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Desarrollo de nuevos métodos para la caracterización estructural de carbohidratos prebióticos y péptidos funcionales de interés alimentario. Estudio de su bioactividad.

OSWALDO HERNÁNDEZ-HERNÁNDEZ

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Memoria presentada por:

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CERTIFICAN:

Que el presente trabajo titulado "Desarrollo de nuevos métodos para la caracterización estructural de carbohidratos prebióticos y péptidos funcionales de interés alimentario. Estudio de su bioactividad.", y que constituye la memoria que presenta D. Oswaldo Hernández-Hernández 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 y en el Departamento de Bioactividad y Análisis de Alimentos del Instituto de Investigación en Ciencias de la Alimentación del C.S.I.C., bajo su dirección.

Y para que así conste firman el presente certificado en Madrid a 9 de enero de 2012.

Fdo. D.ª María Luz Sanz Murias **Fdo. D. Fº Javier Moreno Andújar**

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INDICE GENERAL

INDICE

I. ABREVIATURAS

I.ABREVIATURAS

2-FM-AA	2-furoil-metil-aminoácidos
AGEs	Productos avanzados de la glicación
ALEs	Productos avanzados de la lipo-oxidación
APCI	Ionización química a presión atmosférica
ASE	Extracción acelerada con disolventes
a_w	Actividad de agua
BEH	Fase estacionaria basada en puentes híbridos de etileno con amida enlazada trifuncionalmente
BSA	Albúmina sérica bovina
cDNA	Ácido desoxirribonucleico complementario
CID	Disociación inducida por colisión
CMP	Caseinmacropéptido
Da	Dalton
DP	Grado de polimerización
ECD	Disociación por captura de electrones
EFSA	Agencia Europea de Seguridad Alimentaria
ESI	Ionización por electrospray
ETD	Disociación por transferencia de electrones
FAO	Organización de las Naciones Unidas para la Alimentación y la Agricultura
FID	Detector de ionización de llama
FOS	Fructooligosacáridos
Fru	Fructosa
Gal	Galactosa
GalNAc	*N*-acetil-galactosamina
GC	Cromatografía de gases
GCC	Columna de carbón grafitizado
Glc	Glucosa
GOS	Galactooligosacáridos
GOS-La	Galactooligosacáridos sintetizados a partir de lactosa
GOS-Lu	Galactooligosacáridos sintetizados a partir de lactulosa
GOSLuAa	Galactooligosacáridos sintetizados a partir de lactulosa y β-galactosidasa de *Aspergillus aculeatus*
GOSLuAo	Galactooligosacáridos sintetizados a partir de lactulosa y β-galactosidasa de *Aspergillus oryzae*
GOSLuKl	Galactooligosacáridos sintetizados a partir de lactulosa y β-galactosidasa de *Kluyveromyces lactis*
GRAS	Generalmente reconocido como seguro
hCMP	Hidrolizado de caseinmacropéptido
hCMP:GOS-La	Hidrolizado de caseinmacropéptido conjugado con Galactooligosacáridos sintetizados a partir de lactosa
hCMP:GOS-Lu	Hidrolizado de caseinmacropéptido conjugado con Galactooligosacáridos sintetizados a partir de lactulosa

HILIC	Cromatografía líquida de interacción hidrofílica
HMOs	Oligosacáridos procedentes de leche materna humana
HPAEC	Cromatografía líquida de intercambio aniónico de alta resolución
HPLC	Cromatografía líquida de alta resolución
HPSEC	Cromatografía de Exclusión Molecular de alta resolución
IEC	Cromatografía de intercambio iónico
IL	Interleucinas pleiotrópicas
IT	Trampa iónica
LC	Cromatografía de líquidos
MALDI	desorción/ionización láser asistida por matriz
mRNA	Ácido ribonucleico mensajero
MS	Espectrometría de masas
MS^n	Espectrometría de masas en tándem
MWCO	Molecular weight cut-off
Neu5Ac	Ácido *N*-acetil neuramínico o ácido siálico
NFkB	Factor de transcripción nuclear kappa B
NMR	Resonancia magnética nuclear
NPLC	Cromatografía líquida en fase normal
OMS	Organización Mundial de la Salud
PCR	Reacción en cadena de la polimerasa
PHWE	Extracción con agua caliente presurizada
PLE	Extracción con líquidos presurizados
QqQ	Triple cuadrupolo
Q-TOF	Cuadrupolo acoplado a un tiempo de vuelo
RI	Indice de refracción
RM	Reacción de Maillard
RNA	Ácido ribonucleico
RPLC	Cromatografía Líquida de Fase inversa
SCFA	Ácidos grasos de cadena corta
SCX	Cromatografía de intercambio catiónico
SEC	Cromatografía de Exclusión Molecular
SFE	Extracción con fluidos supercríticos
SHWE	Extracción con agua caliente sobrecalentada
SLM	Membranas de soporte líquido
SWE	Extracción con agua subcrítica
TMSO	Trimetilsililoximas
TNFα	Factor de necrosis tumoral alfa
TOF	Tiempo de vuelo
UV	Ultravioleta
ZIC	Fase estacionaria tipo HILIC basada en una sulfobetaína zwiteriónica

II. RESUMEN - SUMMARY

II. RESUMEN

En la presente memoria se exponen los resultados más relevantes del estudio titulado "**Desarrollo de nuevos métodos para la caracterización estructural de carbohidratos prebióticos y péptidos funcionales de interés alimentario. Estudio de su bioactividad**". En dicho trabajo se han optimizado, validado y aplicado diferentes métodos analíticos basados en cromatografía de gases y de líquidos, acopladas ambas a espectrometría de masas, con el fin de superar muchas de las limitaciones asociadas a las metodologías hoy en uso para el análisis de galactooligosacáridos (GOS) sintetizados a partir de lactosa (GOS-La) y de lactulosa (GOS-Lu), así como de hidrolizados de caseinmacropéptido (CMP). Además, se ha llevado a cabo un estudio de la bioactividad de estos compuestos, centrándose en sus propiedades sobre el funcionamiento del sistema gastrointestinal, analizados individualmente y unidos covalentemente vía Reacción de Maillard.

Desde el punto de vista analítico, inicialmente se estudiaron diferentes técnicas de fraccionamiento (ultrafiltración, cromatografía de exclusión molecular (SEC), tratamiento con levaduras y con carbón activo), con el fin de obtener GOS libres de carbohidratos digeribles, principalmente monosacáridos como glucosa, galactosa y fructosa, y disacáridos como lactosa. En este sentido, la SEC y el tratamiento con carbón activo, resultaron ser los procedimientos más efectivos [**Hernández y col. (2009) Int. Dairy J. 19: 531-536**], aplicándose a las mezclas de GOS-La y GOS-Lu para su posterior empleo. Por otro lado, y debido a la novedad de los GOS-Lu y la escasa información disponible acerca de su estructura, se llevó a cabo su caracterización por GC-MS mediante el estudio de las abundancias relativas de las relaciones m/z características de sus respectivas trimetilsilil-oximas. Asimismo, el grado de polimerización (DP) de dichas mezclas de oligosacáridos fue determinado mediante HPLC-MS. Estos métodos se aplicaron al análisis de diferentes mezclas de GOS-Lu obtenidas empleando tres β-galactosidasas de diferentes orígenes. El mayor rendimiento y variabilidad estructural se obtuvo usando la enzima proveniente de Aspergillus oryzae por lo que dicha mezcla fue seleccionada para los siguientes estudios [**Hernández y col. (2011) J. Chromatogr. A. 1218: 7691-7696**].

También se llevó a cabo el desarrollo de diferentes métodos respectivamente basados en HILIC acoplada a espectrometría de masas en tandem (MS^n) empleando tres fases estacionarias diferentes (sulfobetaína zwiteriónica, polihidroxietil aspartamida y puentes híbridos de etileno con amida enlazada trifuncionalmente) para la caracterización estructural de diferentes mezclas de GOS-La comerciales. La optimización de las condiciones experimentales incluyó la selección del gradiente, composición y aditivos de la fase móvil, etc., siendo la resolución y tiempo de retención los criterios utilizados para la comparación de resultados. En base a los datos experimentalmente obtenidos, la fase de puentes híbridos de etileno resultó ser la más adecuada para la separación de estos compuestos. Además el acoplamiento a MS^n permitió establecer por primera vez criterios para la identificación de los enlaces glicosídicos de dichos oligosacáridos [**Hernández et al. (2012) J. Chromatogr. A. 1220: 57-67**].

Por otro lado, se llevó a cabo un análisis estructural de los O-sialoglicopéptidos presentes en hidrolizados de CMP, desarrollando para ello un nuevo método cromatográfico en modo HILIC con una columna de tipo zwiteriónica acoplada a MS^n. Este método permitió caracterizar 41 *O*-sialoglicopéptidos en un solo análisis además de distinguir entre un mismo péptido glicosilado con glicanos isómeros. En este trabajo se profundizó también en el mecanismo de separación determinándose que no sólo era debido a fenómenos de partición sino también a importantes interacciones electrostáticas tanto de atracción como de repulsión con el grupo sulfonato terminal de la fase estacionaria [Hernández y col. (2010) Proteomics 10: 3699-3711]. De igual forma, el empleo de RPLC acoplada a una trampa iónica y a un cuadrupolo acoplado a un tiempo de vuelo permitió identificar un nuevo sitio de fosforilación en la secuencia del CMP [**Hernández y col. (2009) J. Agric. Food Chem. 59: 10848-10853**].

Tras el estudio analítico de GOS-Lu, GOS-La y del hidrolizado de CMP, se procedió a la evaluación de su bioactividad tanto empleando modelos in vitro como in vivo. Además, dichos compuestos fueron conjugados vía Reacción de Maillard bajo condiciones controladas con el fin de obtener prebióticos de segunda generación que pudieran ser considerados como nuevos ingredientes multifuncionales, evaluándose sus propiedades bioactivas.

Entre los estudios llevados a cabo en sistemas in vitro, en primer lugar se evaluó el efecto de los prebióticos, concretamente los GOS previamente mencionados y la lactulosa, sobre el crecimiento de seis cepas de Lactobacilos y su resistencia a diferentes condiciones gastrointestinales. En general, se observó un efecto cepa-dependiente en todas las condiciones gastrointestinales estudiadas (sales biliares, bajos valores de pH, presencia de α-amilasa y pancreatina). De este modo, determinadas cepas de *Lactobacillus* experimentaron un mayor crecimiento en presencia de los GOS lo que podría incrementar su viabilidad a través del tracto gastrointestinal así como su capacidad de adhesión. [**Hernández y col. (2012) Food Microbiol. DOI: 10.1016/j.fm.2011.12.022**]. Estudios similares se llevaron a cabo para los GOS conjugados con hidrolizados de CMP observándose un comportamiento similar en la mayoría de los casos. [**Muthaiyan y col. (2012). En revisión**].

Posteriormente, y con el fin de determinar el efecto de los carbohidratos de interés no solo sobre cultivos puros, sino sobre la microbiota intestinal, las muestras de GOS-Lu y GOS-La y sus correspondientes conjugados con hidrolizados de CMP se sometieron a sistemas modelo in vitro con heces humanas. Como consecuencia de estos estudios tanto los carbohidratos sin conjugar como los respectivos conjugados mostraron un importante efecto bifidogénico, lo cual, al igual que en los estudios anteriores, permitió concluir que la glicación vía reacción de Maillard no afecta de manera significativa a las propiedades prebióticas de dichos carbohidratos [**Hernández y col. (2011) J. Agric. Food Chem. 59: 11949-11955**].

Por otro lado, se estudió la influencia de estos conjugados y de sus componentes en forma libre, en la adhesión y la respuesta inflamatoria de patógenos intestinales como la *Salmonella enterica* y *Listeria monocytogenes*, encontrándose una marcada reducción en la adhesión de estos patógenos a las mucinas en presencia de los conjugados. En cuanto a la respuesta inflamatoria, se observó una inhibición en la producción de citoquininas pro-inflamatorias en líneas celulares Caco-2 colonizadas por *L. monocytogenes* ejercida por los GOS-Lu y sus respectivos conjugados con CMP [**Laparra y col. (2012) J. Agric. Food Chem. En revisión**].

Una vez evaluada la posible multifuncionalidad de los GOS y sus conjugados mediante sistemas in vitro, se llevaron a cabo estudios *in vivo* usando para ello como modelo biológico ratas masculinas Wistar. Uno de los requisitos imprescindibles para que un compuesto sea considerado prebiótico es que no se digiera en el tracto digestivo, alcanzando el colon para ser fermentado selectivamente por la microbiota. Por tanto, en primer lugar, se evaluó la digestibilidad ileal tanto de GOS-La como de GOS-Lu, siendo la primera vez que se llevaban a cabo estos estudios para estos últimos oligosacáridos. Para ello, se analizaron los carbohidratos suministrados en las dietas a las ratas y los encontrados en la sección del íleon y en heces mediante GC-MS. Como resultado de los análisis realizados se observó un marcado índice de digestibilidad para los GOS-La en comparación con los GOS-Lu. Esta diferente susceptibilidad a la digestión gastrointestinal de los GOS-La y GOS-Lu pudo relacionarse con las características estructurales elucidadas anteriormente, determinándose la mayor resistencia a la acción de las enzimas digestivas de galactosil-fructosas (que es una estructura básica entre los oligosacáridos que conforman los GOS-Lu) frente a la mostrada por las galactosil-glucosas que conforman mayoritariamente los GOS-La. No obstante, se logró determinar un aumento significativo en la población de bifidobacterias en heces, tras la ingesta de ambos tipos de galactooligosacáridos estudiados, siendo este efecto beneficioso mayor en las muestras obtenidas de ratas alimentadas con GOS-Lu [**Hernández y col. (2012) J. Nutrition. En revisión**].

Por otro lado, se observó que los GOS-Lu indujeron un aumento en la población y diversidad de especies de Bifidobacterias presentes el colon de ratas alimentadas con dichos carbohidratos, además de una estimulación de la expresión de interleucinas pleiotrópicas IL-6 e IL-10 y un aumento en la expresión del factor de transcripción nuclear NFkB, implicado en diversos procesos de señalización celular [**Hernández-Hernández y col. (2011). Patente española 201130784**]. Estos resultados indican que los GOS-Lu poseen un efecto no solo prebiótico sino también poseen una serie de propiedades biológicas añadidas en comparación con los GOS-La, dotándoles de un carácter multifuncional.

En conclusión, se ha llevado a cabo un exhaustivo análisis estructural tanto de GOS como de hidrolizados de CMP, demostrando la versatilidad de las columnas de

interacción hidrofílica (HILIC) para su análisis, sin necesidad de derivatizaciones, enriquecimientos o modificaciones previas. Por otro lado, el análisis llevado a cabo por GC-MS de los GOS-Lu demuestra la presencia de diversos GOS con unidades terminales de fructosa, lo cual podría explicar la baja digestibilidad ileal de los GOS-Lu en comparación con los GOS-La, dotándolos de un valor añadido para estos nuevos prebióticos, además de su capacidad inmunomoduladora. Por otro lado, se comprobó mediante estudios in vitro que la glicación de los prebióticos antes mencionados con hidrolizados de CMP, no afecta a sus propiedades prebióticas lo cual permite explorar una nueva vía en la producción de ingredientes alimentarios multi-funcionales que combinen las propiedades bioactivas de los carbohidratos prebióticos y las de los péptidos funcionales.

II. Summary

The present memory describes the most relevant results of the study titled "Development of new methods for the structural characterization of prebiotic carbohydrates and food functional peptides. Study of their bioactivity". In this work different analytical methods based on gas and liquid chromatography coupled to mass spectrometry have been optimized, validated and applied to the analysis of galactooligosaccharides (GOS) synthesized from lactose (GOS-La) and lactulose (GOS-Lu), and enzymatically hydrolyzed bovine caseinmacropeptide (CMP), in order to overcome many of the limitations associated with the existing methodologies used for this purpose. Furthermore, a study of the bioactive properties on the gastrointestinal system of these compounds, both individual and covalently linked via the Maillard reaction, was carried out.

Firstly, four different fractionation techniques (ultrafiltration, size exclusion chromatography (SEC), and activated charcoal and yeast treatments) were evaluated, in order to obtain GOS free from digestible carbohydrates (mostly monosaccharides such as glucose, galactose and fructose, and disaccharides such as lactose). In this regard, SEC and treatment with activated charcoal were the most effective procedures [**Hernández et al. (2009) Int Dairy J. 19: 531-536**], and they were selected for the further fractionation of mixtures of GOS-La and GOS-Lu. On the other hand, due to the novelty of GOS-Lu and the scarce information available about their structure, this mixture was characterized by GC-MS by studying the relative abundances of the *m/z* ratios of their corresponding trimethylsilyl-oximes. Moreover, the degree of polymerization (DP) of these oligosaccharides was determined by HPLC-MS. These methods were applied to the analysis of different mixtures of GOS-Lu obtained using three β-galactosidases of different sources (i.e., *Aspergillus oryzae, Aspergillus aculeatus* and *Kluyveromyces lactis*). The highest concentration of oligosaccharides and structural variability were obtained using the enzyme from *Aspergillus oryzae*, and consequently, this mixture was selected for biological studies [**Hernández et al. (2011) J. Chromatogr. A. 1218: 7691-7696**].

The development of different methods based on HILIC coupled to tandem mass spectrometry (MS^n) for the structural characterization of different mixtures of

commercial GOS-La was also carried out using three different stationary phases (sulfoalkylbetaine zwitterionic, polyhydroxyethyl aspartamide and ethylene bridged hybrid (BEH) with trifunctionally bonded amide). The optimization of experimental conditions included the study of the influence of several factors such as chemical modifiers (formic acid, ammonium acetate and ammonium hydroxide), organic solvent and gradients of the mobile phases. The resolution values and retention times were used for the comparison of the results. Based on the obtained experimental data, the ethylene bridged hybrid phase was the most suitable for separation of these compounds. On the other hand, the coupling with MS^n allowed to establish the criteria for the identification of the glycosidic linkages of these oligosaccharides, not previously reported by other authors [**Hernández et al. (2012) J. Chromatogr. A. 1220: 57-67**].

