *myo*-inositol, DMDP and α-HNJ were identified by comparison of their retention times and MS data with those of corresponding commercial standards. One peak with *m/z* ion compatible with a glycosyl-HNJ structure (probably 7-O-β-*D*-glucopyranosyl-α-HNJ, as previously identified in *Aglaonema* 'Treubii' by Asano *et al*. [11]) was also selected to evaluate HILIC retention. **Figure 5.5** shows the t_R of these compounds under the different isocratic conditions (20-50% water in eluent) assayed. As expected, t_R values were inversely proportional to the percentage of water, and coelution of several carbohydrates was observed at both 40 and 50% of water. The best separation was achieved with 20% of water, but retention times for some carbohydrates were extremely high (e.g. 69.7 min for glycosyl-HNJ). Only slight changes were observed in the elution order of carbohydrates with the different conditions.

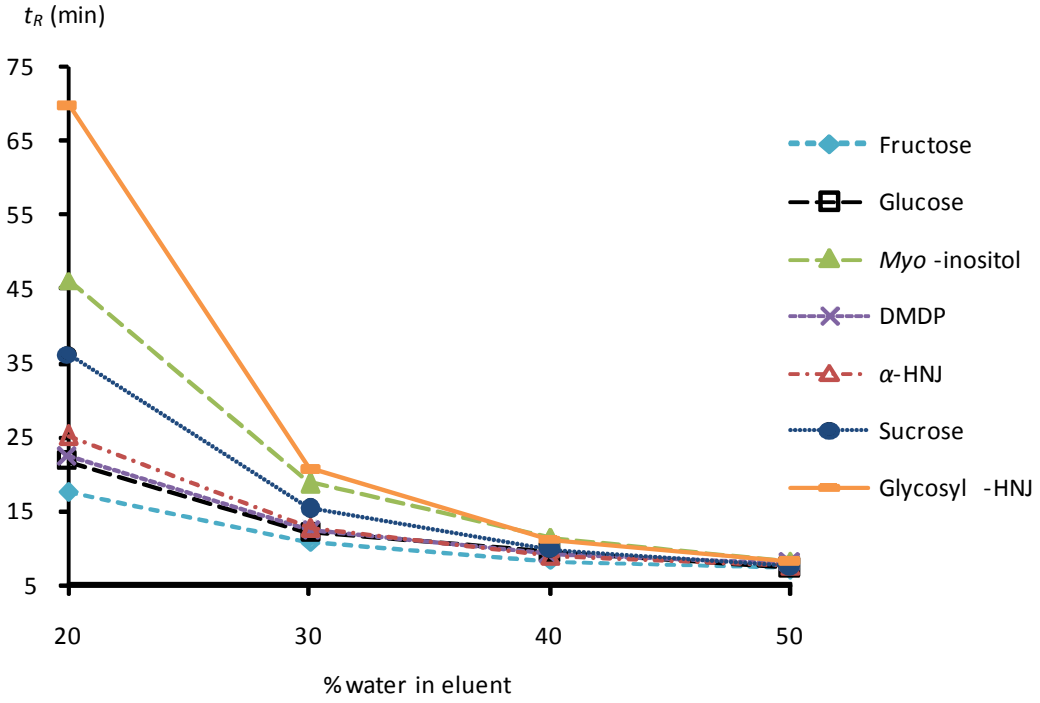


Figure 5.5. Retention times (t_R, min) obtained under isocratic conditions for different water volume ratios in the eluent (acetonitrile:water with 0.1% NH_4OH).

Methanol was also evaluated as organic modifier of the eluent. Ratios of methanol:water between 80:20 and 50:50 (v/v), with 0.1% ammonium hydroxide as additive, were isocratically assayed. As shown in **Figure 5.6**, all carbohydrates were less retained with the use of methanol as modifier and t_R values increased with the percentage of water in the eluent, mainly in the 20-40% range. Contrarily to the separation obtained by using acetonitrile in which HILIC mode seems to be the predominant mechanism, a reverse phase type separation is observed with methanol as organic modifier. The lack of HILIC interactions could also support the similar retention times observed for all the carbohydrates under study, irrespective of the water percentage in the eluent (**Figure 5.6**). Based on these results, binary mixtures acetonitrile:water were selected for further studies.

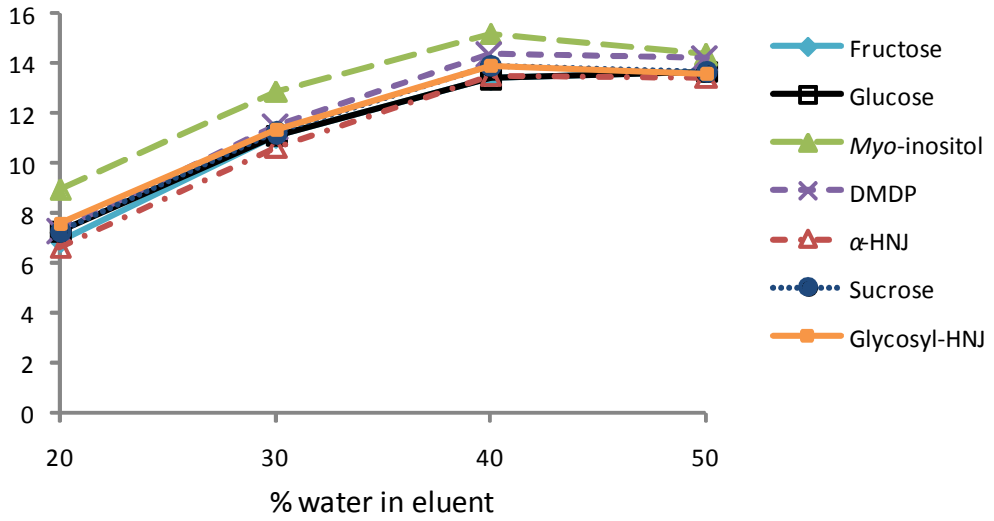


Figure 4.17. Retention times (t_R, min) obtained under isocratic conditions for different water volume ratios in the eluent (methanol:water with 0.1% NH_4OH).

5.2.3.2. Acid and basic mobile phase additives

Different gradients of acetonitrile:water using formic acid, acetic acid and ammonium hydroxide as additives were assayed for the analysis of ACT1 leaf extract. **Table 5.7** shows the best elution conditions for each of these mobile phase additives together with the chromatographic parameters (t_R, w_h, A_s and R_s) obtained for target carbohydrates.

As previously reported in **section 5.1** for mulberry, buckwheat and hyacinth extracts, two eluting zones were clearly distinguished in Aglaonema HILIC profiles using formic acid as additive: (i) sugars and *myo*-inositol (13.0-16.3 min) and (ii) iminosugars (23.7-26.4 min). BEH is considered a neutral stationary phase and, at working pH ($^{S}_{W}pH$ = 2.97-2.85), residual surface silanol groups are not charged. Sugars and inositols are neutral compounds and they elute at shorter retention times. On the contrary, under these acid conditions, iminosugars (pK_b = 7.5-8) are positively charged and their higher retention could be explained as a result of polar hydrophilic interactions through partition mechanism (**section 5.1**)]. The same elution pattern was observed when acetic acid was used as additive, but retention times for iminosugars

were noticeably higher (31.5-33.5 min). Ionization of residual surface silanols under acetic acid conditions ($^{S}_{w}pH$ = 4.46-3.43) confers negative charge to the column and, therefore, electrostatic interactions with positively charged iminosugars could justify the retention behavior observed for these bioactives. For both formic acid and acetic acid conditions, resolution values in the range 1.1-2.0 were obtained for iminosugars and higher than 0.9 for interfering LMWC.

The elution order of iminosugars and LMWC from ACT1 extract was different using ammonium hydroxide as additive. Under these conditions ($^{S}_{w}pH$ = 9.70-9.63), iminosugars are not charged and, therefore, their elution behaviour could be similar to that of neutral sugars and *myo*-inositol. Fructose and glucose were the first to elute followed by DMDP and α-HNJ. Sucrose, *myo*-inositol and glycosyl-HNJ were the most retained compounds, probably due to their higher hydrophilic character. Except for glucose and DMDP, good resolution values (R_s ≥1) were achieved under these conditions.

Regarding peak width and symmetry of iminosugars, the best results were obtained under basic conditions (w_h = 0.4-0.5 min, A_S = 0.9-1.3). However, formic acid conditions allowed the elution of LMWC as the narrowest peaks (w_h = 0.2-0.3 min).

5.2.3.3. Mobile phase ionic strength

Ionic additives, such as ammonium acetate and ammonium formate, are also typically used to control the pH and ionic strength of the mobile phase. In this study, different concentrations (2-10 mM) of ammonium acetate in the aqueous phase were evaluated (**Table 5.8**). Using 2 mM ammonium acetate ($^{S}_{w}pH$ = 7.8-7.5), elution pattern of carbohydrates present in ACT1 extract was similar to that obtained under acidic conditions: sugars and *myo*-inositol eluted first followed by iminosugars. However, separation between both groups was not so drastic and retention order of iminosugars was noticeably affected: *α*-HNJ eluted first (15.4 min) followed by glycosyl-HNJ (17.1 min), whereas DMDP was highly retained (R_s = 20.0 between glycosyl-HNJ and DMDP). Nevertheless, smaller peak widths with better symmetry were obtained under ammonium acetate conditions.

Table 5.7. Chromatographic parameters obtained using different mobile phase additives and acetonitrile:water elution programs.

0.1% ammonium hydroxyde					**0.1% formic acid**					**0.1% acetic acid**				
Gradient of water: 20-50% in 30 min					**Gradient of water: 20-80% in 30 min**					**Gradient of water: 20-80% (5 min) in 30 min**				
Carbohydrate	**t_R (min)**	**w_h (min)**	**A_S**	**R_s**	**Carbohydrate**	**t_R (min)**	**w_h(min)**	**A_S**	**R_s**	**Carbohydrate**	**t_R (min)**	**w_h(min)**	**A_S**	**R_s**
Fructose	14.6	0.8	0.8	2.1	Fructose	13.0	0.2	1.0	2.5	Fructose	13.0	1.0	0.2	0.9
Glucose	16.1	0.6	2.1	0.4	Glucose	13.5	0.2	1.3	5.6	Glucose	13.6	0.4	0.9	4.0
DMDP	16.3	0.5	1.3	2.0	Sucrose	14.9	0.3	3.3	5.6	Sucrose	15.2	0.4	2.2	4.7
α-HNJ	17.2	0.4	1.0	6.0	*Myo*-inositol	16.3	0.2	1.2	9.9	*Myo*-inositol	16.6	0.2	1.6	33.1
Sucrose	19.6	0.4	1.1	6.0	DMDP	23.7	1.3	1.1	1.1	DMDP	31.5	0.7	2.6	2.0
Myo-inositol	22.0	0.4	0.8	2.9	α-HNJ	25.0	1.0	1.8	1.6	α-HNJ	32.7	0.5	1.4	1.6
Glycosyl-HNJ	23.3	0.5	0.9	---	Glycosyl-HNJ	26.4	0.8	2.7	---	Glycosyl-HNJ	33.5	0.5	1.8	---

As ionic strength increased, the retention times of iminosugars decreased while those of sugars and *myo*-inositol were scarcely affected. A change in retention order of *α*-HNJ, which eluted before *myo*-inositol using 5 mM ammonium acetate and before sucrose using 10 mM ammonium acetate, was thus observed. A similar behavior has been previously reported by Liu *et al*. [235] for the simultaneous analysis of 4-(aminomethyl)pyridine and its isomers using a HILIC column and a buffer with different concentrations (10-100 mM) of ammonium formate. As discussed by these authors, the competitive effect of the higher salt concentration in the eluent could affect the electrostatic interaction of protonated basic compounds (iminosugars) with surface silanol groups of the stationary phase and, therefore, their retention behaviour. Consequently, the scarce effect of salt concentration on sugar and inositol retention would point out to the absence of electrostatic interactions between these compounds and the ionized silanol groups of the stationary phase, and would support the HILIC partition as the main mechanism affecting their separation.

Regarding the different salt concentrations, the best peak width and symmetry values were achieved using 5 mM ammonium acetate (w_h = 0.2-0.5 min; A_S = 0.8-1.6). To keep salt concentration constant during analysis, 5mM ammonium acetate was also used as additive of both eluents (**Table 5.9**).

Similar peak widths and asymmetry values to those achieved using 5mM ammonium acetate in the aqueous phase (**Table 5.8**) were in general obtained and only slight differences in retention times and resolution data of iminosugars were found. These last results were also slightly better than those achieved with 0.1% NH_4OH and these conditions were, therefore, used for further experiments.

Table 5.8. Chromatographic parameters obtained using different ammonium acetate concentrations as additive of aqueous phase. acetonitrile:water elution program: 20-80% aq. phase in 25 min, 80% aq. phase for 5 min.

Carbohydrate	2 mM ammonium acetate (aq. phase)				Carbohydrate	5 mM ammonium acetate (aq.phase)				Carbohydrate	10 mM ammonium acetate (aq. phase)			
	t_R (min)	w_h (min)	A_S	R_s		t_R (min)	w_h (min)	A_S	R_s		t_R (min)	w_h (min)	A_S	R_s
Fructose	11.2	0.6	1.2	2.0	Fructose	11.4	0.5	0.9	2.0	Fructose	12.2	0.5	0.9	1.8
Glucose	12.2	0.4	0.7	4.6	Glucose	12.3	0.4	0.8	4.6	Glucose	12.9	0.3	0.7	4.4
Sucrose	13.8	0.3	1.4	4.8	Sucrose	13.9	0.3	1.4	2.7	α-HNJ	14.0	0.2	1.5	1.5
Myo-inositol	15.0	0.2	1.4	1.6	α-HNJ	14.7	0.3	1.6	1.2	Sucrose	14.3	0.2	1.4	6.5
α-HNJ	15.4	0.3	1.0	6.8	*Myo*-inositol	15.0	0.2	1.4	7.5	*Myo*-inositol	15.6	0.2	1.2	3.0
Glycosyl-HNJ	17.1	0.2	1.2	20.0	Glycosyl-HNJ	16.5	0.2	1.3	13.7	Glycosyl-HNJ	16.2	0.2	1.0	5.2
DMDP	26.1	0.7	0.4	---	DMDP	20.6	0.4	0.9	---	DMDP	17.5	0.3	1.4	---

Table 5.9. Chromatographic parameters obtained using 5 mM ammonium acetate as additive in both eluents. Acetonitrile:water elution program: 20-80% aq. phase in 25 min, 80% aq. phase for 5 min.

Carbohydrate	5 mM ammonium acetate (both eluents)			
	t_R (min)	w_h (min)	A_S	R_s
Fructose	11.5	0.6	1.0	1.8
Glucose	12.2	0.2	0.8	8.5
Sucrose	13.9	0.2	1.4	4.0
Myo-inositol	14.9	0.3	1.4	2.0
α-HNJ	15.4	0.2	1.1	9.5
Glycosyl-HNJ	17.3	0.2	1.3	16.3
DMDP	22.2	0.4	0.9	---

5.2.3.4. Column temperature

Four different temperatures (25, 45, 55 and 65ºC) were assayed using acetonitrile:water with 5 mM ammonium acetate in both eluents as mobile phase (**Table 5.10**). As expected a decrease in retention times of all the carbohydrates was observed with temperature (e.g. differences in t_R values of 2 min for fructose and 5 min for DMDP between 25 and 65 ºC), and narrower peaks with better symmetry were also obtained (e.g. w_h =0.1-0.4 min; A_S = 0.9-1.4 at 65 ºC). However, resolution was only slightly affected by temperature and in all cases R_s values were higher than 1. Considering these results and in order to avoid high temperatures which could affect the stability of the stationary phase, 55ºC was selected as a trade-off to decrease total analysis time while optimizing chromatographic parameters for carbohydrates under study.

5.2.3.5. Characterization of Aglaonema leaf extracts

Taking into account the advantages of MS^2 data for both the confirmation of identifications previously reported [117, 227] (**section 5.1**), and the structural characterization of unknowns, a hybrid QTOF mass analyzer was used for subsequent analyses.

Table 5.10. Chromatographic parameters obtained at different column temperatures using 5 mM ammonium acetate as additive in both eluents. acetonitrile:water elution program: 20-80% aq. phase in 25 min, 80% aq. phase for 5 min.

Carbohydrate	**25ºC**				**45ºC**				**55ºC**				**65ºC**			
	t_R **(min)**	w_h **(min)**	A_S	R_s	t_R **(min)**	w_h **(min)**	A_S	R_s	t_R **(min)**	w_h **(min)**	A_S	R_s	t_R **(min)**	w_h **(min)**	A_S	R_s
Fructose	11.2	0.6	1.2	2.0	10.3	0.2	0.5	2.2	9.9	0.3	1.1	2.2	9.3	0.3	1.2	2.7
Glucose	12.2	0.4	0.7	4.6	11.4	0.4	1.1	3.3	11.0	0.3	0.9	4.6	10.3	0.1	0.9	7.3
Sucrose	13.8	0.3	1.4	4.8	13.1	0.2	1.2	3.5	12.7	0.2	1.3	3.7	12.3	0.2	1.4	3.2
Myo-inositol	15.0	0.2	1.4	1.6	14.3	0.2	1.2	1.4	13.7	0.1	1.3	1.2	13.1	0.1	1.3	1.1
α-HNJ	15.4	0.3	1.0	6.8	14.7	0.2	1.2	5.6	14.1	0.2	1.3	4.8	13.5	0.3	1.3	4.8
Glycosyl-HNJ	17.1	0.2	1.2	14.3	16.5	0.2	1.1	9.3	15.8	0.2	1.2	6.5	15.4	0.2	1.2	5.3
DMDP	22.1	0.5	0.8	---	20.2	0.5	0.8	---	18.0	0.6	0.9	---	17.0	0.4	1.0	---

➢ *Qualitative analysis*

Figure 5.7 shows the extracted ion chromatographic profile of ACT1 leaf extract obtained under previously optimized LC conditions. Iminosugar identification was carried out using commercial standards and considering their characteristic MS^2 fragmentation pattern, with successive losses of three water molecules $[(M+H)-18]^+$, $[(M+H)-36]^+$, $[(M+H)-54]^+$ and a subsequent loss of 30 *m*/z units corresponding to a CHOH group (**section 5.1**). MS^2 spectra for these compounds are provided in **Figure 3S** in **Anexo II**. Apart from those LMWC (fructose, peak 1; glucose, peak 2; sucrose, peak 5 and *myo*-inositol, peak 8) and iminosugars (DMDP, peak 15; *α*-HNJ, peak 10 and glycosyl-HNJ, peaks 13) considered for the method optimization, five peaks with ions at *m/z* 194.1 (peaks 6) and a MS^2 fragmentation pattern similar to that of *α*-HNJ were detected. These peaks could correspond to some of the isomers of *α*-HNJ previously detected by Asano *et al*. [11] in the GC-MS analysis of *Aglaonema* 'Treubii' extracts. According to these authors, two glycosyl-HNJ isomers (7-O-*β*-glycopyranosyl-*α*-HNJ and 5-O-*α*-galactopyranosyl-*α*-HNJ) are also present in *Aglaonema* 'Treubii' extracts. In our study, three compounds (t_R = 15.8, 16.0 and 16.3 min; peaks 12 and 13) with quasi-molecular ions at *m/z* 356.1 and a MS^2 fragmentation pattern compatible with that of a glycosyl-HNJ isomer were detected in ACT1 extract. Some disaccharides with retention times between 13.7 and 16.2 min (peaks 7) were also found.

Moreover, different ions with an even mass to charge ratio compatible with the $[M+H]^+$ ion of an iminosugar were also detected and evaluated as potential non previously identified iminosugars. Two peaks (peaks 3) showing a quasi-molecular ion at *m/z* 256.1 were detected at t_R 12.2 and 12.6 min. Their MS^2 spectra were characterized by a base peak at *m/z* 124.0 associated with the loss of 132 *m/z* units from $[M+H]^+$,which might correspond to a pentosyl group.

According to the results previously described in **section 5.1** on iminosugar composition of mulberry leaf extracts, these compounds could be tentatively identified as pentosyl-iminosugars. The glycosylated form of this iminosugar was also found at a retention time of 14.4 min (peak 11), characterized by a quasi-molecular ion at *m/z* 286.1 and by the loss of 162 *m/z* units (glycosyl group).

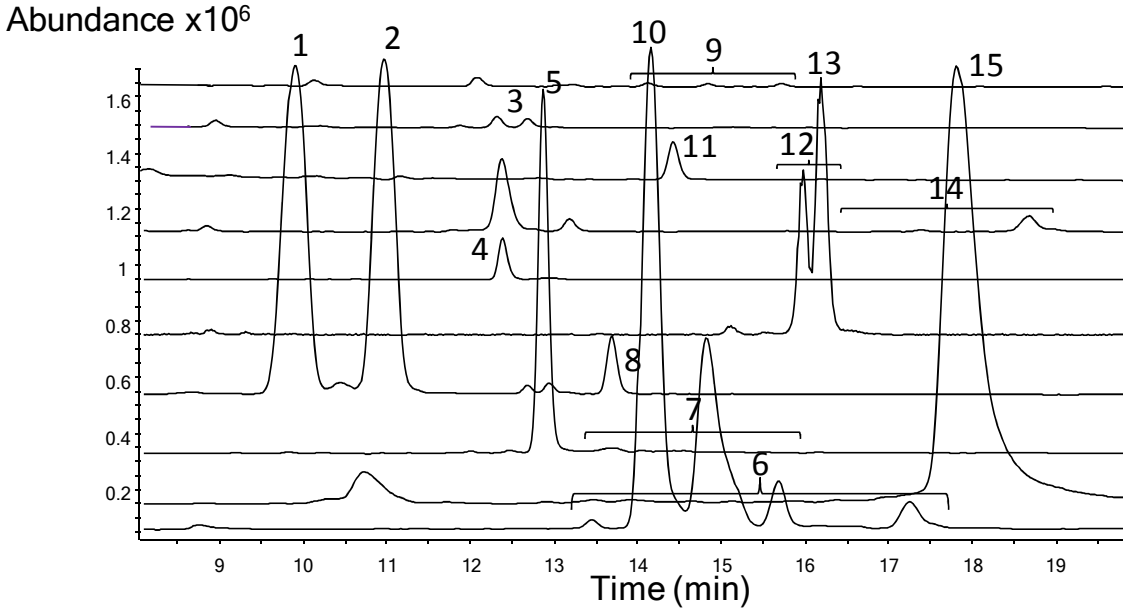


Figure 5.7. HILIC-MS profile of ACT1 leaf extract obtained under the following experimental conditions: 55 ºC, 5 mM ammonium acetate as additive of both aqueous and acetonitrile mobile phases, elution program: 20-80% aq. phase in 25 min, 80% aq. phase for 5 min. Peak numbers refer to those in **Table 5.12.**

A peak eluting at 12.4 min and with quasi-molecular ion at *m/z* 208.1 (peak 4) showed a MS^2 fragmentation pattern similar to that of miglitol standard (characteristic ions at *m/z* 74, 146, 172 and 190); however, its retention time was different. Therefore, this compound could correspond to an isomer of miglitol. Peaks with retention times of 14.1, 14.8, 15.2 and 15.7 min and $[M+H]^+$ 370.2 *m/z* were also detected (peaks 9). These compounds showed a MS^2 spectra characterized by an abundant ion at *m/z* 208.1 corresponding to the loss of a glycosyl group ($[(M+H)-162]^+$) and the consecutive losses of water molecules similarly to miglitol. Therefore, these compounds could be tentatively assigned to four glycosyl-miglitol isomers.

Finally, peaks labelled as 14 in **Figure 5.7**, with t_R of 16.5, 17.2, 18.5 and 18.8 min and $[M+H]^+$ ions at 326.1, were tentatively assigned to different glycosyl-DMDP isomers, considering their MS^2 fragmentation pattern characterized by the loss of a glycosyl group ($[(M+H)-162]^+$) and a *m/z* ion at 164.1 (base peak), compatible with a DMDP group.

- *Quantitative analysis*

As previously described, iminosugars are present in natural sources such as Aglaonema at very low concentrations and, therefore, their analysis requires the development of very sensitive methodologies. Moreover, the intended application of these methodologies to the accurate characterization of different Aglaonema extracts makes necessary that their reproducibility is assured. To this aim, *LOD*, *LOQ* and precision (*RSD*, %) data for a standard mixture including DMDP, α-HNJ, glucose and sucrose were calculated using acetonitrile:water with 5 mM ammonium acetate as mobile phase at 55ºC.

As shown in **Table 5.11**, the lowest *LOD* and *LOQ* values were obtained for DMDP (2.27 and 7.57 ng mL^{-1}, respectively), whereas the highest were found for sucrose (97.36 and 324.53 ng mL^{-1}, respectively). Good precision values were obtained for all standards analyzed (*RSD* ranging 6.0-7.5 %).

In order to compare these values with those obtained in **section 5.1**, the same standard mixture was analyzed using 0.1% ammonium hydroxyde as mobile phase additive. *LOD* and *LOQ* values (**Table 5.11**) considerably increased (between 40 and 500 times) compared to those obtained in the previous section under these basic conditions (**Table 5.5**). As it is known, sensitivity is greatly affected by a number of parameters which might vary as a function of time, even when the same equipment and operating conditions are set. As studies included in **sections 5.1** and **5.2** were carried out in one year time, it was decided to evaluate again the sensitivity of the analysis when 0.1 % ammonium hydroxide is used. Comparing *LOD* and *LOQ* data for both additives (**Table 5.11**), slight differences were only observed. Therefore, subsequent quantitative analyses were carried out following the experimental

conditions optimized in this paper (mobile phase: acetonitrile:water with 5 mM ammonium acetate as additive at 55ºC).

In order to verify if the sample matrix had any effect on quantitative determination of carbohydrates, different dilutions (1:1-1:500, v/v) of ACT1 extract were analyzed. No differences in carbohydrates concentrations associated to a possible matrix effect were found for the different dilutions, so undiluted extracts were considered for reliable quantitation.

Table 5.11. Limits of detection (*LOD*) and of quantitation (*LOQ*) and precision (relative standard deviation, % *RSD*) for a standard mixture analysed underthe following experimental conditions: acetonitrile:water gradient (20-80% aq. phase in 25 min, 80% aq. phase for 5 min), 5 mM ammonium acetate as additive of both eluents, 55 °C.

Compound	**Mobile phase additive**					
	5 mM ammonium acetate			**0.1 % ammonium hydroxide**		
	LOD **(ng mL^{-1})**	***LOQ*** **(ng mL^{-1})**	**Precision (*RSD*, %)**	***LOD*** **(ng mL^{-1})**	***LOQ*** **(ng mL^{-1})**	**Precision (*RSD*, %)**
DMDP	2.27	7.57	6.0	1.07	3.55	5.01
α-HNJ	28.78	95.92	6.1	14.83	49.43	6.54
Glucose	79.91	266.38	7.5	82.97	276.58	4.99
Sucrose	97.36	324.53	6.5	142.86	476.19	4.47

Concentrations of iminosugars and other LMWC in different Aglaonema extracts are shown in **Table 5.12**. In general, *α*-HNJ and DMDP were the most abundant iminosugars in all samples, followed by one of the glycosyl-HNJ isomers (peak 12). Considering the total iminosugar concentration, Aglaonema ACT2 showed the highest value of these bioactives (2.84 mg g^{-1}) followed by ACC2 (2.55 mg g^{-1}), whereas the lowest total iminosugar content was found in Aglaonema ACT1 (1.35 mg g^{-1}). It is worth noting that Aglaonema ACC1 showed the highest concentrations of glycosyl-miglitol isomers (0.42 mg g^{-1}) and Aglaonema ABL had the most abundant glycosyl-HNJ isomers.

Table 5.12. Content (mg g^{-1}) of iminosugars and other LMWC in Aglaonema leaf extracts analyzed under the following experimental conditions: acetonitrile:water gradient (20-80% aq. phase in 25 min, 80% aq. phase for 5 min), 5 mM ammonium acetate as additive of both eluents, 55 °C.

t_R (min)	*m/z***	Compound	Peak No.	AHBL	ACT1	ACEB1	ACC1	ACT2	ACC2
10.0	198.1	Fructose	1	2.36 (0.05)*	2.98 (0.10)	1.94 (0.03)	0.801 (0.004)	1.51 (0.03)	3.11 (0.15)
11.2	198.1	Glucose	2	2.67 (0.08)	2.88 (0.01)	1.955 (0.004)	1.15 (0.01)	1.56 (0.11)	2.96 (0.12)
12.2	256.1	Pentosyl-iminosugar	3	0.033 (0.001)	0.009 (0.001)	0.005 (0.001)	0.057 (0.002)	0.031 (0.001)	0.056 (0.003)
12.4	208.1	Miglitol isomer	4	0.043 (0.001)	0.019 (0.001)	0.099 (0.001)	0.19 (0.01)	0.031 (0.004)	0.21 (0.01)
12.6	256.1	Pentosyl-iminosugar	3	0.008 (0.000)	0.008 (0.000)	0.005 (0.000)	0.004 (0.000)	0.018 (0.002)	0.003 (0.000)
12.8	360.1	Sucrose	5	0.169 (0.004)	0.98 (0.05)	0.56 (0.02)	0.097 (0.001)	0.060 (0.001)	1.38 (0.06)
13.5	194.1	HNJ isomer	6	0.014 (0.001)	0.004 (0.000)	0.010 (0.001)	0.038 (0.001)	0.018 (0.001)	0.030 (0.001)
13.7-16.2	360.2	Disaccharides	7	0.409 (0.006)	0.044 (0.001)	0.071 (0.001)	0.13 (0.02)	0.14 (0.01)	0.27 (0.02)
14.8	198.1	*Myo*-inositol	8	1.48 (0.05)	0.26 (0.01)	0.026 (0.01)	0.15 (0.01)	0.29 (0.03)	0.35 (0.03)
14.1	370.2	Glycosyl-miglitolisomer	9	0.046 (0.002)	0.003 (0.000)	0.028 (0.001)	0.26 (0.01)	0.004 (0.000)	0.25 (0.02)
14.2	194.1	α-HNJ	10	0.46 (0.01)	0.50 (0.01)	0.50 (0.01)	0.371 (0.01)	1.06 (0.15)	0.62 (0.01)
14.4	286.1	Glycosyl-iminosugar	11	0.040 (0.004)	0.019 (0.001)	0.018 (0.001)	0.043 (0.003)	0.044 (0.004)	0.08 (0.01)
14.6	194.1	HNJ isomer	6	0.016 (0.001)	0.005 (0.000)	0.010 (0.001)	0.11 (0.01)	0.040 (0.005)	0.13 (0.01)
14.8	194.1	HNJ isomer	6	0.100 (0.001)	0.138 (0.001)	0.18 (0.01)	0.05 (0.01)	0.124 (0.001)	0.084 (0.006)
14.8	370.2	Glycosyl-miglitol isomer	9	0.005 (0.000)	0.002 (0.000)	0.004 (0.000)	0.042 (0.001)	0.003 (0.001)	0.042 (0.004)
15.2	370.2	Glycosyl-miglitol isomer	9	0.002 (0.000)	-	0.002 (0.000)	0.019 (0.001)	-	0.018 (0.002)
15.7	194.1	HNJ isomer	6	0.042 (0.002)	0.021 (0.001)	0.046 (0.001)	0.049 (0.001)	0.05 (0.01)	0.053 (0.005)
15.7	370.2	Glycosyl-miglitol isomer	9	0.055 (0.005)	0.003 (0.000)	0.014 (0.001)	0.103 (0.001)	0.006 (0.001)	0.10 (0.01)
15.8	356.1	Glycosyl-HNJ isomer	12	0.043 (0.001)	0.059 (0.001)	0.063 (0.003)	0.07 (0.01)	0.10 (0.01)	0.07 (0.01)
16.0	356.1	Glycosyl-HNJ isomer	13	0.49 (0.01)	0.095 (0.004)	0.128 (0.003)	0.174 (0.002)	0.33 (0.02)	0.21 (0.02)
16.3	356.1	Glycosyl-HNJ isomer	12	0.019 (0.001)	0.003 (0.000)	0.013 (0.001)	0.018 (0.001)	0.008 (0.001)	0.041 (0.002)
16.5	326.1	Glycosyl-DMDP	14	0.019 (0.002)	-	0.005 (0.000)	0.010 (0.001)	0.002 (0.000)	0.008 (0.001)
17.2	326.1	Glycosyl-DMDP	14	-	0.002 (0.000)	0.002 (0.000)	-	0.003 (0.000)	-
17.3	194.1	HNJ isomer	6	0.035 (0.001)	0.018 (0.001)	0.028 (0.001)	0.046 (0.001)	0.046 (0.004)	0.050 (0.005)
17.8	164.1	DMDP	15	0.582 (0.002)	0.43 (0.02)	0.57 (0.03)	0.33 (0.01)	0.89 (0.06)	0.43 (0.02)
18.5	326.1	Glycosyl-DMDP	14	0.053 (0.005)	0.011 (0.001)	0.026 (0.001)	0.069 (0.003)	0.027 (0.003)	0.06 (0.01)
18.8	326.1	Glycosyl-DMDP	14	0.004 (0.000)	0.002 (0.000)	0.042 (0.004)	0.007 (0.001)	0.005 (0.001)	0.007 (0.001)
		Total iminosugars		2.11	1.35	1.80	2.06	2.84	2.55

*Standard deviation in brackets (n=3)

**Adducts: $[M+H]^+$ for iminosugars and $[M+NH_4]^+$ for LMWC

5.2.4. Conclusions

In conclusion, a sensitive and reproducible HILIC-QTOF-MS^2 method for analysis of iminosugars and other LMWC in Aglaonema extracts has successfully been developed. Regarding mobile phase strength, binary gradients of acetonitrile:water have proven to be the most appropriate eluents. Among experimental parameters assayed, the mobile phase strength provided by a binary gradient of acetonitrile:water and the use of ammonium acetate as additive of both organic and aqueous phases have been shown to be appropriate to achieve good results in terms of peak width, peak symmetry and resolution. MS^2 data in combination with retention data have allowed the tentative characterization for the first time of several iminosugars (miglitol isomers, glycosyl-miglitol isomers and glycosyl-DMDP isomers) in Aglaonema leaf extracts.

EXTRACCIÓN

6. Optimización de métodos de extracción y fraccionamiento de iminoazúcares bioactivos a partir de fuentes vegetales

Una vez optimizados los métodos de análisis de iminoazúcares y otros carbohidratos de bajo peso molecular (**Secciones 4** y **5**) y caracterizados los extractos convencionales de las muestras objeto de estudio en esta Memoria, se procedió al desarrollo de métodos de extracción con el fin de obtener los mayores rendimientos en iminoazúcares, aspecto éste intrínsecamente relacionado con su bioactividad. Para ello, se seleccionó la PLE por tratarse de una técnica rápida, eficaz y potencialmente de fácil escalado. Aunque esta técnica se había aplicado con anterioridad a la extracción y fraccionamiento de carbohidratos [85, 236], no había sido hasta el momento empleada para la obtención de extractos enriquecidos en carbohidratos bioactivos como son los iminoazúcares, por lo que resultó necesario optimizar las condiciones experimentales (temperatura y tiempo de extracción, cantidad de muestra y número de ciclos).