Additionally, the development of a new chromatographic method based on HILIC mode using a zwitterionic column coupled to MS^n for the structural analysis of *O*-sialoglicopeptides present in hydrolyzed CMP was carried out. This method allowed the characterization of 41 *O*-sialoglicopeptides in a single analysis, as well as the effective separation of isomeric sialylated glycans linked to the same peptide chain. It was also demonstrated that the separation mechanism was not only due to partitioning phenomena but also to important electrostatic interactions with the terminal sulfonate groups of the stationary phase [**Hernández et al. (2010) Proteomics 10: 3699-3711**]. Similarly, the use of RPLC coupled to an ion trap and to a quadrupole time of flight allowed the identification of a new phosphorylation site in the CMP sequence [**Hernández et al. (2011) J. Agric. Food Chem 59: 10848-10853**].

Once the chemical structure of GOS-Lu, GOS-La and the hydrolyzed CMP was evaluated, their bioactive properties using both *in vitro* and *in vivo* systems were determined. Moreover, the bioactive properties of these carbohydrates and peptides conjugated via Maillard reaction under controlled conditions were evaluated, in order to obtain a second generation of prebiotics which could be considered as new multifunctional ingredients.

Firstly, the effect of prebiotics (GOS-La, GOS-Lu and lactulose) on the growth of six strains of *Lactobacillus* and their resistance to various gastrointestinal conditions (bile salts, low (gastric) pH, presence of α-amylase and pancreatin) were evaluated. In general, there was a strain-dependent effect in all studied samples. Thus, the growing

of certain strains of *Lactobacillus* was greater in the presence of GOS that could increase their viability through the gastrointestinal tract and its adhesion ability. [**Hernández-Hernández et al. (2012) Food Microbiol. DOI: 10.1016/j.fm.2011.12.022**]. Similar studies were carried out for the GOS: hydrolyzed CMP conjugates finding an analogous behaviour in the most of cases. [**Muthaiyan et al. (2012). Under revision**].

Subsequently, in order to determine the effect of the GOS not only on pure cultures, but also on the intestinal microbiota, samples of GOS-La and GOS-Lu and their corresponding conjugates with hydrolyzed CMP were fermented using *in vitro* model systems with human faeces. As a result of these studies, both free and conjugated carbohydrates showed a significant bifidogenic effect, which indicated that glycation via Maillard reaction does not significantly affect the prebiotic properties of these carbohydrates [**Hernández et al. (2011) J. Agric. Food Chem 59: 11949-11955**].

Furthermore, the influence in the adhesion and the inflammatory response of intestinal pathogens such as *Salmonella enterica* and *Listeria monocytogenes* of both free and conjugated carbohydrates were studied. A notable reduction in the adhesion of these pathogens to mucins in the presence of the conjugates was found. Regarding the inflammatory response, the inhibition in the production of pro-inflammatory cytokines in Caco-2 cell lines colonized by *L. monocytogenes* exerted by the GOS-Lu and their respective conjugate with hydrolyzed CMP was observed [**Laparra et al. (2012) J. Agric. Food Chem. Under revision**].

After the study of the potential multifunctionality of GOS and their conjugates using the *in vitro* systems, *in vivo* studies were carried out using Wistar male rats as a biological model. A prebiotic compound should not be digestible in the gastrointestinal tract and should reach the colon to be selectively fermented by the microbiota. Therefore, ileal digestibility of both GOS-Lu was evaluated for the first time. Either carbohydrates supplied in the diet of rats, or those found in the ileum section and in faeces were analyzed by GC-MS. A marked GOS-La digestibility compared with GOS-Lu was observed. This different susceptibility to gastrointestinal digestion of GOS-La and GOS-Lu could be related to their chemical structure which was previously elucidated as indicated above. Galactosyl-fructoses (core structure of GOS-Lu) showed a greater resistance to the action of digestive enzymes compared to that shown by galactosyl-glucoses (mainly components of GOS-La). However, a significant increase in the

population of bifidobacteria in faeces was determined after ingestion of both types of GOS; this beneficial effect being greater in samples obtained from rats fed with GOS-Lu [**Hernández et al. (2012) J. Nutrition. Under revision**].

On the other hand, GOS-Lu induced an increase in population and diversity of *Bifidobacterium* species present in the colon and caecum of rats fed with these carbohydrates, as well as the stimulation of the pleiotropic expression of interleukins IL-6 and IL-10 and an increase in the expression of nuclear transcription factor NFkB, which is involved in various cellular signaling processes [**Hernández-Hernández et al. (2011). Spanish patent 201130784**]. These results indicate that GOS-Lu not only have a prebiotic effect but also different and additional biological properties in comparison to the GOS-La, providing them with a multifunctional nature.

In conclusion, a comprehensive structural analysis of both GOS and hydrolyzed CMP was conducted, demonstrating the versatility of the hydrophilic interaction (HILIC) columns for their analysis, without derivatization, enrichment or previous amendments. At the same time, the analysis carried out by GC-MS of the GOS-Lu shows the presence of several terminal fructose units with different linkages, which could explain the low digestibility of GOS-Lu compared with GOS-La. These properties together with their immunomodulatory capabilities provide to GOS-LU a value-added to be used as nutraceutical or functional ingredient. On the other hand, *in vitro* studies showed that glycation of prebiotics with hydrolyzed CMP, does not affect to the prebiotic properties of the carbohydrates. These results open new routes for the production of multi-functional food ingredients that combine the bioactive properties of prebiotic carbohydrates and those of functional peptides.

III. Estructura

III. Estructura

La presente memoria está estructurada en cinco secciones, detalladas a continuación:

Introducción general: donde se detallan los antecedentes correspondientes a cada uno de los objetivos planteados en este trabajo.

Justificación y objetivos: primeramente se presenta detalladamente la justificación para llevar a cabo el trabajo presentado en esta memoria, seguido de los objetivos, presentados en tres bloques, correspondientes a cada sub-sección que constituyen los resultados y discusión.

Plan de trabajo: donde se explica de manera global la metodología aplicada para alcanzar cada uno de los objetivos planteados.

Resultados y discusión: esta sección está dividida en tres diferentes sub-secciones (**4.1, 4.2 y 4.3**), constituidas en primera instancia por un prefacio donde se introduce de manera general y resumida el contenido de la sección y de los resultados obtenidos, seguido por los trabajos científicos generados (publicados, enviados o pendientes de publicación), presentados en inglés, y con el formato convencional de las publicaciones (resumen, introducción, materiales y métodos, resultados y discusión).

Discusión general: esta sección recopila una discusión general de todos los trabajos presentados en el manuscrito.

Conclusiones generales: en esta sección se presentan las conclusiones generales y más relevantes obtenidas de todos los trabajos publicados y discutidos en la sección anterior.

Cabe destacar, que debido a la normativa vigente y con el fin de presentar la memoria en formato de **Tesis Doctoral Europea**, la misma está presentada en dos idiomas oficiales de la Comunidad Europea, **inglés y español**. En el idioma inglés se presentan cada uno de los trabajos publicados en revistas arbitradas internacionales, el resumen de la tesis y las conclusiones; el resto de la memoria está presentada en español.

1. INTRODUCCIÓN GENERAL

1. INTRODUCCIÓN GENERAL

1.1. El Intestino grueso y la flora intestinal humana

Las bacterias son organismos unicelulares microscópicos, sin núcleo definido, que pueden presentarse desnudas o con una cápsula gelatinosa, aisladas o en grupos y presentan orgánulos internos de locomoción. Se estima que en el cuerpo humano hay entre 10 y 20 veces más bacterias que células humanas. En función de su relación con el huésped, las bacterias se pueden clasificar en: mutualistas, donde hay un beneficio entre el hospedador y el microorganismo; comensales donde el microorganismo es beneficiado, pero no el hospedador; y por último, patógenas donde el microorganismo se beneficia, pero causa algún tipo de daño en el hospedador (Candela y col., 2010). La mayor densidad de bacterias mutualistas se encuentra en el intestino humano (entre 10 y 100 trillones). De hecho, se considera al intestino de los mamíferos uno de los ecosistemas más poblados de la Tierra, con una población de 10^{12} organismos/g de materia fecal (Iannitti y col., 2010).

Tanto la distribución longitudinal de bacterias como las condiciones de anaerobiosis del sistema aumentan desde el estómago hasta el colon (**Figura 1.1**). La baja concentración de bacterias en el estómago se debe principalmente a la presencia de un bajo pH (pH 1,5-3,5). Después del estómago se encuentra el intestino delgado, con tres partes diferenciadas, duodeno, yeyuno e íleon, cada sección con una concentración de bacterias mucho mayor a la anterior, básicamente por la disponibilidad de nutrientes (Iannitti y col., 2010). Se sabe además, que el tránsito de alimentos a través del estómago y del intestino delgado es bastante rápido, y en consecuencia, los efectos que pueda ejercer en la microbiota presente en estos órganos son escasos. Sin embargo, en el intestino grueso, las condiciones son más favorables para el crecimiento bacteriano por lo que el número de bacterias llega a ser de entre 10^{10} y 10^{12} células por mL, prevaleciendo las especies anaeróbicas debido a la baja concentración de oxígeno, además de una alta disponibilidad de nutrientes, producto de un tránsito más lento y bajos potenciales de oxido-reducción.

La **tabla 1.1** resume los géneros de bacterias que pueden ser localizados a lo largo del sistema gastrointestinal. En el estómago prevalecen las especies de

lactobacilos con tolerancia a altas concentraciones de ácido, enterococos y bacilos, entre otros, mientras que en el intestino delgado se observa una mayor variabilidad que aumenta considerablemente en el intestino grueso, donde son numerosos los géneros de microorganismos descritos hasta el momento y, en consecuencia, mucho mayor el número de especies. Sin embargo, este ecosistema se caracteriza por una baja diversidad filogenética, siendo las filas dominantes los bacteroidetes (25%) (como bacteroides) y los firmicutes (65%) (tales como lactobacilos o clostridia).

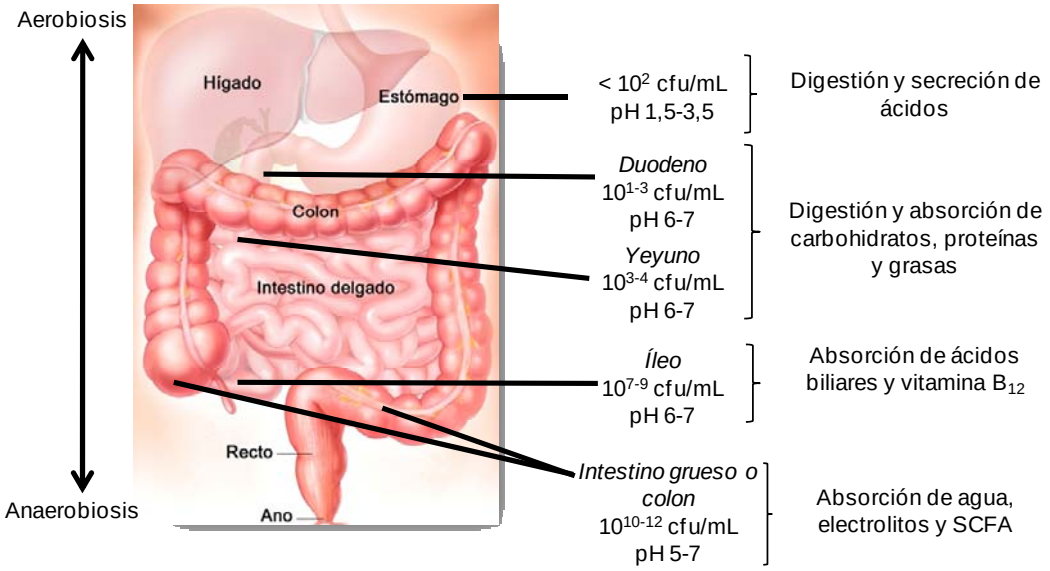


Figura 1.1. Sistema gastrointestinal humano. Adaptado de Dibaise y col. (2008).

Actualmente, se ha descrito un gran número de funciones beneficiosas para el ser humano que ejercen las bacterias que colonizan el intestino grueso, tales como: producción de algunas vitaminas, como la vitamina B por parte de bacterias lácticas; prevención en la adhesión de patógenos debido a fenómenos de competencia; producción de ciertas sustancias como ácidos grasos de cadena corta (SCFA) que, entre otras funciones, inhiben el crecimiento de algunas especies invasoras, además de estimular el desarrollo de ciertos tejidos, como los colonocitos, etc. (Iannitti y col., 2010).

Tabla 1.1. Especies bacterianas presentes en cada sección del tracto gastrointestinal humano. Adaptado de Ianniti y col. (2010).

Tracto gastrointestinal	***Flora bacteriana***
Estómago	*Streptococcus, Lactobacillus, Bifidobacterium, Bacteroides, Enterobacteriaceae.*
Duodeno	*Streptococcus, Lactobacillus, Veillonella, Bacteroides, Bifidobacterium, Enterobacteriaceae.*
Íleon	*Streptococcus, Lactobacillus, Bifidobacterium, Bacteroides, Clostridium, Enterobacteriaceae.*
Intestino grueso	*Bacteroides, Eubacterium, Ruminococcus, Coprococcus, Peptostreptococcus, Bifidobacterium, Streptococcus, Enterobacteriaceae, Lactobacillus, Clostridium*

Como en todo sistema ecológico, en el intestino grueso existe un equilibrio de microorganismos, sin embargo, en algunas ocasiones puede ocurrir un desequilibrio de los mismos, o dibiosis, que tiene como consecuencia una serie de respuestas inmunológicas o metabólicas por parte del hospedador, tales como: flatulencias, hinchazón, dolor abdominal y/o diarrea, entre otras. Esta condición puede generarse por una disminución en la concentración de los organismos mutualistas o un aumento en la concentración de patógenos (Candela y col., 2010, Iannitti y col., 2010). Generalmente, existen diferentes mecanismos a través de los cuales las bacterias beneficiosas que colonizan el intestino grueso pueden devolver a un estado de homeostasis. Entre estos mecanismos se puede destacar la producción de bacteriocinas, la inmunomodulación y la exclusión competitiva de bacterias patógenas. Sin embargo, en algunos casos es posible la restauración de la homeostasis en el intestino grueso, a través de la administración de **probióticos**.

Actualmente, la Organización Mundial de la Salud (OMS) y la Organización de las Naciones Unidas para la Agricultura y Alimentación (FAO) definen a los probióticos como: “microorganismos vivos que cuando son administrados a un huésped en cantidades adecuadas, le confieren beneficios a la salud” (FAO/WHO, 2003). Generalmente se usan como probióticos bacterias similares a las encontradas en el intestino grueso humano o en la leche materna, siendo los más comúnmente usados los géneros *Lactobacillus* y *Bifidobacterium*. En este sentido, los probióticos deben poseer ciertas características para que puedan colonizar el intestino grueso y de esta

forma devolver la homeostasis en dicho órgano. Entre estas características destacan: i) ser capaces de resistir las condiciones gastrointestinales como un bajo valor de pH, y la acción de enzimas digestivas y sales biliares, ii) adherirse a las paredes del intestino grueso, iii) excluir a microorganismos patógenos o evitar su adherencia a los colonocitos, iv) multiplicarse y producir bacteriocinas, ácidos grasos y otros productos beneficiosos para el hospedador, v) ser seguros, no patogénicos ni producir efecto adverso alguno.

Numerosos estudios han descrito el efecto beneficioso de la ingesta de diferentes probióticos en la evolución de enfermedades gastrointestinales, como el síndrome del colon irritable, colitis ulcerosa, infección por *Helicobacter pylori*, además de la modulación de la microbiota intestinal después de condiciones extremas, como la administración de antibióticos. También se han descrito propiedades beneficiosas en enfermedades como la dermatitis atópica e infecciones del tracto respiratorio (Chapman y col., 2011, FAO/WHO, 2003).

Sin embargo, los probióticos en ocasiones presentan un comportamiento variante entre cepas, una limitada colonización del intestino y sólo algunos consiguen sobrevivir al paso del estómago y del intestino delgado. Por tanto, otra alternativa que está cobrando gran importancia en la actualidad para devolver el estado de homeostasis en el intestino grueso es el uso de **prebióticos.**

1.2. Prebióticos

Un prebiótico puede ser definido como un ingrediente, componente o suplemento alimentario capaz de estimular el crecimiento selectivo y/o actividad de uno o un limitado número de género (s)/especie(s) de microorganismos presentes en el intestino grueso que confiere(n) beneficios sobre la salud del hospedador (Roberfroid y col., 2010). Para cumplir con este crecimiento selectivo, y que en consecuencia un compuesto sea considerado como prebiótico, debe cumplir con las siguientes premisas (Gibson y col., 2004, Steed y col., 2009):

i) Resistir a los bajos valores de pH gástricos, a la acción de enzimas digestivas y presencia de sales biliares.

ii) No ser absorbido a nivel del sistema gastrointestinal.

iii) Llegar al intestino grueso, sin variaciones en la estructura molecular.