Considerando también la similitud estructural entre los iminoazúcares y sus correspondientes azúcares y la gran abundancia relativa de estos últimos en las matrices vegetales, la búsqueda de métodos de fraccionamiento que permitan eliminar mono- y disacáridos que pudieran interferir en la bioactividad de los iminoazúcares es indispensable. Además, estos métodos pueden suponer una etapa limitante a la hora de obtener extractos enriquecidos en carbohidratos bioactivos, por lo que en la medida de lo posible deben elegirse métodos rápidos, que no requieran gran manipulación de la muestra y/o sean fácilmente automatizables y, sobre todo, que sean compatibles con su aplicación para la obtención de extractos bioactivos de calidad alimentaria.

Con el fin de facilitar el estudio del comportamientos de los iminoazúcares y de otros carbohidratos de bajo peso molecular durante la optimización de los métodos de extracción y fraccionamiento, se seleccionó la GC-MS como técnica de análisis, aunque es importante tener en cuenta que, como se indica en la **sección 5,** la HILIC-MS proporciona información adicional, al permitir el análisis de diferentes iminoazúcares minoritarios que no se detectan por GC-MS.

Por último, en esta sección se planteó como objetivo no sólo el desarrollo de métodos de extracción y fraccionamiento, sino también la evaluación de la bioactividad, estabilidad, y viabilidad celular de los extractos obtenidos a partir de

matrices no alimentarias. Estos estudios *in vitro* pueden considerarse como una primera aproximación, indispensable en la evaluación del potencial de estos extractos como ingredientes bioactivos de alimentos.

Por tanto, en la **sección 6.1.** se optimiza un método de PLE para la obtención de extractos enriquecidos en iminoazúcares y otros carbohidratos bioactivos tales como inositoles a partir de hojas de morera (*Morus alba*). Los resultados obtenidos son comparados con los de la extracción sólido-líquido convencional, determinándose ventajas y desventajas de estos procedimientos. Asimismo, se evalúa el tratamiento con levaduras (*Saccharomyces cerevisiae*) como método de fraccionamiento alternativo a la cromatografía de intercambio catiónico (CEC) para la eliminación selectiva de otros carbohidratos de bajo peso molecular coextraídos en los extractos y potencialmente interferentes con la bioactividad de los iminoazúcares. Los resultados de estos estudios se recogen en el artículo titulado "**New methodologies for the extraction and fractionation of bioactive carbohydrates from mulberry (*Morus alba*) leaves**" de Rodríguez-Sánchez *y col.*, publicado en *Journal of Agricultural and Food Chemistry* 61 (2013) 4539-4545.

Considerando la diversidad de iminoazúcares previamente detectados por HILIC-MS (**sección 5.2**) en extractos de hojas de Aglaonema, las distintas concentraciones y diferentes estructuras con respecto a los iminoazúcares extraídos de hoja de morera, etc., se consideró necesario optimizar un método de extracción mediante PLE para la obtención de extractos enriquecidos en estos compuestos a partir de hojas y raíces de Aglaonema. Estos estudios se muestran en la **sección 6.2** que incluye el trabajo titulado **"Pressurized liquid extraction of *Aglaonema* sp. iminosugars: bioactivity, cell viability and thermal stability"** de Rodríguez-Sánchez *y col.*, *Journal of Agricultural and Food Chemistry* (2015, enviado). En este estudio se lleva también a cabo la caracterización por GC-MS de los extractos de Aglaonema empleando las condiciones previamente optimizadas en la **sección 4.2.** y usando la información obtenida en la **sección 4.3** sobre la relación estructura química-datos de GC-MS para la identificación de iminoazúcares desconocidos. Los extractos obtenidos se sometieron a un tratamiento con levaduras para su enriquecimiento en iminoazúcares, tal y como se indica en la **sección 6.1**. Dada la relevancia del contenido en iminoazúcares de estos extractos, se han llevado a cabo los estudios preliminares necesarios para su posible

uso como ingrediente funcional: (i) determinación de su actividad inhibidora de *α*-glicosidasas, (ii) evaluación *in vitro* de la viabilidad de células Caco-2 tras el tratamiento con dichos extractos, como indicador de su potencial toxicidad y (iii) evaluación de los posibles cambios en el contenido en iminoazúcares bioactivos durante el almacenamiento en condiciones forzadas de temperatura.

6.1. New methodologies for the extraction and fractionation of bioactive carbohydrates from mulberry (*Morus alba*) leaves.

S. Rodríguez-Sánchez, L. Ruiz-Aceituno, M. L. Sanz, A. C. Soria.

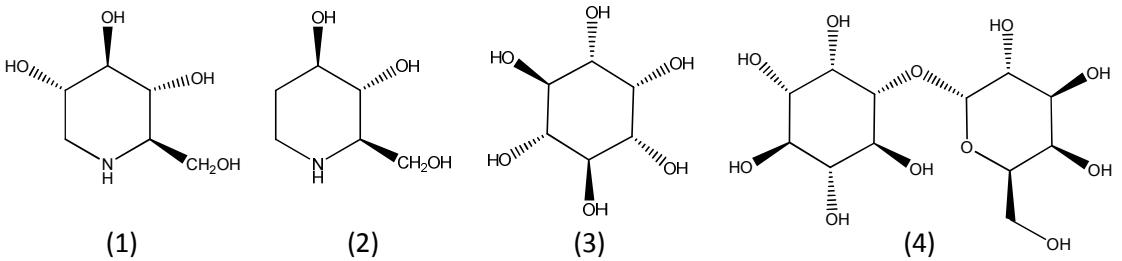
Journal of Agricultural and Food Chemistry (2013) 61: 4539-4545

6.1.1. Introduction

The increasing trend of using functional foods or supplements containing bioactive compounds from natural sources has promoted the search for strategies to obtain plant extracts enriched with these compounds. Among bioactive low molecular weight carbohydrates (LMWC), it is worth noting iminosugars (also called polyhydroxyalkaloids), which have *α*-glucosidase inhibition activity, as they contribute to the regulation of body weight and the prevention of obesity [8, 26], and inositols, which have been described as useful for treating conditions associated with insulin resistance, among others [166, 237, 238].

Over a hundred polyhydroxyalkaloids have been isolated from plants (mainly from Leguminosae, Hyacinthaceae, Solanaceae, Convulaceae, and Moraceae families) and from microorganisms (*Streptomyces* sp.) [10]. Among them, the *Morus* genus draws special attention because different alkaloids, such as 1-deoxynojirimycin (DNJ, **Figure 6.1**), *N*-methyl-DNJ, 2-*O*-*D*-galactopyranosyl-DNJ, and fagomine (**Figure 6.1**), have been found in their leaves, roots, branches, and/or fruits. The amount and type of these polyhydroxyalkaloids vary depending on the species, the harvesting time, plant part, leaf size, etc. [16]. In general, DNJ and fagomine are the most abundant (0.1−0.21% dry weight), and the highest glucosidase inhibitory activity has been attributed to these two compounds [15, 16]. Different food grade products based on mulberry extracts are commercialized in Asia, and mulberry leaf infusions are consumed by diabetes mellitus patients due to their antihyperglycemic properties, which have been mainly attributed to their high iminosugar content [15, 16].

With regard to bioactive inositols, few studies have addressed their content in mulberry extracts. In **section 4.1** is reported the presence of *myo*-inositol and glycosyl-inositols (**Figure 6.1**) in the ranges of 0.75−12.34 and 0.01−3.37 mg g^{-1}, respectively, in leaves, branches, and fruits of different *Morus* species.



(1) (2) (3) (4)

Figure 6.1. Structure of mulberry bioactive LMWC under study: DNJ (1); Fagomine (2); *myo*-inositol (3); galactinol (4).

Pressurized liquid extraction (PLE) has recently emerged as an alternative to conventional solvent extraction techniques, providing faster extractions, lower solvent consumption, and higher selectivity. PLE also helps preserve labile bioactives by maintaining the sample in an oxygen- and light-free environment during extraction. In this respect, there are several studies on the application of PLE to obtain antioxidants from algae and microalgae [239, 240], pomace [241] and birch bark [242]. With regard to carbohydrates, PLE has been mainly used to obtain honey fractions enriched in di- and trisaccharides [236], to purify lactulose from mixtures with lactose [84], and to extract monosaccharides and polyalcohols from woods used in wine aging [85]. However, no study has reported the application of this technique to extract iminosugars or any other mulberry bioactives.

The low amount of bioactives (iminosugars and inositols) present in *Morus* extracts, along with the unavoidable coextraction of other mulberry carbohydrates (glucose, fructose, sucrose, etc.) that can interfere with the bioactivity of mulberry extracts, makes the removal of these soluble sugars a requirement. Among the different procedures described in the literature for iminosugar fractionation, ion-exchange chromatography is one of the most commonly applied. Commercially available anionic and cationic resins with different functionalities, pore sizes, load capacities, etc., have been used; Dowex50 and Amberlite GC120 in their NH_4^+ or H^+ forms have generally been employed for both in-column and in-batch cation-exchange chromatography (CEC) [9, 94, 95]. Furthermore, plant or food extracts may also be subjected to several consecutive treatments with different ion-exchange resins depending on the degree of enrichment or fractionation required [28, 51, 52, 120, 154,

243]. With regard to inositols, several papers [244, 245] describe their purification using ion-exchange resins (Amberjet 4200, Dowex 50, or Dowex 1). Microbiological treatments such as yeast (*Saccharomyces bayanus* and *Saccharomyces cerevisiae*, among others) incubation have also been described to separate interfering carbohydrates (sucrose, fructose, and glucose) from cyclitols present in powdered carob [99] and legume [102] extracts. *S. cerevisiae* has also been used for the selective separation of monosaccharides and disaccharides with different glycosidic linkages [100]. The enrichment obtained by treating honey with *S. cerevisiae* has also been used for different applications, such as the determination of minor honey oligosaccharides [101] or the detection of difructose anhydrides (markers of adulteration with high fructose content syrups or invert syrups) in honey [103].

Therefore, the aims of this work were: i) to optimize the PLE process and to compare it with the solid−liquid extraction of bioactive carbohydrates (iminosugars and inositols) from mulberry leaves, and ii) to evaluate yeast (*S. cerevisiae*) treatment as an alternative to fractionation by CEC for the selective removal of interfering soluble carbohydrates in PLE mulberry extracts.

6.1.2. Materials and methods

6.1.2.1. Samples and standards

Samples of *Morus alba* leaves M1L and M2L were collected under botanical surveillance in Madrid (Spain) in May and August 2010, respectively. Mulberry leaves were air-dried at a controlled temperature (25 °C) and in the absence of light for 3 days.

Carbohydrate standards (fructose, glucose, sucrose, galactose, trehalose, *myo*-inositol, galactinol (*O*-*α*-*D*-galactopyranosyl-(1→1)-*L*-*myo*-inositol), and phenyl-*β*-*D*-glucoside) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DNJ was purchased from Toronto Research Chemicals Inc. (Ontario, Canada).

6.1.2.2. Extraction of low molecular weight carbohydrates

- *Solid–Liquid Extraction*

One gram of dried mulberry leaves (M1L) was ground in a domestic mill (Moulinex) and sieved (500 µm) before extraction at controlled temperature (25 ºC) with 10 mL of different solvents (water, 0.1% HCl (v/v), methanol, ethanol, and mixtures of H_2O/methanol and H_2O/ethanol (50%, v/v)) for 30 min under stirring. Extracts were passed through Whatman no. 4 filters and kept at −20 °C until analysis. For recovery studies, mulberry samples were subjected to four consecutive extraction cycles with the selected solvent. The optimized conditions were further applied to the M2L sample. All extracts were prepared in triplicate.

- *Pressurized Liquid Extraction*

Extractions were carried out in a laboratory-made miniaturized PLE system designed and patented by Ramos *et al*. [246]. This instrument consists of an oven equipped with temperature control and regulation, in which a stainless steel extraction cell (99 mm × 4.4 mm i.d, total volume = 1.5 mL) was placed. This extraction cell was coupled to a model 1050 series isocratic pump (Hewlett-Packard, Palo Alto, CA, USA), which was used to deliver and pressurize the solvent, via a model 7000 six-port Rheodyne valve (Rheodyne L.P., Rohnert Park, CA, USA). Another valve of the same characteristics was connected to the outlet end of the extraction cell to deliver the extract to the collection vial.

Prior to loading the sample into the extraction cell, mulberry (M2L)powders were mixed with sea sand (Panreac, Barcelona, Spain)previously washed with different solvents, to hold the sample in place and to prevent the formation of preferential flow paths. Additional layers of sea sand were also placed at the bottom and at the upper part of the extraction cell to avoid the presence of dissolved gas and to compensate for the differences in sample amount when this parameter was optimized. The extraction cell was sealed with 5 µm stainless steel frits (Supelco, Bellefonte, PA, USA) at its lower and upper ends to avoid blockage of the stainless steel tubing and/or valve by suspended particles from the matrix or sea sand.

PLE was optimized for sample amount (0.05 and 0.10 g), extraction temperature (50−150 °C), time (2−30 min), and number of consecutive extraction cycles (1−2 cycles). All experiments were carried out in triplicate.

6.1.2.3. Carbohidrate fractionation

- *Yeast treatment*

Aqueous PLE mulberry extracts (10 mL) were incubated with 0.125 g of yeast (*S. cerevisiae* type II) (Sigma Chemical Co.) at 37 °C under stirring. Aliquots were taken after 0 (just after adding the yeast), 3, 5, 8, and 12 h of treatment. Yeast removal was immediately carried out by centrifugation at 4400g for 10 min at 4 °C, followed by filtration through Whatman no. 4 paper filters. A PLE mulberry extract sample that had not previously undergone yeast treatment was used as a control in this study. All assays were carried out in triplicate.

- *Cation-exchange chromatography (CEC)*

A glass column filled with a strong acid cation-exchange resin, Dowex 50 W X4 20/50 mesh, sulfonic acid in H^+ form (Fluka, Sigma Chemical Co.), was used. The resin was conditioned by washing with 3 column volumes of 1 N NaOH, followed by subsequent washes with Milli-Q water until neutral pH. Later, 3 column volumes of 1 N HCl were used and conditioning was finished after washing with Milli-Q water until pH 7.

After resin conditioning, aqueous PLE mulberry extract, previously freeze-dried and redissolved in acidulated Milli-Q water (pH = 4), was loaded into the column. Interfering LMWC were separated by consecutive elutions with Milli-Q water, whereas iminosugars were eluted with 2 M NH_4OH. Resin and sample amount and elution volumes were optimized to obtain extracts with the highest recovery and purity of selected iminosugars: DNJ and fagomine. All assays were done in triplicate.

6.1.2.4. Chromatographic analysis

- *Derivatization*

One milliliter of mulberry extract was mixed with 0.5 mL of internal standard (phenyl-*β-D*-glucoside, 1 mg mL^{-1}) and evaporated under vacuum prior to carbohydrate derivatization. Trimethylsilyl oximes (TMSO) were prepared according to the method of Sanz *et al*. [149]. Oximes were obtained by adding 350 µL of a 2.5% solution of hydroxylamine hydrochloride in pyridine, and heating for 30 min at 75 °C. These derivatives were then silylated with hexamethyldisilazane (350µL) and trifluoroacetic acid (35 µL) at 45 °C for 30 min. After reaction, samples were centrifuged at 4400g for 10 min, and 1 µL of supernatant was injected into the GC injection port.

- *Gas chromatography-Mass spectrometry (GC-MS) analysis*

GC-MS analyses (*n* = 3 replicates) were carried out following the validated method of **section 4.2**, using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA). Analyses were carried out on a 30 m × 0.25 mm i.d., 0.25 µm df, methylpolysiloxane TBR-1capillary column (Teknokroma, Barcelona, Spain), using helium at ∼1mLmin^{-1} as carrier gas. The oven temperature was programmed from 100 to 200 °C (15 min) at a heating rate of 15 °Cmin^{-1} and finally programmed to 300 °C (15 min) at 15 °Cmin^{-1}. Injections were carried out in split mode (1:20) at 240 °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 HPChem Station software (Agilent Technologies).

Qualitative analysis was based on the comparison of the obtained spectra with those of the Wiley mass spectral library [247] and with published data and was confirmed, when possible, by using linear retention indices (I^T) as indicated in **section 4.3.** Available standard compounds were also used for further confirmation.

Quantitative data were obtained by the internal standard method. Standard solutions were prepared over the expected concentration range in mulberry extracts to calculate the response factor relative to phenyl-*β-D*-glucoside: fructose and glucose (0.1−4.0 mg mL^{-1}); sucrose (0.02−2.00 mg mL^{-1}); galactose, trehalose, *myo*-inositol (0.05−1.00mg mL^{-1}); galactinol (0.01−1.00 mg mL^{-1}); and DNJ (0.05−0.50 mg mL^{-1}). For

non commercially available standards, such as fagomine and galactinol isomers, response factors were assumed to be those of DNJ and galactinol, respectively. Results were expressed in milligrams per gram of dry mulberry leaves and in milligrams per gram of dry extract. Dry matter content of extracts was gravimetrically determined at 102 °C until constant weight.

6.1.2.5. Statistical analysis

Data were subjected to analysis of variance (simple linear model, ANOVA) by applying Fischer's least significant difference (LSD) test. The software used was Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA) for Windows. The significance of differences was defined as $P < 0.05$.

6.1.3. Results and discussion

6.1.3.1. Extraction of bioactives

- *Solid–Liquid Extraction*

The first step in optimizing conventional extraction was to carry out a comparative study using different solvents. **Table 6.1** lists the content of iminosugars, inositols, and other LMWC determined in mulberry (M1L) extracts after one cycle of extraction (30min) using water, 0.1% HCl, methanol, ethanol, 50% methanol, and 50% ethanol as solvents. On the basis of the previously described interference of several coextracted LMWC on the bioactivity of mulberry extracts, optimal conditions were chosen as a trade-off between a high extraction yield of iminosugars and inositols (data in mg g^{-1} of dry mulberry leaves) and a low content of interfering compounds (mono- and disaccharides) (data in mg g^{-1} dry extract).

Table 6.1. Content (average for *n* = 3) of iminosugars, inositols and other LMWC in mulberry (M1L) extracts obtained by using different solvents (data shown as mg g^{-1} mulberry and as mg g^{-1} dry extract).

Solvent	mg g^{-1} mulberry							
	Fagomine	**DNJ**	**Fructose**	**Glucose**	**Galactose**	***Myo*-inositol**	**Glycosyl-inositols**	**Disaccharides**
H_2O	0.33 (0.04)*[a]	1.25 (0.01)[b]	16.29 (0.22)[b]	6.38 (0.04)[b]	0.43 (0.03)[b]	2.64 (0.13)[b]	0.32 (0.05)[a]	3.55 (0.10)[e]
0.1% HCl	0.34 (0.01)[a]	1.35 (0.02)[a]	20.23 (0.69)[a]	14.15 (0.12)[a]	0.58 (0.05)[a]	3.45 (0.03)[a]	0.28 (0.02)[a]	0.58 (0.37)[f]
50% MeOH	0.23 (0.04)[b]	1.26 (0.06)[b]	1.43 (0.04)[d]	1.60 (0.07)[c]	0.05 (0.01)[c]	2.33 (0.29)[c]	0.05 (0.01)[b]	23.59 (0.29)[a]
MeOH	0.10 (0.01)[d]	0.59 (0.01)[d]	0.81 (0.03)[e]	0.49 (0.03)[d]	0.03 (0.01)[d]	1.35 (0.03)[e]	0.01 (0.01)[d]	17.31 (0.40)[c]
50% EtOH	0.13 (0.01)[c]	0.80 (0.02)[c]	1.70 (0.08)[c]	1.62 (0.12)[c]	0.06 (0.01)[c]	1.84 (0.01)[d]	0.03 (0.01)[c]	22.09 (0.17)[b]
EtOH	tr**[e]	tr[e]	0.35 (0.03)[f]	0.16 (0.03)[e]	tr[e]	0.11 (0.01)[f]	tr[e]	4.39 (0.35)[d]
Solvent	mg g^{-1} mulberry							
	Fagomine		**Fagomine**		**Fagomine**		**Fagomine**	
H_2O	2.52 (0.02)[b]	10.97 (1.23)[ab]	131.94 (14.12)[b]	55.87 (4.70)[b]	3.52 (0.52)[b]	22.37 (0.75)[b]	2.77 (0.27)[a]	32.35 (0.71)[e]
0.1% HCl	2.83 (0.09)[a]	11.34 (0.34)[a]	152.80 (5.74)[a]	107.09 (10.87)[a]	4.22 (0.38)[a]	24.44 (1.05)[a]	2.17 (0.08)[b]	3.42 (0.39)[f]
50% MeOH	2.16 (0.19)[c]	10.28 (0.36)[b]	13.19 (1.00)[d]	12.51 (1.14)[c]	0.60 (0.07)[c]	22.24 (0.86)[b]	0.43 (0.03)[c]	284.04 (16.67)[a]
MeOH	1.53 (0.19)[d]	9.65 (0.15)[c]	11.70 (1.56)[e]	8.73 (0.33)[d]	0.39 (0.02)[d]	18.11 (0.37)[d]	0.23 (0.02)[d]	176.98 (10.49)[d]
50% EtOH	1.28 (0.19)[e]	7.53 (1.53)[d]	16.17 (0.45)[c]	16.18 (1.58)[e]	0.58 (0.08)[c]	18.76 (0.54)[c]	0.25 (0.03)[d]	221.67 (1.81)[c]
EtOH	tr[f]	tr[e]	9.29 (0.45)[f]	7.77 (0.44)[f]	tr[e]	6.66 (0.24)[e]	tr[e]	262.96 (8.10)[b]

*Standard deviation in brackets.

** tr: traces

[a-f] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

With regard to the efficiency of extraction, the highest contents of DNJ and *myo*-inositol (1.35 and 3.45 mg g^{-1} dry mulberry) were extracted by using acidified water (0.1% HCl).There were no significant differences between the amounts of fagomine and glycosyl-inositols extracted by using water or0.1% HCl; the contents of both compounds extracted by the remaining solvents were lower. Whereas significantly higher amounts of monosaccharides (fructose, glucose, and galactose) were extracted with 0.1% HCl followed by water, the highest dissacharide content was extracted by using hydroalcoholic mixtures and methanol (17.31−23.59 mg g^{-1} dry mulberry).

Water, acidified water, and the water-methanol mixture provided extracts with a similar composition in DNJ (10−11mg g^{-1} dry extract). Whereas acidified water showed significantly higher contents of fagomine and *myo*-inositol, water extracted the highest amount of glycosyl-inositols (2.77 mg g^{-1} dry extract). Interfering carbohydrates, fructose and glucose, were also higher for 0.1% HCl. As expected, the amount of disaccharides was the highest when extracted by hydroalcoholic mixtures and alcohols.

On the basis of the results listed in **Table 6.1**, both water and0.1% HCl were the best solvents for extraction of iminosugars and inositols. However, as the total amount of interfering sugars was higher for 0.1% HCl, water was selected as the optimal solvent for further studies.

Data obtained in four consecutive extraction cycles of 30 min were also evaluated to optimize PLE of target compounds. As expected, percentages relative to the total amount of selected bioactives extracted noticeably decreased with the cycle number (64, 22, 12, and 2% on average). The amount extracted in the fourth cycle was considered to be negligible, and thus the combination of extracts 1−3 was chosen for further studies.

- *Pressurized liquid extraction*

PLE temperature, time, and sample amount were optimized for extraction of mulberry bioactives. For comparative purposes, and according to the results previously described for conventional extraction, water was selected as the optimal solvent for PLE.

PLE of 0.1 g of mulberry leaves (M2L) was assayed at different temperatures (50, 100, and 150 °C) for 2 min (**Table 6.2**). According to the ANOVA test (Fischer's LSD) for comparison of mean values, no significant differences were found for the amount of iminosugars extracted or their preferential extraction at the different extraction temperatures assayed. However, 50°C was chosen as optimal because it yielded similar or even higher recoveries of *myo*-inositol and glycosyl-inositols compared to higher extraction temperatures.

Table 6.2. Optimization of temperature in the PLE extraction (2 min) of iminosugars and inositols from mulberry leaves (M2L, 0.1 g).

Temperature (°C)	mg g^{-1} dry mulberry			
	Fagomine	**DNJ**	***Myo*-inositol**	**Glycosyl-inositols**
50	0.36 (0.05) * [a]	1.74 (0.19)[a]	6.78 (0.44)[a]	0.25 (0.05)[a]
100	0.38 (0.04)[a]	2.00 (0.20)[a]	5.78 (0.15)[b]	0.18 (0.02)[b]
150	0.33 (0.19)[a]	1.87 (0.74)[a]	5.88 (0.19)[b]	0.20 (0.01)[ab]

Temperature (°C)	mg g^{-1} dry extract			
	Fagomine	**DNJ**	***Myo*-inositol**	**Glycosyl-inositols**
50	1.43 (0.22)[a]	7.02 (1.08)[a]	27.41 (2.87)[a]	1.02 (0.21)[a]
100	1.68 (0.03)[a]	8.69 (0.32)[a]	25.30 (2.53)[ab]	0.80 (0.14)[a]
150	1.28 (0.67)[a]	7.28 (2.51)[a]	23.18 (1.22)[b]	0.77 (0.07)[a]

* Standard deviation in brackets.
[a-b] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

Extraction time was not a determinant parameter when using PLE to extract iminosugars and inositols from mulberry leaves(0.1 g of sample extracted at 50 °C, **Table 6.3**). As significant differences in the preferential extraction of DNJ and fagomine (mg g^{-1} dry extract) were only found at 5 min, this time was considered the most favorable.

Table 6.3. Optimization of time in the PLE extraction (50 ºC) of iminosugars and inositols from mulberry leaves (M2L; 0.1 g).

Time (min)	mg g^{-1} dry mulberry			
	Fagomine	**DNJ**	***Myo*-inositol**	**Glycosyl-inositols**
2	0.36 (0.05)*[a]	1.74 (0.19)[a]	6.78 (0.44)[ab]	0.25 (0.05)[a]
5	0.34 (0.08)[a]	1.72 (0.37)[a]	5.73 (1.20)[b]	0.22 (0.07)[a]
15	0.29 (0.14)[a]	1.91 (0.60)[a]	7.26 (0.68)[b]	0.30 (0.06)[a]

Time (min)	mg g^{-1} dry extract			
	Fagomine	**DNJ**	***Myo*-inositol**	**Glycosyl-inositols**
2	1.43 (0.22)[b]	7.02 (1.08)[b]	27.41 (2.87)[a]	1.02 (0.21)[a]
5	2.03 (0.30)[a]	10.11 (1.69)[a]	30.23 (5.04)[a]	1.06 (0.14)[a]
15	1.12 (0.54)[b]	8.27 (1.42)[b]	29.54 (3.23)[a]	1.26 (0.15)[a]

* Standard deviation in brackets.
[a-b] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

To optimize the sample amount, 0.05 and 0.10 g of sample were considered (**Table 6.4**). To keep the solvent volume within the extraction cell constant (1.3 mL), a higher amount of sea sand was used to compensate for the smaller sample amount. No significant differences were observed either in the amount extracted per sample amount (mg g^{-1} dry mulberry) or in the concentration of iminosugars in the mulberry dry extract (mg g^{-1} dry extract). However, because *myo*-inositol and glycosyl-inositol contents extracted were significantly higher for 0.05 g of mulberry leaves, this sample amount was used in subsequent experiments.

Under the selected PLE conditions, a second extraction cycle was also carried out. As the only compounds extracted invariable percentages, ranging from 1 to 2%, were *myo*-inositol and sucrose, a single extraction cycle was chosen for PLE of mulberry bioactives.

For comparative purposes, **Table 6.5** summarizes the amount of iminosugars/inositols extracted from mulberry leaves (M2L) by conventional solid−liquid extraction (three cycles) and by PLE (one cycle) under optimized conditions (**Figure 6.2**). For a similar sample amount/total solvent volume ratio (approximately

1:30 (w/v)), both extraction procedures gave rise to similar yields of DNJ, fagomine, *myo*-inositol, and glycosyl-inositols.

Table 6.4. Optimization of sample amount in the PLE extraction (50 ºC; 5 min) of iminosugars and inositols from mulberry leaves (M2L).

Sample amount (g)	mg g^{-1} dry mulberry			
	Fagomine	**DNJ**	***Myo*-inositol**	**Glycosyl-inositols**
0.05	0.48 (0.15)* [a]	2.44 (0.50)[a]	8.90 (1.88)[a]	0.35 (0.06)[a]
0.10	0.34 (0.08)[a]	1.72 (0.37)[a]	5.73 (1.20)[b]	0.22 (0.07)[b]

Sample amount (g)	mg g^{-1} dry extract			
	Fagomine	**DNJ**	***Myo*-inositol**	**Glycosyl-inositols**
0.05	1.64 (0.38)[a]	8.66 (2.54)[a]	40.67 (10.97)[a]	1.41 (0.48)[a]
0.10	2.03 (0.30)[a]	10.11 (1.69)[a]	30.23 (5.04)[a]	1.06 (0.14)[a]

* Standard deviation in brackets.
[a-b] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

However, extraction of these compounds required three cycles of 30 min in the case of conventional extraction, whereas similar results were obtained by PLE after only one cycle of 5 min. Moreover, total recovery of mulberry bioactives by PLE required a slightly lower volume of solvent (1.39 mL for 0.05 g of sample) as compared to conventional extraction (30 mL for 1 g of sample). Further advantages of PLE for obtaining extracts rich in bioactives that can be applied in the food industry include the possibility of using automated equipment that requires less sample manipulation and is easily scalable.

Table 6.5. Content of iminosugars and inositols in extracts of mulberry (M2L) leaves obtained by solid-liquid extraction and by PLE.

Extraction procedure	mg g^{-1} dry mulberry			
	Fagomine	**DNJ**	***Myo*-inositol**	**Glycosyl-inositols**
Solid-liquid extraction (3 cycles)	0.47 (0.01)*[a]	2.44 (0.04)[a]	7.88 (0.15)[a]	0.30 (0.07)[a]
PLE (1 cycle)	0.48 (0.15)[a]	2.52 (0.89)[a]	8.90 (1.88)[a]	0.35 (0.06)[a]

*Standard deviation in parentheses

[a]Entries followed by the same letter (a) in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

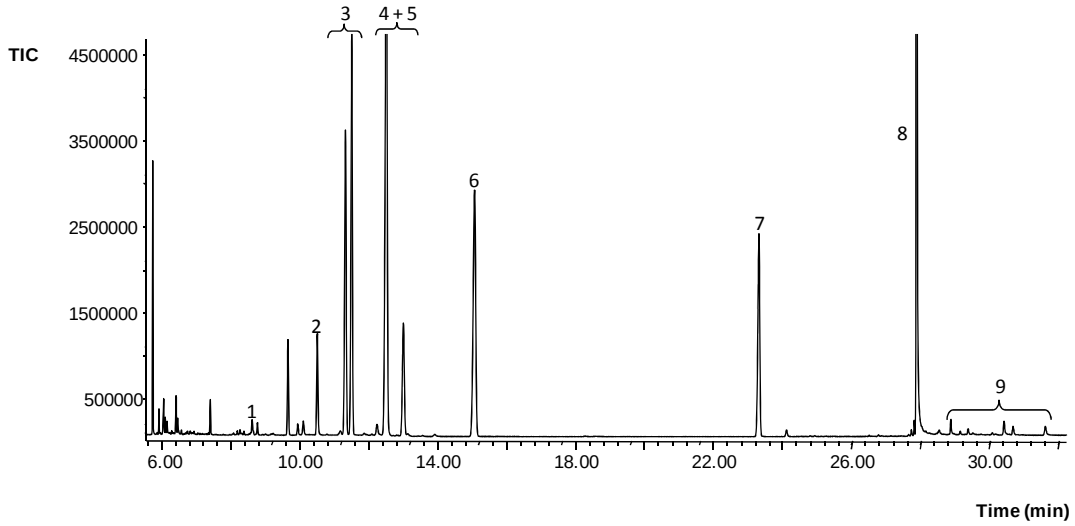


Figure 6.2. Chromatographic profile of a PLE extract of *Morusalba* (M2L) under optimized conditions. Peaks: 1, fagomine; 2, DNJ; 3, fructose; 4, glucose; 5, galactose; 6, *myo*-inositol; 7, phenyl-β-D-glucoside (I.S.); 8, sucrose; 9, glycosyl-inositols.

6.1.3.2. Fractionation procedures

- *Yeast treatment*

For *S. cerevisiae* treatment, a yeast/carbohydrate ratio (w/w) equal to 2:1 was selected by considering the previously determined carbohydrate content in mulberry extracts and in agreement with data reported in the literature [101-103, 105]. As indicated by Ruiz-Aceituno *et al*. [102], 37 °C was selected as an appropriate temperature for yeast incubations.

With regard to optimization of incubation time, **Figure 6.3** shows the evolution over time of iminosugars and inositoles (**Figure 6.3A**) and of other LMWC (glucose, fructose, galactose, sucrose, and trehalose) (**Figure 6.3B**) during fermentation with *S. cerevisiae* at 37 °C of the PLE extract from mulberry leaves (ML2). In these figures, data for control (extract not treated with yeast) and of extract taken just after the addition of yeast (0 h) are also included for comparison.

Iminosugar and inositol contents (**Figure 6.3A**) remained practically invariable irrespective of incubation time. However, noticeable differences were observed for the remaining carbohydrates (**Figure 6.3B**). Sucrose was digested at the beginning of the treatment due to the action of the extracellular invertase (*β*-fructosidase) present in yeast, as has been previously described by Ruiz-Matute *et al*. [84].

The main action of this enzyme is to break the glycosidic linkage of sucrose into glucose and fructose; this explains the increase in fructose with respect to the control extract. A similar trend should be expected for glucose. However, this monosaccharide decreases from the beginning of the treatment. Prior studies have shown that glucose is metabolized more quickly than fructose by this yeast [103].

Although both sugars are linked to the same transporters, they present a higher affinity for glucose than fructose [248]. In addition, it has been reported that glucose can suppress the expression of several transporters specific for fructose [249].

After 5 h of yeast treatment, nearly all glucose and fructose are removed from the extract. However, low amounts of galactose (1.47 mg g^{-1} dry mulberry) remain constant after 12 h of treatment. As a consequence of yeast metabolism, the appearance of trehalose was also observed; however, concentration of this carbohydrate decreased with digestion time. After 5 h of yeast treatment, very low levels of trehalose and galactose were detected. Galactose was completely fermented after 12 h, whereas a negligible amount of trehalose (0.79 mg g^{-1} dry mulberry) was determined at this time in mulberry extract.