Diversos estudios, tanto *in vitro* como *in vivo*, incluyendo algunas intervenciones en humanos, han descrito las numerosas propiedades beneficiosas de diferentes prebióticos (Patel y col., 2010). Sin embargo, aún no se entiende totalmente el mecanismo por el cual los prebióticos estimulan selectivamente el crecimiento de microorganismos en el intestino grueso (Roberfroid y col., 2010). Una alternativa que presentan algunos estudios es la presencia de enzimas en algunos microorganismos de la flora intestinal, que hidrolizan los prebióticos en el medio extracelular y cuyos productos (monosacáridos) son posteriormente asimilados por las bacterias. Otra posibilidad es la presencia de transportadores específicos de oligosacáridos en dichos microorganismos, llevándose a cabo la hidrólisis en el medio intracelular, y en consecuencia los monosacáridos liberados son sólo aprovechados por la bacteria que lo hidroliza y no por otras especies competidoras (Rastall, 2010).

Entre las propiedades beneficiosas que pueden ejercer los prebióticos se encuentran (Rastall, 2010, Roberfroid y col., 2010, Rycroft y col., 2001, Steed y col., 2009):

1) Prevención en la producción de compuestos genotóxicos y promotores de tumores, por parte de bacterias no beneficiosas, a través del incremento de bifidobacterias y lactobacilos.

2) Disminución en los síntomas causados por enfermedades inflamatorias del colon como el síndrome de *Crohn* y la colitis ulcerosa, mediadas por la microbiota intestinal.

3) Disminución de la probabilidad de colonización de bacterias patógenas, como *Clostridium difficile*, causante de diarrea, por la producción de compuestos antimicrobianos como SCFA, que disminuyen el pH del colon, y bacteriocinas.

4) Mejora en la absorción de minerales, como el calcio, posiblemente por la disminución del pH causado por la producción de SCFA por parte de la microbiota intestinal, lo cual aumenta la biodisponibilidad del calcio o por la

apertura, por parte de algunos prebióticos, de canales epiteliales por donde es absorbido el mismo.

5) Reducción en la incidencia de enfermedades atópicas tales como: dermatitis o urticaria alérgica, presumiblemente por el aumento de bifidobacterias en el intestino grueso.

Si bien es conocido el efecto de los prebióticos en la modulación de la microbiota intestinal, sobre todo en la estimulación en el crecimiento de bifidobacterias y lactobacilos, existen discrepancias entre algunos trabajos y opiniones de expertos sobre muchos de estos efectos beneficiosos. En consecuencia, se deben llevar a cabo más estudios, sobre todo *in vivo*, que permitan confirmar dichos efectos y otros que están surgiendo recientemente como los relacionados con la obesidad y el tratamiento de diabetes tipo 2 (Roberfroid y col., 2010).

1.2.1. Tipos de prebióticos

Los prebióticos son, principalmente, oligosacáridos de diversos grados de polimerización, aunque también se pueden considerar algunos disacáridos o polisacáridos.

1.2.1.1. Prebióticos comerciales

En Europa existen actualmente cuatro tipos de productos con reconocidas propiedades prebióticas establecidos en el mercado. Estos son: **inulina**, polisacáridos con monómeros de fructosa unidos por enlaces β(1→2) con una unidad inicial de glucosa, enlazados de forma linear, ramificada o cíclica; **fructooligosacáridos** (FOS), obtenidos por hidrólisis enzimática de la inulina o sintetizados con β-fructofuronosidasas usando como substrato sacarosa y compuestos como la 1-kestosa, 1-nistosa y 1-β-fructofuranosil-nistosa; **lactulosa** (Gal-β(1→4)-Fru)**,** disacárido sintético obtenido a través de la isomerización de la lactosa **y galactooligosacáridos** (GOS), mezclas complejas de oligosacáridos basados en unidades de galactosil-galactosas y galactosil-glucosas, producidos mediante transgalactosilación de la lactosa, usando

como catalizador β-galactosidasas de diferentes orígenes biológicos (Collins y col., 2008, Gosling y col., 2010, Olano y col., 2009, Rastall, 2010, Roberfroid y col., 2010).

Sin embargo, recientemente la Autoridad Europea de Seguridad Alimentaria (EFSA) en su evaluación sobre las propiedades prebióticas de estos carbohidratos ha concluido que no existe una relación causa-efecto entre el consumo de estos ingredientes y el efecto fisiológico beneficioso relacionado con el aumento del número de microorganismos gastrointestinales y la disminución de patógenos (Artículo 13 (1) de Regulación (EC) Nº 1924/20061). Únicamente a la lactulosa se le han reconocido sus propiedades para regular el tiempo de tránsito intestinal (http://www.efsa.europa.eu/en/efsajournal/pub/1806.htm). Por el contrario, Japón posee una legislación más abierta en este campo, de forma que en su mercado se encuentran disponibles éstos y otros oligosacáridos con reconocidas propiedades prebióticas, tales como **xilooligosacáridos**, compuestos por monómeros de xilosa unidos a través de enlaces tipo β(1→4), **oligosacáridos procedentes de soja**, cuyos principales componentes son **rafinosa** y **estaquiosa**, **isomaltooligosacáridos** y **gentiooligosacáridos** formados por monómeros de glucosa y enlazados via α(1→6) y β(1→6), respectivamente, y **lactosacarosa**, trisacárido no reductor (Gal- β(1→4)-Glc-β(1→2)-Fru) sintetizado enzimáticamente usando sacarosa y β-fructosidasas. Existen también algunos polisacáridos como almidones resistentes y polidextrosas a los que se les atribuyen ciertas propiedades prebióticas.

- *Galactooligosacáridos (GOS)*

Actualmente existen numerosos GOS sintetizados a partir de lactosa mediante β-galactosidasas procedentes de *Aspergillus oryzae*, *Aspergillus niger*, *Aspergillus aculeatus*, *Bacillus circulans*, *Kluyveromyces lactis*, *Kluyveromyces fragilis*, *Streptococcus thermophilus*, *Escherichia coli*, *Lactobacillus* spp, *Bifidobacteria* spp, *Sulfolobus solfataricus*, *Pyrococcys furiosus*, entre otros (Gosling y col., 2010, Rastall, 2010, Torres y col., 2010).

El proceso de obtención de GOS usando β-galactosidasas se basa en una cinética enzimática, donde la transgalactosilación y la hidrólisis entran en competencia, estando esta última más favorecida termodinámicamente (Torres y col., 2010, Tzortzis y col., 2009). El proceso de transgalactosilación se basa en dos mecanismos moleculares, uno intramolecular, donde directamente la galactosilación se lleva a cabo en los residuos de glucosa, y otra intermolecular, mediante el cual los residuos de galactosa se unen a otros similares permitiendo el aumento en el grado de polimerización de estos compuestos (**Figura 1.2**).

Investigaciones recientes demuestran que el rendimiento de los diferentes oligosacáridos presentes en las mezclas de GOS varía dependiendo del origen biológico de la enzima y de las condiciones de reacción. Por ejemplo, el Oligomate 55® (Yakult, Japón), obtenido a partir de β-galactosidasas procedentes de *A. oryzae* y *S. thermophilus* (Crittenden y col., 1996) predominantemente contiene enlaces β(1-6), mientras que el Vivinal-GOS® (Borculo-Domo, Holanda) sintetizado con enzimas procedentes de *Bacillus circulans* contiene principalmente enlaces β(1-4), y en el caso de BiMuno® (Clasado, Reino Unido), cuyas β-galactosidasas son reproducidas por *Bifidobacterium bifidum*, contiene una mezcla de enlaces β(1-6), β(1-4) y β(1-3) (Depeint y col., 2008, Tzortzis y col., 2005). Es conocido que la estructura de los oligosacáridos prebióticos puede afectar a sus propiedades biológicas, tal como se explicará en la sección 1.2.3.

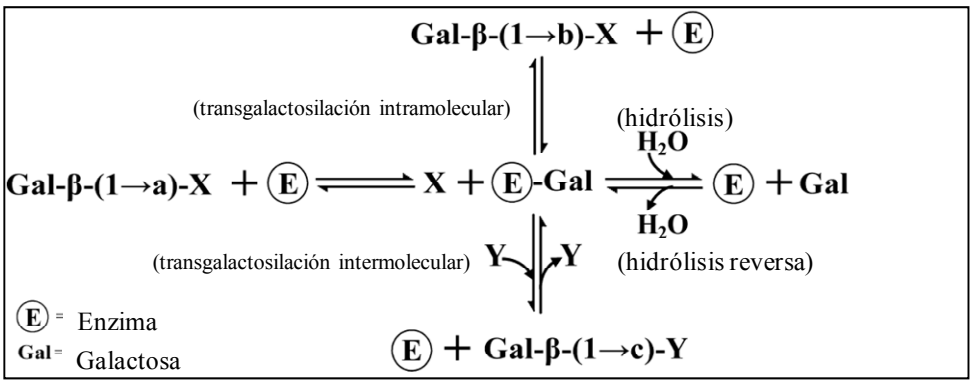


Figura 1.2. Esquema de galactosilación enzimática a partir de lactosa. a, b, c. indican el tipo de enlace (2, 3, 4, 6; a≠b). X: glucosa; Y: aceptor de galactosa. Adaptado de Torres y col. (2010).

1.2.1.2. Nuevos prebióticos

Actualmente existe un gran interés en la obtención de nuevos productos con propiedades prebióticas. En este sentido están emergiendo nuevos oligosacáridos y algunos polisacáridos con posibles propiedades prebióticas que requieren de más estudios para confirmar su utilidad. Entre ellos se pueden citar: glucooligosacáridos sintetizados empleando sacarosa como donante y distintos aceptores (gentiobiosa, maltosa, etc.) mediante glicosil-transferasas como altenansacarasas o dextransacarasas (Côté, 2009, Côté y col., 2006, Côté y col., 2009), oligosacáridos derivados de pectinas (Olano-Martin y col., 2002), arabinoxilooligosacáridos (Hughes y col., 2007, Pastell y col., 2009) y oligosacáridos procedentes de polisacáridos de plantas, después de su hidrólisis mediante glicasas, como endogalactanasas para producir arabinogalactooligosacáridos o ramnogalacturonasas para producir ramnogalaturooligosacáridos (Rastall y col., 2002, Van Laere y col., 2000).

Recientemente, se han sintetizado galactooligosacáridos, usando β-galactosidasas de diferentes orígenes biológicos y empleando como sustrato lactulosa, en lugar de lactosa (Cardelle-Cobas, 2009, Martinez-Villaluenga y col., 2008). Diferentes estudios *in vitro* empleando tanto cultivos puros como mezclas de bacterias procedentes de muestras fecales han descrito las propiedades bifidogénicas de estos carbohidratos (Cardelle-Cobas y col., 2011, Cardelle-Cobas y col., 2009). Sin embargo, hasta el momento no se ha realizado una caracterización exhaustiva de sus estructuras químicas y sólo se ha descrito la presencia de dos trisacáridos (Gal-β(1→4)-Fru-β(1→1)-Gal y Gal-β(1→6)-Gal-β(1→4)-Fru) en la mezcla. Asimismo, no se han estudiado sus propiedades bifidogénicas y/o prebióticas así como su digestibilidad en sistemas *in vivo*.

Uno de los objetivos principales en la obtención de nuevos oligosacáridos prebióticos es que dichos carbohidratos consigan aumentar su persistencia a través del intestino grueso, es decir, que lleguen a las zonas más distales del colon, donde tienen lugar la mayor parte de las afecciones intestinales (Roberfroid y col., 2010). Para ello, se han intentado llevar a cabo distintas estrategias como la obtención de oligosacáridos con grados de polimerización relativamente elevados que mantengan el

efecto en la flora intestinal pero que se digieran lentamente en el intestino (Rastall y col., 2002) o la obtención selectiva de oligosacáridos con enlaces glicosídicos específicos, aunque hasta el momento este objetivo no se ha alcanzado con éxito.

1.2.2. Multifuncionalidad

Otro de los puntos de especial interés en la obtención de oligosacáridos prebióticos es que, además de cumplir con los requisitos comentados anteriormente para el desarrollo de la flora intestinal, también posean otras propiedades adicionales beneficiosas para la salud, es decir, que puedan ser considerados como ingredientes multifuncionales. En este sentido, Sinclair y col. (2009) han descrito la capacidad de las fracciones con grados de polimerización 6 y 7 procedentes de GOS comerciales, de inhibir la adhesión de la tóxina de *Vibrio cholerae* y de la enterotóxina lábil de *Escherichia coli*. Por otro lado, se han descrito diferentes funciones gastrointestinales como: el control de tiempo de tránsito, la motilidad de la mucosa intestinal y la modulación de la proliferación de células intestinales usando fructooligosacáridos (Kolida y col., 2007). Otras propiedades de los oligosacáridos, tales como actividades anti-adherentes por la incorporación de receptores con estructuras glicosídicas específicas, propiedades atenuantes de patógenos gastro-intestinales, propiedades tecnológicas como edulcorantes, surfactantes o espesantes, etc., son, en los últimos años, objeto de estudio (Gibson, 2004).

1.2.3. Relación estructura-función

La composición estructural de los oligosacáridos (grado de polimerización, tipos de enlaces y composición monomérica) está estrechamente relacionada con su efecto sobre la flora intestinal (Rastall y col., 2005). En consecuencia, la determinación de la estructura química de estos compuestos es de gran importancia para poder establecer relaciones con funciones específicas en el tracto gastrointestinal. En este sentido, Barboza y col. (2009) describieron la especificidad en el crecimiento de diferentes especies de bifidobacterias en presencia de GOS comerciales en función de su grado de polimerización. Por otro lado, fructooligosacáridos de alto peso molecular son más

lentamente fermentados por las bacterias intestinales y, en consecuencia, más persistentes en el intestino grueso que los de peso molecular inferior (Rastall y col., 2005). Sanz y col. (2006) describieron que maltooligosacáridos con grado de polimerización igual a 3 mostraban una mayor selectividad de fermentación, similar a los de grados de polimerización (DP) 6-7. En el caso de gentiooligosacáridos comerciales la mayor selectividad la presentaron los carbohidratos de DP 2-3, contrario a los oligosacáridos sintetizados a partir de gentiobiosa usando alternansacarasas, donde los de DP 4-5 fueron los más selectivos (Sanz y col., 2006).

Diversos estudios *in vitro* han relacionado también el tipo de enlace glicosídico de algunos carbohidratos no digeribles con sus propiedades prebióticas. Sanz y col. (2005) estudiaron la influencia de la estructura de diversos disacáridos sobre su selectividad en el crecimiento de bacterias intestinales describiendo que enlaces 1-2, 1-4 y 1-6 producían un aumento en el número de bacterias beneficiosas. A su vez estos autores observaron que existía una alta dependencia de la composición monomérica con dicho efecto (unidades de glucosa, fructosa, galactosa o manosa).

1.2.4. Análisis de prebióticos

Con el fin de entender las múltiples propiedades de los prebióticos, tanto fisicoquímicas como biológicas, es importante caracterizar sus estructuras y llevar a cabo tanto análisis cualitativos como cuantitativos, según las necesidades de cada caso.

El análisis de carbohidratos prebióticos no es una tarea trivial debido principalmente a la similitud estructural de estos compuestos, a la ausencia de grupos fluorescentes o cromóforos en sus estructuras y a su falta de volatilidad. Por otra parte, estos oligosacáridos a menudo se encuentran presentes en forma de mezclas complejas, que incluyen dichos compuestos en muy diversa concentración. El hecho de que prácticamente no existan patrones comercialmente disponibles dificulta aún más su caracterización. Además, en algunos casos, se encuentran en presencia de otros compuestos formando parte de matrices complejas, por lo que es necesaria una previa

preparación de muestra que incluya diferentes etapas de fraccionamiento y/o enriquecimiento, tal y como se discutirá en las secciones 4.1.1 y 4.1.2 de esta memoria.

Para el análisis de carbohidratos prebióticos se han empleado desde métodos químicos o enzimáticos hasta métodos instrumentales.

Los **métodos químicos** se basan en su reacción con determinados agentes que dan lugar a complejos o precipitados coloreados que se pueden determinar por gravimetría, espectrofotometría o mediante valoraciones colorimétricas (Brummer y col., 2005). Los **métodos enzimáticos** son específicos para un determinado tipo de carbohidrato, lo que resulta problemático para el análisis de mezclas de estos compuestos. Los **métodos instrumentales** se basan en el empleo de diversas técnicas como polarimetría, refractometría, densitometría, resonancia magnética nuclear (NMR), etc. aunque son las técnicas de separación (cromatográficas y electroforéticas) las más utilizadas por sus ventajas en cuanto a resolución, selectividad y sensibilidad (Sanz y col., 2009). De entre ellas, la cromatografía de gases (GC) y de líquidos (LC) son las más empleadas y se detallarán a continuación. Por su parte la espectrometría de masas (MS) puede ser usada bien directamente o acoplada a estas técnicas de separación para el análisis de prebióticos.

La **tabla 1.2** muestra un resumen de las técnicas analíticas más comunes en el análisis de oligosacáridos prebióticos y algunos ejemplos recientes de sus aplicaciones.

1.2.4.1. Cromatografía de gases

La cromatografía de gases (GC) ha sido ampliamente usada en el análisis de carbohidratos, tanto con fines cuantitativos como cualitativos. El alto poder de resolución, sensibilidad y selectividad de la GC la convierten en una técnica versátil para el análisis de carbohidratos prebióticos. Su acoplamiento a la espectrometría de masas porporciona además información adicional sobre su estructura química (Sanz y col., 2009).

Un requisito imprescindible para el análisis por GC-MS de carbohidratos prebióticos es su derivatización previa, debido a su baja volatilidad producida por la

presencia de múltiples grupos polares (grupos hidroxilo). En este proceso los hidrógenos activos de estos grupos se substituyen por otros grupos más apolares.