These results indicate that incubating mulberry extracts with *S. cerevisiae* for 12 h at 37 °C allows the selective fractionation of interfering carbohydrates while preserving the contents of bioactive iminosugars and inositols.

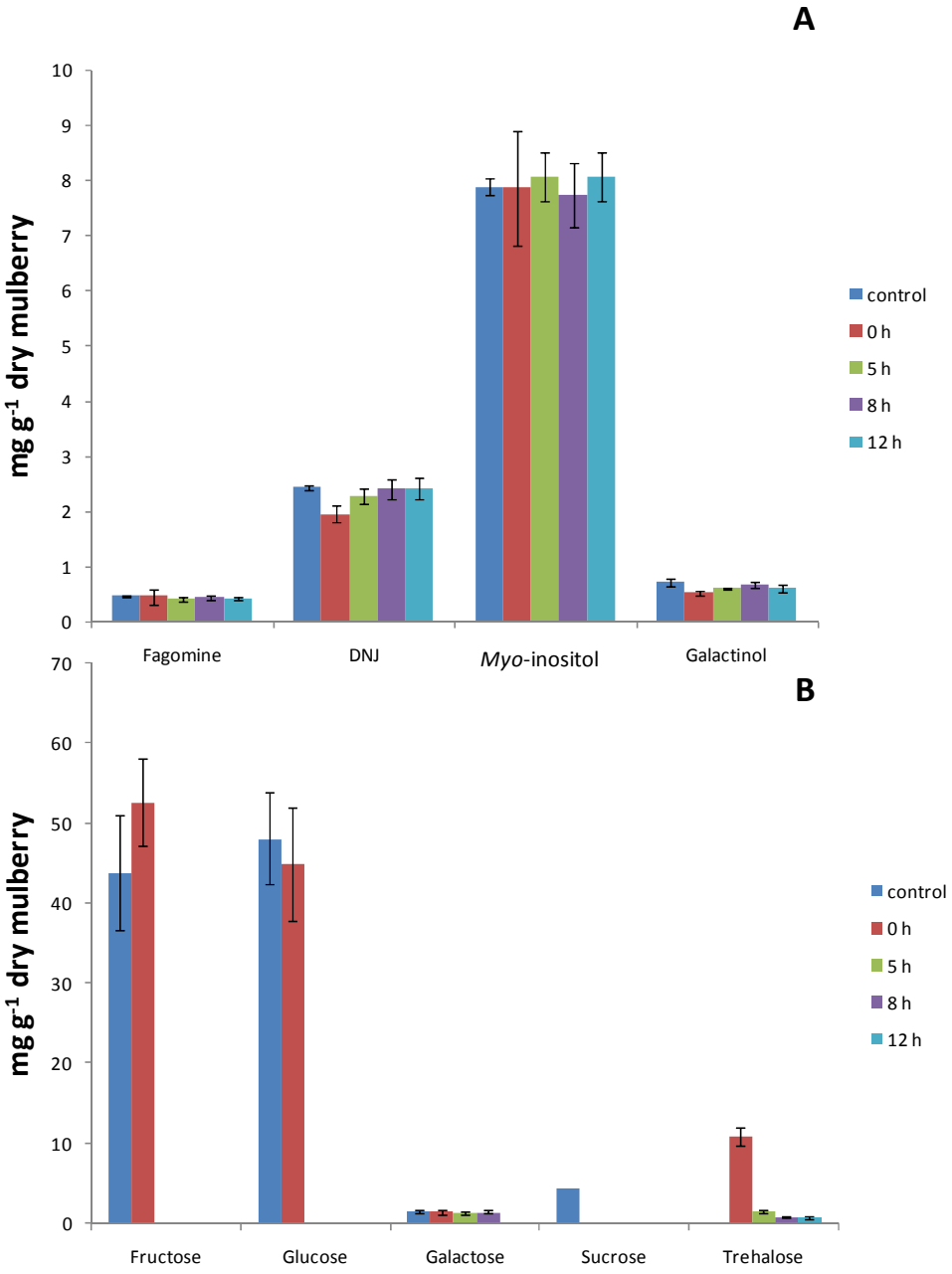


Figure 6.3. Evolution of iminosugar and inositol content (A) and of other LMWC (B) with time (0-12 h) of *S. cerevisiae* treatment at 37 ºC of PLE mulberry leaf (M2L) extract. Data labeled as control correspond to the extract not subjected to incubation with yeasts.

➢ *Ion-exchange chromatography*

The PLE extract of mulberry M2L was subjected to ion-exchange chromatography to remove interfering carbohydrates, according to the method of Nash *et al*. [243] with with slight modifications. For a fixed Dowex 50 resin weight (3 g), the amount of

extract to be loaded into the column and the volume of eluents (water for elution of carbohydrates and NH_4OH for iminosugars) were optimized.

Several volumes of PLE extract, corresponding to 90, 180,300, 600, and 800 mg of mulberry leaves, were evaluated, and 600 mg was found to be the optimal amount to avoid low recoveries or resin saturation. Water volume (12−42 mL) was also evaluated for the removal of carbohydrates. Whereas complete elution of fructose, glucose, and galactose was obtained after two to three washing stages (12−18 mL), total removal of sucrose required the number of column volumes to be increased in 6 (total volume = 36 mL of H_2O).

For iminosugar elution, volumes of NH_4OH ranging from 12 to 24 mL were tested. Eighteen milliliters (3 column volumes) allowed total recovery of the DNJ and fagomine present in the control extract. The highest concentrations of these iminosugars were recovered after the second elution stage, 82 and 86%for fagomine and DNJ, respectively. In this fraction, removal of interfering carbohydrates was practically complete. However, *myo*-inositol and glycosyl-inositols were not recovered in these fractions, and they eluted at the same time as the other LMWC.

Comparison of yeast and CEC treatments (**Table 6.6**) shows that both procedures preserved the initial content of iminosugars in mulberry extract; the goal of removing interfering carbohydrates (fructose, glucose, galactose, and sucrose) was also achieved. Under optimized conditions, yeast treatment provided minimal amounts of trehalose, whereas CEC treatment gave rise to the loss of inositols and derivatives; this is a disadvantage of CEC compared to yeast treatment, as inositols also play a role in the control of diseases related to carbohydrate metabolism, such as diabetes mellitus. However, more pure iminosugars were obtained by CEC, although their elution was achieved using a nonfood grade solvent (NH_4OH).Further research should be carried out to find an alternativesolvent that allows the implementation of this procedure in thefood industry.

Table 6.6. Content of iminosugars, inositols and other LMWC in the mulberry (M2L) extract obtained by conventional extraction before (control) and after yeast/CEC treatment.

	mg g^{-1} dry mulberry								
Sample	**Fagomine**	**DNJ**	**Fructose**	**Glucose**	**Galactose**	***Myo*-inositol**	**Sucrose**	**Trehalose**	**Glycosyl-inositols**
Control	0.47 (0.01)*[a]	2.44 (0.04)[a]	43.89 (7.17)[a]	48.10 (5.69)[a]	1.47 (0.16)[a]	7.88 (0.15)[a]	4.36 (0.04)[a]	-	0.32 (0.07)[a]
Yeast (12h)	0.41 (0.04)[b]	2.27 (0.14)[a]	-	-	-	8.06 (0.44)[a]	-	0.79 (0.08)[a]	0.30 (0.01)[a]
CEC (NH_4OH fraction)	0.48 (0.03)[a]	2.36 (0.19)[a]	-	-	-	-	tr**[b]	-	-
	mg g^{-1} dry extract								
Sample	**Fagomine**	**DNJ**	**Fructose**	**Glucose**	**Galactose**	***Myo*-inositol**	**Sucrose**	**Trehalose**	**Glycosyl-inositols**
Control	2.55 (0.05)[b]	13.80 (0.60)[b]	279.69 (19.88)[a]	308.16 (35.92)[a]	9.40 (1.16)[a]	46.08 (3.91)[a]	28.13 (6.32)[a]	-	1.87 (0.16)[a]
Yeast (12h)	2.58 (0.06)[b]	14.28 (0.60)[b]	-	-	-	50.42 (7.35)[a]	-	5.18 (0.29)[a]	1.79 (0.06)[a]
CEC (NH_4OH fraction)	11.36 (1.17)[a]	53.84 (5.97)[a]	-	-	-	-	-	-	-

*Standard deviation in brackets

**tr: traces

[a-b] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

6.1.4. Conclusions

In conclusion, once temperature, time, and sample amount are optimized, PLE has proved to be an appropriate technique for the extraction of bioactive carbohydrates (inositols and iminosugars) from plant tissues (e.g., mulberry leaves). This work also demonstrates that both yeast treatment and CEC are useful procedures for removing interfering carbohydrates; the selection of one or the other depends on the required composition of the plant extracts.

6.2. Pressurized liquid extraction of *Aglaonema* sp. iminosugars: Bioactivity, cell viability and thermal stability.

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Journal of Agricultural and Food Chemistry (enviada)

6.2.1. Introduction

The number of people suffering from carbohydrate metabolism disorders, such as diabetes mellitus and obesity, has dramatically increased over the last years. Among different drugs, iminosugars (also called polyhydroxyalkaloids) are generally used for treating these disorders [38, 69, 233, 250], as they are described to be potent *α*-glucosidase inhibitors [21, 232].

Several works have been focused on the chemical synthesis of these carbohydrates [61, 218, 234]; however, their extraction from natural sources is preferred if incorporation of these bioactive compounds as ingredients of functional foods is intended by the food industry.

A number of plant families (Euphorbiaceae, Moraceae, Campanulaceae, etc.) have been reported to contain iminosugars [52, 150, 251]. Among them, the wide variety of iminosugars present in *Aglaonema* sp. (Araceae), including homonojirimycin (HNJ), homomannojirimycin (HMJ), 2,5-dideoxy-2,5-imino-*D*-mannitol (DMDP), etc. [9, 21] (**section 5.2)**, has made of this plant an interesting source of these bioactive compounds.

Among other enhanced extraction procedures, pressurized liquid extraction (PLE) has gained wide acceptance for the rapid and efficient extraction of different metabolites, with low solvent consumption [84, 88]. However, the number of papers devoted to PLE extraction of carbohydrates is still scarce [84, 87], and only a previous paper has aimed its application to the improved extraction of iminosugars from mulberry (*Morus alba*) leaves (**section 6.1**). Moreover, the presence in plant extracts of LMWC, coextracted along with iminosugars and interfering with their bioactivity, made mandatory the development of appropriate clean-up protocols. In this sense, the use

of *Saccharomyces cerevisiae* has been shown to be advantageous for the selective removal of interfering LMWC in different food/plant extracts [99, 102] (**section 6.1**).

On the other hand, the incorporation of Aglaonema extract as ingredient of a functional food requires a previous evaluation of the thermal stability of target bioactives, as well as the evaluation of the potential toxicity of coextracted compounds other than iminosugars. To the best of our knowledge, there are few reports dealing with thermal stability of iminosugars [252-254]. In most of these high temperature-short time assays, iminosugars such as DNJ and miglitol (*N*-(2-hydroxymethyl)-1-deoxynojirimycin) have been shown to be stable upon heating to temperatures well above ambient temperature. Regarding safety studies, iminosugars have been described to be well tolerated drugs [255, 256], and plant extracts including iminosugars have been reported to show lower side effects (flatulence, diarrhea, abdominal cramping, etc) as compared to other α-glucosidase inhibitors [257]. However and as far as we know, no reference has yet addressed the evaluation for the first time of the effect on cell viability of whole Aglaonema extracts.

The aims of the present paper are: (i) optimization of a PLE method for extraction of iminosugars from *Aglaonema* sp., (ii) the characterization of iminosugar composition of PLE extracts of different Aglaonema cultivars; and (iii) the study of α-glucosidase inhibition activity, thermal stability and effect on cell viability of Aglaonema PLE extracts with a view to evaluate its potential application as ingredients of functional foods.

6.2.2. Materials and methods

6.2.2.1. Standards

Analytical standards of α-HNJ and DMDP were purchased from Dextra Laboratories (Reading, UK). *Myo*-inositol, mannitol, *chiro*-inositol, fructose, glucose, sucrose, galactose and phenyl-β-*D*-glucoside were obtained from Sigma Chemical Co. (St. Louis, USA). 1-Deoxynojirimycin (DNJ) was purchased from Toronto Research Chemicals Inc. (Ontario, Canada). Miglitol (Distabol, Sanofi-Aventis) was acquired in a local pharmacy.

6.2.2.2. Samples

Seven Aglaonema cultivars (*A. commutatum* 'Treubii' 1 and 2 (ACT1, ACT2), *A. commutatum* 'Emerald Beauty' 1 and 2 (ACEB1, ACEB2), *A.commutatum* 'Crete' 1 and 2 (ACC1, ACC2) and *A*. hybrid 'Black Lance' (AHBL)) were acquired in local garden centers in Madrid (Spain). For Aglaonema plants yielding enough sample weight, both roots and leaves (sample codes ending in R and L, respectively) were considered.

Aglaonema samples were air-dried under controlled temperature (25°C) and in the absence of light for 5 days. Dried samples were ground in a domestic mill (Moulinex), sieved (<500 µm) and stored under dry conditions and protected from direct light until analysis.

6.2.2.3. Pressurized liquid extraction

Extractions were carried out in the laboratory-made miniaturized PLE system previously described in **section 6.1.2.2**. Two cells of different dimensions were considered: a small cell (100 mm × 4.6 mm i.d., total volume = 1.5 mL) for method optimization and a big cell (75 mm × 9 mm i.d., volume = 4.75 mL) for higher volume extraction of the different Aglaonema samples under optimized conditions.

Based on previous results (**section 6.1**), water was selected as optimal solvent for PLE extraction of Aglaonema iminosugars. PLE was optimized for the following experimental parameters: sample amount (10, 20, 40, 80 and 160 mg), extraction temperature (50, 100 and 150 ºC), time (2, 5and 10 min) and number of cycles (1-3). The extraction pressure was always set at 10 MPa. All experiments were carried out in triplicate.

6.2.2.4. Chromatographic analysis

- *Derivatization*

A two-step derivatization procedure (oximation + silylation) [149] was followed prior to GC-MS analysis of iminosugars and other coextracted LMWC. One milliliter of PLE Aglaonema extract was mixed with 0.5 mL of internal standard (phenyl-*β*-*D*-glucoside, 1 mg mL^{-1}) and evaporated under vacuum prior to derivatization. Oximes

were obtained by adding 350 µL of a 2.5% solution of hydroxylamine hydrochloride in pyridine, which was then heated for 30 min at 75°C. These derivatives were then silylated with hexamethyldisilazane (350 µL) and trifluoroacetic acid (35 µL) at 45°C for 30 min. Under these derivatization conditions, iminosugars give rise to a single chromatographic peak corresponding to the *O*-persilylated form, and *N*-silylated derivatives are not obtained, as previously indicated in **section 4.1**. After reaction, samples were centrifuged at 4400g for 10 min, and 1 µL of supernatant was injected into the GC injection port.

- Gas Chromatography-Mass Spectrometry (GC-MS) analysis

GC-MS analyses (*n* =3 replicates) were carried out following the validated method of **section 4.2**, using a 7890A gas chromatograph coupled to a 5975C quadrupole mass detector (Agilent Technologies, Palo Alto, CA, USA). Analyses were done on a crosslinked methylpolysiloxane TBR-1 capillary column (Teknokroma, Barcelona, Spain) (30 m length × 0.25 mm i.d., 0.25 µm d.f.), using helium at ~1 mL min^{-1} as carrier gas. The oven temperature was programmed from 100 °C to 200°C (15 min) at a heating rate of 15°C min^{-1} and finally programmed to 300 °C (15 min) at 15°C min^{-1}. Injections were carried out in split mode (1:20) at 240 °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 *m/z* mass range 35−650. Acquisition was done using HPChem Station software (Agilent Technologies).

Qualitative analysis was based on the comparison of experimental spectra with published data [11, 53, **sections 4.1, 4.3** and **6.1**] was confirmed, when possible, by using linear retention indices (I^T). Available standards were also used for further confirmation of identifications.

Quantitative data were obtained by the internal standard method. Standard solutions were prepared over the expected concentration range in Aglaonema extracts to calculate the response factor relative to phenyl-*β*-*D*-glucoside: glucose, fructose and sucrose (0.05−0.50 mg mL^{-1}); DMDP and *α*-HNJ (0.01-0.04 mg mL^{-1}); *myo*-inositol (0.01−0.5mg mL^{-1}). For other iminosugars, response factors were assumed to be that of *α*-HNJ.

6.2.2.5. Yeast treatment

Yeast treatment of Aglaonema extracts was carried out following the method described in **section 6.1**, although optimization of incubation time was required considering the different sugar composition of the samples. Aqueous Aglaonema PLE extracts (10 mL) were incubated with 0.1 g of yeasts (*S. cerevisiae* type II) (Sigma Chemical Co., St. Louis, USA) at 37 °C under stirring. Aliquots were taken after 1, 2, 3, 5, 7, and 48 h of treatment. Yeast removal was immediately carried out by centrifugation at 4400*g* for 10 min at 4 °C, followed by filtration through Whatman no. 4 paper filter. PLE Aglaonema extracts that had not previously undergone yeast treatment were used as controls in this study. All assays were carried out in triplicate.

6.2.2.6. *α*-Glucosidase inhibition assays

α-Glucosidase inhibitory activity was determined according to the procedure previously described by Tao *et al*. [258] and Feng *et al*. [259], after optimization of enzyme and substrate concentrations. 20 µL of a 0.25 U mL^{-1} solution of *α*-glucosidase from *Saccharomyces cerevisiae* (Type II) (Sigma Chemical Co.) in phosphate buffer (pH 6.8) (Sigma Chemical Co.) were premixed with 20 µL of Aglaonema extracts of different concentrations (0.001-0.6 mg total iminosugars mL^{-1}) in 96-well plates. Controls were conducted by replacing inhibition solutions with water. Similar concentrations of DNJ and *α*-HNJ were used as positive controls.

After incubation at 37 ºC for 10 min, 40 µL of 0.5 mM *p*-nitrophenyl-*α*-*D*-glucopyranoside (pNPG) (Sigma) in phosphate buffer were added as enzyme substrate. The reaction was incubated at 37 ºC for 30 min and stopped by adding 80 µL of 0.2 M Na_2CO_3 in phosphate buffer. After soft stirring of the 96-well plate, the *α*-glucosidase activity was determined by measuring the *p*-nitrophenol released from pNPG at 405 nm.

The percent inhibition was determined according to the following equation:

$$\%\ \text{inhibition}_{\alpha-\text{glucosidase}} = [(\Delta A_{\text{control}} - \Delta A_{\text{sample}})/\Delta A_{\text{control}}] \times 100 \quad \text{Eq. (7)}$$

where:

$$\Delta A_{\text{control}} = A_{\text{test}} - A_{\text{blank}}$$

$$\Delta A_{\text{sample}} = A_{\text{test}} - A_{\text{blank}}$$

IC_{50} value, defined as the concentration of α-glucosidase inhibitor to inhibit 50 % of its activity under the assayed conditions, was obtained from non-linear curve-fitting of inhibition percentage *vs* logarithm of inhibitor concentration.

6.2.2.7. Cell culture and Aglaonema extracts treatments

Human Caco-2 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) F-12 medium (Lonza), supplemented with10% fetal bovine serum (FBS) (Lonza) and 50 mg L^{-1} of each of the following antibiotics, gentamicin, penicillin, and streptomycin (Sigma). Cells were maintained at 37ºC in a humidified atmosphere of 5% CO_2.

Cells were changed to serum-free medium 24 h before the assay in order to avoid the influence of the growth factors contained in the FBS on the results. To study the cellular effect of PLE Aglaonema extracts, different concentrations (5-500 µg mL-1), diluted in serum-free culture medium, were added to the cell plates for 24 h. At the end of the incubation period, cell cultures were processed according to the assay shown below.

6.2.2.8. Evaluation of cell viability

Cell viability was determined by using the crystal violet assay [260]. Caco-2 cells were seeded ($2x10^5$cells per well) in 24-well plates (Falcon, Cajal), grown for 20 h, treated with different PLE Aglaonema extracts, and then incubated with crystal violet (Sigma) (0.2% in ethanol) for 20 min. Plates were rinsed with distilled water and allowed to dry; 1% sodium dodecyl sulfate (Panreac) was later added. The absorbance of each well was measured using a microplate reader (Bio-Tek, Winooski, VT, USA) at 570 nm. Cell viability was determined as relative percent of Crystal Violet stained control cells.

6.2.2.9. Thermal stability

ACEB2L extract (12 mL) was selected as a representative sample for accelerated assays storage. Aliquots (2 mL) were freeze-dried and stored in closed 5 mL vials at controlled temperature (50 ± 5 °C) for 1 month to simulate storage under non-favorable conditions. Sampling was carried out in duplicate after 0, 3, 7, 14, 21 and 30 days of storage.

6.2.2.10. Statistical analysis

Data were subjected to analysis of variance (ANOVA, Fischer's least significant difference (LSD) test) by using Statistica 7.0 software for Windows (StatSoft, Inc., Tulsa, OK, USA). The significance of differences was defined as $P < 0.05$.

6.2.3. Results and discussion

6.2.3.1. Characterization of *Aglaonema* sp. extracts

The selection of optimal operating parameters in the pressurized liquid extraction of Aglaonema iminosugars requires a prior characterization of the composition of Aglaonema extracts. For this purpose, extracts previously derivatized were analyzed by gas chromatography-mass spectrometry (GC-MS).

Figure 6.4 shows the GC-MS profile of extracted carbohydrates from ACEB1L extract previously derivatized. GC-MS profiles of both Aglaonema leaves and roots were qualitative similar. Fructose, glucose, sucrose and *myo*-inositol were the most abundant LMWC detected in both Aglaonema extracts. Two other polyalcohols (mannitol and *chiro*-inositol) were also present as minor compounds.

Different chromatographic peaks with mass spectra compatible with iminosugar structures were also detected. **Table 6.7** shows I^T data and abundances of characteristic *m/z* ratios of the 7 *O*-persilylated iminosugars found in extracts of Aglaonema samples under study. Full scan (EI) mass spectra for these compounds are included in **Figure 4S** of **Anexo III**.

As expected, molecular ions of persilylatediminosugars were not observed in (EI) mass spectra; however, $[M-15]^+$ ions were always present. Peaks 2 and 11in **Figure 6.4** were assigned as DMDP and *α*-HNJ, respectively, by comparison of experimental I^T and characteristic fragment ions with data for corresponding standards.

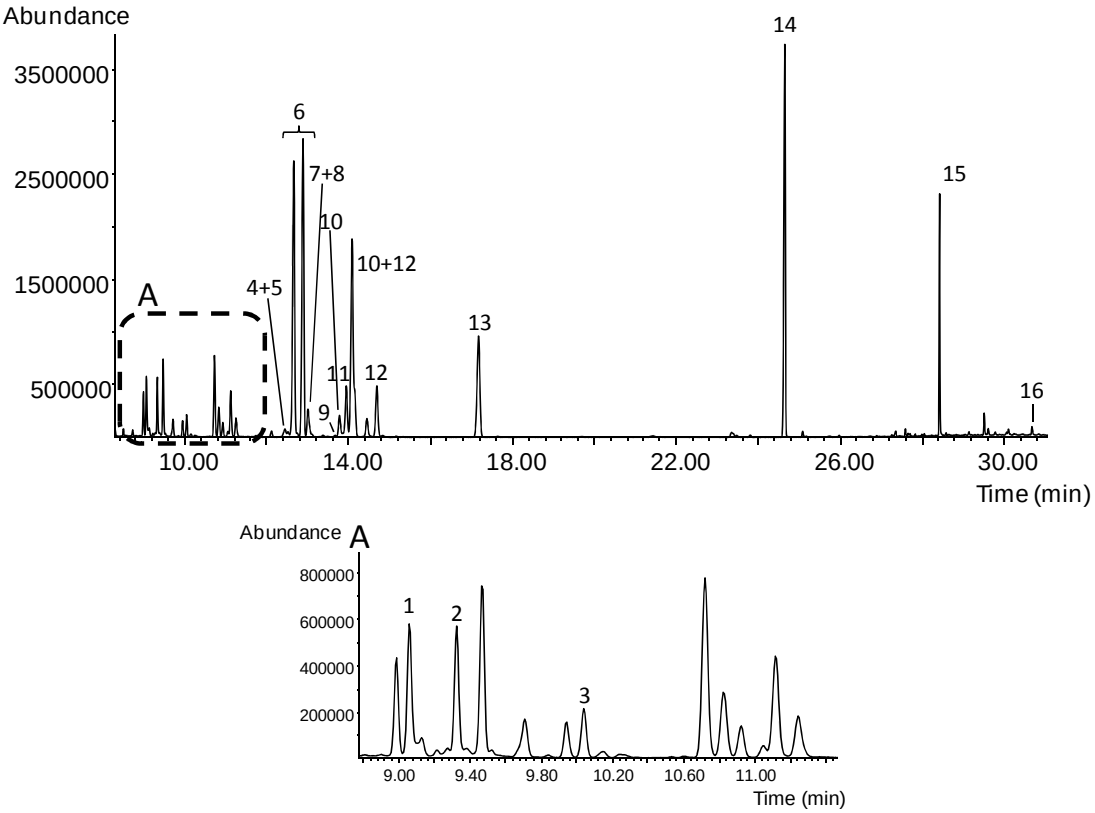

Figure 6.4. GC-MS profile of ACEB1L extract previously derivatized. 2,3,4-trihydroxy-5-hydroxymethyl piperidine, 1; DMDP, 2; 2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine, 3; *α*-HMJ, 4; mannitol, 5; fructose, 6; *α*-*allo*-HNJ, 7; *chiro*-inositol, 8; miglitol isomer, 9; galactose, 10; *α*-HNJ, 11; glucose, 12; *myo*-inositol, 13; phenyl-*β*-*D*-glucoside (I.S.), 14; sucrose, 15; glycosyl-HNJ, 16.

Table 6.7. Linear retention indices (I^T) and relative abundances for characteristic *m/z* ions of TMS iminosugars detected in Aglaonema extracts.

Iminosugar	Molecular weight of TMS derivatives (*u*)	I^T	*m/z*
2,3,4-trihydroxy-5-ydroxymethyl piperidine	451	1783	73 (100), 144 (1), 147 (31), 168 (1), 217 (21), 258 (1), 191 (12), 320 (19), 348 (3), 436 (32)
DMDP	451	1806	73 (87), 144 (15), 147 (18), 168 (10), 217 (15), 258 (15), 348 (100), 436 (3)
2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine	539	1857	73 (100), 147 (44), 191 (11), 204 (7), 217 (9), 319 (17), 346 (2), 408 (18), 436 (72), 524 (1)
α-HMJ	553	1987	73 (100), 147 (23), 217 (21), 246 (18), 270 (7), 360 (7), 450 (47), 538 (1)
α-allo-HNJ	553	2040	73 (100), 147 (36), 217 (36), 246 (21), 270 (7), 360 (5), 450 (42), 538 (1)
Miglitol isomer	567	2056	73 (100), 147 (24), 260 (16), 374 (11), 464 (44), 552 (2)
α-HNJ	553	2063	73 (100), 147 (28), 217 (26), 246 (26), 270 (10), 360 (6), 450 (55), 538 (2)
Glycosyl-HNJ	931	3036	73 (100), 147 (30), 217 (35), 244 (21), 361 (13), 450 (17), 492 (3), 624 (1)

Several peaks were tentatively identified or characterized based on their mass spectral and chromatographic retention data. Thus, two peaks with mass spectra similar to that of α-HNJ and I^T of 1987 and 2040 (peaks 4 and 7) were tentatively assigned as α-HMJ and α-*allo*-HNJ, according to retention data previously reported by Asano *et al.* [18, 58]. Peak 1 with I^T of 1783 was tentatively assigned as 2,3,4-trihydroxy-5-hydroxymethyl piperidine based on $I^T$and mass spectra previously indicated in **section 4.3**. Peak 9 showing a mass spectrum similar to that of miglitol standard (**section 4.3**), but with a different linear retention index (I^T of 2056), was here tentatively identified as a miglitol isomer. Finally, peak 3 with a I^T of 1857 and an abundant *m/z* ion at 436 ($[M\text{-}CH_2OTMS]^+$) was identified for the first time in this paper as a $C_6NO_5H_{13}$iminosugar. The presence of characteristic *m/z* ions at 346 ($[436\text{-}TMSOH]^+$), at 319 ($[436\text{-}(CH_2)_2OTMS)]^+$ and at 408 ($[M\text{-}(CH_2)_2\text{-}CH_2OTMS]^+$) allowed us to tentatively identify this compound as 2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine.

6.2.3.2. Pressurized liquid extraction

Optimization of PLE extraction of bioactive iminosugars was done using AHBLR sample. Yield of iminosugar extraction was considered for selection of optimal PLE conditions, as removal of major interfering carbohydrates (glucose, fructose and sucrose) would be further addressed by yeast (*S. cerevisiae*) incubation of Aglaonema extracts (see **section 6.2.3.3**).

First, optimization of sample amount was carried out at 100 °C for 5 min (**Table 6.8**). According to the ANOVA test for comparison of mean values, significant differences were found for the amount of iminosugars extracted with Aglaonema weight (10, 20, 40, 80 and 160 mg). Eighty mg was chosen as optimal PLE amount as it yielded the highest recoveries for all iminosugars. Optimal extraction temperature was then evaluated for 80 mg of Aglaonema sample and 5 min of extraction time. Similar to results obtained in **section 6.1**, as shown in **Table 6.9**, both 50 and 100 ºC provided similar yields for iminosugars. Only significant differences in the extraction of α-HNJ and miglitol isomer were obtained. These iminosugars were better extracted at 100ºC; therefore, this last temperature was selected for optimization of extraction time (2, 5

and 10 minutes) (**Table 6.10)**. Although most iminosugars were equally extracted at 2 and 5 minutes, *α*-HMJ and *α-allo*-HNJ showed higher recoveries at 2 minutes.

Under optimized conditions (80 mg, 100 ºC and 2 min), the number of extraction cycles was also evaluated. Although two cycles were necessary to maximize the extraction of miglitol isomer and the compound tentatively identified as 2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine (recoveries for the first and second extractions in the range 92-95% and 8-5%, respectively); a single cycle was enough to extract more than 96 % of the remaining Aglaonema iminosugars and, therefore, one extraction was selected for further PLE extractions of Aglaonema samples.

6.2.3.3. Yeast treatment

In order to achieve the enrichment in iminosugars of Aglaonema extracts, removal of major interfering carbohydrates by incubation with *Saccharomyces cerevisiae*for different times (1-48 h) was assayed using ACT1L PLE extract. In agreement with previous studies on *S. cerevisiae* treatment of carbohydrate-rich extracts [101-103], a yeast/carbohydrate (w/w) ratio of 2:1 and a temperature of 37 °C were used. Five hours of yeast fermentation were enough for digestion of carbohydrates (glucose, fructose and sucrose) interfering with the *α*-glucosidase inhibitory activity of Aglaonema extracts with no loss of target bioactive iminosugars. Trehalose formation derived from yeast metabolism was not significant after 5 hours of yeast treatment.

Table 6.8. Optimization of sample amount in the PLE extraction (100 ºC; 5 min) of iminosugar and other LMWC from AHBLR.

Sample amount (mg)	mg g^{-1} dry AHBLR											
	2,3,4-trihydroxy-5-hydroxymethyl piperidine	DMDP	2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine	α-HMJ	α-*allo*-HNJ	Miglitol isomer	α-HNJ	Fructose	Glucose	*Myo*-inositol	Sucrose	Glycosyl-HNJ
10	0.07 (0.02)*[a]	0.23 (0.10)[a]	0.32 (0.12)[a]	-	0.07 (0.02)[a]	0.07 (0.02)[a]	0.43 (0.15)[a]	5.84 (0.26)[a]	3.43 (0.32)[a]	1.56 (0.09)[a]	13.29 (0.01)[a]	0.18 (0.02)[a]
20	0.11 (0.01)[b]	0.28 (0.06)[a]	0.37 (0.11)[a]	-	0.079 (0.002)[a]	0.08 (0.02)[a]	0.57 (0.06)[a]	3.25 (0.32)[b]	3.87 (0.29)[ab]	1.73 (0.14)[b]	5.83 (1.20)[b]	0.35 (0.02)[b]
40	0.10 (0.03)[ab]	0.47 (0.09)[a]	0.30 (0.07)[a]	-	0.08 (0.03)[a]	0.05 (0.01)[a]	0.57 (0.23)[a]	8.14 (1.63)[cd]	3.92 (1.69)[ab]	1.42 (0.13)[a]	7.30 (0.43)[b]	0.19 (0.01)[a]
80	0.12 (0.01)[b]	1.16 (0.19)[b]	0.37 (0.04)[a]	0.017 (0.002)[a]	0.13 (0.01)[b]	0.07 (0.01)[a]	1.00 (0.01)[b]	9.20 (0.83)[d]	5.78 (0.31)[c]	1.78 (0.08)[b]	15.40 (1.70)[c]	0.37 (0.10)[b]
160	0.098 (0.002)[ab]	0.79 (0.16)[ab]	0.25 (0.08)[a]	0.010 (0.002)[b]	0.11 (0.01)[b]	0.06 (0.01)[a]	0.99 (0.26)[b]	7.56 (0.77)[c]	4.51 (0.17)[b]	1.04 (0.06)[c]	13.07 (0.70)[ac]	0.38 (0.08)[d]

* Standard deviation is given in brackets.
[a-d] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

Table 6.9. Optimization of extraction temperature in the PLE extraction (80 mg; 5 min) of iminosugar and other LMWC from AHBLR.

Temperature (°C)	mg g^{-1} dry AHBLR											
	2,3,4-trihydroxy-5-hydroxymethyl piperidine	DMDP	2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine	α-HMJ	α-*allo*-HNJ	Miglitol isomer	α-HNJ	Fructose	Glucose	*Myo*-inositol	Sucrose	Glycosyl-HNJ
50	0.10 (0.03)*[a]	1.01 (0.15)[a]	0.31 (0.04)[a]	0.01 (0.03)[a]	0.11 (0.02)[a]	0.017 (0.001)[a]	0.87 (0.04)[a]	5.57 (0.02)[a]	4.55 (0.31)[a]	1.76 (0.06)[a]	14.97 (0.37)[a]	0.55 (0.02)[b]
100	0.12 (0.01)[a]	1.16 (0.19)[a]	0.37 (0.04)[a]	0.017 (0.002)[a]	0.13 (0.01)[a]	0.07 (0.01)[b]	1.00 (0.01)[b]	9.20 (0.83)[b]	5.78 (0.31)[b]	1.78 (0.08)[a]	15.40 (1.70)[a]	0.37 (0.10)[a]
150	0.01 (0.01)[b]	0.45 (0.04)[b]	0.04 (0.02)[b]	-	0.07 (0.01)[b]	0.006 (0.001)[c]	0.33 (0.02)[c]	5.78 (1.22)[a]	3.22 (0.69)[c]	0.79 (0.10)[b]	4.92 (2.80)[b]	0.63 (0.04)[c]

* Standard deviation is given in brackets.
[a-c] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

Table 6.10. Optimization of extraction time in the PLE extraction (80 mg; 100 ºC) of iminosugar and other LMWC from AHBLR.

Time (min)	mg g^{-1} dry AHBLR											
	2,3,4-trihydroxy-5-hydroxymethyl piperidine	DMDP	2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine	α-HMJ	α-*allo*-HNJ	Miglitol isomer	α-HNJ	Fructose	Glucose	*Myo*-inositol	Sucrose	Glycosyl-HNJ
2	0.14 (0.01)*[a]	1.08 (0.28)[a]	0.26 (0.07)[a]	0.021 (0.001)[a]	0.19 (0.01)[a]	0.068 (0.001)[a]	1.05 (0.05)[a]	3.68 (0.28)[a]	4.49 (0.37)[a]	1.64 (0.07)[a]	7.22 (2.98)[a]	0.36 (0.08)[a]
5	0.12 (0.01)[a]	1.16 (0.19)[a]	0.37 (0.04)[a]	0.017 (0.002)[b]	0.13 (0.01)[b]	0.07 (0.01)[a]	1.00 (0.01)[a]	9.20 (0.83)[b]	5.78 (0.31)[b]	1.78 (0.08)[b]	15.40 (1.70)[b]	0.37 (0.10)[a]
10	0.025 (0.004)[b]	0.78 (0.19)[a]	0.08 (0.02)[b]	0.004 (0.006)[c]	0.12 (0.03)[b]	0.02 (0.01)[b]	0.62 (0.15)[b]	4.53 (0.27)[c]	5.64 (0.27)[b]	2.20 (0.02)[c]	19.28 (0.64)[c]	0.21 (0.01)[b]

* Standard deviation is given in brackets.
[a-c] Entries followed by the same letter in the same column showed no statistically significant differences for their mean values at the 95.0% confidence level.

6.2.3.4. Quantitative analysis of Aglaonema extracts

Table 6.11 shows the content of bioactive iminosugars present in different Aglaonema PLE extracts after yeast incubation.

In general, *α*-HNJ was the most abundant iminosugar in all Aglaonema extracts under study (0.74-1.94 mg g^{-1} dry leaves and 1.35-3.87mg g^{-1} dry roots), followed by DMDP (0.29-0.82 mg g^{-1} dry leaves and 1.08-3.16mg g^{-1} dry roots). Whereas average content of *α-allo*-HNJ and glycosyl-HNJ was found to be similar irrespective of the Aglaonema part (leaves or roots) considered for PLE extraction, concentration of *α*-HNJ, DMDP, miglitol isomer and 2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine was higher in root extracts. On the contrary, leaf extracts showed slightly higher content of *α*-HMJ.

6.2.3.5. *α*-Glucosidase inhibitory assays

Once characterized the composition of the different PLE Aglaonema extracts, evaluation of the *α*-glucosidase inhibitory activity associated with their iminosugar composition was carried out. Despite total iminosugar content was higher for root extracts, the higher yield (on a weight basis) of Aglaonema leaves makes of them a more feasible source for PLE extraction of iminosugars and, therefore, they were selected for further studies.

α-Glucosidase inhibitory capacity of Aglaonema leaf extracts was evaluated together with that of two iminosugar standards: DNJ as one of the most active *α*-glucosidase inhibitors [257] and *α*-HNJ as the major compound present in all Aglaonema extracts (**Table 6.11**).

Table 6.11. Iminosugar content (mg g^{-1} dry sample) of PLE extracts of different Aglaonema samples after 5 hour-incubation with *Saccharomyces cerevisiae*.

Sample	mg g^{-1} dry Aglaonema								Total iminosugars
	2,3,4-trihydroxy-5-hydroxymethyl piperidine	DMDP	2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine	α-HMJ	α-*allo*-HNJ	Miglitol isomer	α-HNJ	Glycosyl-HNJ	
AHBLL*	0.36 (0.03)**	0.37 (0.02)	0.081 (0.001)	0.09 (0.01)	0.45 (0.04)	-	0.74 (0.07)	0.93 (0.14)	3.02
AHBLR*	0.22 (0.01)	1.08 (0.28)	0.53 (0.04)	0.034 (0.002)	0.56 (0.05)	0.142 (0.004)	2.25 (0.26)	0.36 (0.05)	5.18
ACT1L	0.32 (0.04)	0.41 (0.03)	0.12 (0.01)	0.066 (0.001)	0.10 (0.01)	-	1.68 (0.04)	0.23 (0.03)	2.93
ACT2L	0.28 (0.08)	0.39 (0.02)	0.08 (0.00)	0.06 (0.03)	0.09 (0.01)	-	1.94 (0.34)	0.19 (0.01)	3.03
ACT2R	2.26 (0.59)	1.45 (0.36)	0.33 (0.15)	-	0.07 (0.02)	-	1.82 (0.59)	0.19 (0.01)	6.12
ACEB1L	0.49 (0.11)	0.82 (0.16)	0.19 (0.01)	0.07 (0.01)	0.19 (0.02)	0.04 (0.01)	0.87 (0.10)	0.098 (0.001)	2.77
ACEB1R	0.51 (0.08)	1.49 (0.04)	0.12 (0.04)	0.12 (0.03)	0.06 (0.00)	0.16 (0.01)	1.35 (0.27)	0.24 (0.04)	4.05
ACEB2L	0.81 (0.13)	0.80 (0.04)	0.23 (0.01)	0.09 (0.03)	0.20 (0.04)	0.03 (0.01)	1.80 (0.31)	0.08 (0.01)	4.04
ACEB2R	0.58 (0.21)	1.74 (0.67)	0.73 (0.18)	0.12 (0.01)	0.09 (0.02)	0.18 (0.01)	2.46 (0.67)	0.16 (0.03)	6.06
ACC1L	0.30 (0.03)	0.30 (0.03)	0.09 (0.04)	0.51 (0.04)	0.37 (0.01)	0.33 (0.04)	1.35 (0.04)	0.06 (0.01)	3.31
ACC1R	0.58 (0.03)	3.16 (0.08)	1.94 (0.02)	0.10 (0.00)	0.12 (0.00)	1.17 (0.03)	3.87 (0.12)	0.85 (0.03)	11.79
ACC2L	0.22 (0.03)	0.29 (0.08)	0.04 (0.001)	-	0.36 (0.02)	0.27 (0.02)	0.92 (0.16)	0.05 (0.01)	2.15

*Sample codes ending in L and R correspond to leaf and root Aglaonema samples, respectively.

**Standard deviation is given in brackets.

As expected, inhibitory effect on *α*-glucosidase of both Aglaonema extracts and iminosugars was found to decrease in a dose-dependent manner (data not shown). As shown in **Table 6.12**, half maximal inhibitory concentration (IC_{50}) values were significantly lower for Aglaonema leaf extracts when compared to data for iminosugar standards experimentally obtained and previously reported in the literature [261, 262]. Different factors should be considered for explanation of these results. First, the synergic effect of the different iminosugars should always be considered to explain the bioactivity of natural extracts. In this sense, despite the similar inhibitory pattern of both *α*-HNJ and Aglaonema extracts which could point out to a noticeable contribution of this major iminosugar to the bioactivity of Aglaonema extracts, other minor iminosugars could also play an important role on *α*-glucosidase inhibition. This could be supported by data obtained for ACEB1L and ACT1L extracts which showed the highest and lowest inhibition, respectively. Although the total iminosugar content (3.30 mg g^{-1} dry leaves) and *α*-HNJ concentration (1.68 mg g^{-1} dry leaves, respectively) were higher for ACT1L sample, ACEB1L extract with a total iminosugar concentration of 2.77 mg g^{-1} dry leaves and an *α*-HNJ content of 0.87 mg g^{-1} dry leaves was more bioactive. On the other hand, and according to the results previously reported for mulberry iminosugars [263], the significantly different IC_{50} values determined for ACT1L and ACT2L could also be attributed to the different ripening stage of these two samples from the same cultivar.

Table 6.12. IC_{50} values (mg mL^{-1}) for iminosugar standards and different Aglaonema leaf PLE extracts.

Sample/Standard	IC_{50} (mg mL^{-1})
DNJ	0.169[a]
α-HNJ	0.110[b]
AHBLL	0.032[c]
ACT1L	0.079[d]
ACT2L	0.013[f]
ACEB1L	0.010[g]
ACC1L	0.019[h]
ACC2L	0.017[h]

Furthermore, it was also found that bioactivity of Aglaonema extracts was significantly higher as compared to that of iminosugar standards. These results agree with data previously reported by Kwon *et al*. [257] and Kim *et al*. [264], who postulated that active unabsorbed components other than iminosugars in mulberry leaf extracts might also contribute to reduce intestinal glucose digestion and absorption, as compared to equivalent amounts of pure iminosugars(e.g. DNJ).

6.2.3.6. Cell viability assays

With the aim of evaluating their potential application as functional ingredients of foods, *in vitro* studies on viability of Caco-2 cells after treatment with Aglaonema leaf extracts was done as a measurement of the potential toxicity of coextractives other than iminosugars. As shown in **Figure 6.5**, the percentage of alive Caco-2 cells decreased after treatment with concentrations higher than 125 μg mL^{-1} of PLE extracts of both samples of *A. commutatum* 'Treubii', whereas the rest of Agloanema leaf extracts showed no toxicity in the whole concentration range assayed.

As it can be observed, the IC_{50} values obtained for *α*-glucosidase inhibition (**Table 6.12**) are included within the non-toxic range of Aglaonema extracts (**Figure 6.5)**. This fact contributes to support the potential of these extracts as bioactive to be included in the elaboration of safe functional foods.

6.2.3.7. Thermal stability

Potential changes in the iminosugar content of Aglaonema extracts with storage were measured as a way to evaluate the feasibility of these compounds as stable bioactives. As shown in **Figure 6.6**, no significant differences in the content of iminosugars were found after storage of ACEB2L extract under accelerated conditions (50 ºC, 1 month). In agreement with these results, Yatsunami *et al.* [252] reported the stability of DNJ upon accelerated heating (121 °C for up to 15 min). Miglitol has also been described to minimally degrade under conditions well above those usually employed for storage of plant bioactives [254]. The thermal stability of Aglaonema iminosugars is another favourable feature that might contribute to the promising incorporation of Aglaonema

PLE extracts as functional ingredients of foodstuffs stored or processed under mild heating conditions.

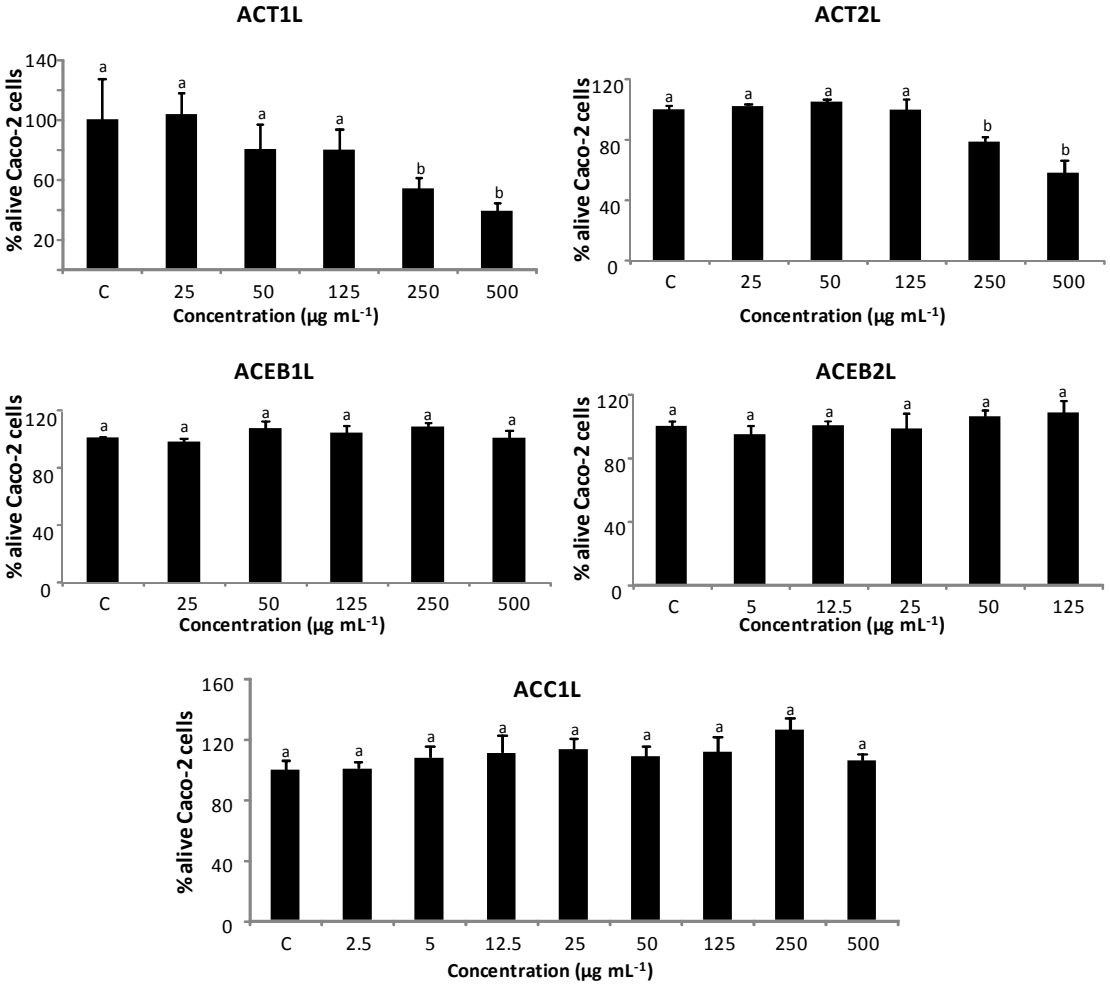

Figure 6.5. Percentage of alive Caco-2 cells *vs* total iminosugar concentration of the different Aglaonema leaf extracts under study. Bars labeled with the same letter (a, b) over the same Aglaonema extract showed no statistically significant differences for their mean values at the 95.0% confidence level. Cell viability was determined as relative percent of Crystal Violet stained control cells.

6.2.4. Conclusions

To conclude, the PLE method here optimized has allowed the complete extraction of most iminosugars present in Aglaonema extracts. Based on GC-MS analyses (mass spectral and chromatographic retention data) several iminosugars corresponding to iminosugars were tentatively identified or characterized for the first time.

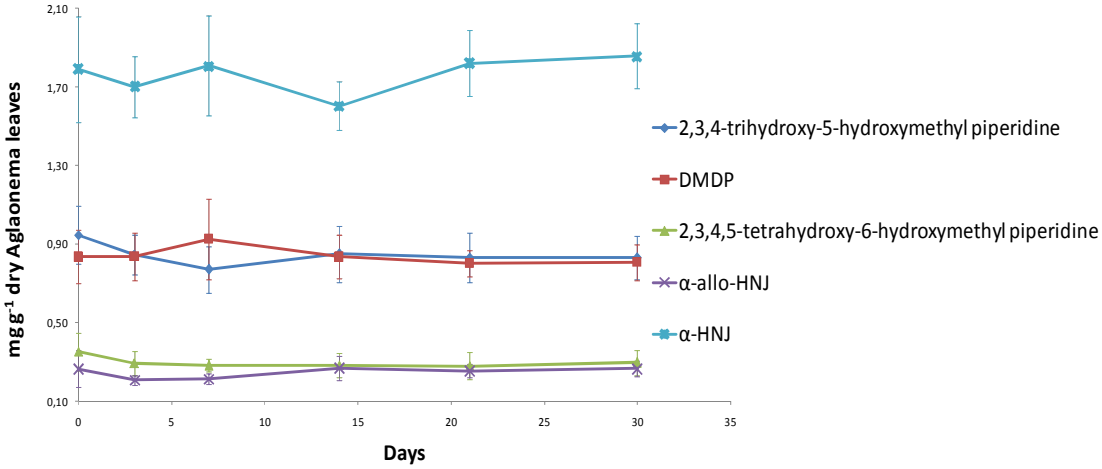

Figure 6.6. Evolution of iminosugar content of ACT1L PLE extract with storage at 50 ºC for 30 days.

The Aglaonema extracts were thermically stable under accelerated storage conditions here assayed. They presented a higher than iminosugar standards (DNJ and □-HNJ), possibly due to the synergic effect of other bioactive compounds present in the samples.

Although the PLE extracts resulted non toxic at concentrations below 125 µg mL^{-1}, further *in vivo* studies should be carried out to establish if the effective dose of Aglaonema extract is well below the concentrations here shown to decrease alive Caco-2 cells and, therefore, Aglaonema extracts can be incorporated as safe ingredients of functional foods.

DISCUSIÓN GENERAL

7. Discusión general

Como se ha comentado en secciones anteriores, el desarrollo de nuevos ingredientes alimentarios con carácter funcional es un reto actual para la investigación en Ciencia y Tecnología de los Alimentos. Para abordarlo, es necesario optimizar los procesos de extracción de los ingredientes bioactivos, así como desarrollar metodologías analíticas que permitan una caracterización lo más completa posible de dichos extractos. Estos estudios deben ir siempre complementados con los correspondientes ensayos, *in vitro* en primera instancia, que permitan determinar inequívocamente su bioactividad, estabilidad y seguridad.

Así, en esta Tesis Doctoral se ha llevado a cabo una investigación multidisciplinar orientada tanto al desarrollo de metodologías avanzadas para la obtención de extractos vegetales enriquecidos en iminoazúcares con actividad antiglucosidasa, como a la optimización y validación de métodos analíticos que permitan la caracterización exhaustiva de los mismos.

Aunque son numerosas las fuentes vegetales de iminoazúcares bioactivos [1], esta Tesis Doctoral se ha centrado fundamentalmente en el estudio de cuatro muestras: los granos de trigo sarraceno y las hojas de morera, como ejemplos de fuentes vegetales reconocidas, en el primer caso, o aceptadas en el segundo, para su consumo alimenticio, y los bulbos de jacinto y hojas/raíces de Aglaonema, como ejemplo de muestras complejas que incluyen una gran variedad de iminoazúcares.

La optimización del proceso de extracción de iminoazúcares bioactivos constituye una etapa determinante, ya que dichos compuestos suelen encontrarse en relativamente bajas concentraciones en fuentes vegetales, junto con otros carbohidratos de bajo peso molecular interferentes con su bioactividad y que, además, se encuentran en concentraciones bastante más elevadas.

Dicha optimización generalmente incluye la evaluación del efecto de diversas variables (cantidad de muestra, temperatura y tiempo de extracción y número de ciclos) sobre el rendimiento y selectividad de la extracción, ambos relacionados con la bioactividad de los extractos obtenidos. En este sentido, y como se describe en la **sección 6.1.3.1**, se ha optimizado un método por PLE para la extracción de

iminoazúcares a partir de muestras de morera. Al comparar el extracto obtenido mediante PLE con el generado en la extracción sólido-líquido convencional, se ha comprobado que si bien los rendimientos obtenidos en ambos casos son similares, la primera aporta una serie de ventajas como son la rapidez de extracción y el ser una técnica respetuosa con el medioambiente por su menor consumo de disolventes. Además, la PLE es una técnica fácilmente automatizable y con posibilidad de escalado a nivel industrial.

Por otra parte, la optimización de las previamente mencionadas condiciones de operación debe llevarse a cabo para cada muestra en estudio, pues si bien los compuestos extraídos (en este caso los iminoazúcares) son comunes tanto en muestras de morera (**sección 6.1.3.1**) como de Aglaonema (**sección 6.2.3.2**), las diferencias en cuanto a composición de ambas muestras, las distintas matrices extraídas e incluso el planteamiento de una extracción conjunta de iminoazúcares y otros compuestos bioactivos pueden hacer distintas las condiciones seleccionadas como óptimas, principalmente la temperatura de extracción.

Una vez optimizado el proceso de extracción, debe abordarse el requerido fraccionamiento o enriquecimiento en iminoazúcares de los extractos obtenidos, a través de la eliminación parcial o total de otros carbohidratos interferentes con su bioactividad. La **sección 6.1.3.2** hace referencia a la evaluación para este fin de dos procedimientos: (i) la incubación con levaduras (*Saccharomyces cerevisiae*), y (ii) el uso de resinas de intercambio catiónico. Si bien ambos procedimientos resultaron adecuados para el objetivo aquí planteado, la posibilidad de fraccionamiento conjunto de iminoazúcares y otros compuestos bioactivos como los inositoles, junto con la pretendida utilización de extractos de morera y otros vegetales como ingredientes funcionales de alimentos, determinaron la elección del tratamiento con levaduras como método de enriquecimiento rápido, efectivo, de grado alimentario y de fácil escalado.

Por otra parte, la utilización de extractos vegetales enriquecidos en iminoazúcares como ingredientes funcionales requiere del desarrollo de metodologías analíticas que permitan una caracterización lo más completa posible de su composición. Dichos métodos, además de emplearse para la determinación sensible y reproducible de

iminoazúcares, deberán también posibilitar el análisis simultáneo de estos compuestos con el de otros carbohidratos de bajo peso molecular e interferentes con su bioactividad, como forma de controlar la eficacia de los procesos de enriquecimiento previamente comentados.

Dada la heterogeneidad en cuanto a variedad estructural y la carencia de patrones comerciales, la baja abundancia de estos compuestos en extractos vegetales, y la problemática en cuanto a coeluciones observada en su análisis conjunto con el de otros carbohidratos de bajo peso molecular, este objetivo se ha abordado mediante el desarrollo de métodos por dos técnicas diferentes: GC-MS y HPLC-MS^2.

La GC-MS es una técnica muy apropiada para el análisis de carbohidratos de bajo peso molecular presentes en extractos de origen vegetal, ya que combina la buena resolución y sensibilidad de la GC, con la información estructural proporcionada por la MS. Sin embargo, el análisis de dichos compuestos por esta técnica requiere una etapa previa de derivatización, en la que conferir a los mismos la necesaria volatilidad y estabilidad térmica. Mientras que la derivatización de carbohidratos de bajo peso molecular tales como aldosas o cetosas es un proceso muy controlado y optimizado [2], no ocurre lo mismo en el caso de los iminoazúcares. En este sentido, resulta determinante la optimización del proceso de derivatización (reactivos, preparación de la muestra, tiempos de reacción, etc.) para conseguir que el proceso sea total y los derivados formados estables. Además, dada la complejidad de los extractos vegetales y la formación de distinto número de derivados en función del tipo de carbohidrato, es importante seleccionar aquellos derivados que contribuyan a simplificar la separación cromatográfica de dichas mezclas. Estos requisitos fueron exitosamente conseguidos mediante la formación de oximas para los azúcares reductores y la consecutiva sililación con HMDS y TFA de todos los azúcares presentes en los extractos vegetales en estudio (**sección 4.1.3**).

En lo referente al análisis, el desarrollo de todo método cromatográfico debe incluir la optimización de diversos parámetros experimentales (temperaturas del inyector y detector, elección de fase estacionaria, etc). En este sentido, y como se describe en la **sección 4.2,** uno de los parámetros más determinantes en el análisis de iminoazúcares resultó ser la temperatura del inyector. Aunque es sabido que ésta

puede afectar a compuestos termolábiles (degradación a temperaturas altas) o muy pesados (baja volatilidad a temperaturas altas), el comportamiento de los iminoazúcares resultó ser completamente diferente al de sus cetosas/aldosas homólogas. Por este motivo, y tras su optimización, se seleccionó 240 °C como temperatura óptima del inyector para permitir, tanto el análisis reproducible de iminoazúcares, como el de otros carbohidratos de mayor peso molecular (glicosil-inositoles).

Respecto a la cuantificación de iminoazúcares, no hay que olvidar que se trata de matrices complejas en las que los compuestos bioactivos de interés se encuentran presentes en bajas concentraciones, y estando éstas generalmente muy por debajo de las de otros componentes del extracto (principamente carbohidratos de bajo peso molecular como glucosa, fructosa y sacarosa). Por este motivo, se consideró relevante la evaluación del efecto sobre la sensibilidad de la cuantificación del uso de distintos detectores: FID y MS, operando éste último tanto en modo SIM como en SCAN (**sección 4.2.3**).

Dado que el FID no proporciona información estructural y, por tanto, muestra limitaciones en su aplicación a la caracterización de extractos complejos de composición relativamente desconocida, y su sensibilidad es menor que la del detector de masas (modo SIM), se seleccionó este último como el más adecuado para la cuantificación de iminoazúcares presentes en bajas concentraciones (e.g. extractos de morera, trigo sarraceno y jacinto). Sin embargo, para el caso de extractos con concentraciones mayores de iminoazúcares como los de Aglaonema, el uso del detector de MS en modo SCAN resultó suficientemente sensible para la cuantificación de estos compuestos bioactivos (**secciones 4.3 y 6.2**).

Otra cuestión de especial interés en el proceso de optimización de un método cromatográfico, que se aborda en la **sección 4.4**, es el estudio de la fase estacionaria. Recientemente han salido al mercado nuevas fases estacionarias basadas en ILs a las que se les atribuye una selectividad diferente a la de las columnas de GC convencionales. Se consideró, por tanto, de interés evaluar su posible empleo para el análisis de mezclas complejas de carbohidratos de bajo peso molecular, aplicación no contemplada hasta el momento en la bibliografía.

Sin embargo, antes de la evaluación de la utilidad de estas nuevas fases para el análisis de los compuestos de interés, fue necesaria una caracterización previa de las propiedades de retención de dichas fases. Este objetivo se desarrolla en la **sección 4.4.1**, y se ha llevado a cabo aplicando el Modelo de los Parámetros de Solvatación (SPM) a los datos cromatográficos obtenidos para un elevado número de compuestos de distinta funcionalidad, analizados en un rango de temperatura entre 80-160 ºC. De los resultados de este estudio puede extraerse que la acidez de las columnas de líquidos iónicos estudiadas es muy distinta a la de las fases estacionarias convencionales, justificándose así la distinta selectividad de las primeras en la separación de analitos con carácter básico como los iminoazúcares.

En base a estas conclusiones, y como se aborda en la **sección 4.4.2**, se procedió al análisis de mezclas complejas de patrones de carbohidratos de bajo peso molecular, incluyendo iminoazúcares, inositoles, mono-, di- y trisacáridos, empleando las 5 columnas de líquidos iónicos comercializadas hasta ese momento. De entre ellas, se seleccionó la SLB-IL82 como la más adecuada para el análisis conjunto de todos los compuestos anteriormente mencionados. Sin embargo, esta fase presenta como desventaja la baja capacidad de carga asociada a su limitado espesosr de fase, lo que hace difícil el análisis de mezclas complejas que incluyan compuestos en muy diferente concentración, como puede ser el caso de determinados extractos vegetales. A pesar de esto, el diferente patrón de elución proporcionado con respecto a las fases convencionales justifica su empleo para futuras aplicaciones relacionadas con el análisis por GC-MS de mezclas de carbohidratos de bajo peso molecular que incluyan, entre otros, iminoazúcares.

En cuanto a la anteriormente mencionada problemática de la identificación/caracterización de iminoazúcares presentes en extractos vegetales, hay que señalar que ésta se debe principalmente a la falta de patrones comerciales, así como a la escasez de datos bibliográficos sobre retención cromatográfica y espectros de masas para estos compuestos bioactivos. Es por ello que la **sección 4.1.3** de esta Tesis se focalizara en la obtención de espectros por (EI)MS e índices de retención lineales (I^T) para 12 patrones de iminoazúcares analizados en dos de las fases estacionarias más comúnmente empleadas para el análisis de carbohidratos de bajo

peso molecular (HP-1 y BPX-50). Dicha información, que se ha tratado de correlacionar con la estructura de los patrones analizados, ha permitido la caracterización/identificación tentativa de algunos iminoazúcares desconocidos en extractos de Aglaonema, siendo estos datos también de aplicación general a la elucidación estructural de iminoazúcares desconocidos presentes en otras muestras reales.

A pesar de los buenos resultados conseguidos mediante GC-MS para el análisis conjunto de iminoazúcares y otros carbohidratos de bajo peso molecular presentes en extractos vegetales, se decidió evaluar lo beneficioso y/o complementario para este fin del empleo de la HPLC (en modo HILIC) acoplada a MS^2, técnica especialmente indicada para el análisis de compuestos polares, y que no requiere su derivatización previa al análisis. En este caso, la optimización de métodos incluyó la evaluación, llevada a cabo en dos fases (**secciones 5.1 y 5.2**), de parámetros experimentales como la fase estacionaria hidrofílica, el modificador orgánico, el aditivo de la fase móvil, el gradiente de elución, la temperatura de la fase estacionaria, etc.

La optimización del método HILIC para el análisis de iminoazúcares y otros carbohidratos de bajo peso molecular en extractos vegetales (trigo sarraceno, jacinto y morera) señaló a la fase estacionaria amina híbrida como la más adecuada para el análisis de estos compuestos bioactivos (**sección 5.1**). Asimismo, un estudio en mayor profundidad de las condiciones cromatográficas empleando esta vez Aglaonema como extracto rico en iminoazúcares bioactivos (**Sección 5.2**) concluyó que, tanto la temperatura como los aditivos de la fase móvil, tienen una influencia moderada sobre la sensibilidad y reproducibilidad del método optimizado, teniendo en cuenta que para el caso particular del análisis conjunto de iminoazúcares y otros carbohidratos de bajo peso molecular, los aditivos que mejores resultados ofrecieron en cuestión de sensibilidad y resolución fueron la sales o el medio ácido. Asimismo, la temperatura influye de manera importante en la resolución, de modo que será necesario en cada caso particular optimizar este parámetro para llegar a un compromiso entre temperatura de la columna y resolución.

La aplicación del método optimizado al análisis de extractos de Aglaonema reveló además la presencia de algunos iminoazúcares no detectados previamente en el

análisis por GC-MS de estas muestras, probando así la complementariedad de los resultados obtenidos por ambas técnicas. Por otra parte, el acoplamiento de la HILIC a espectrometría de masas tándem (MS^2) proporcionó también información estructural de gran utilidad para la identificación tentativa/caracterización por primera de vez de varios iminoazúcares desconocidos en los extractos vegetales analizados.

Una vez caracterizados cuali- y cuantitativamente los extractos vegetales objeto de estudio, la posible aplicación de éstos como suplementos bioactivos o su incorporación como ingredientes funcionales de alimentos requiere la comprobación de que verdaderamente poseen la bioactividad que se les atribuye, evaluada mediante ensayos de inhibición de α-glucosidasas, además de los pertinentes estudios de estabilidad y seguridad de los extractos (**sección 6.2**). Dichos estudios se han centrado en los extractos de Aglaonema por ser esta muestra de origen no alimentario y por no haberse evaluado con anterioridad la posible toxicidad de sus extractos, asociada a compuestos coextraídos junto con los iminoazúcares.

En este sentido, y respecto a la capacidad de inhibición de glucosidasas de los extractos, se ha confirmado mediante estudios *in vitro* la dependencia directa concentración-efecto inhibitorio. Además, la capacidad de inhibición de los extractos probó ser mayor que la de patrones individuales, para ensayos llevados a cabo con concentraciones equivalentes de iminoazúcares. Este hecho puede deberse, por un lado, al efecto sinérgico de distintos iminoazúcares, o bien a la presencia en los extractos de otros componentes distintos a los iminoazúcares y que contribuyen de manera favorable a la inhibición.

Por otra parte, y como primera aproximación para la evaluación de la seguridad de extractos de Aglaonema, se han llevado a cabo estudios *in vitro* sobre la viabilidad celular de células CaCo-2 tras su tratamiento con dichos extractos. Como se puede comprobar en la **sección 6.2.3.6**, la totalidad de extractos de Aglaonema de distintos cultivares estudiados resultaron no tóxicos en concentraciones inferiores a 125 μg mL^{-1}, siendo esta concentración muy superior a la requerida para conseguir el 50% de inhibición de *α*-glucosidasas ($IC_{50)}$ en las condiciones ensayadas. Si bien estos estudios deberían completarse con la evaluación *in vivo* de la seguridad de extractos que contengan las dosis efectivas para conseguir el efecto biológico deseado, los resultados

aquí expuestos prueban una vez más el enorme potencial de los extractos de Aglaonema enriquecidos en iminoazúcares, entre otros de los aquí estudiados, para su aplicación en la elaboración de alimentos funcionales con actividad antiglucosidasas o para su comercialización como extractos naturales bioactivos.

Por último, los resultados incluidos en la sección **6.2.3.7** han demostrado la estabilidad térmica de los iminoazúcares presentes en extractos de Agalonema, tras su almacenamiento acelerado (50°C, 1 mes), siendo éste otro punto a favor de la posible explotación comercial de estos extractos bioactivos o de su potencial incorporación a alimentos funcionales procesados en condiciones suaves de temperatura.

En base a lo arriba expuesto, cabe concluir que los resultados incluidos en la presente Tesis Doctoral han contribuido de manera significativa al avance en el conocimiento sobre los procesos de obtención, enriquecimiento y análisis de extractos vegetales ricos en iminoazúcares, objetivo éste encuadrado dentro de los ámbitos de Ciencia y Tecnología de Alimentos y de Química Analítica.

CONCLUSIONES

8. Conclusiones

- El método de GC-MS optimizado ha permitido el análisis simultáneo de iminoazúcares y otros carbohidratos de bajo peso molecular presentes en extractos vegetales (morera, jacinto, trigo sarraceno y Aglaonema) con la requerida sensibilidad y reproducibilidad, siendo la formación de TMSO el procedimiento de derivatización más adecuado y la temperatura del inyector uno de los parámetros cromatográficos más relevantes.
- El estudio de las relaciones entre estructura química y datos obtenidos mediante GC-MS (índices de retención lineales y abundancias relativas de los iones fragmento característicos) ha permitido establecer criterios de validez generalpara lacaracterización o identificación tentativa de iminoazúcares desconocidos.
- Se ha demostrado que las fases estacionarias comerciales para GC basadas en ILs presentan alta polaridad y carácter ácido, lo que las diferencian de las fases estacionarias convencionales. Ambas características les confieren una selectividad única y abren nuevas vías de exploración para el análisis de compuestos de distinta naturaleza.
- Por primera vez se ha analizado con éxito mezclas de iminoazúcares y otros carbohidratos de bajo peso molecular en columnas de GC con fases estacionarias basadas en ILs. La columna SLB^{TM}-IL82 ha resultado ser una buena alternativa frente a las convencionalmente empleadas para el análisis de mezclas complejas de estos compuestos.
- El método de HILIC-MS^2 desarrollado, empleando una columna de BEH amida y mezclas binarias acetonitrilo:agua con acetato amónico o hidróxido amónico como aditivos, ha permitido la separación eficaz y detección sensible de iminoazúcares y otros carbohidratos de bajo peso molecular en los extractos vegetales en estudio. Los espectros obtenidos por MS^2 han sido de utilidad para la caracterización estructural de iminoazúcares desconocidos, siendo estos resultados complementarios a los obtenidos por GC-MS.
- La PLE se ha aplicado por primera vez a la extracción de iminoazúcares a partir de fuentes vegetales (morera y Aglaonema), proporcionando recuperaciones similares a las de la extracción sólido-líquido convencional en un tiempo notablemente menor.