Existen numerosos procedimientos de derivatización para el análisis de carbohidratos por GC, siendo la obtención de trimetilsilil éteres uno de los más comunes y que presenta mayores ventajas en cuanto a volatilidad y estabilidad de los compuestos formados. Sin embargo, con estos derivados se pueden obtener hasta un máximo de cinco picos por cada carbohidrato reductor correspondientes a las distintas formas anoméricas del carbohidrato (α y β piranosa, α y β furanosa y cadena abierta), lo cual puede suponer una desventaja al poder existir problemas de coeluciones. Una alternativa para resolver este problema es la oximación del carbono anomérico previo a la sililación, obteniéndose así trimetilsilil-oximas (TMSO) en dos sencillos pasos de derivatización y reduciéndose a dos el número de picos cromatográficos para cada carbohidrato reductor correspondientes a las formas *syn*- (*E*) y *anti*- (*Z*). Los carbohidratos no reductores son convertidos a sus TMS éteres preservando el anillo hemiacetálico y dando lugar a un solo pico cromatográfico (Ruiz-Matute y col., 2011).

La GC normalmente se emplea para el análisis de mono-, di- y trisacáridos mediante columnas generalmente de polimetildisiloxano, con dimensiones que oscilan entre 10-50 m longitud, 0,1-0,5 mm de diámetro y 0,02-2 µm de fase y con un rango de temperaturas que varía entre 60 ºC y 330 ºC. Sin embargo, el empleo de columnas con grupos carborano presentes en la estructura principal del polisiloxano, incluso fabricadas en capilares de sílice recubiertos de aluminio en lugar de poliimida, resistentes a temperaturas elevadas, ha permitido extender su aplicación hasta oligosacáridos de grado de polimerización de 7 (Montilla y col., 2006).

El detector más usado en GC es el de ionización de llama (FID), que posee una alta sensibilidad. Sin embargo, el empleo de patrones para la identificación de los carbohidratos es imprescindible. Como se ha comentado anteriormente, el acoplamiento a **MS** convierte a la GC en una técnica aún más versátil que facilita la caracterización de los compuestos. La MS está basada en la producción de iones que, posteriormente, son separados o filtrados según su relación masa/carga (*m/z*) y detectados.

Tabla 1.2. Técnicas y ejemplos de aplicaciones para el análisis de carbohidratos prebióticos.

Técnica analítica	Analito	Condiciones de análisis	Finalidad	Referencia
Cromatografía en capa fina de alta resolución	Oligosacáridos procedentes de leche materna humana (HMOs)	Eluyente butanol: ácido acético: agua (2,5:1:1 v/v) y como sistema de visualización 0,1% de Orcinol en ácido sulfúrico	Detectar fracciones de HMOs, purificados con diferentes técnicas cromatográficas	Kunz y col. (1996)
Cromatografía de gases (GC)	GOS sintetizados a partir de lactosa	Columna: Metilsilicona. Derivados: TMSO Programa de temperatura: 200ºC durante 15 min, aumento a 15ºC /min a 280 ºC, aumento a 1ºC/min a 290 ºC, aumento a 15ºC/min hasta 300 ºC y mantenida 35 min. Detección: MS	Identificar, usando tiempos de retención y espectros de masas, la estructura de los GOS (DP 2-3) presentes en dos muestras obtenidas con enzimas de diferente origen biológico	Cardelle-Coba y col. (2009)
Cromatografía de líquidos (LC)	GOS comerciales sintetizados a partir de lactosa	Columna: ZIC-HILIC Eluyentes: **A**:MeOH **B**: 5% NH_4OAc en H_2O Método: 95% **A** hasta 50% de fase móvil **A** en 40 min. Detección: ESI-MS	Analizar fracciones de diferentes DP provenientes de los GOS	Sinclair y col. (2009)
Electroforesis capilar	GOS comerciales sintetizados a partir de lactosa	Capilar cubierto con polivinil-alcohol (50 µm x 50,2 cm). Derivatizado con ácido 6-pireno-sulfónico y cianoborhidruro de sodio. Detección por fluorescencia inducida por láser	Cuantificar GOS	Albrecht y col. (2010)
Resonancia Magnética Nuclear (NMR)	Galactooligosacáridos sintetizados a partir de lactulosa	293K disueltas en agua deuterada y una sonda fría de HCN de 5mm. ^1H.	Identificar la estructura de trisacáridos purificados	Martinez-Villaluenga y col. (2008)
Espectrometría de masas (MS)	FOS e Inulina	Ionización: desorción/ionización láser asistida por matriz. Detección: espectrometría de resonancia ciclotrónica de iones (MALDI-FT-ICR-MS)	Cuantificar y caracterizar cualitativamente FOS e Inulina remanente tras fermentación con bifidobacterias	Seipert y col. (2008)

Dependiendo de la fuente de ionización empleada se consigue una mayor o menor fragmentación de la molécula, pudiéndose determinar el peso molecular del compuesto cuando la ionización es suave (ionización química) o proporcionando información sobre la estructura (impacto electrónico). La información estructural proporcionada por el sistema de MS también depende del analizador empleado, permitiendo en algunos casos, como en el de las trampas iónicas, la obtención de fragmentos sucesivos a partir de una misma molécula (MS^n). Sin embargo, en el caso de los carbohidratos, su similitud estructural hace que su caracterización por MS sea compleja, obteniéndose patrones de fragmentación con características muy similares para distintos compuestos.

1.2.4.2. Cromatografía de líquidos

La cromatografía de líquidos (LC) es la técnica más usada para la caracterización de carbohidratos y en especial de oligosacáridos prebióticos, por su alta versatilidad y múltiples modos de operación.

Para el fraccionamiento de dichos oligosacáridos se emplean columnas abiertas de intercambio iónico, carbón activo y exclusión molecular, como se verá en la sección 4.1.1.1 de esta memoria, sobre todo con fines preparativos o previos a su análisis. Por otro lado, existen diversos modos de operación de la cromatografía de líquidos de alta resolución (HPLC) empleadas para la caracterización de oligosacáridos.

La **cromatografía de intercambio aniónico de alta eficacia (HPAEC)** es una de las técnicas más difundidas para la caracterización de carbohidratos (Avila-Fernandez y col., 2011, Blanch y col., 2011, Borromei y col., 2010, Coulier y col., 2009). Este tipo de cromatografía se lleva a cabo a elevados valores de pH, a los que los carbohidratos son ionizados para ser separados en resinas de intercambio aniónico. Sin embargo, esta técnica presenta dos grandes desventajas: (i) un comportamiento de elución de los compuestos, relacionada con la estructura de los carbohidratos, lo cual hace imprescindible el uso de patrones para la posible identificación de dicho compuesto y (ii) su difícil acoplamiento a la espectrometría de masas debido a la presencia de altos

contenidos de sales, como el acetato de sodio, usado como modificador en la fase móvil, o de altos contenidos de hidróxido de sodio.

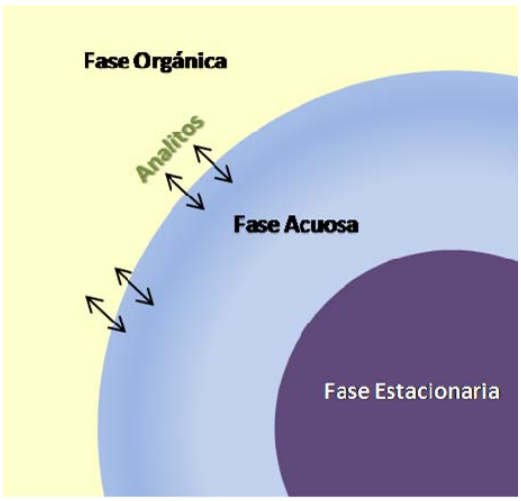
No obstante, existen otros modos de operación que se usan para el análisis de carbohidratos prebióticos como:

i) **Cromatografía de fase inversa (RPLC)**: se emplean columnas alquil-enlazadas con silica, siendo las más comunes las C18. En este tipo de cromatografía el mecanismo de retención viene gobernado por interacciones hidrofóbicas de los analitos con la fase estacionaria de la columna. Dichas interacciones dependerán de la naturaleza de los analitos y de las fases móviles a usar. En el caso de carbohidratos, el agua es el eluyente más usado, pero la aplicabilidad de este modo de operación es muy limitada debido a la poca retención de estos compuestos. Sólo FOS son separados con una resolución aceptable (Brokl y col., 2011, El Rassi, 2002).

ii) **Columnas de carbón grafitizado (GCC)**: su mecanismo de separación no es del todo conocido aunque se sabe que tienen lugar diversas interacciones, tales como: repulsión hidrofóbica entre eluyente y analito, interacción de los grupos funcionales polarizados del analito con el grafito, etc. Una de las ventajas de este tipo de cromatografía es su capacidad de separar isómeros en presencia de modificadores básicos, como el hidróxido de amonio, así como la estabilidad de la fase estacionaria en todo el rango de pH y el uso de fases móviles totalmente compatibles con MS (Koizumi, 2002). Recientemente, se han empleado columnas de carbón grafitizado para la separación de carbohidratos prebióticos, resultando ser adecuadas para la separación de isómeros, aunque se observaron coeluciones entre oligosacáridos de distinto grado de polimerización dando problemas en el análisis de mezclas complejas (Brokl y col., 2011).

iii) **Columnas de interacción hidrofílica (HILIC)**: su mecanismo de retención aún sigue siendo motivo de debate, aunque, principalmente, se basa en la partición del analito entre una fase acuosa, inmovilizada parcialmente en la fase estacionaria, y el eluyente orgánico (**Figura 3**) (Alpert, 1990, Hemström y col.,

2006, Jandera, 2011). En 1990, Alpert le dio nombre a la cromatografía de interacción hidrofílica para así distinguirla de la cromatografía líquida en fase normal (NPLC), convirtiéndose, en la última década, en uno de los modos de cromatografía más usuales para separar compuestos altamente polares (Ikegami y col., 2008, Jandera, 2011). Algunos autores denominan la HILIC como una fase normal donde se usan composiciones de fases móviles típicas de RPLC. En la actualidad existe una alta variedad de columnas con fases polares y modo de operación HILIC. Aunque todas cumplen con el mecanismo de partición antes mencionado, 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 (Hemström y col., 2006) (**Figura 4**). Existen algunas aplicaciones para la separación de carbohidratos con distintas fases estacionarias (BEH amida (Brokl y col., 2011); poliacrilamida modificada (Ikegami y col., 2008); zwiteriónica (Sinclair y col., 2009)), sin embargo, la comparación entre ellas o con otras nuevas es todavía un estudio pendiente de realizar, que puede resultar de gran utilidad para la caracterización de mezclas tan complejas como los carbohidratos prebióticos.



.**Figura 1.3**. Esquema de partición propuesto para la separación en HILIC.

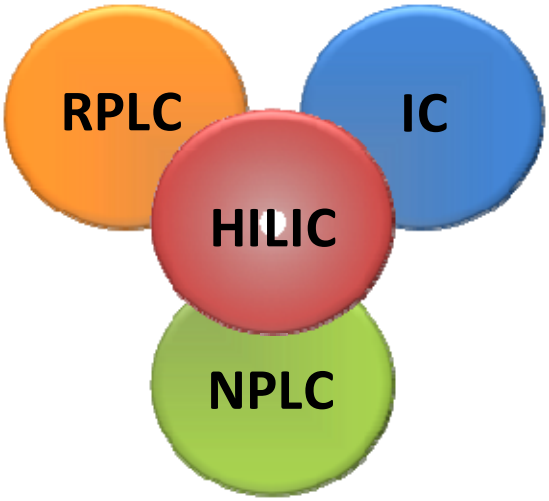


Figura 1.4. Representación esquemática de la cromatografía de interacción hidrofílica (HILIC) con otros mecanismos de retención. (RPLC) cromatografía líquida en fase inversa, (NPLC) cromatografía líquida en fase normal, (IC) intercambio iónico. Adaptado de Sequant-Merck.

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 solo se puede emplear cuando el modo de elución es en isocrático. Los detectores de ultravioleta y fluorométrico son también ampliamente utilizados, pero en estos casos se debe llevar a cabo una derivatización previa debido a la ausencia de grupos cromóforos o fluoróforos en los carbohidratos. 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 del orden de picomoles (Sanz y col., 2009).

Actualmente, el acoplamiento de espectrómetros de masas a equipos de HPLC se usa ampliamente para el análisis de carbohidratos ya que, como se ha comentado en el caso de GC, proporciona una información estructural adicional. Uno de los requisitos principales para llevar a cabo dicho acoplamiento es el uso de disolventes volátiles y agua, adecuados para ser introducidos en el espectrómetro. En el caso de la RPLC, los eluyentes utilizados son totalmente compatibles con MS, con la precaución de no usar ciertos tipos de ácidos, como el ácido trifluoroacético, para evitar cualquier tipo de supresión iónica. Esto también es aplicable a HILIC, ya que usa los mismos

eluyentes que RPLC pero con la ventaja adicional de que pueden emplearse fases móviles con una elevada concentración de solvente orgánico proporcionando así una mayor respuesta en MS.

La fuente de ionización más común para el análisis de carbohidratos, en general, es la electronebulización (ESI), aunque también se emplea la ionización química a presión atmosférica (APCI), que se pueden acoplar a numerosos analizadores, como cuadrupolos (Q), trampas iónicas (IT), tiempo de vuelo (TOF), y variantes de los mismos como el triple cuadrupolo (QqQ), o un cuadrupolo acoplado a tiempo de vuelo (Q-TOF), entre otros.

Dependiendo del analizador utilizado, se puede aplicar la espectrometría de masas MS/MS o tándem MS, basada en la fragmentación de un ión precursor, en iones más pequeños, y acompañada por la pérdida de un fragmento neutro. Generalmente, los iones precursores y productos son separados en el tiempo si se realizan diferentes etapas secuenciales en el mismo analizador (i.e. IT) o separados en el espacio usando analizadores conectados en línea (i.e. QqQ y Q-TOF). Sin embargo, en el caso de la trampa iónica existe una ventaja adicional debido a la capacidad de este analizador para realizar MS^n. En este sentido, la espectrometría de masas en tándem representa una alternativa ventajosa en el análisis estructural de oligosacáridos como los prebióticos. Zhang y col. (2008) establecieron pautas para la identificación del enlace glicosídico y de la composición monomérica de algunos disacáridos a partir del patrón de fragmentación de cada uno de ellos y, en consecuencia, de su espectro de masas característico. Sin embargo, es necesario profundizar más en este estudio con distintos carbohidratos de estructura conocida para su aplicación a la caracterización de oligosacáridos desconocidos.

1.3. Péptidos

1.3.1. Modificaciones post-traduccionales.

La glicosilación enzimática es el proceso de la unión covalente de carbohidratos a péptidos o proteínas. Es una de las modificaciones post-traduccionales más comunes que ocurren en todos los sistemas vivos, y está relacionada con múltiples funciones biológicas de gran importancia, como por ejemplo, inmunomoduladoras (anticuerpos) u hormonales (hormona estimulante de la glándula tiroidea), entre muchas otras. Asimismo, puede jugar un papel importante en la conformación estructural de la glicoproteína. Los carbohidratos enlazados covalentemente a la secuencia aminoacídica son numerosos y variados, pudiéndose encontrar mono-, di- y oligosacáridos de distinta longitud y grado de ramificación. Estos carbohidratos se sintetizan en el aparato de Golgi y se unen covalentemente a la proteína en el retículo endoplasmático (Nelson y col., 2008).

Principalmente, existen dos tipos de glicoproteínas: *O*-glicoproteínas y *N*-glicoproteínas. Si el carbohidrato se enlaza a través de su carbono anomérico, vía enlace glucosídico, al grupo hidroxilo de los residuos de serina o treonina, se obtienen las llamadas *O*-glicoproteínas. Por otro lado, si el carbohidrato se une a través de un enlace *N*-glicosil al nitrógeno de la amida del residuo de asparagina resulta en las llamadas *N*-glicoproteínas (**Figura 1.5**). El grado de glicosilación es muy variable y puede constituir entre el 1% y el 70% de la masa de la glicoproteína (Nelson y col., 2008, Vance y col., 1997).

Otra modificación post-traduccional común en péptidos y proteínas es la fosforilación, que consiste en la unión de un grupo fosfato a residuos de serina, treonina y tirosina, en el caso de los organismos eucariotas (**Figura 1.6**). Dicha modificación participa en múltiples funciones biológicas en la naturaleza, incluyendo: metabolismo, regulación traduccional y de transcripción, degradación de proteínas, homeostasis, señalización celular, diferenciación; supervivencia celular, entre otras (Boersema y col., 2009, Reinders y col., 2005).

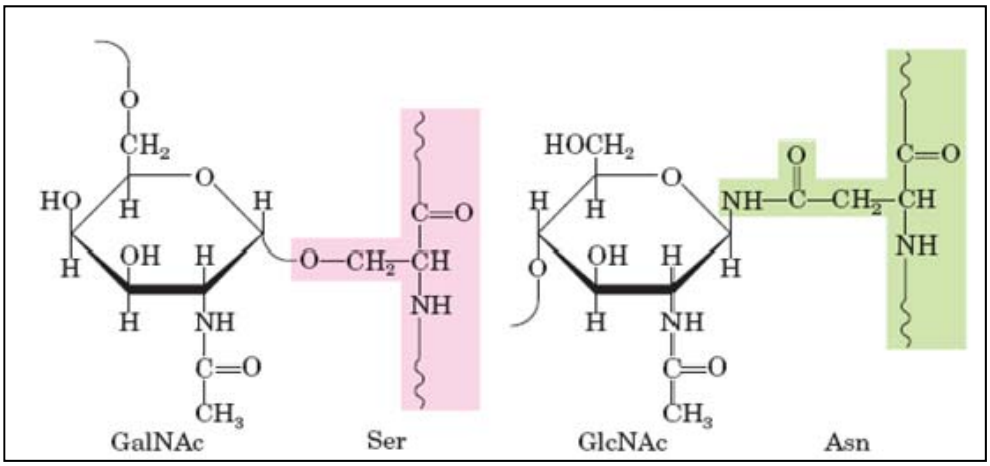


Figura 1.5. Tipos de glicosilación enzimática de proteínas. Adaptado de Cox y col. (2010).