La PLE es, además, una técnica fácilmente automatizable y escalable para su potencial aplicación industrial.

- El tratamiento con *Saccharomyces cerevisiae* de los extractos vegetales ha sido apropiado para la eliminación selectiva de carbohidratos de bajo peso molecular interferentes en la bioactividad de los iminoazúcares. Como ventajas frente a la CEC, este procedimiento permite la recuperación de inositoles bioactivos, emplea disolventes de grado alimentario y puede ser fácilmente escalable.
- Los extractos de Aglaonema presentaron alta actividad inhibidora de □-glucosidasas, adecuada estabilidad térmica y baja toxicidad frente a células Caco-2, evidenciando lo prometedor de su empleo para el desarrollo de ingredientes o complementos alimenticios con carácter funcional.

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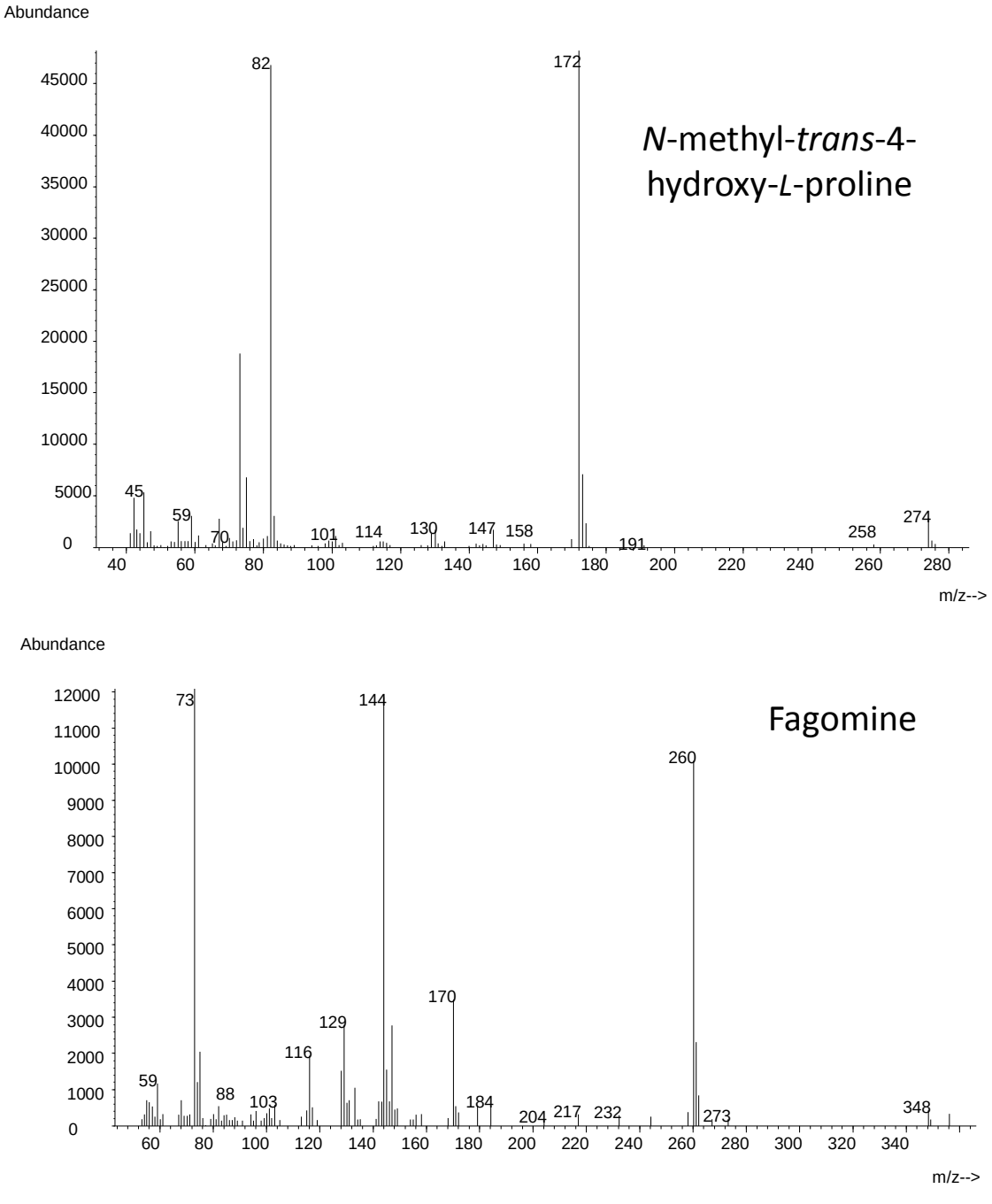
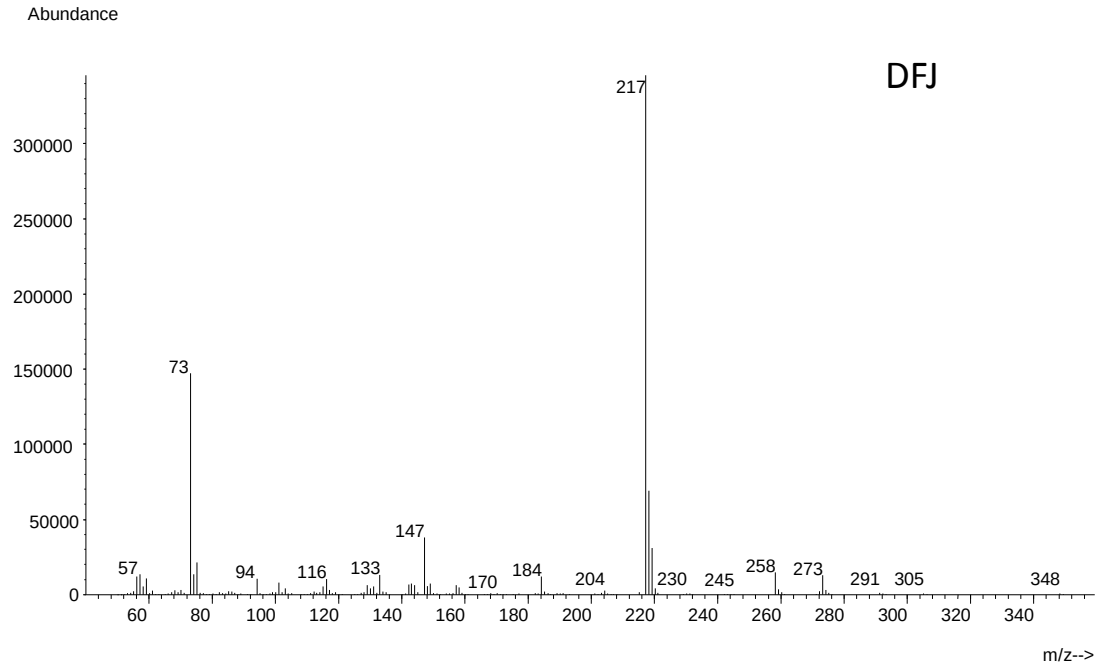
ANEXOS

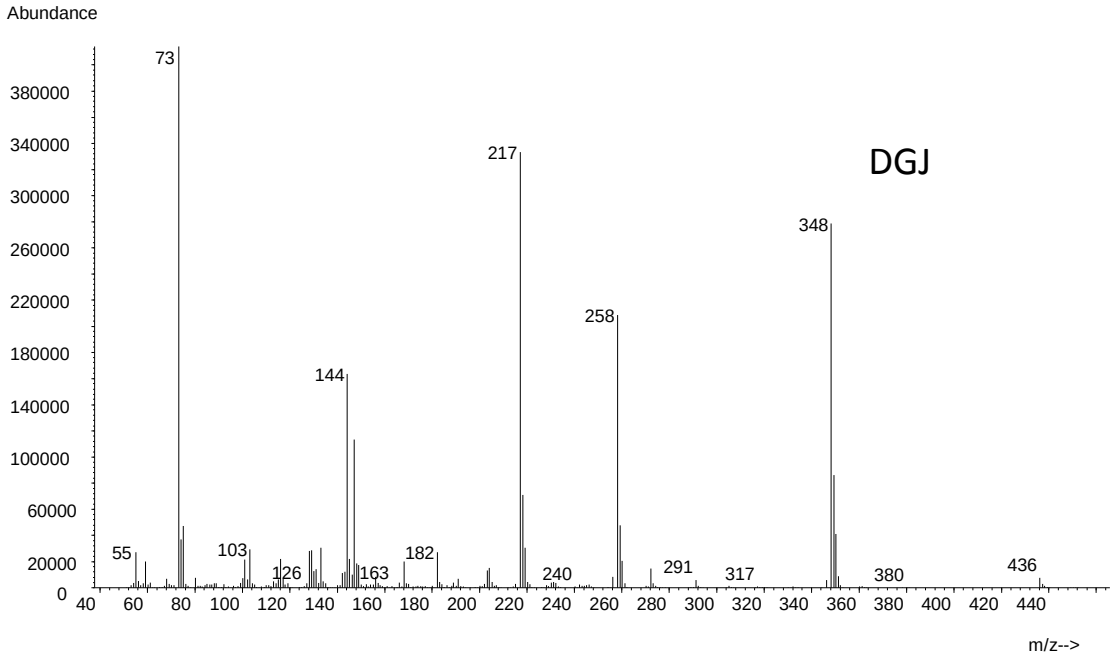
10. Anexos

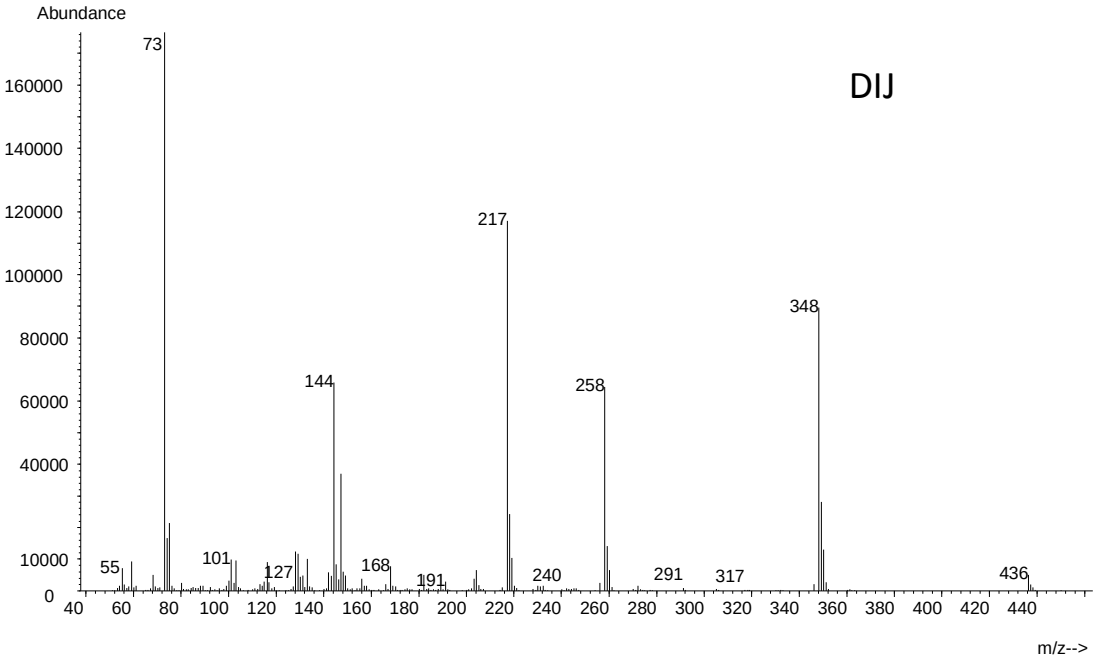
ANEXO I

Figure 1S. EI mass spectra of derivatised iminosugars and other related compounds.




Abundance
DFJ
217
73
147
57
94
116
133
170
184
204
230
245
258
273
291
305
348
300000
250000
200000
150000
100000
50000
0
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
m/z-->


Abundance
DGJ
73
217
348
258
144
55
103
126
163
182
240
291
317
380
436
380000
340000
300000
260000
220000
180000
140000
100000
60000
20000
0
40
60
80
100
120
140
160
180
200
220
240
260
280
300
320
340
360
380
400
420
440
m/z-->


Abundance
DIJ
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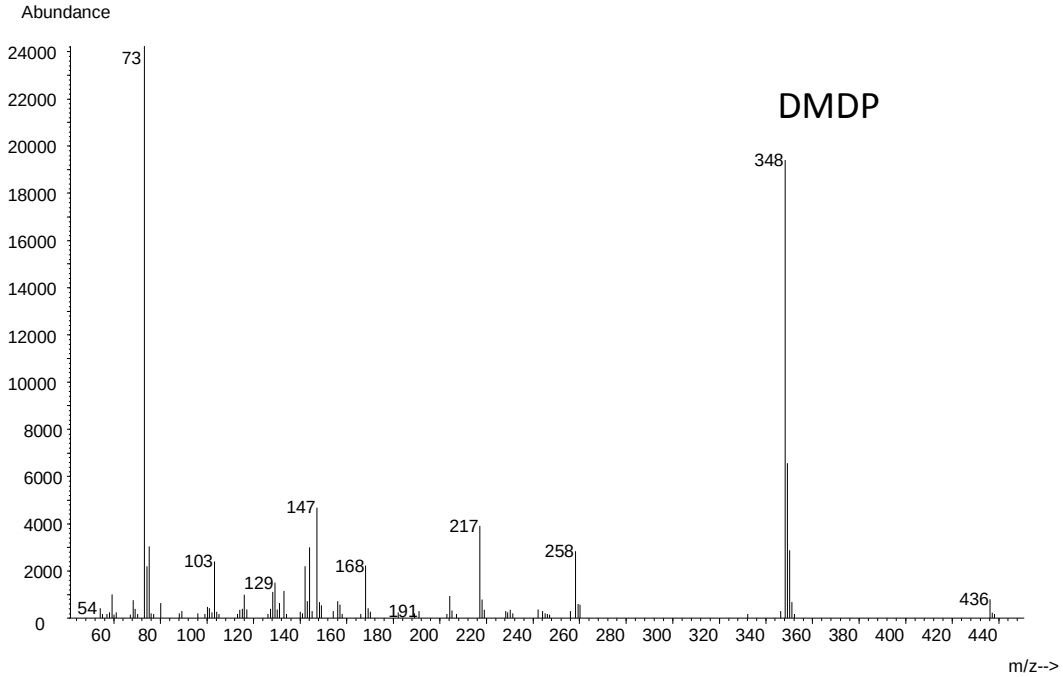
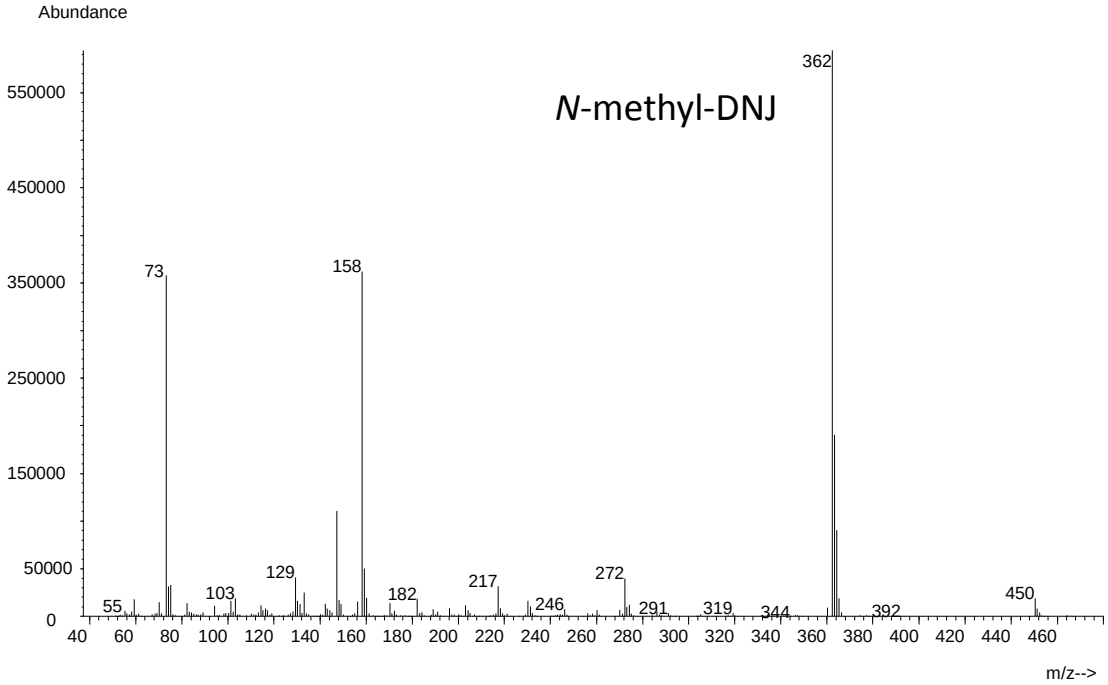
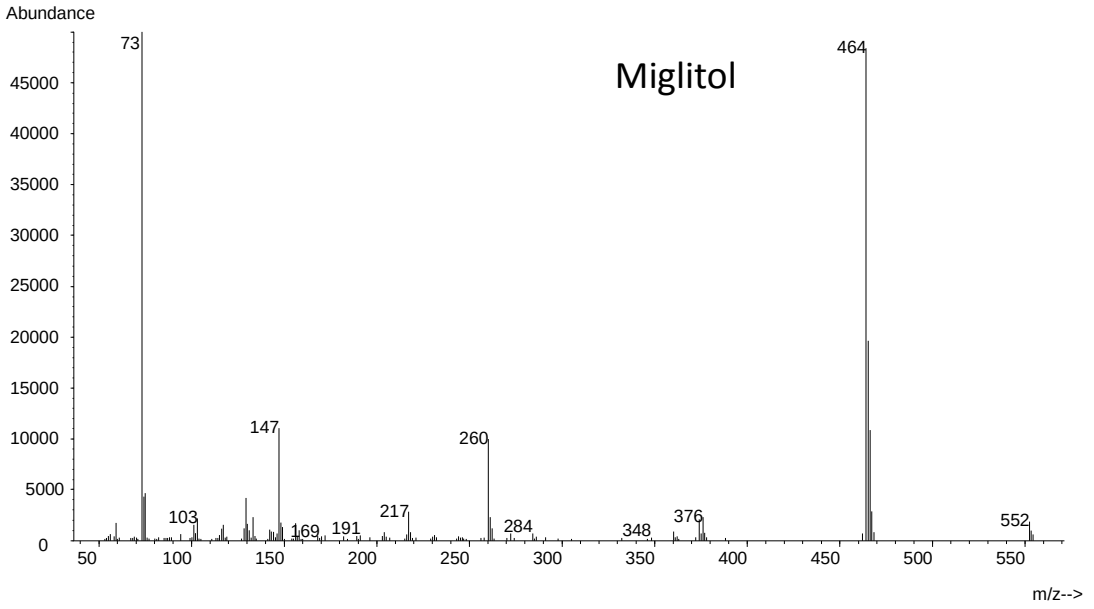
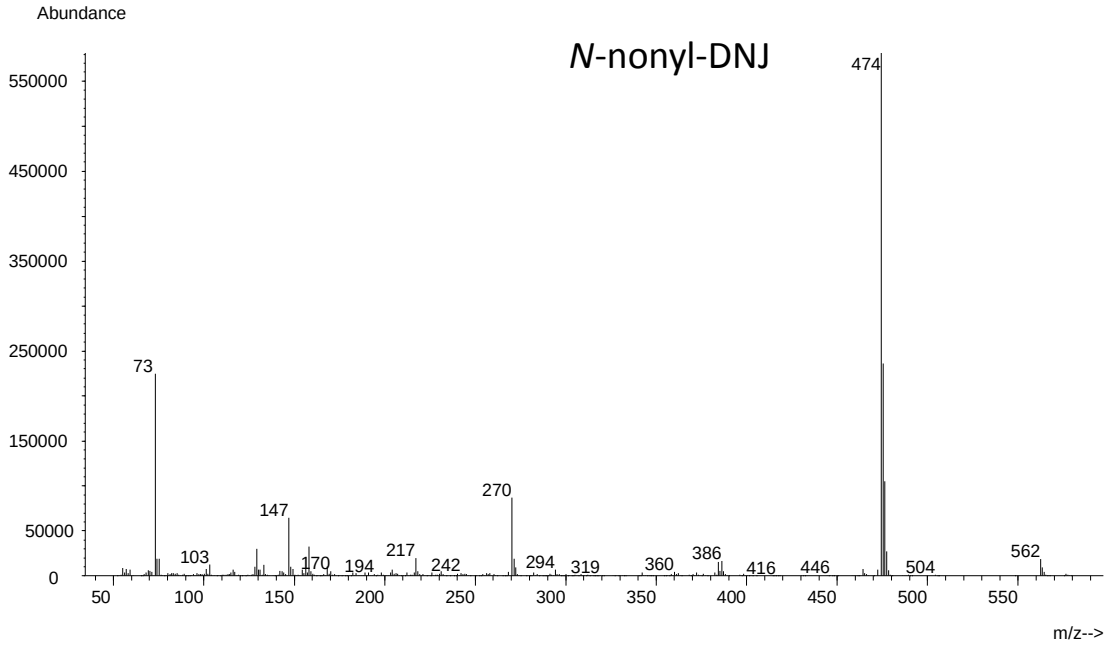
217
348
144
258
55
101
127
168
191
240
291
317
436
m/z-->


Abundance
DMDP
73
348
147
217
258
103
168
129
54
191
436
m/z-->


Abundance
N-methyl-DNJ
362
73
158
129
103
182
217
246
272
291
319
344
392
450
550000
450000
350000
250000
150000
50000
0
40 60 80 100 120 140 160 180 200 220 240 260 280 300 320 340 360 380 400 420 440 460
m/z-->


Abundance
Miglitol
73
464
147
260
103
217
169
191
284
348
376
552
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
50 100 150 200 250 300 350 400 450 500 550
m/z-->


Abundance
N-nonyl-DNJ
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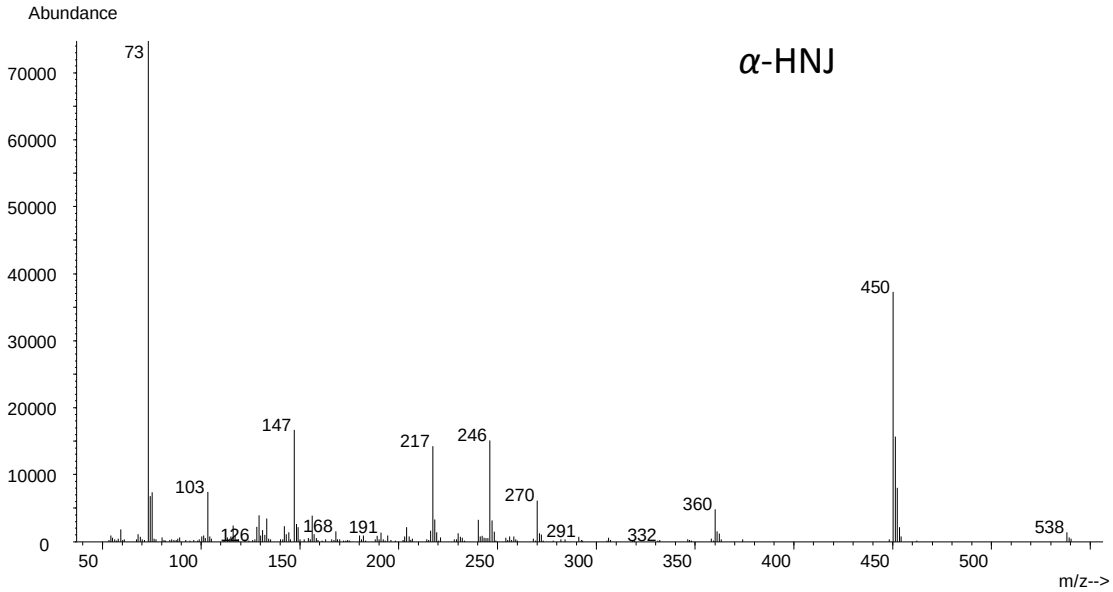
450000
350000
250000
150000
50000
0
73
103
147
170
194
217
242
270
294
319
360
386
416
446
474
504
562
50
100
150
200
250
300
350
400
450
500
550
m/z-->


Abundance
α-HNJ
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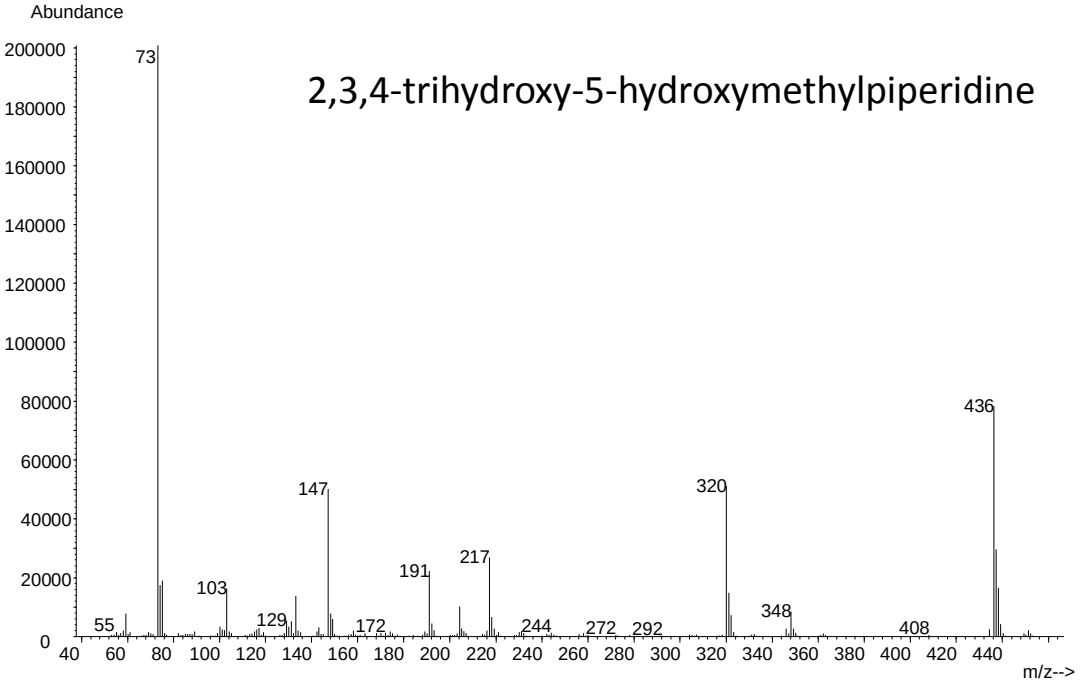
60000
50000
40000
30000
20000
10000
0
73
103
126
147
168
191
217
246
270
291
332
360
450
538
50
100
150
200
250
300
350
400
450
500
m/z-->


Abundance
2,3,4-trihydroxy-5-hydroxymethylpiperidine
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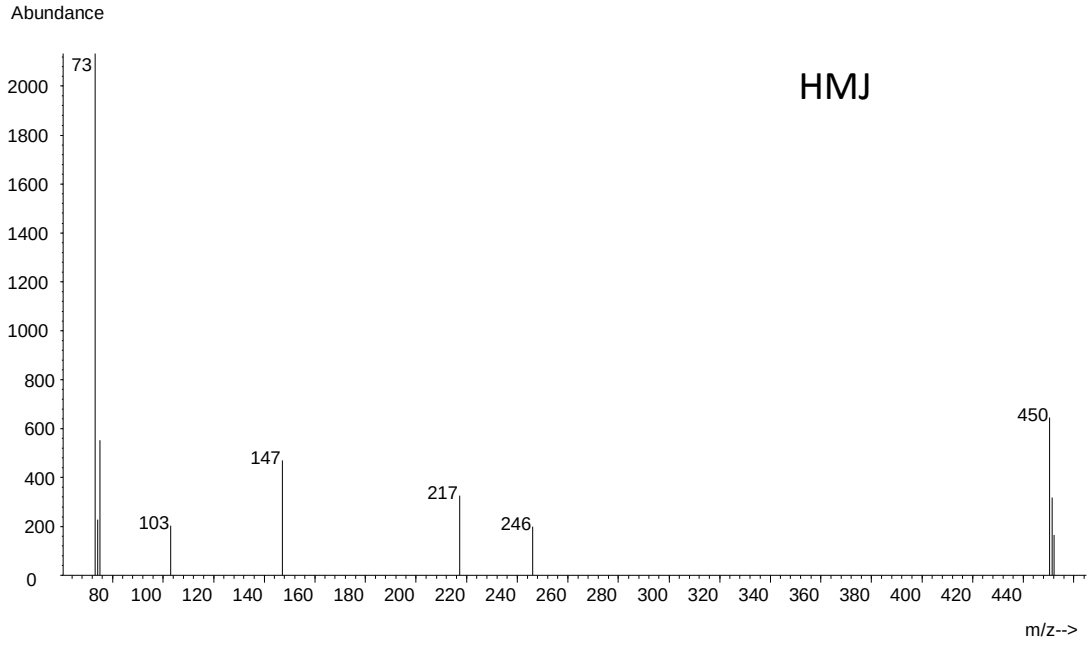
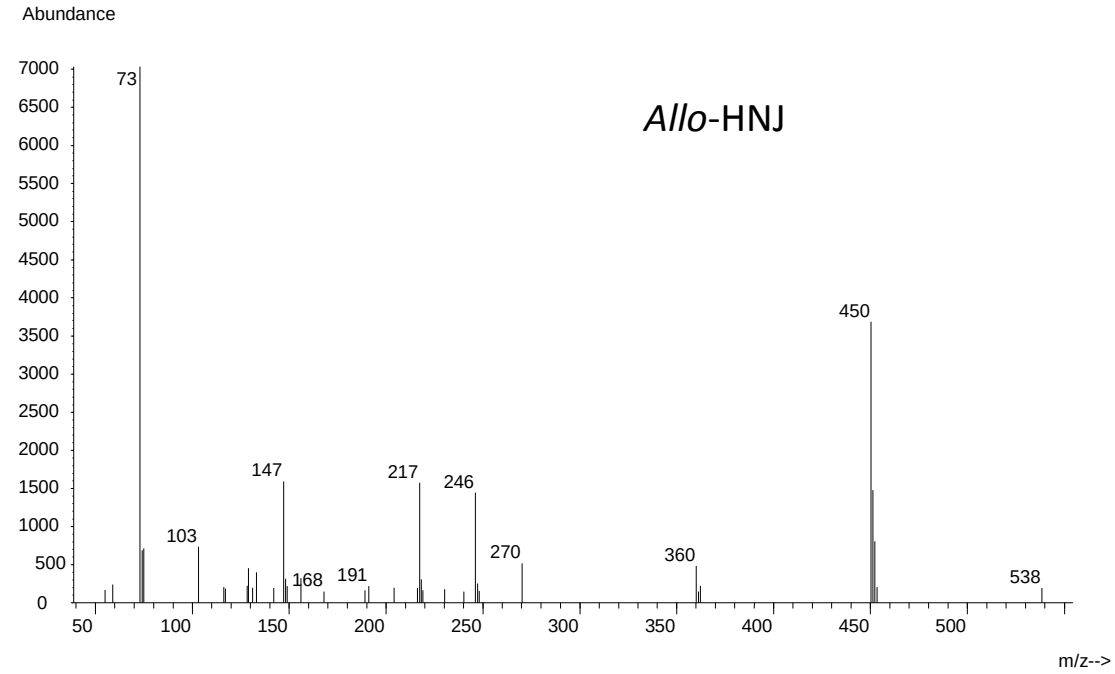
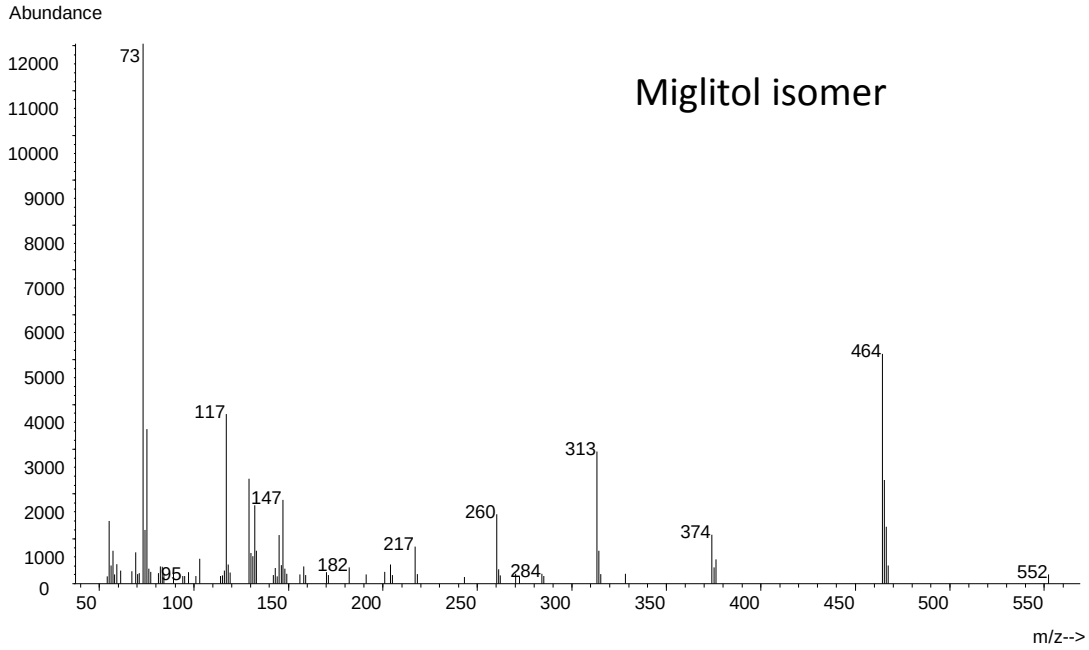
147
103
129
55
172
191
217
244
272
292
320
348
408
436
m/z-->


Abundance
HMJ
73
147
103
217
246
450
m/z-->


Abundance
Allo-HNJ
73
147
103
168
191
217
246
270
360
450
538
m/z-->


Abundance
Miglitol isomer
73
117
147
95
182
217
260
284
313
374
464
552
m/z-->

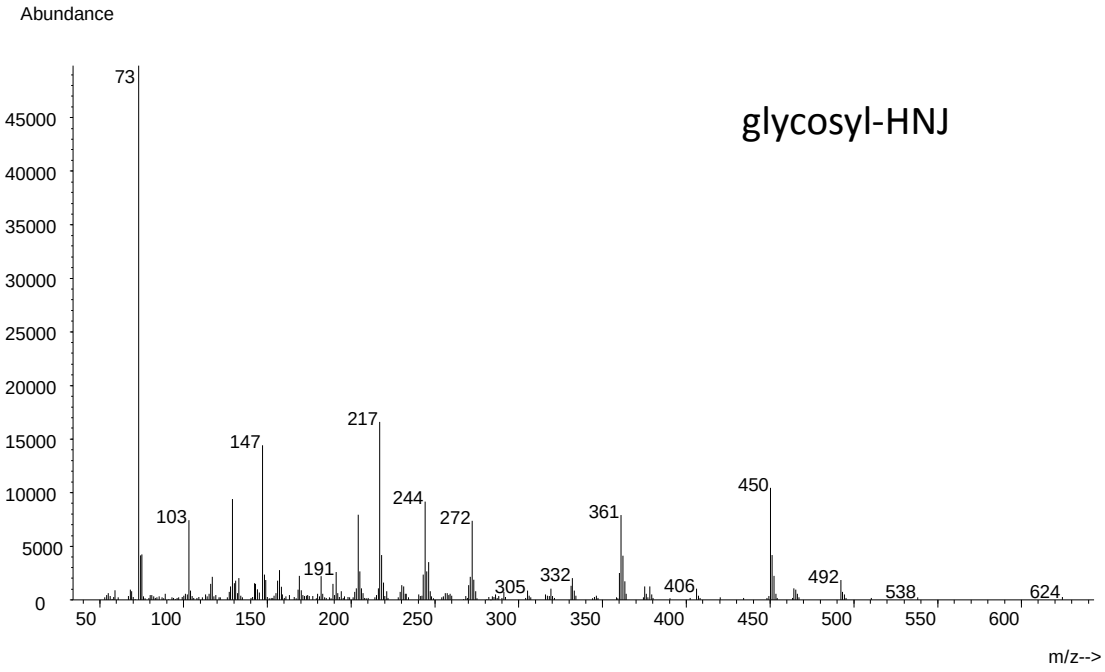
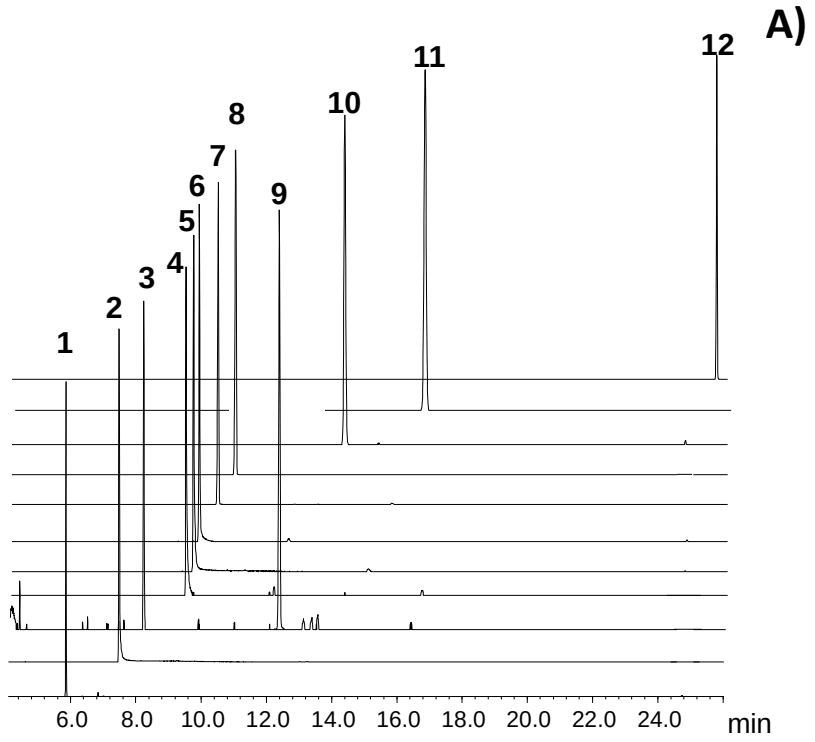

Abundance
glycosyl-HNJ
73
147
217
103
244
272
361
450
191
305
332
406
492
538
624
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
50
100
150
200
250
300
350
400
450
500
550
600
m/z-->

Figure 2S. GC-MS profiles of derivatised iminosugars eluted on HP-1 (A) and BPX-50 (B) columns. (1) *N*-methyl-*trans*-4-hydroxy-*L*-proline, (2) DFJ, (3) Fagomine, (4) DMDP, (5) DIJ, (6) DMJ, (7) DGJ, (8) *N*-methyl-DNJ, (9) DNJ, (10) *α*-HNJ, (11) Miglitol, (12) *N*-nonyl-DNJ.



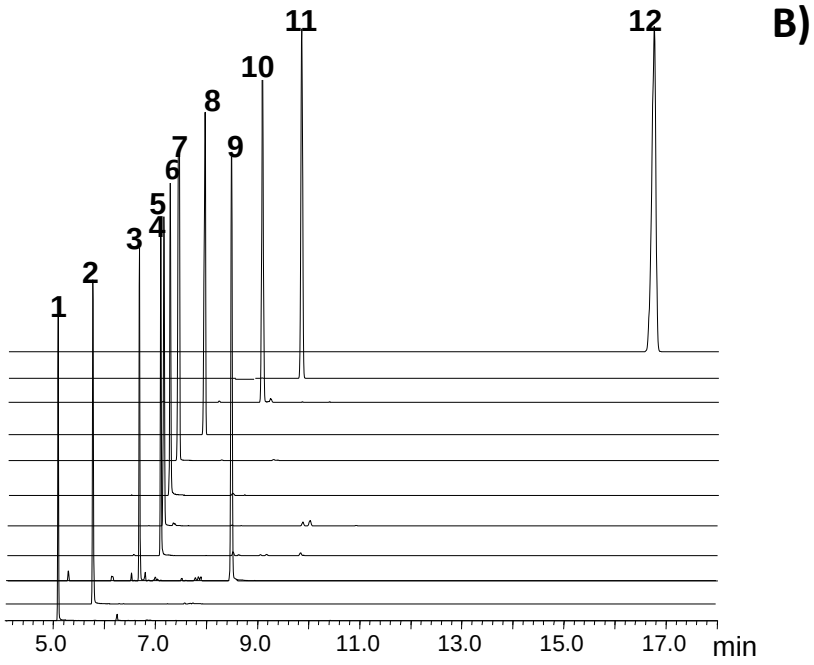


Table 1S. Probes and their corresponding solute descriptors [a,b] employed in the study of the IL columns using the solvation parameter model.

Probe	Solute	E	S	A	B	L
1	Benzene	0.610	0.52	0.00	0.14	2.786
2	Toluene	0.601	0.52	0.00	0.14	3.325
3	Ethylbenzene	0.613	0.51	0.00	0.15	3.778
4	Propylbenzene	0.604	0.50	0.00	0.15	4.230
5	Butylbenzene	0.600	0.51	0.00	0.15	4.730
6	Pentylbenzene	0.594	0.51	0.00	0.15	5.230
7	Propanone	0.179	0.70	0.04	0.49	1.696
8	2-Butanone	0.166	0.70	0.00	0.51	2.287
9	2-Pentanone	0.143	0.68	0.00	0.51	2.755
10	2-Hexanone	0.136	0.68	0.00	0.51	3.286
11	2-Heptanone	0.123	0.68	0.00	0.51	3.760
12	2-Octanone	0.108	0.68	0.00	0.51	4.257
13	2-Nonanone	0.119	0.68	0.00	0.51	4.735
14	2-Decanone	0.108	0.68	0.00	0.51	5.245
15	2-Undecanone	0.101	0.68	0.00	0.51	5.732
16	Cyclopentanone	0.373	0.86	0.00	0.52	3.221
17	Cyclohexanone	0.403	0.86	0.00	0.56	3.792
18	Cycloheptanone	0.436	0.86	0.00	0.56	4.376
19	Cyclooctanone	0.474	0.86	0.00	0.56	4.981
20	1-Propanol	0.236	0.42	0.37	0.48	2.031
21	1-Butanol	0.224	0.42	0.37	0.48	2.601
22	1-Pentanol	0.219	0.42	0.37	0.48	3.106
23	1-Hexanol	0.210	0.42	0.37	0.48	3.610
24	1-Heptanol	0.211	0.42	0.37	0.48	4.115
25	1-Octanol	0.199	0.42	0.37	0.48	4.619
26	1-Nonanol	0.193	0.42	0.37	0.48	5.124
27	1-Decanol	0.191	0.42	0.37	0.48	5.610
28	Cyclohexanol	0.460	0.54	0.32	0.57	3.758
29	Cycloheptanol	0.513	0.54	0.32	0.58	4.407
30	Cyclooctanol	0.566	0.54	0.32	0.58	5.054
31	2-Methyl-2-pentanol	0.169	0.30	0.31	0.60	3.081
32	Methyl acetate	0.142	0.64	0.00	0.45	1.911
33	Ethyl acetate	0.106	0.62	0.00	0.45	2.314
34	Propyl acetate	0.092	0.60	0.00	0.45	2.819
35	Butyl acetate	0.071	0.60	0.00	0.45	3.353
36	Methyl pentanoate	0.108	0.60	0.00	0.45	3.392
37	Methyl hexanoate	0.080	0.60	0.00	0.45	3.874
38	Methyl octanoate	0.065	0.60	0.00	0.45	4.838
39	Methyl nonanoate	0.056	0.60	0.00	0.45	5.321
40	Methyl decanoate	0.053	0.60	0.00	0.45	5.803

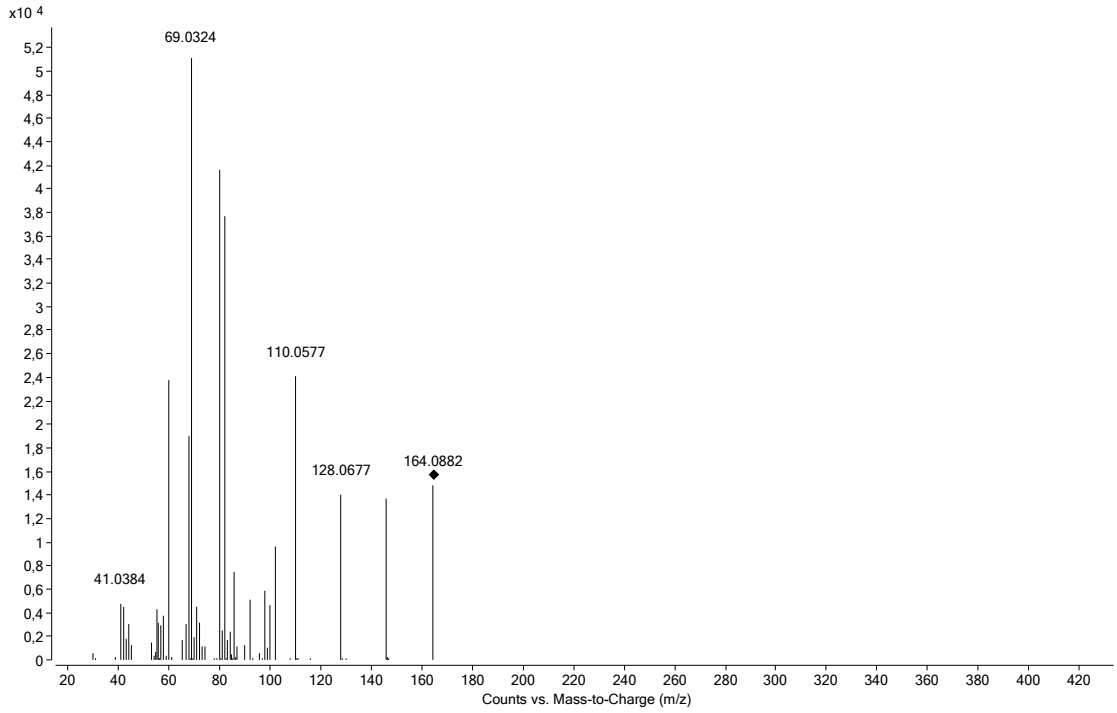
Probe	Solute	E	S	A	B	L
41	Methyl undecanoate	0.050	0.60	0.00	0.45	6.285
42	Methyl dodecanoate	0.037	0.60	0.00	0.45	6.767
43	Acetonitrile	0.237	0.90	0.07	0.32	1.739
44	Propanenitrile	0.162	0.90	0.02	0.36	2.082
45	Butanenitrile	0.188	0.90	0.00	0.36	2.548
46	Pentanenitrile	0.177	0.90	0.00	0.36	3.108
47	Hexanenitrile	0.166	0.90	0.00	0.36	3.608
48	Octanenitrile	0.162	0.90	0.00	0.36	4.585
49	Benzonitrile	0.742	1.11	0.00	0.33	4.039
50	1-Nitropropane	0.242	0.95	0.00	0.31	2.894
51	1-Nitrobutane	0.227	0.95	0.00	0.29	3.415
52	1-Nitropentane	0.212	0.95	0.00	0.29	3.938
53	1-Nitrohexane	0.203	0.95	0.00	0.29	4.416
54	Nitrobenzene	0.871	1.11	0.00	0.28	4.557
55	1-Chlorobutane	0.210	0.40	0.00	0.10	2.722
56	1-Chloropentane	0.208	0.38	0.00	0.09	3.223
57	1-Chlorohexane	0.201	0.39	0.00	0.09	3.708
58	1-Chloroheptane	0.194	0.40	0.00	0.09	4.208
59	1-Chlorooctane	0.191	0.40	0.00	0.09	4.708
60	1-Chlorononane	0.188	0.40	0.00	0.09	5.208
61	1-Chlorodecane	0.185	0.40	0.00	0.09	5.708
62	1-Iodobutane	0.628	0.40	0.00	0.14	3.628
63	Fluorobenzene	0.477	0.57	0.00	0.10	2.788
64	Chlorobenzene	0.718	0.65	0.00	0.07	3.657
65	Bromobenzene	0.882	0.73	0.00	0.09	4.041
66	Acetic acid	0.265	0.64	0.62	0.44	1.816
67	Propanoic acid	0.233	0.65	0.61	0.44	2.276
68	Butanoic acid	0.210	0.64	0.61	0.45	2.750
69	Pentanoic acid	0.205	0.63	0.62	0.45	3.227
70	Hexanoic acid	0.174	0.60	0.60	0.45	3.920
71	Pyridine	0.631	0.84	0.00	0.52	3.022
72	Aniline	0.955	0.96	0.26	0.41	3.993
73	N,N-dimethylaniline	0.957	0.84	0.00	0.42	4.701
74	Tetrahydrofuran	0.289	0.52	0.00	0.48	2.636
75	Heptane	0.000	0.00	0.00	0.00	3.173
76	Octane	0.000	0.00	0.00	0.00	3.677
77	Nonane	0.000	0.00	0.00	0.00	4.182
78	Decane	0.000	0.00	0.00	0.00	4.686
79	Undecane	0.000	0.00	0.00	0.00	5.191
80	Dodecane	0.000	0.00	0.00	0.00	5.696
81	Tridecane	0.000	0.00	0.00	0.00	6.200
82	Tetradecane	0.000	0.00	0.00	0.00	6.705
83	Pentadecane	0.000	0.00	0.00	0.00	7.209

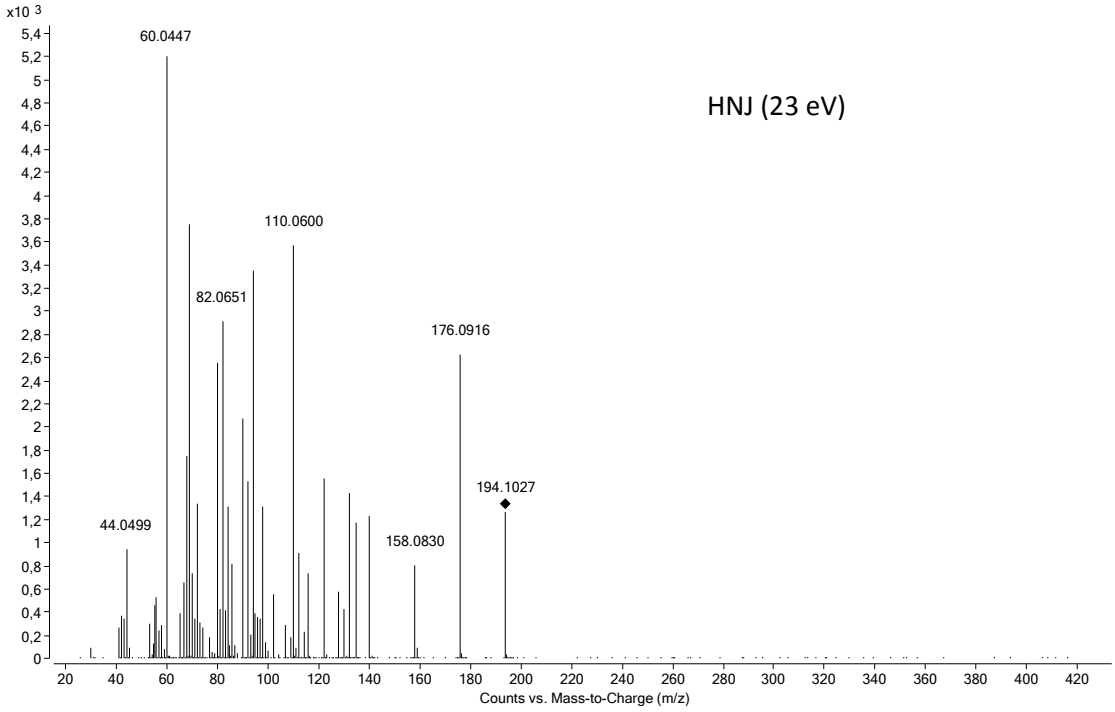
Probe	Solute	E	S	A	B	L
84	Hexadecane	0.000	0.00	0.00	0.00	7.714
85	Heptadecane	0.000	0.00	0.00	0.00	8.218
86	Octadecane	0.000	0.00	0.00	0.00	8.722
87	Nonadecane	0.000	0.00	0.00	0.00	9.226
88	Eicosane	0.000	0.00	0.00	0.00	9.731
89	Heneicosane	0.000	0.00	0.00	0.00	10.236
90	Docosane	0.000	0.00	0.00	0.00	10.740
91	Cyclohexane	0.305	0.10	0.00	0.00	2.964
92	*cis*-Hydrindane	0.439	0.25	0.00	0.00	4.635
93	1-Hexyne	0.166	0.22	0.10	0.12	2.510
94	1-Heptyne	0.160	0.23	0.09	0.10	3.000
95	2-Octyne	0.225	0.30	0.00	0.10	3.850

[a] M.H. Abraham, Chem. Soc. Rev. 22 (1993) 73.
[b] M.H. Abraham, J. Andonian-Haftvan, G.S. Whiting, A. Leo, R.S. Taft, J. Chem. Soc. Perkin Trans. 2 (1994) 1777.

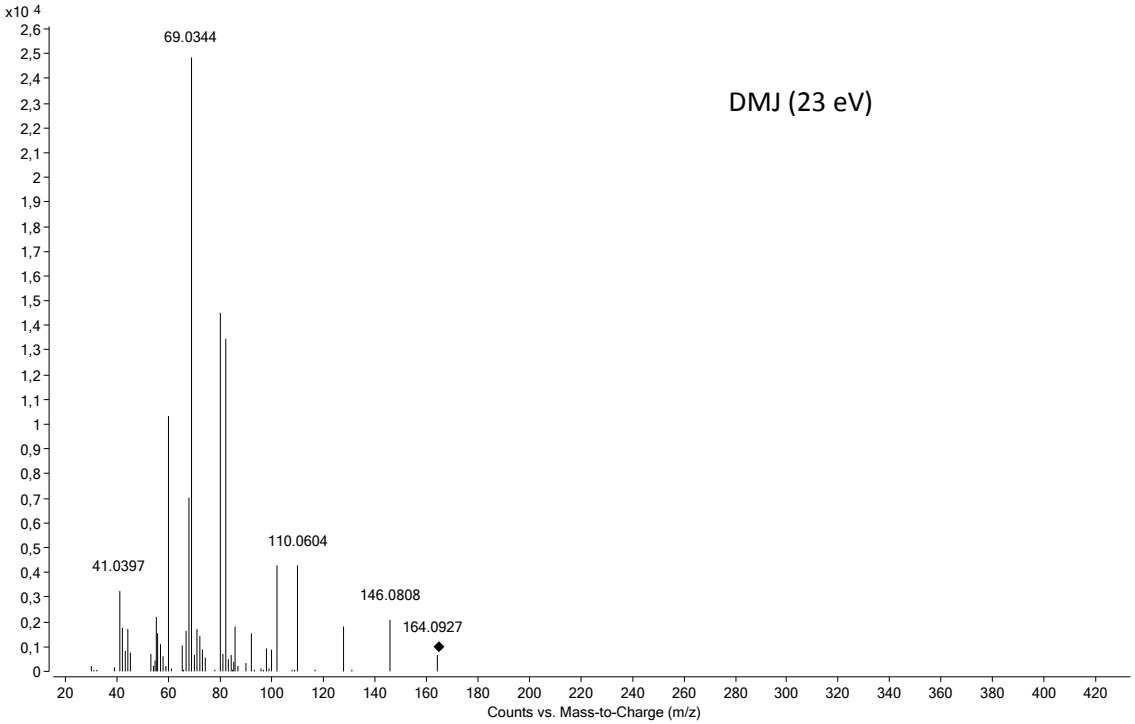
ANEXO II

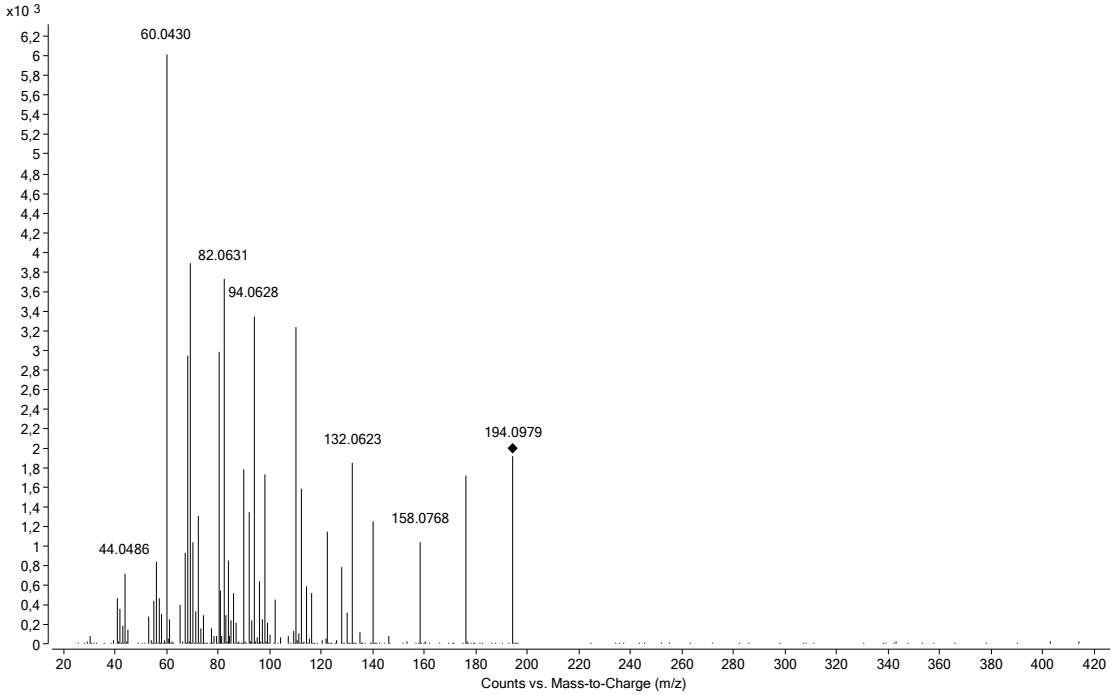
Figure 3S. MS^2 mass spectra of iminosugars and other related compounds.

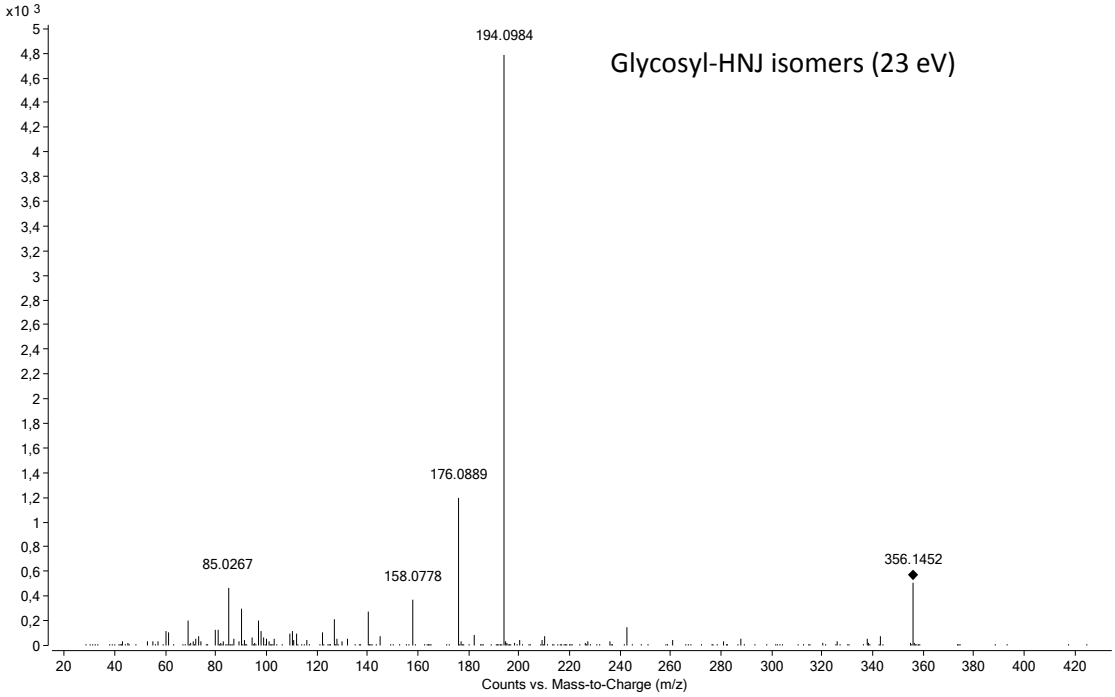


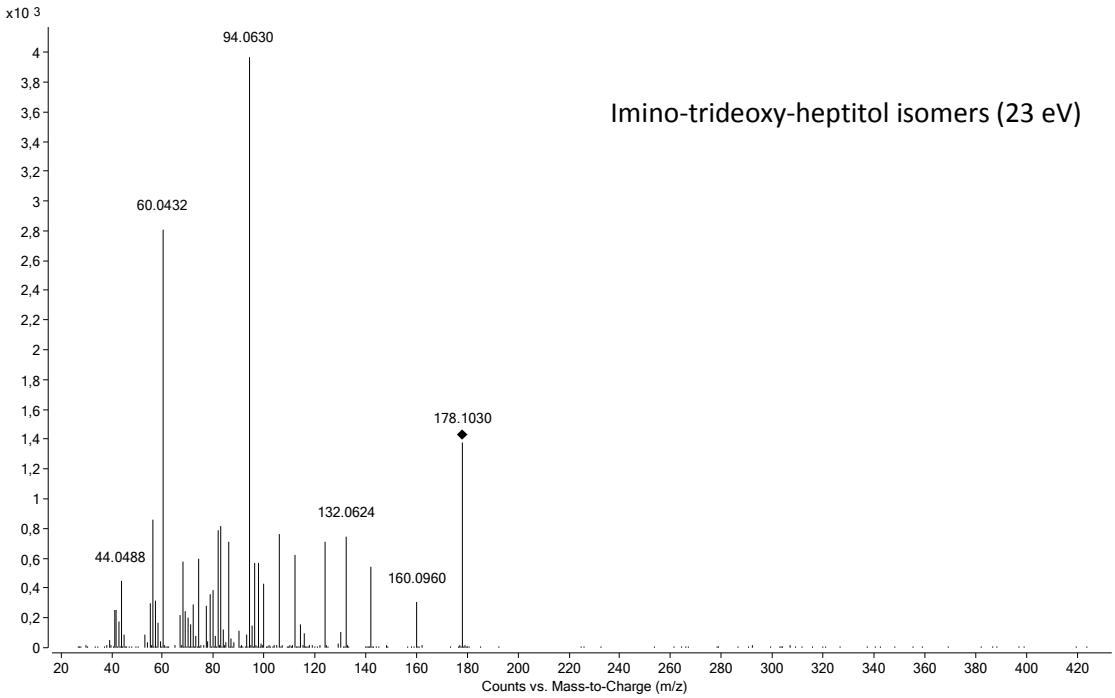


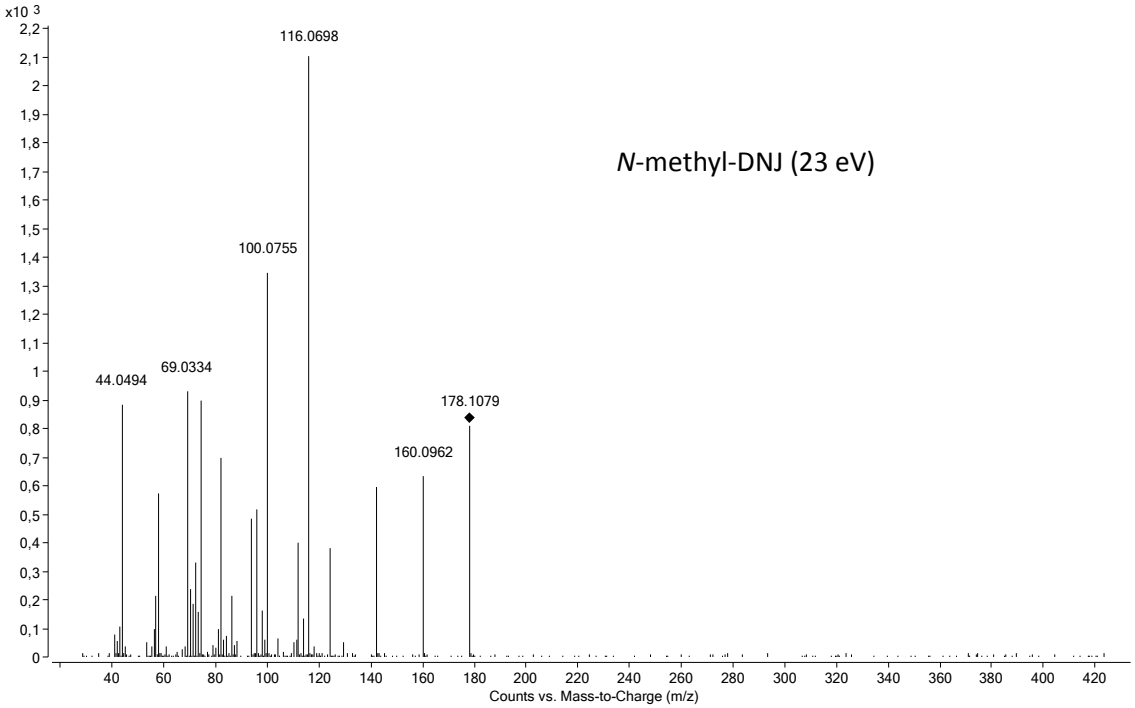
HNJ (23 eV)


DMJ (23 eV)
x10 4
69.0344
110.0604
41.0397
146.0808
164.0927
Counts vs. Mass-to-Charge (m/z)

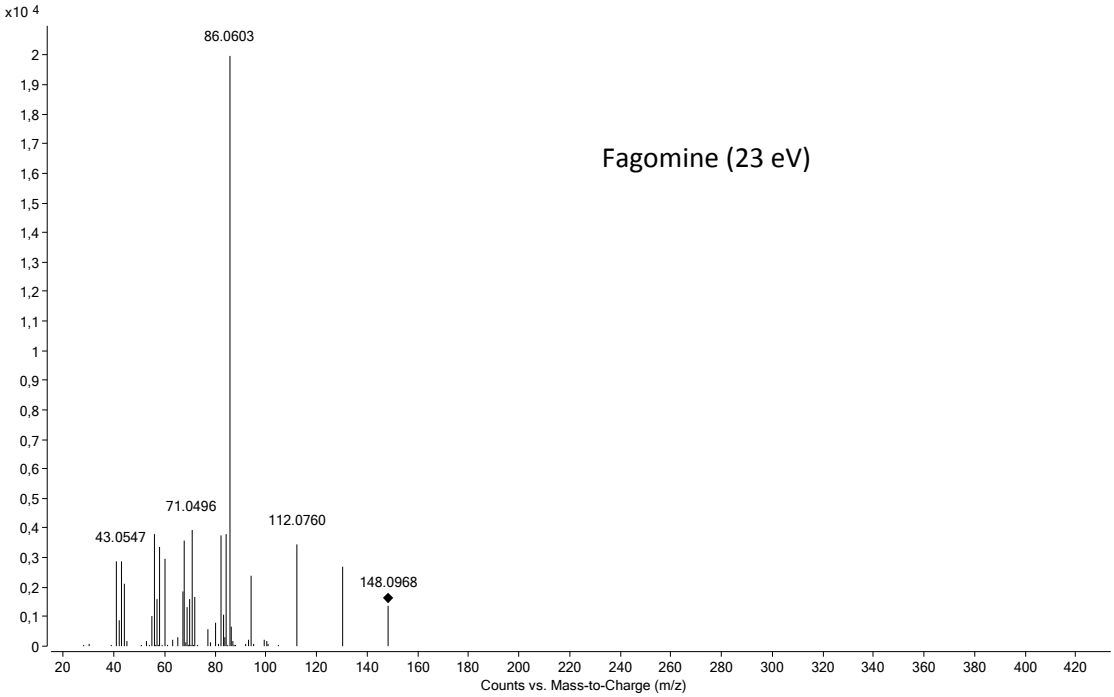

x10 3
60.0430
82.0631
94.0628
132.0623
194.0979
158.0768
44.0486
Counts vs. Mass-to-Charge (m/z)


Glycosyl-HNJ isomers (23 eV)
x10 3
194.0984
176.0889
85.0267
158.0778
356.1452
Counts vs. Mass-to-Charge (m/z)