La fosforilación se caracteriza por ser una modificación reversible, en consecuencia la actividad de la proteína puede ser eficazmente controlada por el proceso de fosforilación/desfosforilación. Asimismo, dependiendo en el residuo de aminoácido que ocurra le confiere a una misma secuencia proteica diferentes cambios conformacionales y posiblemente diferentes actividades biológicas (Thingholm y col., 2009).

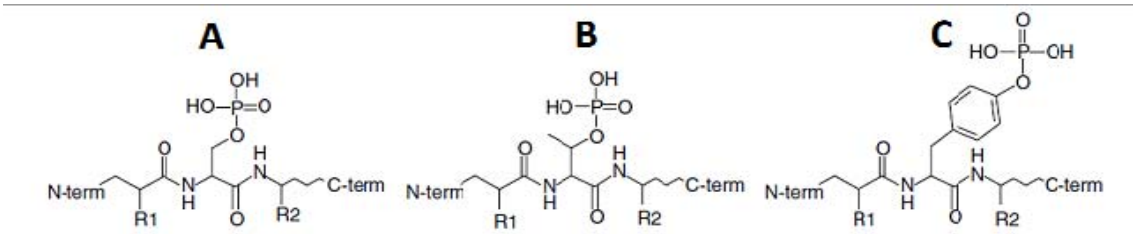


Figura 1.6. Estructura química de (A) fosfoserina, (B) fosfotreonina y (C) fosfotirosina. Adaptado de Boersema y col. (2009).

1.3.2. Caseinmacropéptido (CMP)

1.3.2.1. Definición y estructura

El caseinmacropéptido (CMP) es el fragmento C-terminal liberado después de la acción proteolítica de la quimosina sobre la κ-caseína durante las etapas iniciales en la fabricación de quesos, o por la acción de la pepsina durante el proceso de digestión gástrica. La κ-caseína se hidroliza por el enlace ^{105}F-M^{106}, formándose dos polipéptidos muy diferentes: la para-κ-caseína (residuos 1-105), ligeramente catiónica a pH 6,6, hidrofóbica y poco soluble y el CMP (residuos 106-169), fuertemente polar por lo que difunde hacia la fase acuosa, eliminándose durante el desuerado con el suero de quesería (Delfour y col., 1965).

El CMP es un grupo heterogéneo de péptidos debido a que contiene todas las modificaciones post-traduccionales (fosforilación y glicosilación) y algunas variantes genéticas de la κ-caseína de partida. Por ello, la heterogeneidad del CMP, debida al polimorfismo genético de la κ-caseína, causa variaciones en la secuencia de aminoácidos, y afecta al número, localización y contenido de residuos glicosídicos y grupos fosfato unidos a la cadena peptídica (Abd-El-Salam y col., 1996). Por tanto, se entiende por CMP como una mezcla compleja de macropéptidos glicosilados y no glicosilados, con distintos grados de fosforilación. Esta heterogeneidad afectará tanto a las propiedades del CMP como a los péptidos derivados de él por hidrólisis enzimática u otro tipo de modificaciones.

En el caso del CMP bovino, cerca del 60% está representado por las formas glicosiladas y es conocido comúnmente como glicomacropéptido (Vreeman y col., 1986). En la **tabla 1.3** se recoge a modo de resumen las variantes genéticas con las sustituciones de aminoácidos que las caracterizan, así como los lugares de glicosilación y fosforilación que contribuyen a la heterogeneidad del CMP bovino.

Se han identificado cinco carbohidratos diferentes de tipo-mucina formadas por residuos de galactosa (Gal), *N*-acetil-galactosamina (GalNAc) y ácido siálico, concretamente *N*-acetil neuramínico (Neu5Ac), (Saito y col., 1992). Dichos carbohidratos son: GalNAc-O-R; Gal-β(1→3)-GalNAc-O-R; Neu5Ac-α(2→3)-Gal-

β(1→3)-GalNAc-O-R; Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc-O-R; Neu5Ac-α(2→3)-Gal-β(1→3)(Neu5Ac-α(2→6))-GalNAc-O-R. Estas estructuras glicosídicas se encuentran unidas por enlaces *O*-glicosídicos, entre la GalNAc, presente en todos estos carbohidratos, y los residuos de treonina (**Figura 1.7**). Actualmente, hay descritos 6 lugares diferentes de glicosilación en el CMP, T^{121}, T^{131}, T^{133}, T^{136}, T^{142}, T^{165} (Holland y col., 2004, Pisano y col., 1994, Takeuchi y col., 1985) y tres lugares de fosforilación, las serinas S^{127}, S^{149} (Mercier, 1981) y, más recientemente, T^{145} (Holland y col., 2006) (**Tabla 1.3**).

1.3.2.2. Propiedades

El CMP presenta múltiples funcionalidades biológicas, como han puesto de manifiesto distintos trabajos de revisión publicados durante los últimos 15 años (Abd-El-Salam y col., 1996, Brody, 2000, Dziuba y col., 1996, Manso y col., 2004, Thomae-Worringer y col., 2006). Diversos estudios que han abordado la relación estructura-actividad del CMP han determinado la importancia de ciertos aspectos estructurales sobre la función biológica ejercida. Particularmente, las bioactividades basadas en las interacciones con componentes celulares están estrechamente relacionadas con el contenido y estructura de los oligosacáridos, especialmente con los residuos de ácido siálico, mientras que otras actividades que pueden ser ejercidas por pequeños péptidos contenidos en la cadena aminoacídica dependen exclusivamente de la estructura primaria (Moreno y col., 2009). De este modo, se ha descrito la capacidad del CMP para interaccionar con toxinas, virus y bacterias, ejerciendo una serie de actividades beneficiosas para la salud mediadas fundamentalmente por la fracción glicosídica. Esto es debido a que un gran número de patógenos y enterotoxinas pueden adherirse a ciertas células tras ser reconocidos por receptores compuestos por oligosacáridos (Dziuba y col., 1996). Asimismo, el CMP glicosilado humano y bovino puede fomentar el crecimiento de bacterias del género *Bifidobacterium* en cultivos puros de *B. breve*, *B. bifidum*, y *B. infantis* (Azuma y col., 1984, Idota y col., 1994, Metwally y col., 2001). La administración de leche con un 2% de CMP produjo un aumento *in vitro* de *B. lactis* en comparación con leches no enriquecidas en CMP (Janer

y col., 2004). La ingesta de CMP como fuente de ácido siálico resultó en un incremento del contenido de este carbohidrato en la saliva de lechones, afectando a su viscosidad y capacidad de protección frente a microorganismos (Wang y col., 2007). Así, se ha confirmado que el CMP inhibe la adhesión de bacterias cariogénicas como *Streptococcus sanguis*, *Streptococcus mutans* y *Streptococcus sobrinus* a la cavidad oral (Neeser y col., 1988, Neeser y col., 1994, Vacca Smith y col., 2000) y de modular la composición de la microbiota de la placa dental (Guggenheim y col., 1999, Schupbach y col., 1996).

Por otra parte, al CMP también se le han atribuido propiedades inmunomoduladoras (Otani y col., 1996, Otani y col., 1995, Otani y col., 2005), así como anti-inflamatorias (Daddaoua y col., 2005), anti-trombóticas (Jollès y col., 1986, Léonil y col., 1990) y anti-hipertensivas (Manso y col., 2003, Miguel y col., 2007). Además, debido a su composición en aminoácidos, el CMP puede ser incluido en dietas especiales. El CMP no contiene aminoácidos aromáticos (F, W, Y) por lo que resulta adecuada su inclusión en alimentos para personas que padezcan fenilcetonuria (Nielsen y col., 1994, Smithers y col., 1991). Igualmente, su relativamente alto porcentaje de aminoácidos de cadena ramificada (V e I) es de interés en dietas para personas con alteraciones hepáticas, donde los aminoácidos ramificados son utilizados como fuente de energía (Abd-El-Salam y col., 1996).

Al CMP no sólo se le han atribuido propiedades biológicas, sino también algunas propiedades funcionales de tipo tecnológico, como una alta solubilidad en soluciones acuosas, y termoestabilidad, además de una alta capacidad emulsionante (Martín-Diana y col., 2005), que contribuyen al interés de su uso como ingrediente funcional por la industria alimentaria.

1.3.3. Análisis de glico- y fosfopéptidos

Hasta el momento se han empleado un gran número de técnicas y estrategias analíticas para el análisis de glico- y fosfopéptidos. La variada complejidad de este tipo de estructuras hace que, dependiendo del objetivo que se plantee, se puedan usar unas u otras. La espectrometría de masas (MS) se ha convertido, actualmente, en la técnica más usada para la identificación y caracterización de péptidos en general. En múltiples ocasiones, es necesario el enriquecimiento y/o separación previa de los compuestos que se deseen analizar (Wuhrer y col., 2007), siendo la técnica de separación más empleada la cromatografía líquida de alta resolución (HPLC) en diferentes modalidades como: la cromatografía de afinidad usando lectinas como fase estacionaria (Wang y col., 2006), exclusión molecular (Alvarez-Manilla y col., 2006), cromatografía de interacción hidrofílica (Takegawa y col., 2008), uso de columnas de carbón grafitizado (Larsen y col., 2005), sistemas en fase normal (Gonzalez de Paredo y col.) y en fase inversa (Satomi y col., 2004), etc.

Otra técnica empleada actualmente en el análisis de péptidos es la electroforesis capilar acoplada a MS. Numerosos trabajos han investigado la presencia de péptidos y proteínas en múltiples muestras biológicas como, por ejemplo, orina (Zamfir y col., 2001), muestras de antitrombina (Amon y col., 2006) al igual que en muestras de interés alimentario, como la soja (Garcia-Ruiz y col., 2007, Saz y col., 2007) usando dicha técnica.

1.3.3.1. *Cromatografía de líquidos*

- *Cromatografía líquida de fase inversa (RPLC).*

La RPLC es una de las técnicas analíticas de mayor uso para el análisis de péptidos procedentes, en su mayoría, de digeridos enzimáticos de proteínas. En líneas generales, si se desea hacer un análisis exhaustivo de la muestra, la cromatografía de fase inversa es acoplada a espectrómetros de masas, aunque en algunos casos, pueden usarse detectores simples de espectrofotometría.

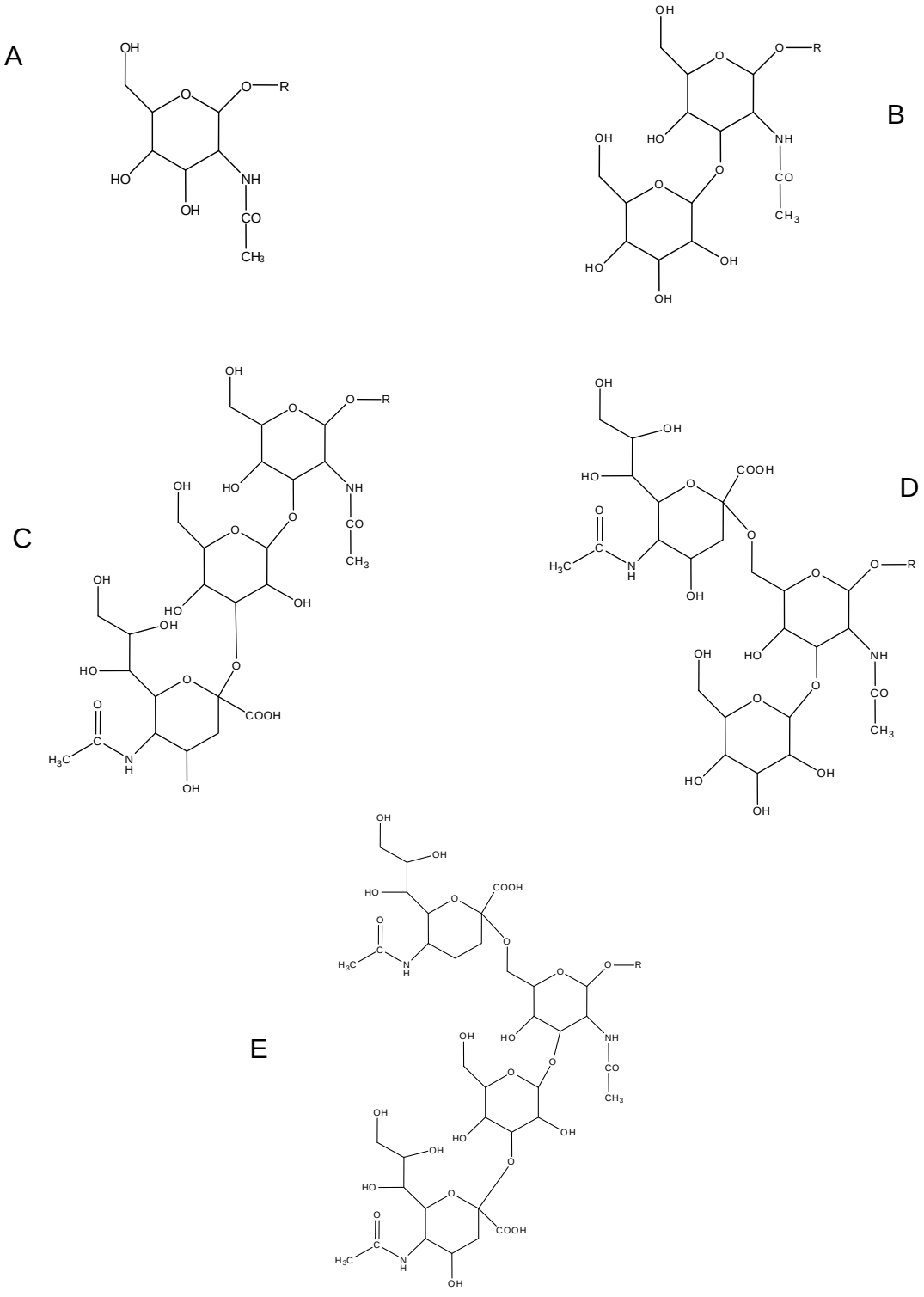


Figura 1.7. Estructuras de los *O*-glicanos identificados en el caseinmacropéptido bovino. A) GalNAc-O-R, B) Gal-β(1→3)-GalNAc-O-R, C) Neu5Ac-α(2→3)-Gal-β(1→3)-GalNAc-O-R, D) Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc-O-R, E) Neu5Ac-α(2→3)-Gal-β(1→3)(Neu5Ac-α(2→6))-GalNAc-O-R.

Tabla 1.3. Composición aminoacídica del CMP bovino, variantes genéticas y modificaciones post-traduccionales implicadas en su heterogeneidad. La nomenclatura está basada en la secuencia de aminoácidos de la κ-caseína bovina. Tomada de Moreno y López-Fandiño (2009).

Estructura Primaria (variante A)	**Polimorfismo genético**	**Lugares de glicosilación**	**Lugares de fosforilación**
κ-CN f(106-169) Met-Ala-Ile-Pro-Pro-Lys-Lys-Asn-Gln-Asp-Lys-Thr-Glu-Ile-Pro-Thr-Ile-Asn-Thr-Ile-Ala-Ser-Gly-Glu-Pro-Thr-Ser-Thr-Pro-Thr-Thr-Glu-Ala-Val-Glu-Ser-Thr-Val-Ala-Thr-Leu-Glu-Asp-Ser-Pro-Glu-Val-Ile-Glu-Ser-Pro-Pro-Glu-Ile-Asn-Thr-Val-Gln-Val-Thr-Ser-Thr-Ala-Val	A/F) T^{135} T^{136} D^{148} I^{153} S^{155} B/C=D) T^{135} I^{136} A^{148} I^{153} S^{155} B2) T^{135} I^{136} A^{148} T^{153} S^{155} E) T^{135} T^{136} D^{148} I^{153} G^{155} G/H) I^{135} T^{136} D^{148} I^{153} S^{155}	T^{121} T^{131} T^{133} T^{136} T^{142} T^{165}	S^{127} S^{149} T^{145}
Mercier y col. (1973)	Alexander y col. (1988) Coolbear y col. (1996) Prinzenberg y col. (1999)	Claverol y col. (2003) Holland y col. (2004) Minkiewicz y col. (1996) Mollé y Léonil (1995) Pisano y col. (1994) Vreeman y col. (1986)	Holland y col. (2006) Claverol y col. (2003) Holland y col. (2004) Mercier (1981) Minkiewicz y col. (1996) Mollé y Léonil (1995) Talbo y col. (2001)

En el caso de los glicopéptidos, la polaridad no solo vendrá dada por la estructura polar del glicano, sino también por la secuencia de aminoácidos (Dalpathado y col., 2008). Como fase móvil se suelen emplear gradientes binarios de agua y acetonitrilo con ácido trifluoroacético o fórmico como modificadores orgánicos, aumentado la concentración del acetonitrilo a medida que avanza el gradiente. Bajo estas condiciones, los glicopéptidos eluirán antes que los péptidos sin glicosilar, siempre que posean la misma secuencia de aminoácidos, debido a su mayor carácter hidrofílico (Wuhrer y col., 2005).