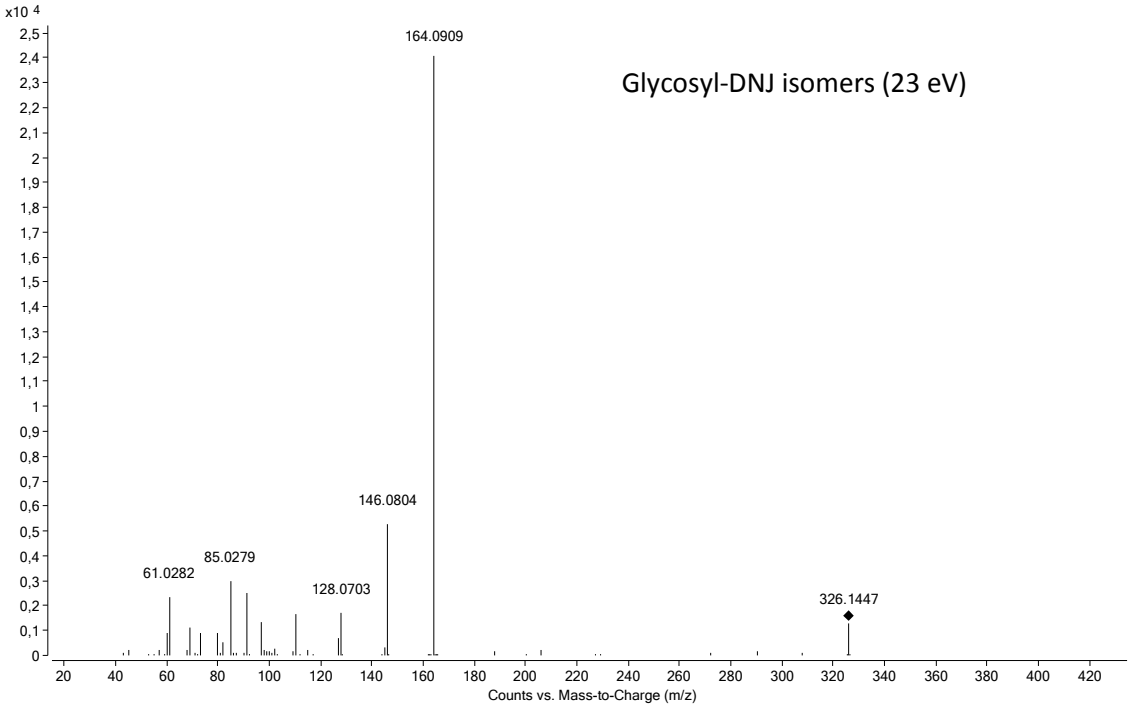

Imino-trideoxy-heptitol isomers (23 eV)
x10 3
94.0630
60.0432
178.1030
132.0624
44.0488
160.0960
Counts vs. Mass-to-Charge (m/z)



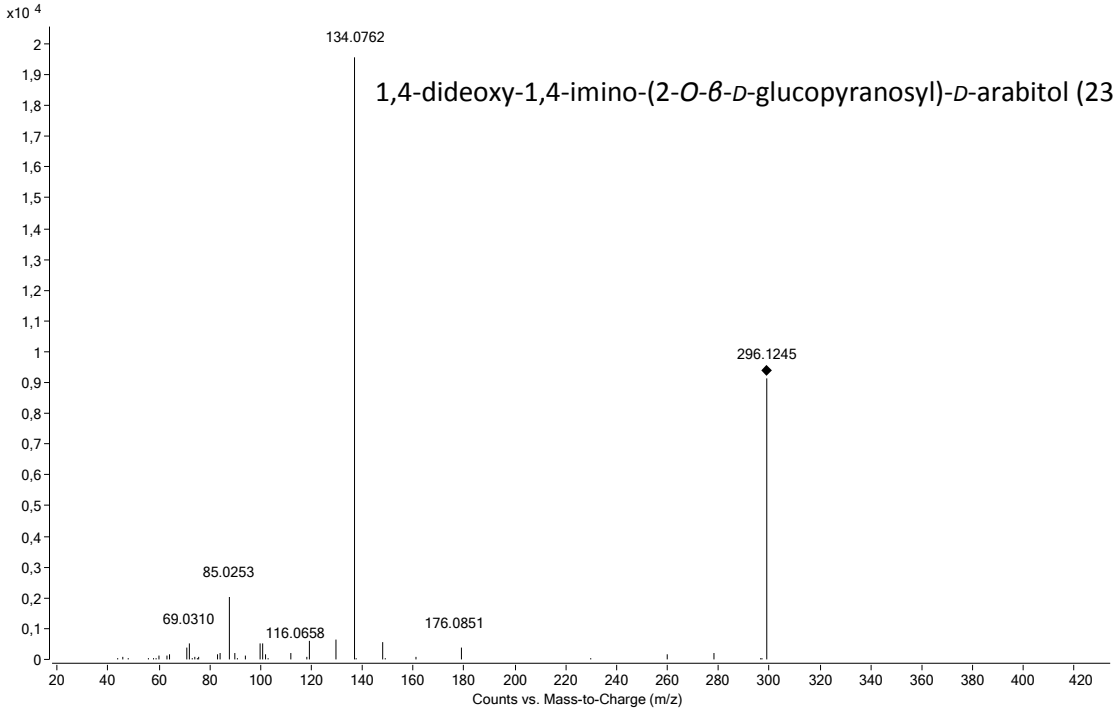
N-methyl-DNJ (23 eV)



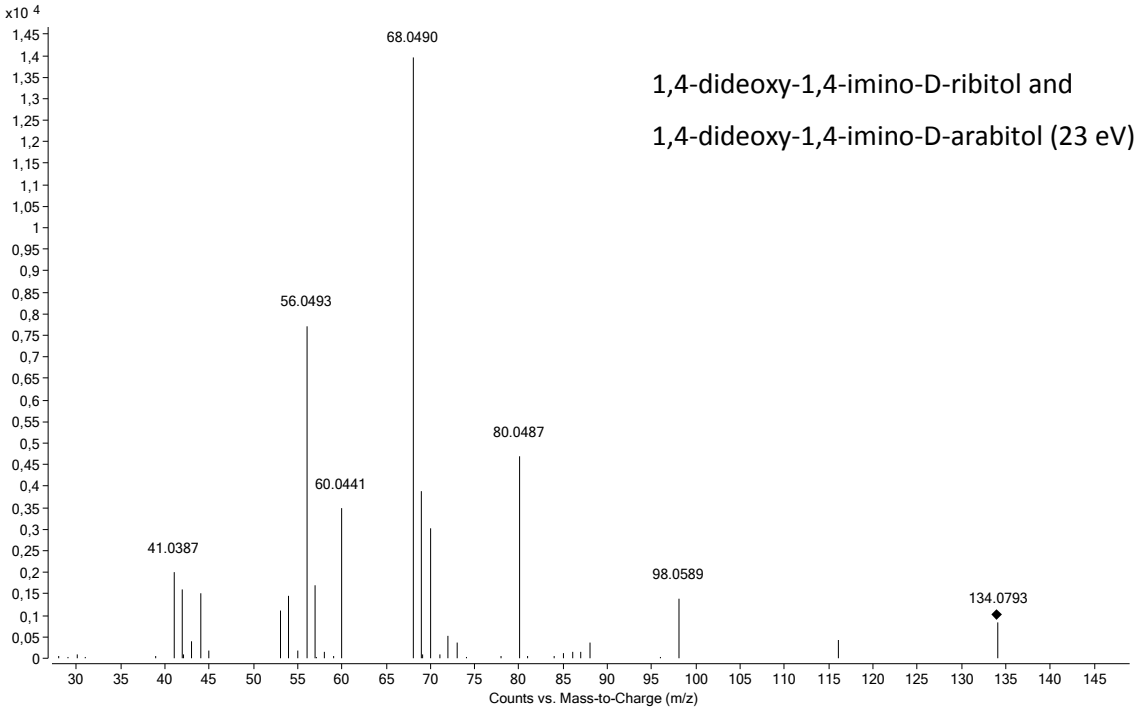
Fagomine (23 eV)



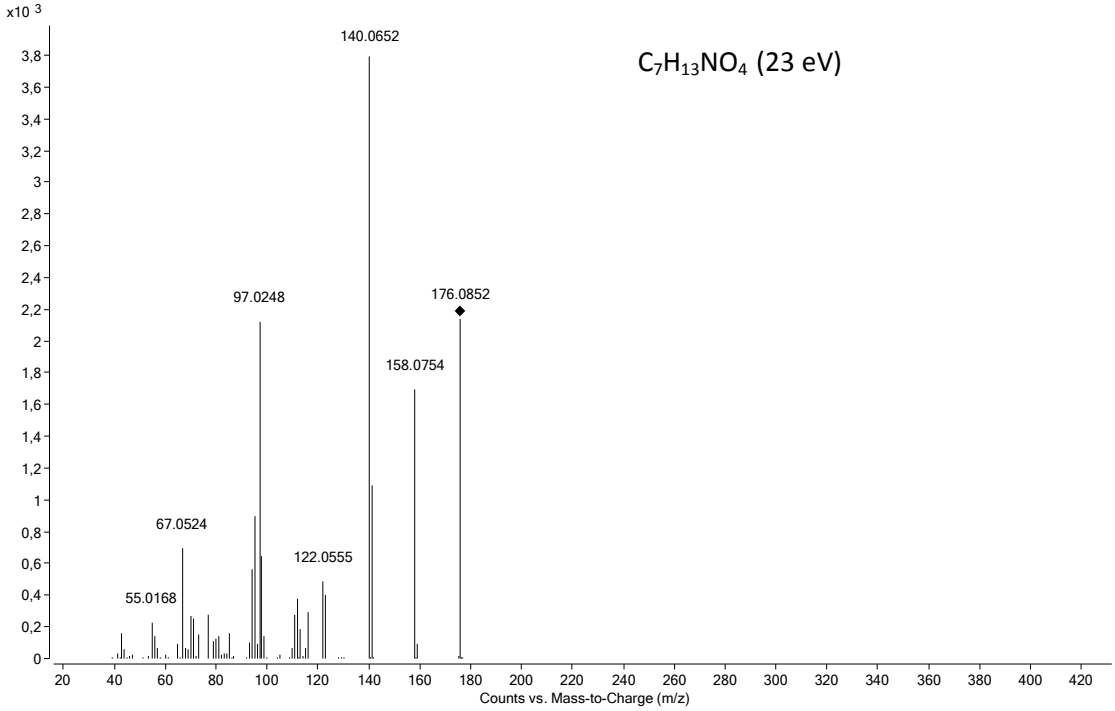
Glycosyl-DNJ isomers (23 eV)



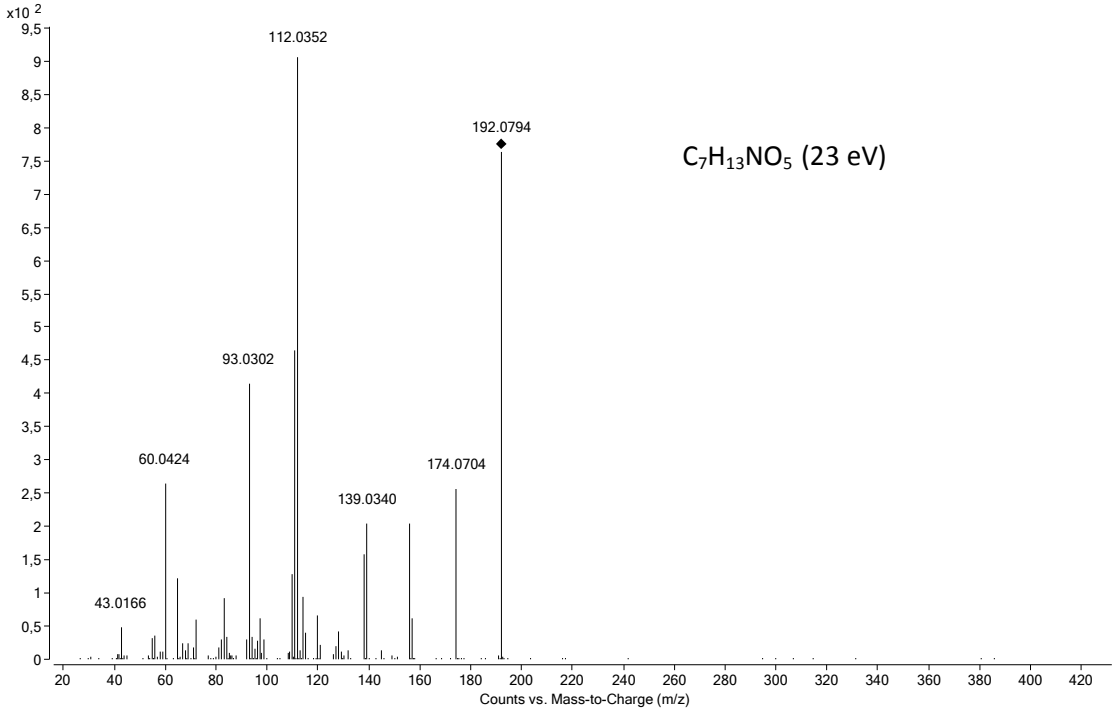
1,4-dideoxy-1,4-imino-(2-*O*-*β*-*D*-glucopyranosyl)-*D*-arabitol (23 eV)



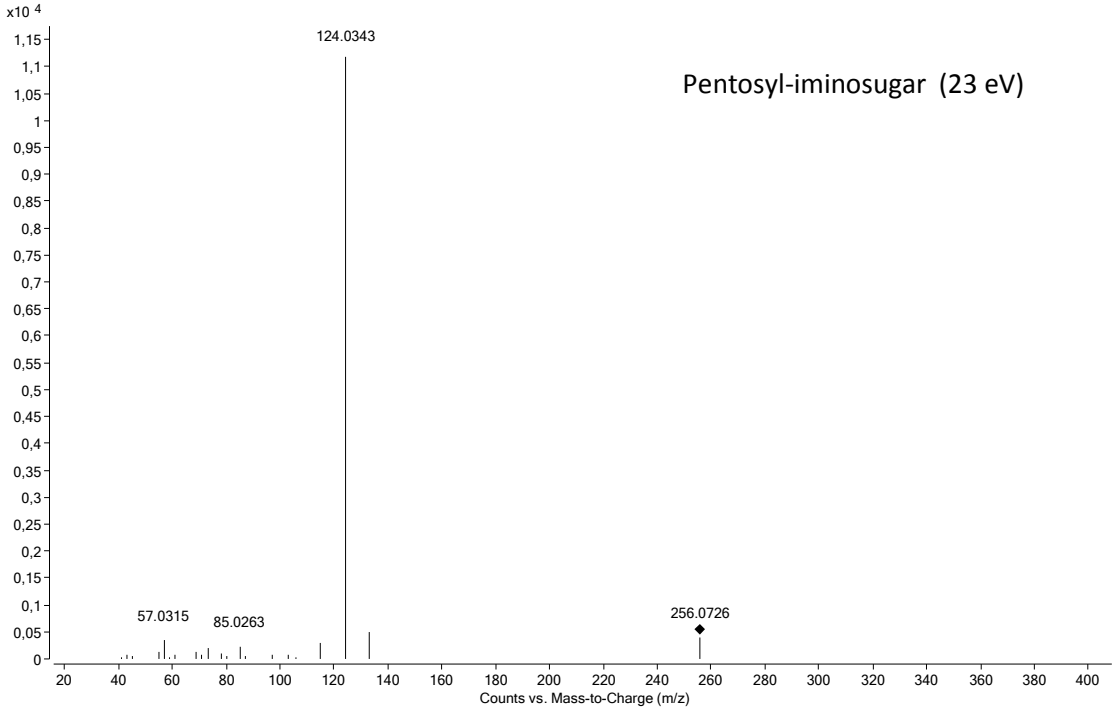
1,4-dideoxy-1,4-imino-D-ribitol and
1,4-dideoxy-1,4-imino-D-arabitol (23 eV)



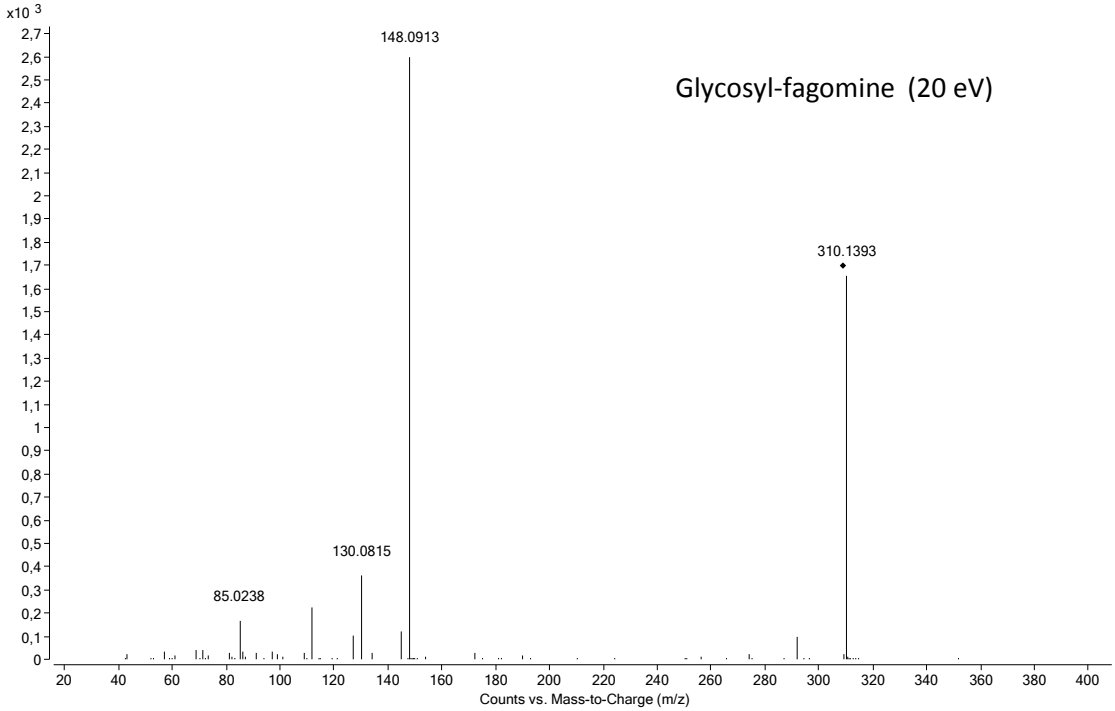
$C_7H_{13}NO_4$ (23 eV)



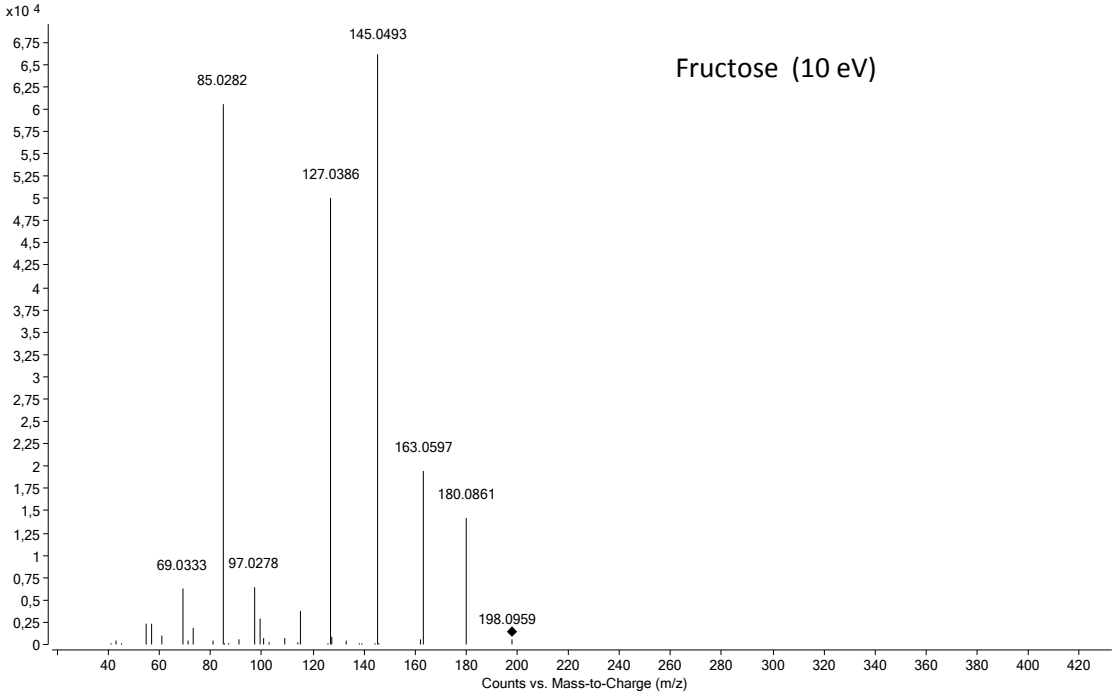
$C_7H_{13}NO_5$ (23 eV)



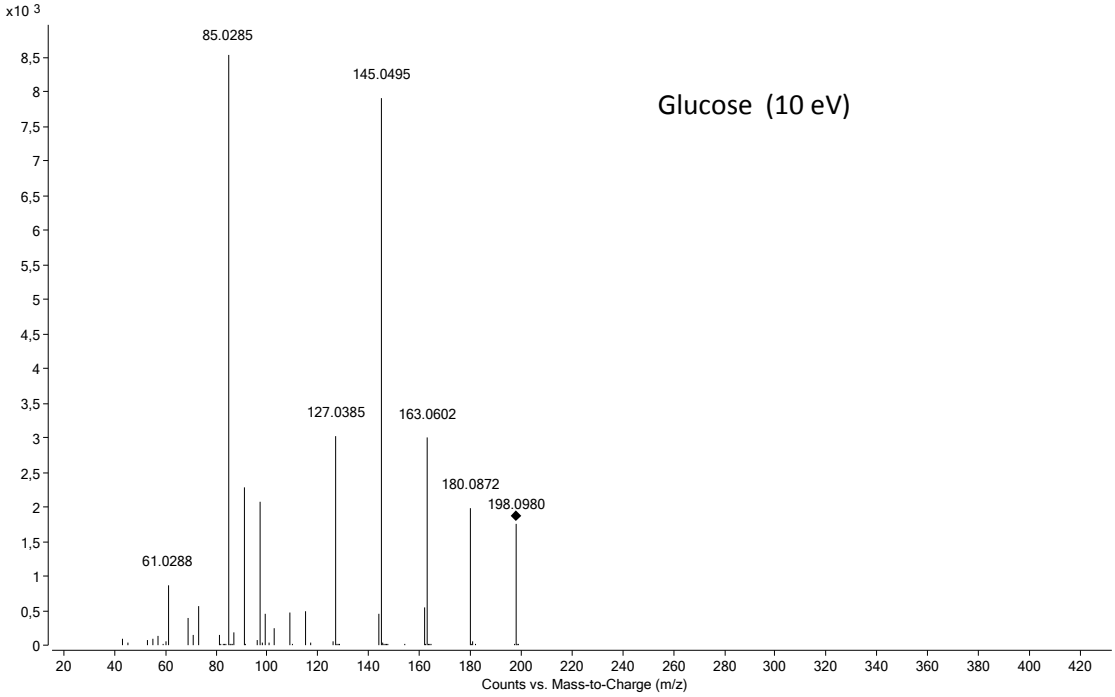
Pentosyl-iminosugar (23 eV)



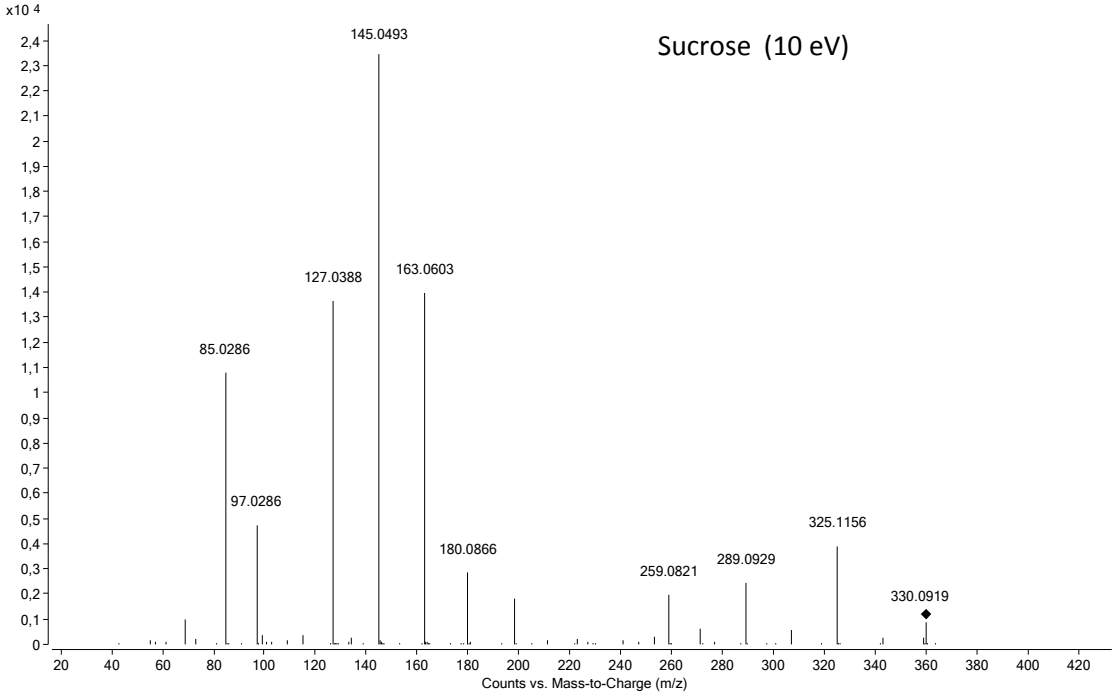
Glycosyl-fagomine (20 eV)



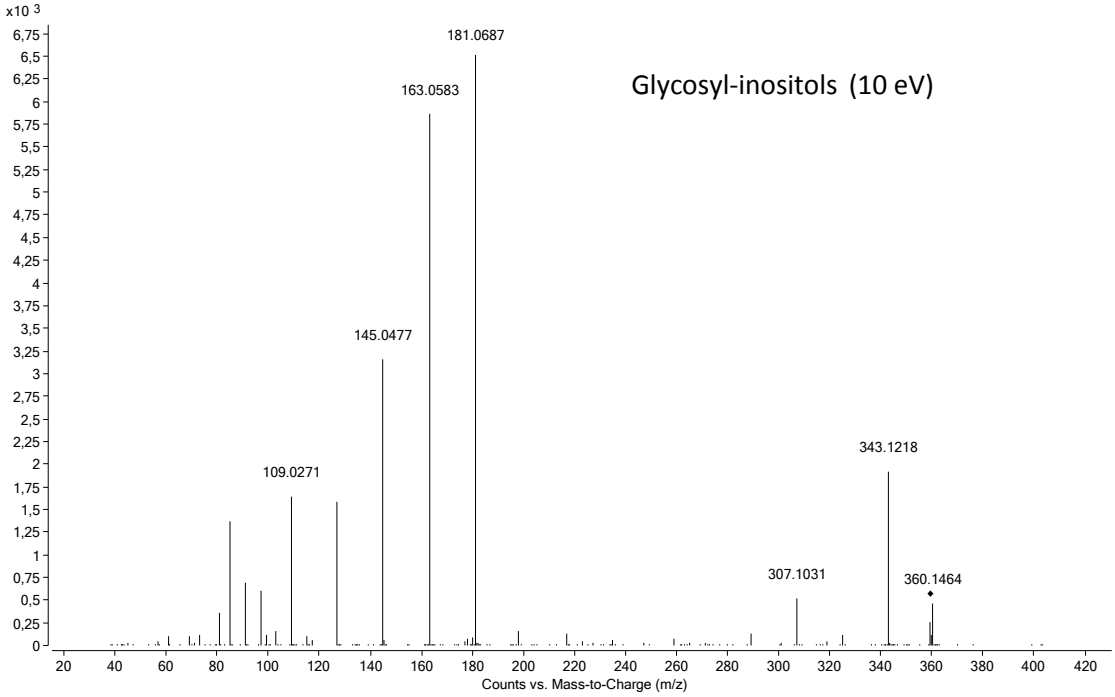
Fructose (10 eV)

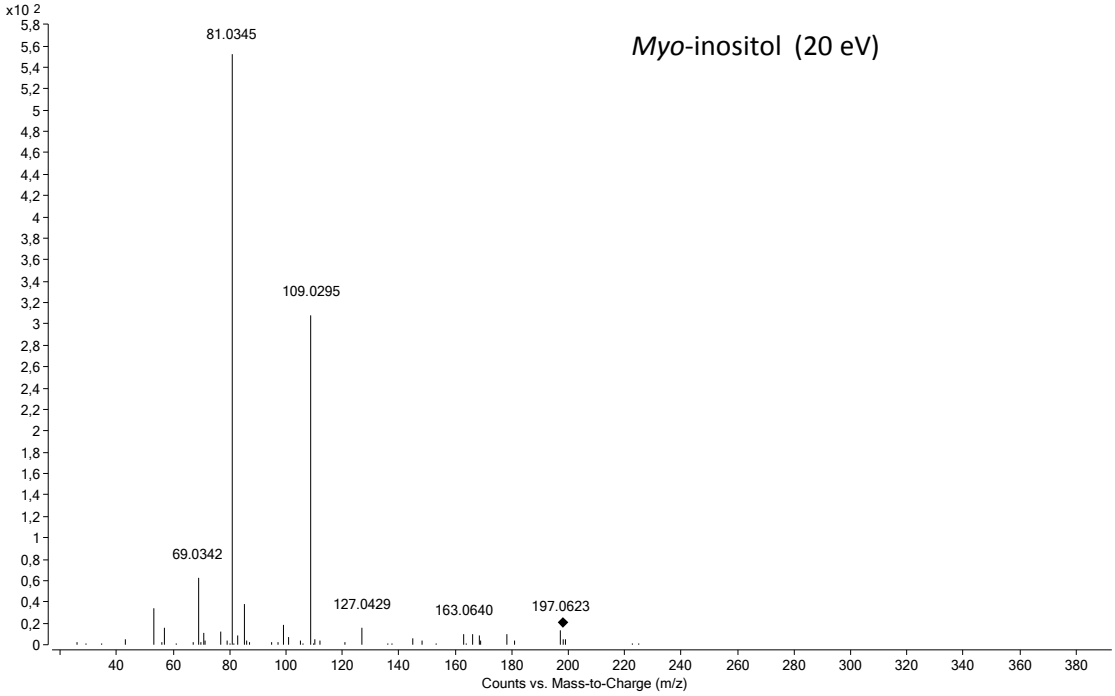


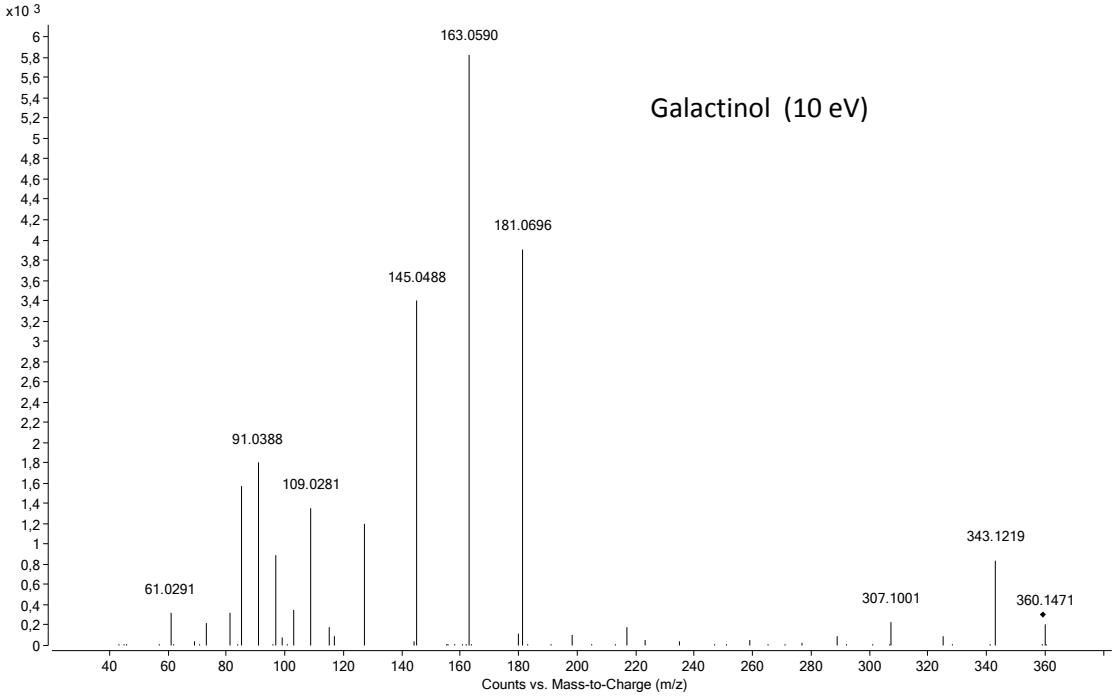
Glucose (10 eV)



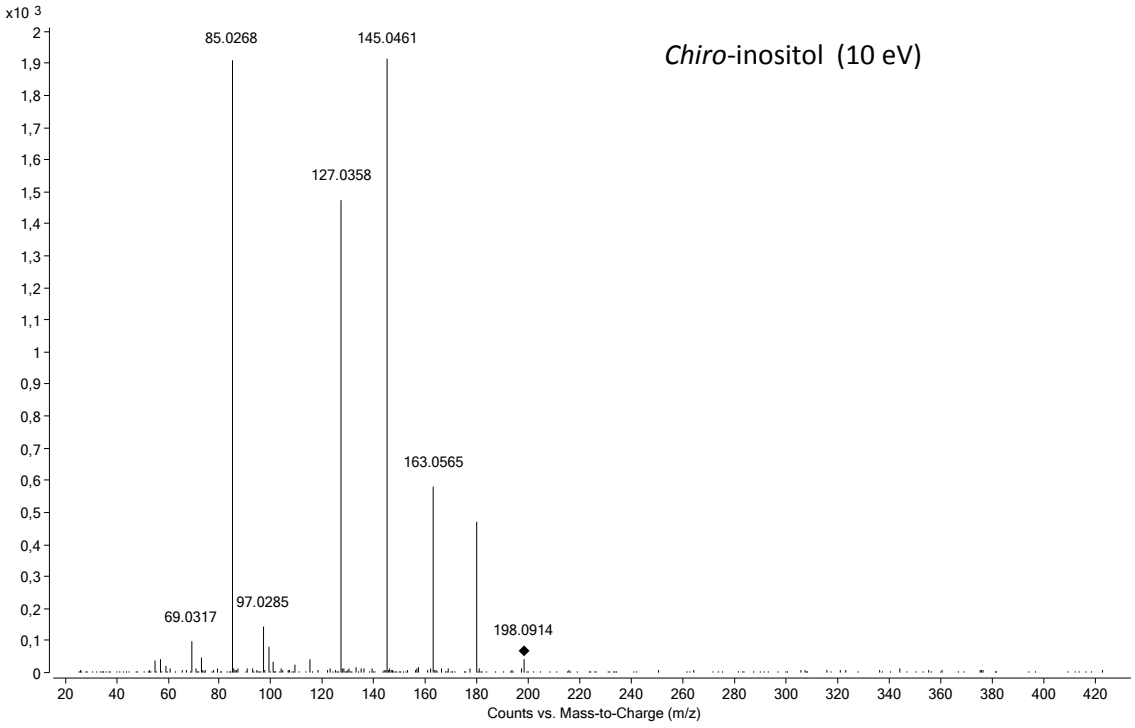
Sucrose (10 eV)


Glycosyl-inositols (10 eV)
x10 3
181.0687
163.0583
145.0477
109.0271
343.1218
307.1031
360.1464
Counts vs. Mass-to-Charge (m/z)

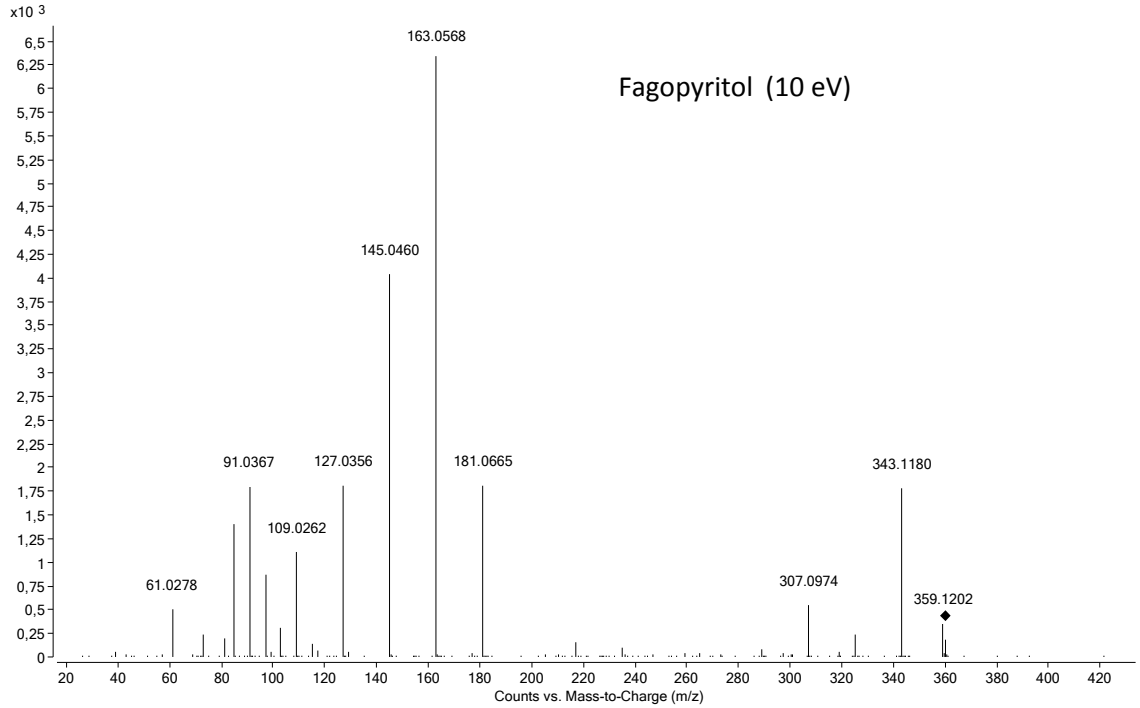

Myo-inositol (20 eV)
x10 2
81.0345
109.0295
69.0342
127.0429
163.0640
197.0623
Counts vs. Mass-to-Charge (m/z)

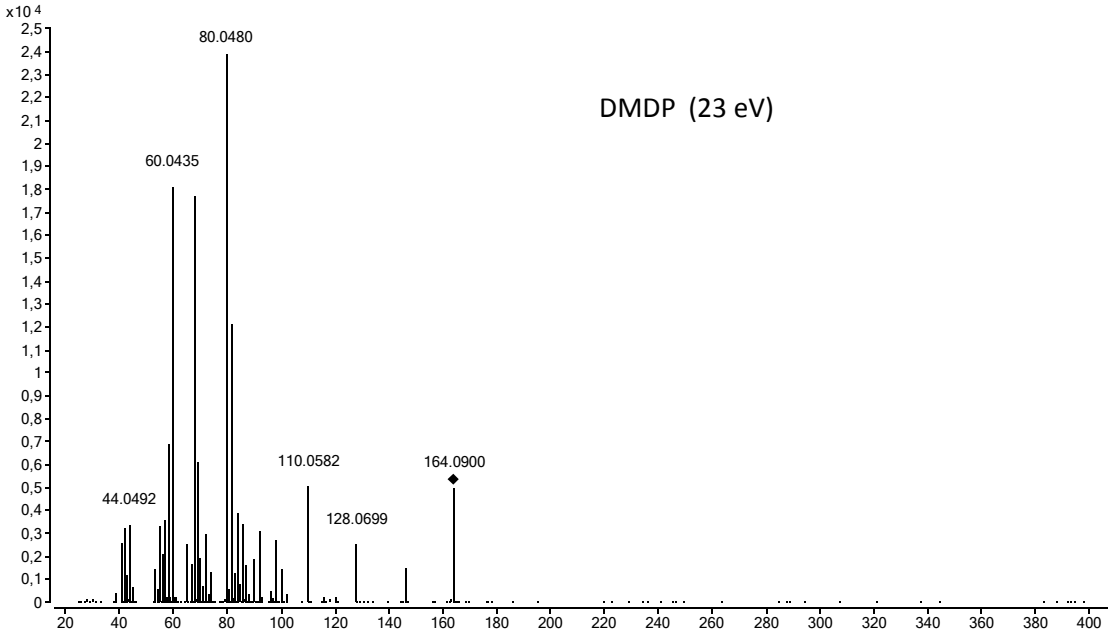


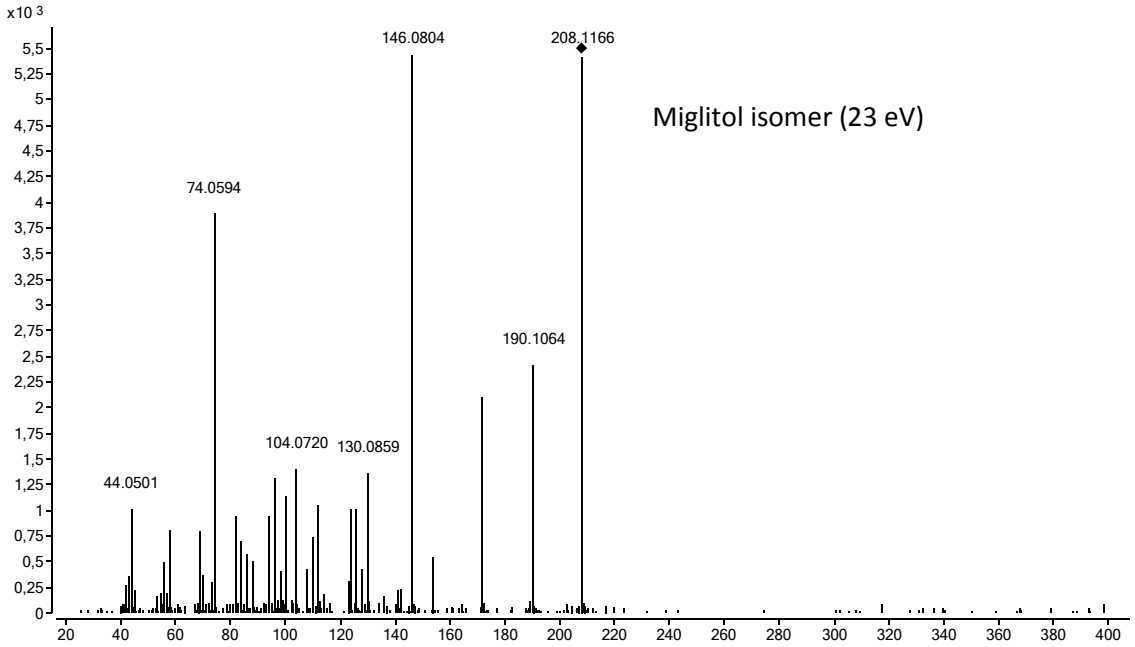
Galactinol (10 eV)



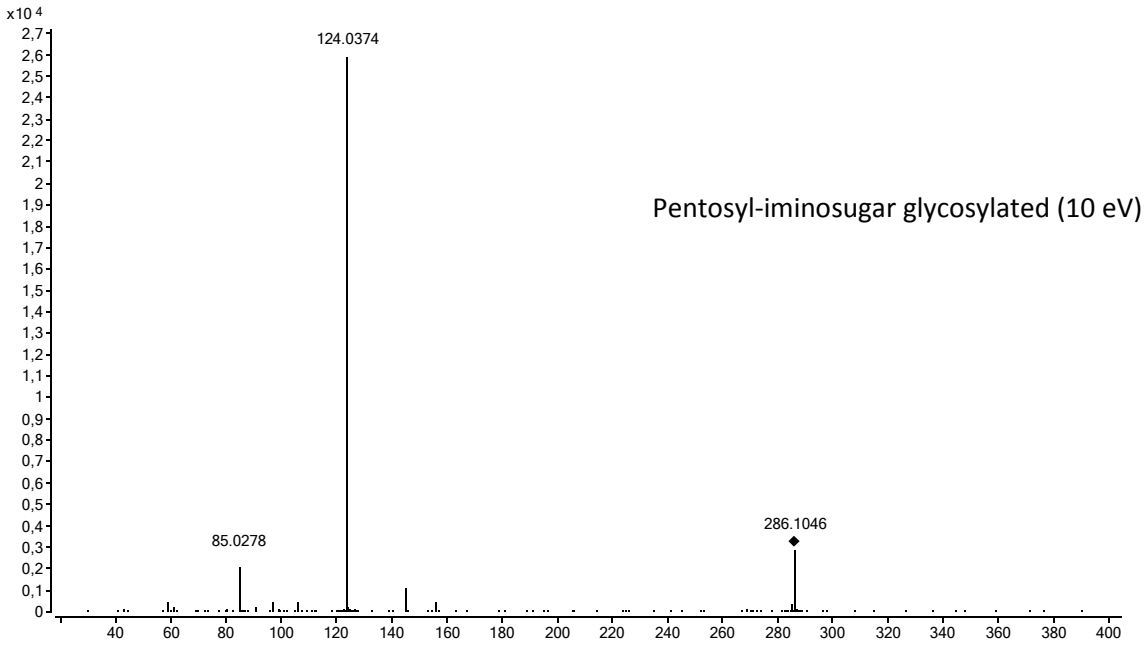
Chiro-inositol (10 eV)


Fagopyritol (10 eV)
x10 3
163.0568
145.0460
91.0367
127.0356
181.0665
343.1180
109.0262
61.0278
307.0974
359.1202
Counts vs. Mass-to-Charge (m/z)

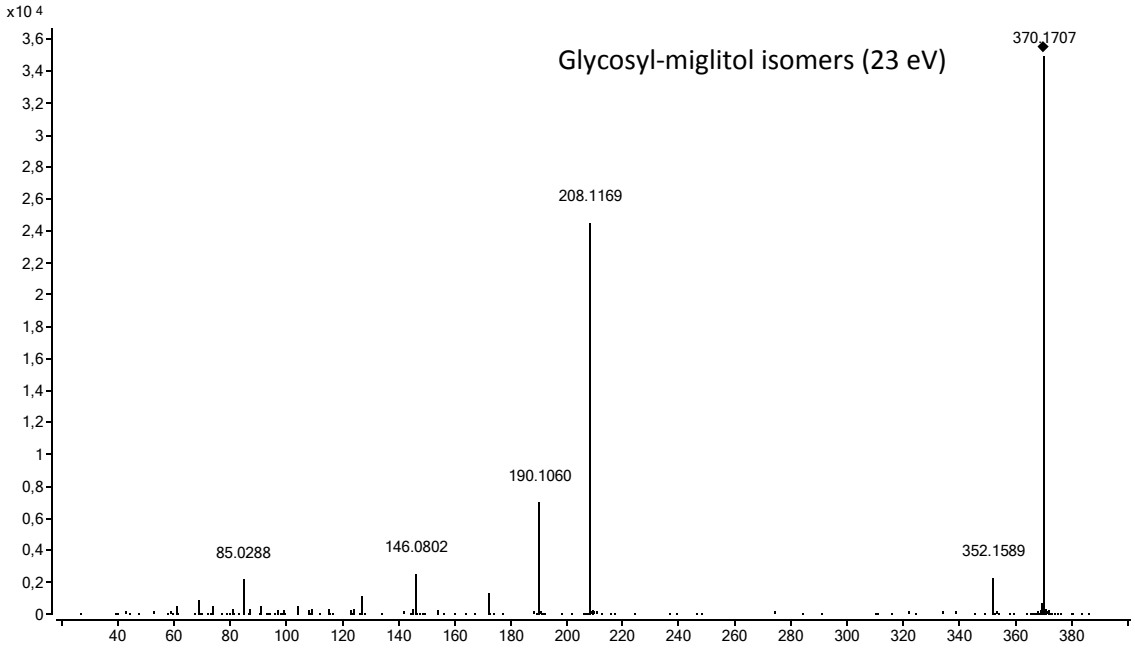

DMDP (23 eV)
x10 4
80.0480
60.0435
110.0582
164.0900
44.0492
128.0699

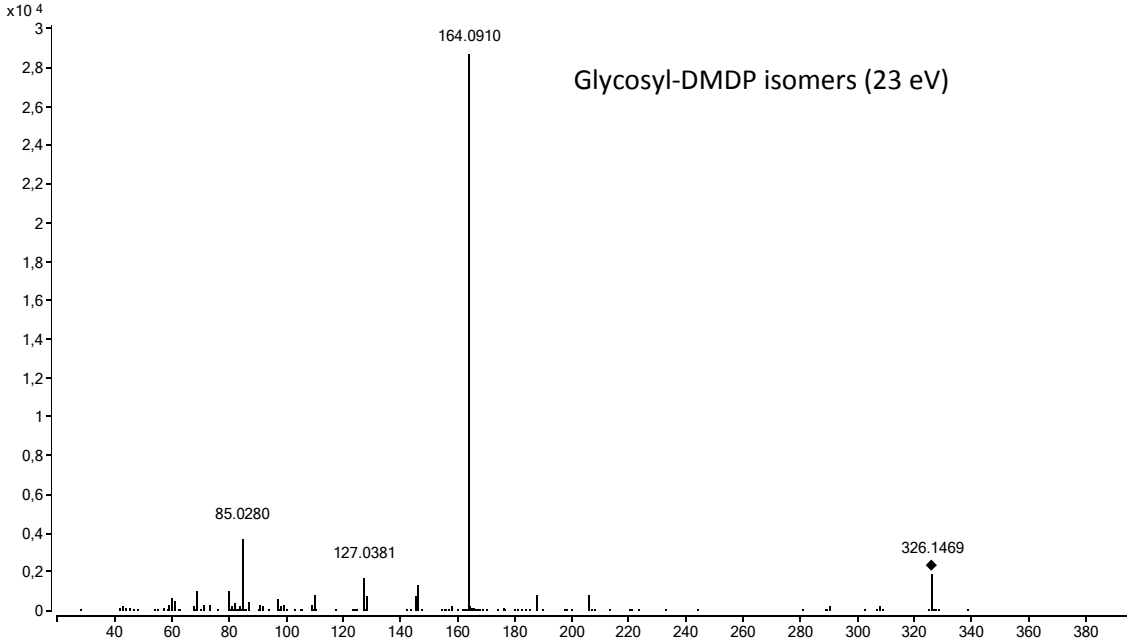


Miglitol isomer (23 eV)



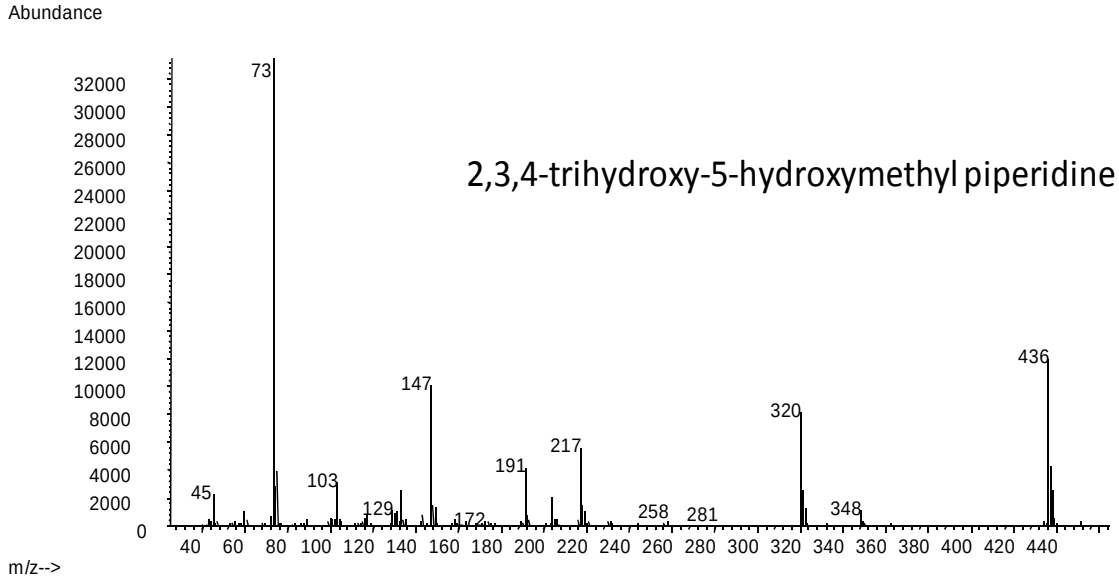
Pentosyl-iminosugar glycosylated (10 eV)

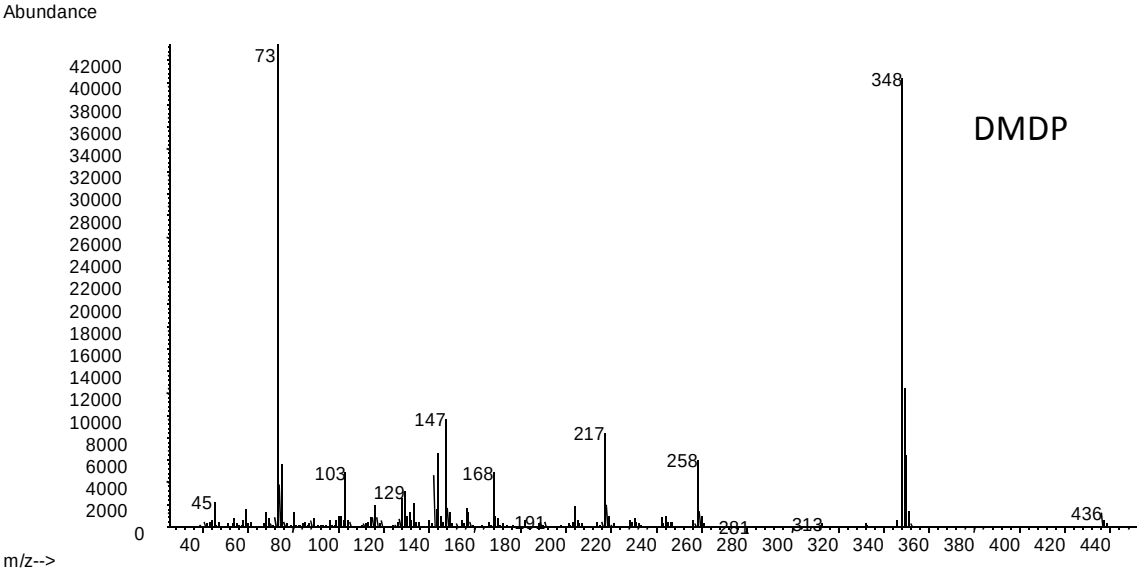

Glycosyl-miglitol isomers (23 eV)
x10 4
370.1707
208.1169
190.1060
85.0288
146.0802
352.1589

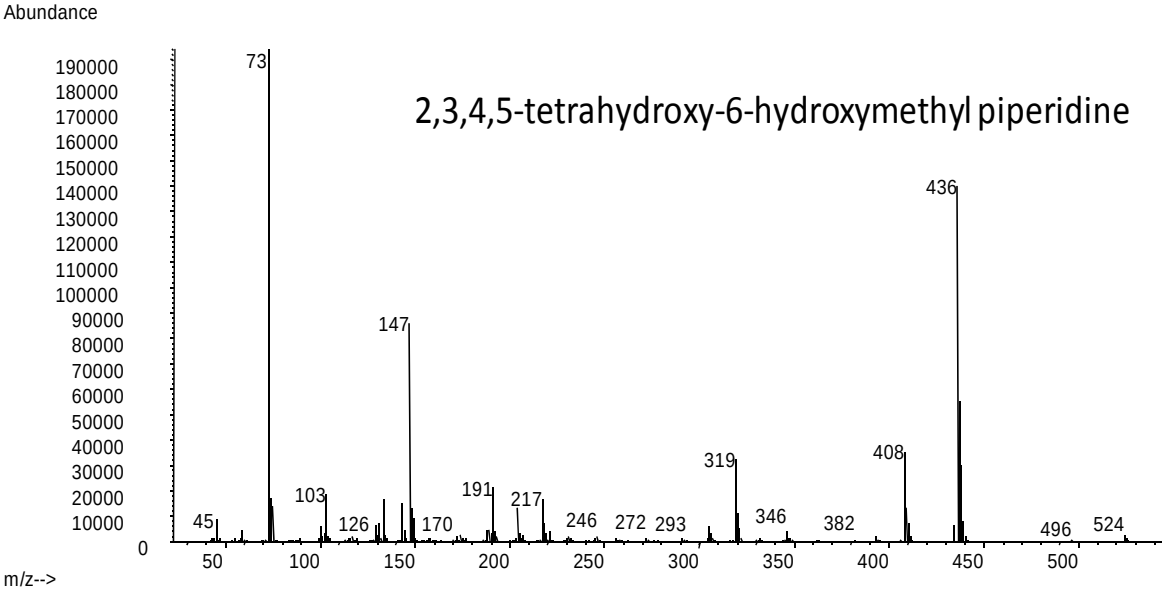

Glycosyl-DMDP isomers (23 eV)
x10 4
164.0910
85.0280
127.0381
326.1469

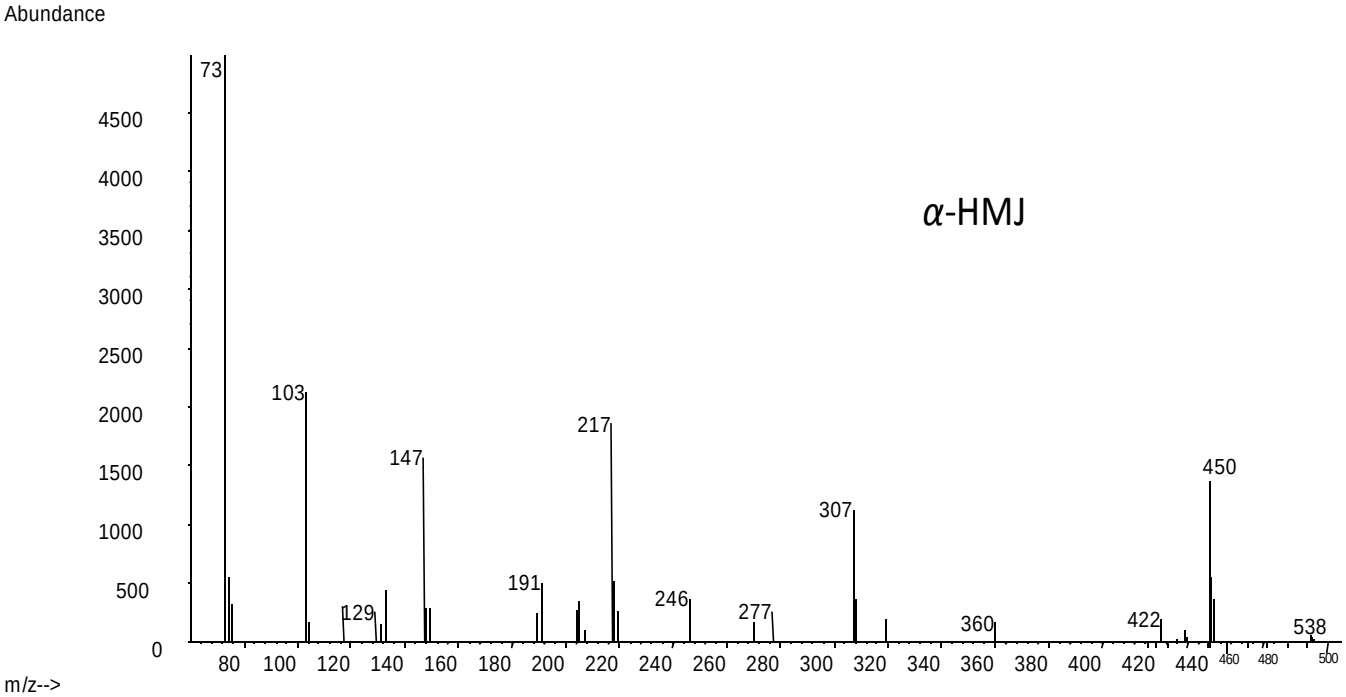
ANEXO III

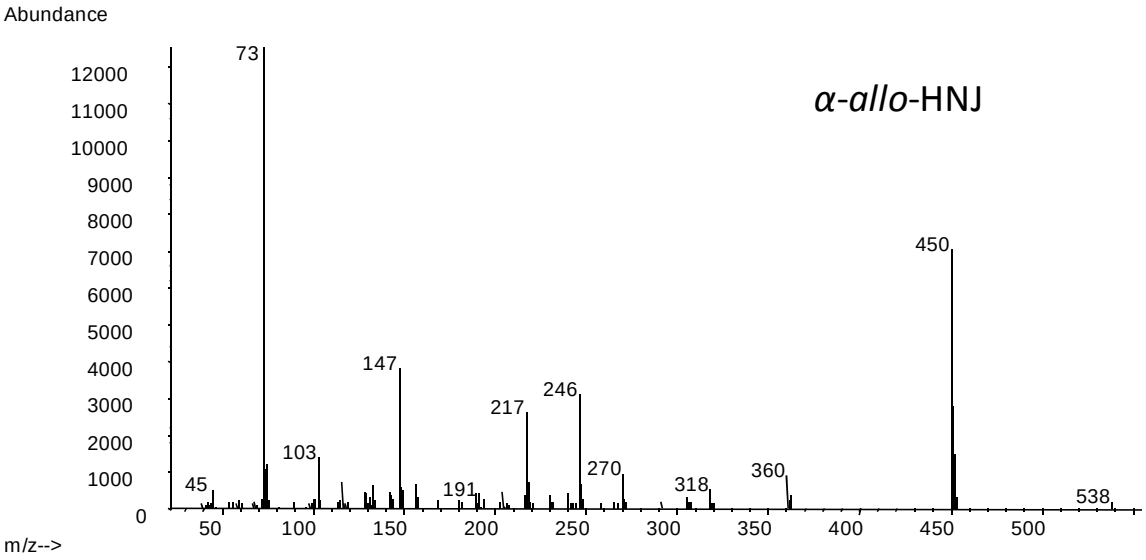
Figure 4S. EI mass spectra of derivatized iminosugars and other related compounds.

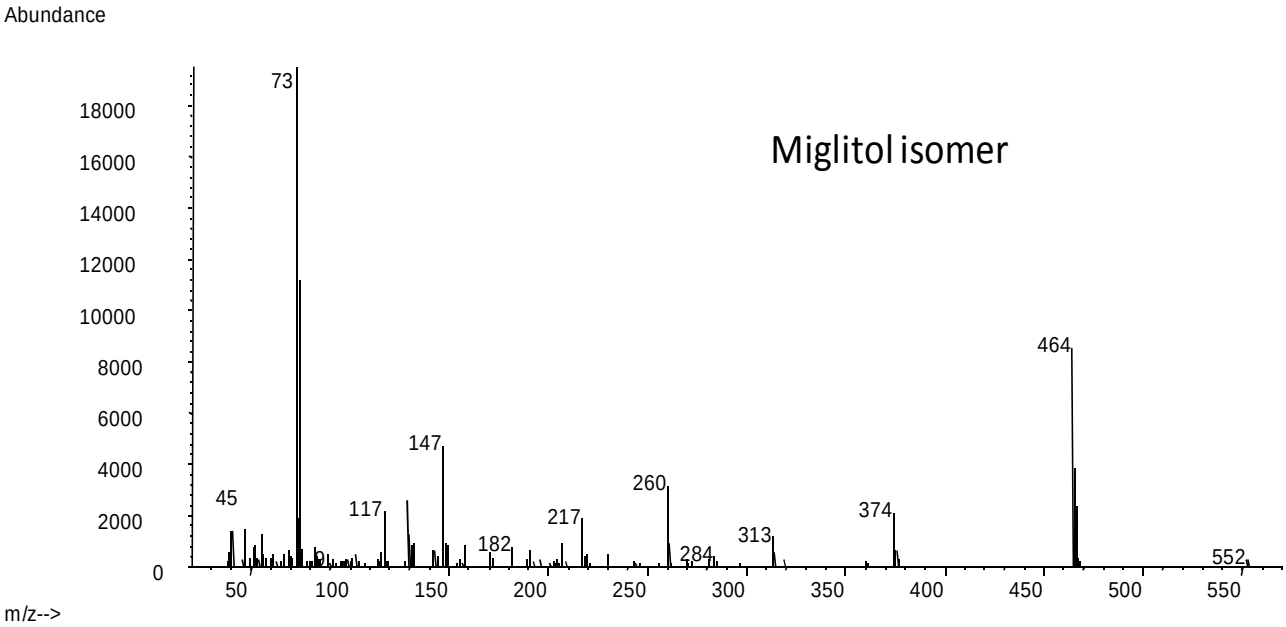


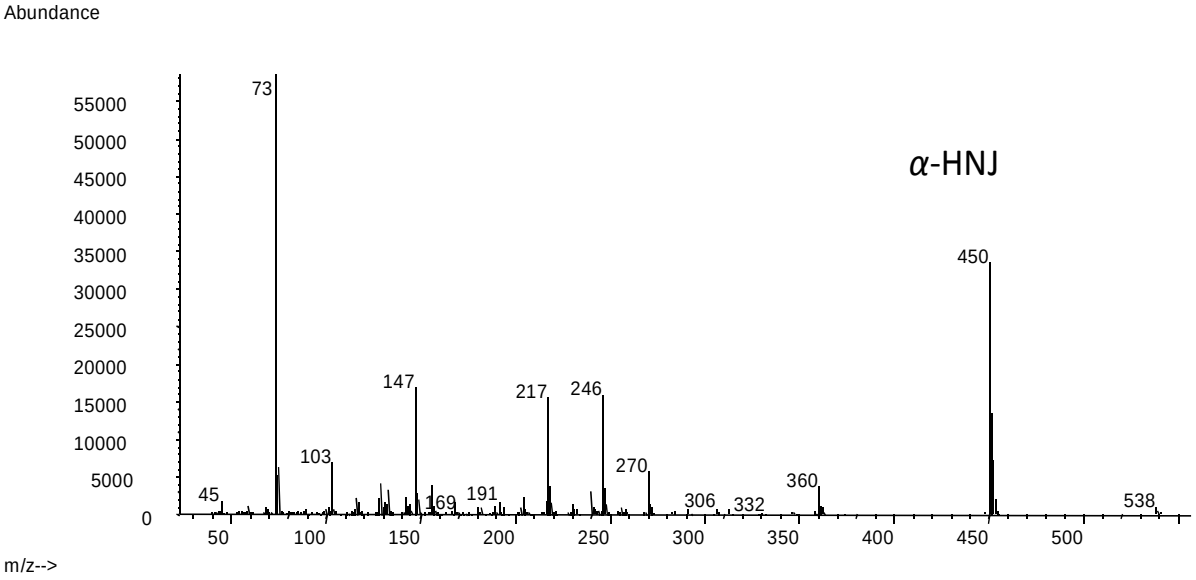



Abundance
2,3,4,5-tetrahydroxy-6-hydroxymethyl piperidine
m/z-->

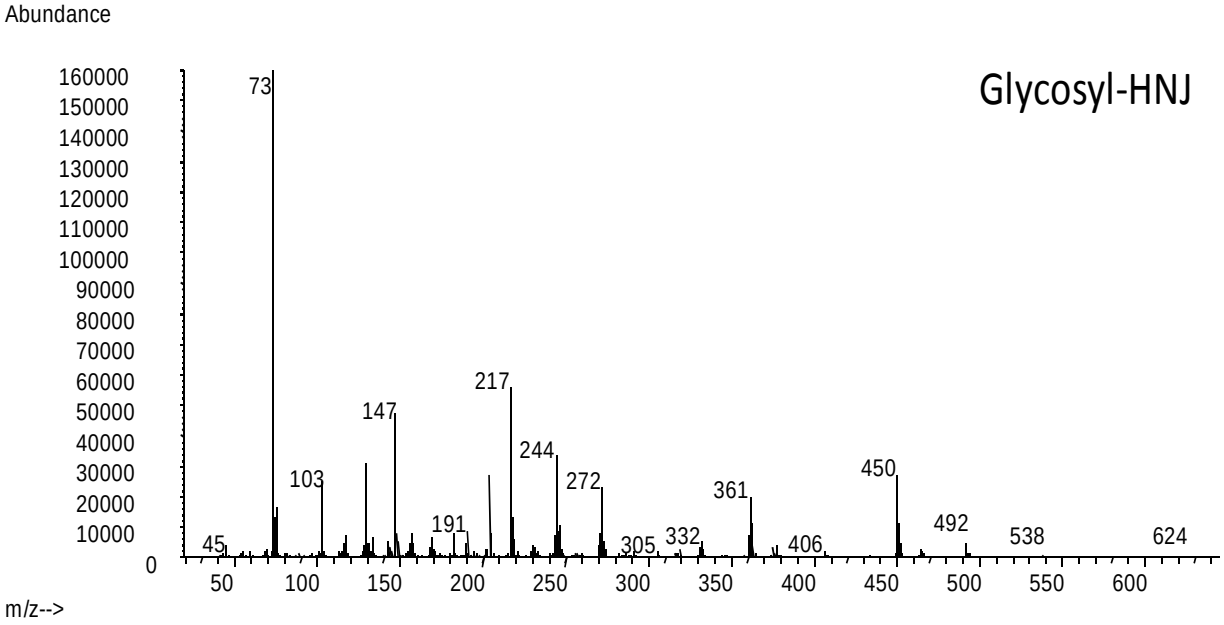

Abundance
α-HMJ
m/z-->


Abundance
12000
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
73
147
103
45
191
217
246
270
318
360
450
538
50
100
150
200
250
300
350
400
450
500
m/z-->
α-allo-HNJ


Abundance
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
73
147
45
117
182
217
260
284
313
374
464
552
50
100
150
200
250
300
350
400
450
500
550
m/z-->
Miglitol isomer



α-HNJ



Glycosyl-HNJ