Existen numerosos trabajos que utilizan la RP para caracterizar glicopéptidos. La fase estacionaria más empleada para caracterizar tanto glicoformas intactas como péptidos derivados de la hidrólisis del CMP ha sido la C18. De este modo, Mollé y Léonil (1995)(Molle y col., 1995) emplearon tres columnas C18, de diferentes casas comerciales, acopladas a espectrometría de masas para caracterizar 14 formas glicosiladas distintas de CMP de vaca, variante genética A, sin hidrolizar. Igualmente, Moreno y col. (2000, 2001) emplearon una C18 acoplada a espectrometría de masas para caracterizar hasta 8 y 10 formas glicosiladas intactas de CMP ovino y caprino, respectivamente. Manso y López-Fandiño (2003) emplearon columnas C18 para el análisis de péptidos sin glicosilar procedentes del CMP bovino, ovino y caprino tras tripsinólisis, mientras que Boutrou y col. (2008) utilizaron una C18 para evaluar el efecto de la glicosilación del CMP sobre el grado de proteólisis de enzimas gastrointestinales, identificando un número muy reducido de glicopéptidos.

Otros trabajos han descrito el fraccionamiento de glicopéptidos usando cromatografía en modo mixto basada en la utilización de diferentes sorbentes como fase estacionaria. Gilar y col. (2008) usaron un sorbente de sílice funcionalizado, con grupos de pentafluorofenil, obteniendo una columna con las propiedades de la fase inversa pero con una relativa capacidad de intercambio catiónico (SCX). La combinación de diferentes solventes, los idóneos para RP como para SCX, convierte a este tipo de separación en una cromatografía pseudo-bidimensional, obteniendo una buena separación entre los glicopéptidos negativamente cargados (como los sialilados) y aquellos neutros o sin glicosilar.

También se ha descrito el uso de cromatografía en dos dimensiones para la purificación de glicopéptidos. Geng y col. (2000) usaron una columna de afinidad acoplada a una segunda de fase inversa obteniendo una suficiente resolución para caracterizar, por medio de la espectrometría de masas, digeridos trípticos de muestras biológicas complejas, como puede ser el suero humano.

El uso de RPLC también ha sido aplicado al análisis de fosfopéptidos procedentes de diferentes matrices biológicas. En este sentido, Iwase y col. (2010) usaron RPLC para el análisis de los fosfopéptidos presentes en hidrolizados trípticos de proteínas complejas. Este mismo modo de operación ha sido empleado para el análisis bidimensional de fosfopéptidos, usando en ambas dimensiones, columnas de fase inversa (Song y col., 2010).

- *Cromatografía líquida de Interacción Hidrofílica (HILIC)*

La retención de compuestos polares puede suponer un problema cuando se usan técnicas como la RPLC, como se ha comentado para el caso de los carbohidratos. De hecho, en los trabajos descritos en la sección anterior referentes a la identificación de péptidos derivados de hidrólisis del CMP, la identificación de glicopéptidos fue limitada. Una de las alternativas a dicha técnica es la cromatografía de líquidos en fase normal (NPLC). Sin embargo, en algunas ocasiones, los compuestos polares son altamente retenidos en estas fases estacionarias o no son suficientemente solubles en las fases móviles típicas en NPLC. Por otra parte, también se han descrito problemas de reproducibilidad debidos principalmente a la sensibilidad de la técnica y a la presencia de pequeñas concentraciones de contaminantes polares en la fase móvil (Dalpathado y col., 2008). En este sentido, la HILIC podría resolver estos inconvenientes, debido a que usa conjuntamente soluciones acuosas y orgánicas como fases móviles y columnas que proporcionan una mejora de la reproducibilidad (Jandera, 2008).

Dentro del campo de la peptidómica, el empleo de HILIC para el análisis de péptidos ha cobrado gran importancia en la última década, siendo considerables los trabajos en los que se emplea dicha técnica (Yoshida, 2004). Existen, por tanto, varios

estudios que se han enfocado a la caracterización de *N*-glicopéptidos mediante HILIC (Takegawa y col., 2008, Takegawa y col., 2006), sin embargo, existen muy pocos trabajos enfocados al análisis de *O*-glicopéptidos. Recientemente, Takegawa y col. (2008) han descrito el uso de una fase zwiteriónica para la caracterización de *N*- y *O*-glicopéptidos procedentes de la hidrólisis de eritropoyetina recombinante, logrando identificar 113 glicopéptidos, aunque únicamente 8 de ellos fueron *O*-glicopéptidos. Estos autores observaron que los péptidos *N*-glicosilados, independientemente del enlace péptido-glicano, presentaron una mayor retención que los *O*-glicopéptidos, y éstos a su vez tuvieron una mayor retención que los péptidos sin glicosilar, indicando que la fase zwiteriónica podría ser adecuada para purificar y separar los glicopéptidos de los péptidos sin glicosilar. Según estos resultados y los revisados por Wuhrer y col. (2005), tanto HILIC como RP pueden emplearse de forma ortogonal. En este sentido, Boersema y col. (2007) acoplaron una columna zwiteriónica como primera dimensión, y otra de RP como segunda dimensión. El empleo combinado de ambas columnas permitió identificar alrededor de 1000 proteínas procedentes de lisados celulares mediante técnicas proteómicas.

Aunque mucho menos extendido que el análisis de glicopéptidos, HILIC también ha sido utilizada para el análisis de fosfopéptidos, en este sentido, McNulty y col. (2008) describen el uso de este modo de operación en el análisis de digeridos con tripsina de diferentes proteínas, concluyendo que la presencia del grupo fosfato influye en un aumento de la retención en HILIC debido a su alto carácter hidrofílico.

- *Acoplamientos a la Espectrometría de Masas (MS)*

Actualmente la MS juega un papel principal dentro del área de la proteómica y la peptidómica, sobre todo después de la aparición de la ionización por electronebulización (ESI) y la desorción/ionización láser asistida por matriz (MALDI). Generalmente, el análisis de péptidos usando MS conlleva una etapa de separación, como las anteriormente mencionadas, aunque algunas veces se pueden analizar también directamente mezclas complejas (Careri y col., 2003, Wuhrer y col., 2007).

Sin embargo, a pesar de la gran utilidad de la MS, la identificación de glicopéptidos mediante esta técnica no es sencilla. En este sentido, la MS^n ha

demostrado ser una herramienta eficaz para la caracterización de glicopéptidos gracias a la información adicional obtenida sobre la fragmentación de la estructura glicosídica y/o peptídica (Wuhrer y col., 2007). No obstante, es necesario señalar que la interpretación de los espectros depende altamente del tipo de analizador y fragmentación empleados, debido a que la naturaleza de los iones generados varía según los tipos de espectrómetros de masas utilizados.

Así, dependiendo del analizador utilizado, y los objetivos planteados para el análisis de glicopéptidos, se pueden emplear diferentes mecanismos de fragmentación. Algunos trabajos describen la caracterización de glicopéptidos usando la disociación inducida por colisión (CID), promovida por diferentes analizadores como QqQ (Huddleston y col., 1993), IT (Amon y col., 2006), Q-TOF (Stimson y col., 1999), etc. La ventaja de estos sistemas es la capacidad de fragmentación controlada de los analitos, lo cual permiten su futura identificación por la pérdida de iones característicos (Dalpathado y col., 2008).

A parte de la CID, existen otro tipo de técnicas de fragmentación válidas, como la disociación por captura de electrones (ECD) o la disociación por transferencia de electrones (ETD) (Wuhrer y col., 2007).

Por otra parte, la caracterización completa de los *O*-glicopéptidos entraña dificultades adicionales en comparación con los *N*-glicopéptidos respecto a la identificación específica del residuo glicosilado. Esto es debido a que, a diferencia de los *N*-glicopéptidos, en las *O*-glicoproteínas no existe una secuencia aminoacídica consenso que determine el lugar de glicosilación. Esto implica que los *O*-glicanos pueden unirse a cualquier residuo de treonina o serina a lo largo de la secuencia peptídica. En consecuencia, es de esperar que con la introducción de nuevas técnicas cromatográficas, como HILIC, y el uso de la MS^n pueda realizarse una caracterización más exhaustiva de los *O*-glicopéptidos. Esta nueva información podría suponer un avance en el conocimiento de la relación estructura-actividad biológica de *O*-glicoproteínas de interés en el ámbito alimentario o biomédico.

En este mismo sentido, la fosforilación ha sido ampliamente estudiada usando MS, siendo los modos de ionización más usados el MALDI y ESI. Sin embargo, en

cuanto al uso de analizadores, para conocer el sitio de fosforilación es necesaria la aplicación de MS en tándem o en su defecto MS/MS, con el fin de localizar las pérdidas neutrales del grupo fosfato y de detectar los diferentes fragmentos derivados de la cadena peptídica, que permitan elucidar el lugar exacto de fosforilación (Dunn y col., 2009, Reinders y col., 2005).

1.4. Glicosilación (no-enzimática) de péptidos y proteínas vía Reacción de Maillard

1.4.1. Definición y etapas

La reacción de Maillard (RM) engloba una serie de reacciones complejas que aún no están definidas completamente (Fayle y col., 2002). Descrita por primera vez por Louis-Camille Maillard en 1912, los trabajos posteriores de Amadori (1931), Kuhn y Weygand (1937) y Heyns y col (1957) lograron describir las primeras fases de la reacción, pero no fue hasta 1953 cuando Hodge realizó, por primera vez, un esquema global de la reacción, con formación de productos a partir de las etapas iniciales de la misma (**Figura 1.8**), y donde puede observarse que la RM engloba una serie de reacciones complejas, que ocurren de forma secuencial y/o paralela. No obstante, y con el fin de simplificar, estas reacciones pueden ser clasificadas en tres grandes etapas: iniciales, avanzadas y finales, definidas a continuación (Silvan y col., 2006):

1. **Etapas iniciales**. Son las etapas donde ocurre la glicosilación no enzimática o glicación, mediante la unión covalente de un grupo primario amino libre, normalmente de un aminoácido, péptido o proteína con un grupo carbonilo, generalmente de un carbohidrato reductor. El producto formado sufre una deshidratación, lo que da lugar a la generación de una base de *Schiff* (**Figura 1.9**). Esta base de *Schiff* se transforma en una glicosilamina *N*-sustituida que a su vez sufre un reordenamiento molecular, denominado reordenamiento de Amadori (procedente de aldosas) o de Heyns (procedente de cetosas). El compuesto de Amadori (1-amino-1-desoxi-2-cetosa) o el compuesto de Heyns (2-amino-2-desoxi-aldosa) son los primeros compuestos estables de la reacción.

2. **Etapas avanzadas**. Estas etapas se caracterizan por la formación de compuestos dicarbonilos y AGEs (Productos avanzados de la glicación), a partir de la fragmentación de los azúcares, degradación de aminoácidos y del compuesto de Amadori o Heyns. Estos últimos, a pesar de ser estables, se degradan por intensos calentamientos o tiempos de almacenamiento prolongados, además de por las condiciones físicas del medio, como la actividad de agua. Entre los compuestos α-dicarbonilos, altamente reactivos y con importancia biológica se encuentran: glioxal, metilglioxal y 3-desoxiglucosona, entre otros. Son compuestos altamente tóxicos y precursores, previa reacción con cadenas de lisina y arginina presentes en péptidos y proteínas, de los denominados AGEs, también de alta importancia biológica (Silvan y col., 2006).

3. **Etapas finales**. Se caracterizan por la formación de compuestos coloreados de tipo polimérico denominados melanoidinas. Si bien se ha asumido que son compuestos de alto peso molecular, recientemente se ha descrito que fracciones de bajo peso molecular también se pueden incluir en esta etapa (Wang y col., 2011). Las melanoidinas se forman a través de ciclaciones, deshidrataciones, retroaldolización, rearreglos moleculares, isomerización y condensación de compuestos de bajo peso molecular procedentes de las fases iniciales y avanzadas de la RM, sin embargo, aún no se conoce una elucidación completa de estos compuestos, lo que representa uno de los campos más estudiados actualmente de la RM (Bekedam y col., 2008, Kim y col., 2009, Wang y col., 2011).

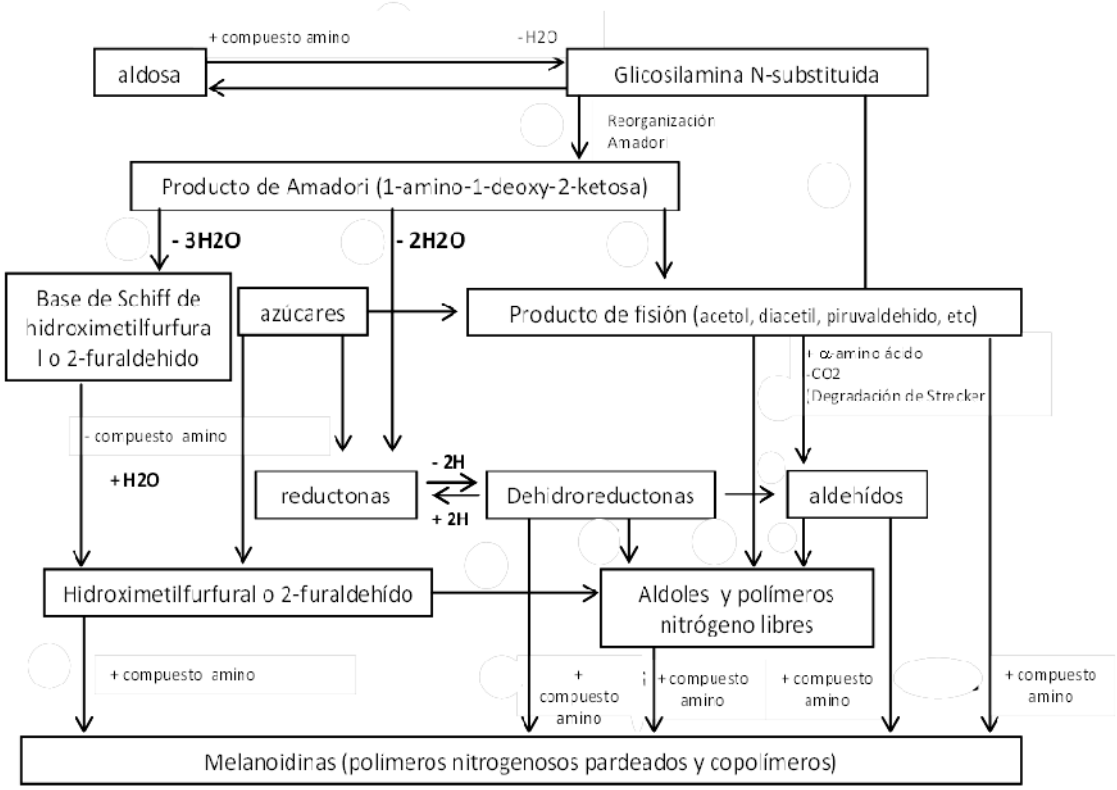


Figura 1.8. Esquema de la reacción de Maillard basado en el trabajo de Hodge (1953). Adaptado de Ames (1998).

1.4.2. Factores que afectan a la RM

La RM se puede emplear para mejorar las propiedades tecnológicas y biológicas de ciertos ingredientes alimentarios, sobre todo proteínas (Oliver y col., 2006). Sin embargo, existen una serie de factores que afectan a dicha reacción y que es necesario controlar, como: la actividad de agua (a_w), el pH, la temperatura y la concentración y estructura de los reactantes.

i) *Actividad de agua*: En líneas generales un aumento de la a_w produce un aumento en la cinética de la RM, hasta un máximo donde los reactantes son diluidos y dicha cinética disminuye (Pereyra Gonzales y col., 2010). Diversos trabajos describen que el intervalo de a_w donde la reacción se encuentra favorecida es entre 0,3 y 0,7 (Burvall y col., 1978, Desrosiers y col., 1989).

Sin embargo, diversos trabajos concluyen que una a_w de 0,44 es óptima para la obtención de un máximo rendimiento en la reacción (Labuza y col., 1982, Sanz y col., 2007).

ii) *pH del medio:* la evolución de la RM se ve altamente afectada por el pH del medio, debido a que en diversas etapas intervienen reacciones tipo ácido-base. Además, es necesario para que se inicie la reacción que el carbohidrato esté en forma abierta y el grupo amino no protonado (van Boekel, 2001). En este sentido, se ha descrito que un pH comprendido entre 6 y 8, es el más favorable para que se lleve a cabo la RM. Para controlar el pH, el uso de soluciones tampón está ampliamente aceptado, siendo los más usados fosfatos y acetatos, que además actúan como catalizadores de la misma (Bell, 1997).

iii) *Temperatura del medio:* La diversidad de etapas que implica la RM, la hace altamente dependiente de la temperatura. El efecto de la temperatura es diferente para cada etapa de la reacción y, en general, un aumento de la misma favorece la actividad de los reactantes y, en consecuencia, la velocidad de la reacción.

iv) *Concentración y tipos de reactantes:* en líneas generales, se ha demostrado que a medida que aumenta la concentración de azúcares reductores y la relación molar grupo carboxilo:grupo amino, la RM se ve favorecida (Warmbier y col., 1976). Por otro lado, dependiendo de la naturaleza de los reactantes, la velocidad de la reacción se ve afectada. En el caso de los carbohidratos la velocidad disminuye siguiendo el orden: aldopentosas > aldohexosas > cetohexosas > disacáridos > polisacáridos, con algunas excepciones, como la fructosa cuya reactividad, según describen algunos trabajos, es mayor que la de la glucosa (Laroque y col., 2008), aunque existen resultados contradictorios al respecto (Baxter, 1995, Naranjo y col., 1998). En el caso de los grupos amino, son más reactivos los grupos ε-amino como el situado en la cadena lateral de la lisina y los α-amino libres situados en el extremo amino terminal de aminoácidos, péptidos y proteínas. En

consecuencia, una mayor concentración de estos grupos se traduce en un mayor grado de glicación.

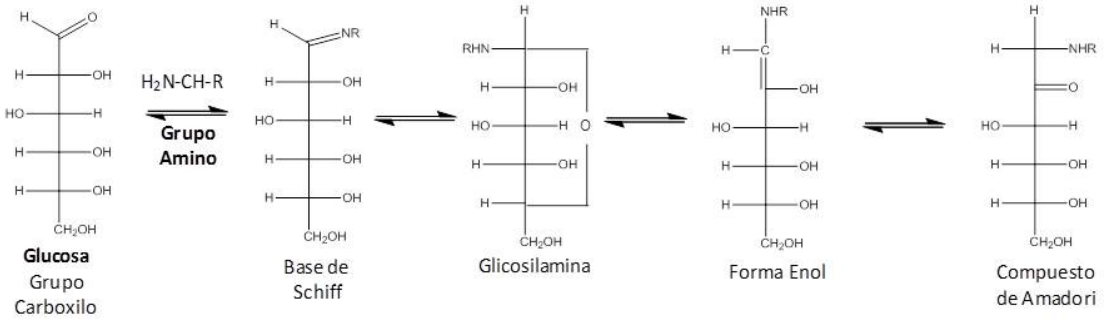


Figura 1.9. Esquema de formación del compuesto de Amadori.

1.4.3. Efectos en la salud humana

La RM no sólo es una reacción compleja de múltiples pasos, sino también una reacción que ocurre de forma espontánea en sistemas *in vivo* o durante el procesado de alimentos. Sin embargo, como se ha comentado anteriormente, en algunas ocasiones puede llevarse a cabo de forma controlada con el fin de obtener mejoras tecnológicas y/o funcionales en algunos alimentos. Diversos estudios han descrito diferentes efectos, tanto potencialmente beneficiosos como adversos, de los productos de la reacción de Maillard sobre la salud humana (Oliver y col., 2006, van Boekel y col., 2010).

- *Efectos negativos*

Uno de los efectos negativos de la RM, es la reducción de la calidad proteíca, consecuencia del bloqueo de aminoácidos esenciales (como lisina, metionina, triptófano, arginina e histidina) por los grupos carbonilos tanto de azúcares como de lípidos oxidados, además de por cambios conformacionales que hacen a la proteína menos accesible a la hidrólisis enzimática (Somoza, 2005). Numerosos estudios describen este comportamiento empleando diferentes sistemas modelo. En este sentido, se ha descrito en sistemas *in vivo* e *in vitro* la baja digestibilidad de los productos iniciales de la reacción de Maillard (Erbersdobler y col., 2001, Finot, 2005, Sanz y col., 2007).

También se ha descrito una disminución del valor nutritivo de los alimentos, debido a interacciones de algunos productos de la RM con aminoácidos esenciales, vitaminas y minerales como calcio, zinc, magnesio, cobre y hierro (Mossine y col., 2007, Obrien y col., 1989, Rufian-Henares y col., 2009, Seiquer y col., 2000). Dichas interacciones tienen como consecuencia una menor biodisponibilidad de estos compuestos, lo que podría derivar en una serie de problemas en la salud, como, por ejemplo, osteoporosis por falta de calcio y magnesio, o descenso en los niveles de hemoglobina por falta de hierro (Delgado-Andrade y col., 2011, Garcia y col., 2009, Seiquer y col., 2010).

También se ha descrito una serie de efectos negativos de los AGEs y ALEs (productos avanzados de la lipo-oxidación; cuando el grupo carbonilo implicado en la glicación proviene de un lípido). En este sentido, dichos compuestos, producidos *in situ* en los tejidos o ingeridos con la dieta, pueden acumularse en los tejidos, produciendo estrés oxidativo, envejecimiento celular y complicaciones en enfermedades, como la diabetes, además de poseer actividades mutagénicas que pueden, finalmente, afectar al ácido desoxirribonucleico (DNA) (Edeas y col., 2010, Robert y col., 2010).

- *Efectos positivos*

Existe un número considerable de los trabajos que describen propiedades biológicas beneficiosas, entre las cuales podemos mencionar:

(i) *Capacidad antioxidante*. Numerosos trabajos muestran la capacidad antioxidante de algunos compuestos derivados de la RM, usando para ello sistemas modelo sencillos de aminoácidos y azúcares o con sistemas más complejos usando para ello proteínas como la caseína, albúmina sérica bovina y ovoalbúmina (Alaiz y col., 1997, Chevalier y col., 2001, McGookin y col., 1991, Nakamura y col., 1992). Esta actividad radica en la capacidad que tienen estos compuestos de quelar metales oxidantes, como se mencionó anteriormente (Somoza, 2005, van Boekel y col., 2010, Wang y col., 2011).

Si bien es cierto que los AGEs poseen capacidad de ocasionar estrés oxidativo en las células, recientemente se ha demostrado que su ingesta moderada no implica un grave riesgo oxidativo, sino por el contrario favorece el mantenimiento del balance oxidativo y activación de vías quimiopreventivas (Zill y col., 2003).

(ii) *Capacidad antiinflamatoria*. Enomoto y col. (2009) describen la supresión en la producción de citoquinas pro-inflamatorias, después de la glicación de α-lactoalbúmina con maltopentaosa. Sin embargo, los trabajos que describen esta propiedad son escasos.

(iii) *Capacidad de modulación de la flora intestinal*. Se ha descrito que algunos compuestos de la RM de Maillard, como el compuesto de Amadori y las melanoidinas, no son metabolizados por el sistema digestivo de mamíferos. Por tanto, estos compuestos podrían alcanzar el intestino grueso y posiblemente ser fermentados por la microbiota intestinal (Erbersdobler y col., 2001, Faist y col., 2001). Estas hipótesis han sido parcialmente confirmadas para las melanoidinas procedentes del café y de pan, demostrándose cierta capacidad prebiótica de estos compuestos (Borrelli y col., 2005, Gniechwitz y col., 2008, Reichardt y col., 2009). Sin embargo, son escasos los trabajos que describen los posibles efectos del compuesto de Amadori o de Heyns sobre la microbiota intestinal. En este sentido, Wiame y col. (2002; 2005) lograron descubrir la fructosamina-6-quinasa y la glucosillisina-6-fosfato deglucosidasa, enzimas capaces de metabolizar dichos compuestos, y que están presentes en *E. coli* y otras especies de bacterias. Por otro lado, Hernández-Hernández y col. (2011) describen la capacidad bifidogénica de hidrolizados de β-lactoglobulina conjugados con carbohidratos prebióticos, vía RM, usando para ello muestras fecales de humanos sanos.

También se ha descrito la capacidad antimicrobiana de los productos de la RM, sin embargo, algunos resultados son contradictorios. Recientemente, Rufián-Henares y col. (2009) han descrito la capacidad antimicrobiana de

melanoidinas derivadas del café sobre diferentes cepas patógenas, encontrando que dicho efecto se debe al poder quelante de las melanoidinas, en este caso, de hierro y magnesio. Summa y col. (2008) publicaron resultados similares tras observar una disminución en el crecimiento de patógenos en presencia de melanoidinas. Por el contrario, Chevalier y col. (2001) no encontraron efectos antimicrobianos para estos compuestos usando sistemas modelos de glicación basados en albúmina sérica bovina y diferentes monosacáridos, e incluso se ha descrito un aumento en bacterias patógenas usando sistemas de glicación similares en pacientes con colitis ulcerosa (Mills y col., 2008).

Por tanto, el estudio de las bioactividades que presentan los compuestos procedentes de la RM, y más concretamente los compuestos de Amadori, es un campo que requiere de más investigaciones que podrían aportar nuevas vías para la obtención de ingredientes multi-funcionales.

2. JUSTIFICACIÓN Y OBJETIVOS

2. Justificación y Objetivos

El creciente interés de los consumidores por alimentos que además de satisfacer las necesidades nutricionales proporcionen beneficios para la salud y reduzcan el riesgo de contraer enfermedades ha originado un cambio considerable en el desarrollo de nuevos productos. Hoy día, el estudio de la relación entre un alimento (o uno de sus componentes) y la salud, así como el desarrollo y caracterización estructural de nuevos ingredientes funcionales, son unos de los principales retos a los que se enfrentan la Ciencia y Tecnología de Alimentos y la Nutrición.

El estado del intestino grueso y, en particular, del colon tiene una gran importancia en la salud humana debido en parte a las actividades metabólicas de la microbiota que lo coloniza. Un gran número de enfermedades (diarrea, inflamación, cáncer, etc.) están relacionadas con alteraciones en la composición de la microbiota del colon, mientras que cepas de los géneros *Bifidobacterium* y *Lactobacillus* pueden ejercer efectos beneficiosos sobre estos trastornos mediante modulación de las funciones fisiológicas, metabólicas e inmunológicas del hospedador. Desde que hace más de 15 años se introdujera el concepto de "prebiótico", la investigación en este campo se ha centrado en el desarrollo y caracterización de nuevos carbohidratos que fueran resistentes a la digestión gastrointestinal y favorecieran selectivamente el crecimiento y/o actividad de especies bacterianas beneficiosas en el colon.

En los últimos años se está demandando la producción de una “segunda generación” de ingredientes prebióticos que posean una serie de propiedades que incluyan: i) la capacidad de ser fermentados en las zonas más distales del colon, ya que es en esta región del intestino grueso donde tienen su origen diversas enfermedades crónicas como el cáncer de colon y la colitis ulcerosa y ii) una bioactividad añadida que les permita ejercer otras funciones saludables en el tracto gastrointestinal y/o sistema inmunológico. En este sentido, la combinación de propiedades prebióticas y antimicrobianas en un mismo ingrediente es muy prometedora ya que su ingesta podría producir un aumento del nivel de bacterias beneficiosas al mismo tiempo que impediría la colonización del tracto gastrointestinal por microorganismos patógenos.

Así, esta tesis doctoral tiene como punto de partida el proyecto Intramural de Fronteras **SIALOBIOTIC** (**200870F010**) financiado por el CSIC y basado en el desarrollo de nuevos ingredientes prebióticos multifuncionales con actividad antimicrobiana producidos a partir de la interacción covalente de *O*-glicopéptidos sialilados procedentes de proteínas alimentarias con carbohidratos prebióticos. Dicha conjugación, vía Reacción de Maillard, tiene una gran influencia en la industria alimentaria para obtener productos con propiedades beneficiosas tanto tecnológicas como biológicas. Estos nuevos ingredientes podrían, por un lado, alcanzar las zonas más distales del colon donde serían fermentados por la microbiota (según el biometabolismo descrito para los compuestos de Amadori) y, por otro lado, tendrían la capacidad de inhibir la adhesión de los microorganismos patógenos más frecuentes en el tracto gastrointestinal debido a la actividad antiadherente atribuida a los residuos de ácido siálico que forman parte de ciertas *O*-glicoproteínas.

Sin embargo, previo al estudio de estos conjugados es necesario conocer tanto la estructura química como las propiedades bioactivas de los compuestos independientes (carbohidratos y péptidos). Unos de los carbohidratos prebióticos con mayor relevancia en la industria alimentaria son los GOS obtenidos a partir de lactosa. Asimismo, recientemente se han sintetizado GOS a partir de lactulosa cuyas estructuras químicas y actividad prebiótica están aún por determinar. Por otra parte el CMP posee propiedades bioactivas con capacidad de ejercer efectos beneficiosos sobre la salud gastrointestinal del consumidor.

Para llevar a cabo la caracterización estructural tanto de carbohidratos prebióticos como de péptidos y proteínas se han descrito diversas técnicas analíticas tal y como se indica en la introducción general. Entre ellas, las técnicas cromatográficas son las más usadas por su alta resolución y sensibilidad, así como por la posibilidad de ser acopladas a la espectrometría de masas que proporciona información cualitativa adicional sobre la estructura de dichos compuestos. Sin embargo, la caracterización de sus estructuras no es trivial siendo necesario el desarrollo de nuevos métodos que mejoren los ya existentes.

El estudio analítico-estructural de carbohidratos prebióticos y péptidos funcionales se debe complementar con el estudio de la bioactividad de estos compuestos, abarcando desde estudios *in vitro*, empleando sistemas modelos, hasta estudios *in vivo*, usando para ello modelos animales y, finalmente, estudios en humanos.

En este sentido el objetivo general de esta tesis es el **desarrollo de diferentes métodos analíticos para la caracterización estructural de galactooligosacáridos sintetizados enzimáticamente a partir de lactosa o lactulosa y de péptidos derivados de la hidrólisis del CMP, y el estudio de su bioactividad *in vitro* e *in vivo* a nivel del sistema gastrointestinal, en primer lugar de manera individual y posteriormente conjugados vía reacción de Maillard.**

Partiendo del objetivo general, se plantearon los siguientes **objetivos parciales**, que se corresponden, tal y como se indica, con las distintas secciones del apartado de Resultados y Discusión de esta Memoria (**Sección 4**).

1. *Desarrollar métodos cromatográficos y espectrométricos para el análisis de GOS* (**Sección 4.1**).

- Comparar diferentes técnicas de fraccionamiento, para purificar GOS prebióticos a partir de una fuente con alto contenido de carbohidratos digeribles (**Sección 4.1.2**).

- Desarrollar un método mediante HILIC-MS^n para caracterizar estructuralmente GOS comerciales sintetizados a partir de lactosa (**Sección 4.1.3**).

- Caracterizar estructuralmente GOS sintetizados a partir de lactulosa (GOS-Lu) y β-galactosidasas mediante HPLC-MS y GC-MS (**Sección 4.1.4**).

2. *Desarrollar métodos cromatográficos y espectrométricos para el análisis de hidrolizados de CMP* (**Sección 4.2).**

- Desarrollar y validar un método basado en HILIC-MS^n para la caracterización de *O*-sialoglicopéptidos procedentes de hidrolizados de CMP (**Sección 4.2.2)**.

- Identificar fosfopéptidos y nuevos lugares de fosforilación en hidrolizados de CMP mediante RPLC-MS^2, usando dos analizadores, una trampa iónica (IT) y un cuadrupolo acoplado a un tiempo de vuelo (Q-TOF) (**Sección 4.2.3**).

3. *Evaluar la actividad prebiótica tanto in vitro como in vivo de GOS y de péptidos derivados de hidrolizados de CMP, libres y conjugados vía Reacción de Maillard* (**Sección 4.3**).

- Determinar la influencia de galactooligosacáridos sintetizados a partir de lactulosa (GOS-Lu) y lactosa (GOS-La) y lactulosa en el crecimiento de diferentes cepas de Lactobacilos y su resistencia a las condiciones gastrointestinales (**Sección 4.3.2.1**).

- Evaluar el efecto de la glicación de péptidos derivados de hidrolizados de CMP conjugados con GOS-Lu, GOS-La y lactulosa sobre el crecimiento de diferentes cepas de Lactobacilos y su resistencia a las condiciones gastrointestinales (**Sección 4.3.2.2**).

- Estudiar el efecto sobre la microbiota intestinal de la glicación de péptidos derivados de hidrolizados de CMP conjugados con GOS-Lu, GOS-La y lactulosa usando para ello sistemas de fermentación *in vitro* (**Sección 4.3.2.3).**

- Determinar el potencial de GOS-Lu, GOS-La y lactulosa, además de sus respectivos glicoconjugados con hidrolizados de CMP, como inhibidores de procesos inflamatorios en células epiteliales del intestino (**Sección 4.3.2.4**).

- Determinar la digestibilidad ileal *in vivo* de GOS-Lu, GOS-La y lactulosa (**Sección 4.3.3.1**).

- Estudiar el efecto prebiótico *in vivo* de GOS-Lu, GOS-La y lactulosa (**Sección 4.3.3.1 y 4.3.3.2**).

-Determinar la biodiversidad de Bifidobacterias en muestras de inestino grueso de ratas alimentadas con GOS-Lu y GOS-La (**Sección 4.3.3.2**).

- Estudiar la actividad inmunomoduladora *in vivo* de galactooligosacáridos derivados de lactulosa y lactosa (**Sección 4.3.3.2**).

3. PLAN DE TRABAJO

3. Plan de trabajo

Según los antecedentes expuestos y los objetivos planteados en esta Tesis, se siguió el siguiente plan de trabajo, presentado de forma esquemática en la **Figura 3.1** y clasificado en función de las secciones presentadas en el apartado de Resultados y Discusión de esta Memoria.

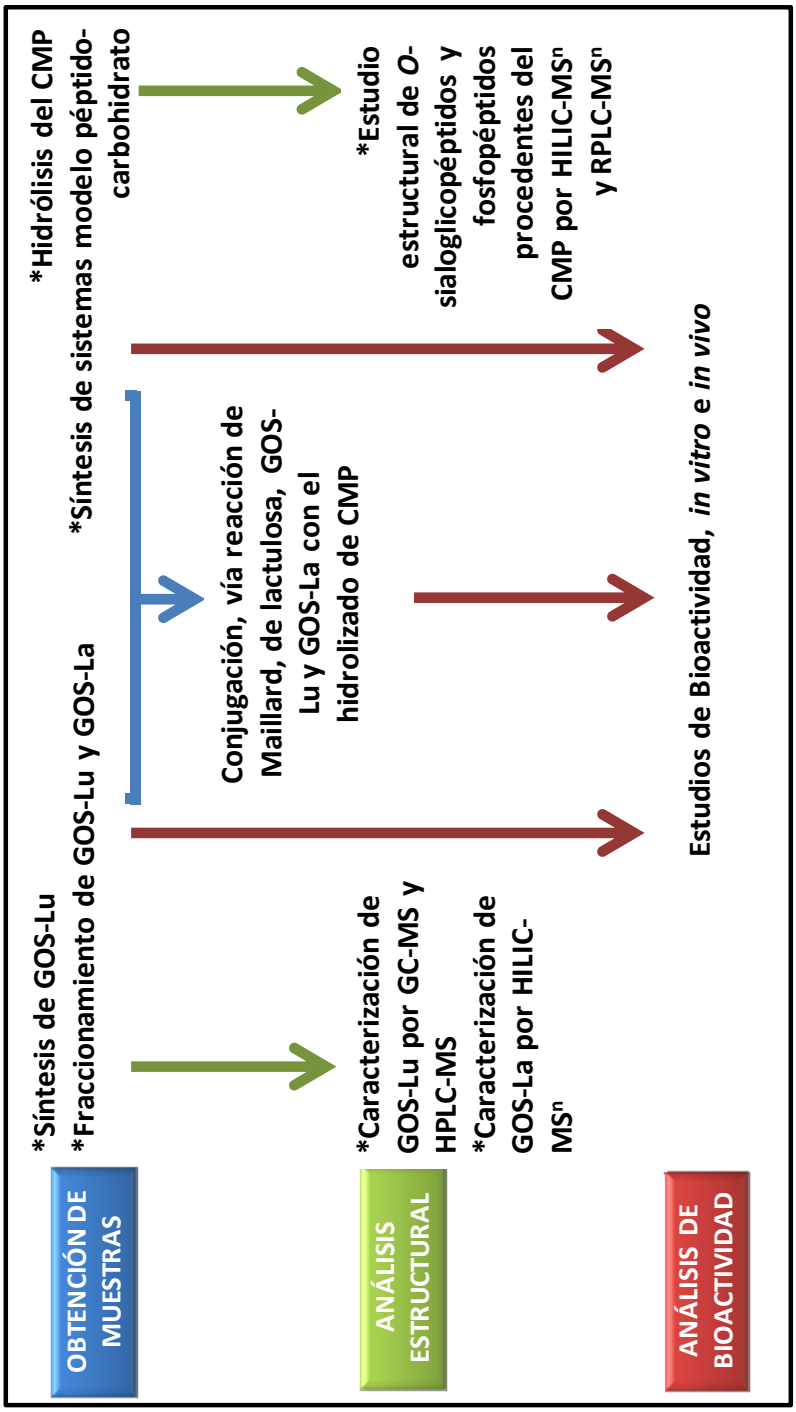


Figura 3.1. Esquema general del plan de trabajo llevado a cabo en la presente memoria.

Sección 4.1. Desarrollo de métodos para el análisis de GOS

- Estudio comparativo de técnicas cromatográficas, de membrana y microbiológicas para el fraccionamiento de oligosacáridos. Aplicación de las mejores condiciones de fraccionamiento a distintas mezclas de GOS con el fin de obtener fracciones puras del mismo peso molecular y/o eliminar carbohidratos digeribles de bajo peso molecular (**Sección 4.1.2**).

- Desarrollo y comparación de métodos basados en HPLC y MS para la caracterización de diferentes GOS comerciales sintetizados a partir de lactosa. Empleo de diferentes fases estacionarias HILIC acopladas a MS^n usando un analizador de tipo trampa iónica (IT). Estudio de la fragmentación por MS^n de diferentes patrones de carbohidratos comerciales y aplicación de estos resultados para la identificación de GOS (**Sección 4.1.3**).

- Síntesis de GOS empleando lactulosa como sustrato y β-galactosidasas procedentes de diferentes especies de hongos. Caracterización de los GOS obtenidos mediante GC-MS y LC-ESI-MS (**Sección 4.1.4**).

Sección 4.2. Desarrollo de métodos para el análisis de péptidos procedentes del CMP

- Hidrólisis del CMP bovino mediante la acción combinada de tripsina y quimotripsina (**Sección 4.2.2**).

- Obtención de glicopéptidos mediante glicosilación no enzimática (Reacción de Maillard) como sistemas modelo de péptidos sintéticos con carbohidratos neutros y sialilados (**Sección 4.2.2**).

- Optimización de métodos cromatográficos basados en la interacción hidrofílica zwiteriónica (ZIC-HILIC) para la separación de *O*-glicopéptidos procedentes de la hidrólisis del CMP (**Sección 4.2.2**).

- Identificación de fosfopéptidos y péptidos sin modificaciones post-traduccionales por RPLC-MS, usando una trampa iónica (IT) y un cuadrupolo acoplado a un tiempo de vuelo (Q-TOF). Estudio del efecto del analizador sobre los espectros de masas de fosfopéptidos (**Sección 4.2.3**).

Sección 4.3. Evaluación de la bioactividad mediante estudios *in vitro* e *in vivo* de GOS y de péptidos procedentes del CMP, así como de sus respectivos glicoconjugados.

- Estudio del efecto de GOS-La y GOS-Lu, previamente fraccionados siguiendo los métodos optimizados en la **sección 4.1.2**, sobre el crecimiento de diferentes cepas de *Lactobacillus*. Evaluación de la resistencia de estos microorganismos frente a diferentes condiciones gastrointestinales (**Sección 4.3.2.1**).

- Síntesis de glicoconjugados, vía reacción de Maillard bajo condiciones controladas de actividad de agua y temperatura, con el fin de obtener un producto enriquecido en compuestos de Amadori o Heyns, empleando hidrolizados de CMP y como fuente de carbohidratos GOS-Lu, GOS-La y lactulosa, respectivamente (**Secciones 4.3.2.2 y 4.3.2.3**).

- Estudio del efecto de los glicoconjugados CMP-GOS-La y CMP-GOS-Lu sobre el crecimiento y resistencia a sales biliares de diferentes cepas de Lactobacillus (**Sección 4.3.2.2**).

- Evaluación mediante sistemas *in vitro* del efecto sobre la microbiota intestinal en humanos de los glicoconjugados CMP-GOS-La, CMP-GOS-Lu y CMP-lactulosa. Comparación de dicho efecto con el de los GOS-La y GOS-Lu y lactulosa para determinar la influencia de la glicación sobre la actividad prebiótica de los carbohidratos (**Sección 4.3.2.3**).

- Evaluación del efecto sobre la respuesta inflamatoria generada por patógenos intestinales humanos en sistemas celulares tipo Caco-2 de los glicoconjugados CMP-GOS-La y CMP-GOS-Lu. Comparación de dicho efecto con el de los GOS-La

y GOS-Lu para determinar la influencia de la glicación sobre la respuesta inflamatoria intestinal (**Sección 4.3.2.4**).

- Estudio de la ingesta de lactulosa, GOS-Lu y GOS-La, previamente fraccionados, sobre el crecimiento y desarrollo de órganos vitales de ratas Wistar. Obtención de muestras de íleon e intestino grueso (**Sección 4.3.3.1**).

- Evaluación de la digestibilidad ileal de la lactulosa, GOS-La y GOS-Lu, usando las muestras de ileon de las ratas alimentadas con dichos carbohidratos, mediante GC-MS (**Sección 4.3.3.1**).

- Estudio del efecto prebiótico *in vivo* de lactulosa, GOS-La y GOS-Lu. Estudio de la biodiversidad bacteriana intestinal mediante qPCR (**Sección 4.3.3.2**).

- Estudio de la actividad inmunomoduladora *in vivo* de los GOS-Lu mediante la monitorización de la expresión (mRNA) de marcadores del control homeostático de la inflamación y respuesta inmune por transcripción inversa y qPCR (**Sección 4.3.3.2**).

4. RESULTADOS Y DISCUSIÓN

4.1. Desarrollo de métodos analíticos para el análisis de GOS

4.1.1. Prefacio

Debido a la relación existente entre la actividad prebiótica de los GOS y su estructura química, ya mencionada en la **Sección 1** de esta Memoria, su caracterización resulta de especial importancia. Sin embargo, dada la similitud estructural de estos compuestos y la ausencia de patrones comerciales, su análisis tanto cuantitativo como cualitativo supone un importante reto de investigación.

Dado que los carbohidratos se encuentran normalmente formado por mezclas complejas, previo a su análisis, es necesario, en ciertas ocasiones, llevar a cabo un fraccionamiento de la muestra, con el fin de obtener fracciones de un grado de polimerización determinado y simplificar así su caracterización. Así mismo, para poder evaluar la actividad de dichos compuestos mediante sistemas *in vitro* o para su empleo como ingredientes funcionales, es imprescindible eliminar aquellos carbohidratos digeribles presentes en las mezclas, principalmente mono- y disacáridos, que enmascararán sus propiedades. Por tanto, la selección de la técnica de fraccionamiento más eficaz para estos propósitos, que sea además rápida y rentable económicamente, presenta gran interés en este campo.

Como se ha descrito en la Introducción General (**Sección 1**), existen diversos carbohidratos con actividad prebiótica, siendo los GOS unos de los más conocidos y comercializados a nivel mundial. Sin embargo, su estructura química varía con la enzima y condiciones empleadas en su síntesis, no existiendo una técnica que permita su determinación directa.

En este sentido, el objetivo general de esta sección se basó en el desarrollo y aplicación de diferentes técnicas de fraccionamiento y análisis para el enriquecimiento y la caracterización estructural de GOS, tanto comerciales como obtenidos en el laboratorio empleando lactosa y lactulosa como sustratos y diferentes β-galactosidasas de origen microbiano.

Debido al amplio número de técnicas empleadas para el fraccionamiento de carbohidratos se ha considerado de interés incluir un apartado en este prefacio que resuma sus modos de operación y usos principales (**apartado 4.1.1.1**). Dicho apartado

está basado en el artículo de revisión titulado "**Fundamentos y aplicaciones de las técnicas de fraccionamiento de carbohidratos**" de Hernández, O. y Ruiz-Matute, A. publicado en *Cromatografía y Técnicas Afines*, 2008, 29 (1) 3-11. Algunas de estas técnicas de fraccionamiento (cromatografía de exclusión molecular, carbón activo, tratamiento con levaduras y ultrafiltración) se aplicaron para la obtención de GOS libres de mono- y disacáridos, los cuales incluyen carbohidratos digeribles como la glucosa, galactosa y lactosa. La comparación de los resultados obtenidos mediante estas técnicas constituye la **sección 4.1.2** que dio lugar al trabajo titulado "**Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides**" de Hernández y col. publicado en *International Dairy Journal*, 2009, 19, 531-536. Las técnicas de fraccionamiento más adecuadas para la eliminación de mono- y disacáridos fueron la cromatografía de exclusión molecular (SEC) y el tratamiento con carbón activo. Los oligosacáridos obtenidos mediante estas técnicas se emplearon tanto para el desarrollo de métodos de análisis (**Sección 4.1.4**) como para el estudio de sus propiedades bioactivas (**Sección 4.3**).

La **sección 4.1.3** recoge el trabajo de Hernández-Hernández y col. titulado: "**Characterization of galactooligosaccharides derived from lactulose**" publicado en la edición especial "Food Analysis" del *Journal of Chromatography A*, 2011, 1218, 7691-7696. En dicho trabajo, se elucida la estructura de di- y trisacáridos presentes en GOS sintetizados a partir de lactulosa y β-galactosidasas procedentes de tres especies diferentes de hongos, previamente convertidos en sus TMSO (trimetilsililoximas) y usando para ello GC-MS. Se empleó también HPLC-MS con una columna de carbón grafitizado para el análisis de los diferentes grados de polimerización de estos carbohidratos. Es de destacar, que debido a la novedad de estos carbohidratos, es la primera vez que se realiza, de manera exhaustiva, una caracterización de este tipo de galactooligosacáridos. Los GOS-Lu obtenidos mediante β-galactosidasa de *Aspergillus oryzae*, fueron los que presentaron mayor grado de polimerización y variabilidad estructural por lo que se seleccionaron para posteriores estudios de bioactividad (Sección 4.3)

Seguidamente, en la **sección 4.1.4** se incluyen los resultados obtenidos del desarrollo de un método mediante HILIC-MS^n para la caracterización de diferentes GOS

comerciales sintetizados a partir de lactosa. Este trabajo surgió de la necesidad de caracterizar diferentes GOS procedentes de distintas casas comerciales y, en consecuencia, con diferentes protocolos de síntesis y distintas estructuras. Esta sección está basada en el artículo publicado por Hernández-Hernández y col. titulado "**Hydrophilic Interaction Liquid Chromatography (HILIC) coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides**" y publicado en *Journal of Chromatography A*, 2012, En prensa, DOI: 10.1016/J.chroma.2011.11.047. En dicho trabajo, se comparan por primera vez diferentes fases estacionarias HILIC, para la caracterización de este tipo de carbohidratos, además se logra realizar una identificación tentativa de la estructura que presentan los mismos por MS^n, sin necesidad de derivatización previa. Es la primera vez que esta técnica se emplea para la caracterización de GOS, pudiendo ser aplicada a otros carbohidratos de alto interés nutricional y económico.

4.1.1.1 Fundamentos y aplicaciones de las técnicas de fraccionamiento de carbohidratos

Hernández, O y Ruiz-Matute, A.

Cromatografía y Técnicas Afines 29 (1) (2008) 3-14.

4.1.1.1.1. Técnicas de Fraccionamiento

Las diferentes técnicas usadas para fraccionar carbohidratos que se detallan a continuación, abarcan desde técnicas cromatográficas, hasta técnicas de separación con membranas, tratamientos biológicos y fisicoquímicos. En función de la naturaleza fisicoquímica de los carbohidratos, se podrán utilizar una o más de estas técnicas para obtener muestras purificadas.

Técnicas Cromatográficas

La cromatografía ha sido una de las técnicas más usadas para el fraccionamiento de carbohidratos, permitiendo no solo la obtención de muestras con

alto grado de pureza, sino también su análisis. Por ello, a continuación se describen algunas de las técnicas cromatográficas más utilizadas para el fraccionamiento de carbohidratos, haciendo también mención algunas de sus aplicaciones analíticas para el estudio de estos compuestos.

- Cromatografía de Exclusión Molecular (SEC)

Es una técnica de cromatografía líquida basada en la separación de moléculas en función de su tamaño, su volumen hidrodinámico y el tamaño de poro del gel utilizado (Park, y col., 2007; Sanz, y col., 2007). En esta técnica las moléculas de mayor peso molecular eluyen primero de la columna seguidas por las moléculas de menor tamaño, consecuencia de su distribución en la fase estacionaria.

Debido a las características de este tipo de cromatografía, esta técnica ha sido utilizada ampliamente para el fraccionamiento de carbohidratos de distinto peso molecular con aplicaciones tanto analíticas como industriales. Dependiendo del tamaño de las moléculas que se necesiten fraccionar, se debe elegir un gel o fase estacionaria con un tamaño de poro adecuado; en este sentido, existen una serie de fases estacionarias fabricadas con diferentes materiales, tales como polisacáridos entrecruzados (dextranos y agarosa) y poliacrilamida, que abarcan un amplio rango de fraccionamiento, además de poseer propiedades fisicoquímicas diferentes, pudiéndose elegir el gel más adecuado en función de las características químicas de la muestra y del eluyente. El fraccionamiento de oligosacáridos ha sido uno de los usos más comunes que se le ha dado a este tipo de cromatografía. Knutsen y col. (2001) emplearon dos geles formados por dextranos y agarosa, para purificar y analizar oligosacáridos, obtenidos tras hidrólisis enzimática de carragenanos. En la **figura 4.1.** se observa el hidrolizado de kappa-carragenanos, donde el polisacárido no hidrolizado eluye en el volumen muerto (“Void”) seguido por los distintos oligosacáridos (neocarrobiosa) obtenidos a partir de dicha hidrólisis y cuyos números representan el grado de polimerización.

Como puede observarse, el tiempo de retención es inversamente proporcional al peso molecular del oligosacárido eluido. Por otra parte, Chonan y col. (2004) purificaron los di-, tri- y tetrasacáridos procedentes de GOS preparados a partir de la

transgalactosilación de lactosa usando una fase estacionaria con rango de fraccionamiento comprendido entre 100 y 1800 Da.

Si bien el uso de SEC para el fraccionamiento de polisacáridos ha sido utilizado en ciertas ocasiones, no es recomendable aplicar esta técnica para estos compuestos debido a que su naturaleza cristalina dificulta su solubilización (Eremeeva, 2003; Sanz y col., 2007). Sin embargo, se emplean algunas metodologías capaces de disolver estos carbohidratos, tales como la gelatinización en el caso del almidón usando soluciones alcalinas como KOH 2M (Hernández y col., 2008) o el empleo de SEC de alta temperatura (Park, y col., 2007). En el estudio de polisacáridos como el almidón, la SEC permite el fraccionamiento entre amilosa y amilopectina proporcionando información sobre la relación existente entre ambos compuestos, e incluso sobre la heterogeneidad de tamaño de estas moléculas, lo que influye en las propiedades tecnológicas de este polisacárido. Hernández y col. (2008) determinaron la relación de amilosa y amilopectina en almidones de plátano, sagú, patata y maíz, al igual que Sandhu y Lim (2008), en almidones de mango. Por otra parte, Guerra y col. (2007) determinaron el comportamiento en SEC de la lignina extraída de eucalipto, trigo, picea y abeto.

Una de las modificaciones de la SEC convencional es la cromatografía de exclusión molecular rápida, que emplea columnas de menor longitud (50 mm), fases estacionarias con menor tamaño de partícula (5-10 µm) y volúmenes inferiores de eluyente (20-1000 mL), lo que se traduce en tiempos de análisis más cortos, pero implica una pérdida de resolución (Popovici y col., 2005). También existen columnas de HPLC que cumplen con el principio de exclusión molecular; a esta cromatografía se le conoce comúnmente como cromatografía de exclusión molecular de alta eficacia HPSEC. Es frecuente observar acoplamientos de HPSEC con detectores de diferentes tipos, siendo los más comunes los refractómetros y espectrofotómetros de UV y visible, entre otros. Charles y col. (2008) utilizaron HPSEC con un detector de luz láser de multiángulos de dispersión (Multiangle Laser Light Scattering) y otro de índice de refracción (RI) para caracterizar mucopolisacáridos provenientes de tubérculos de yuca (*Manihot esculenta* Crantz L.).

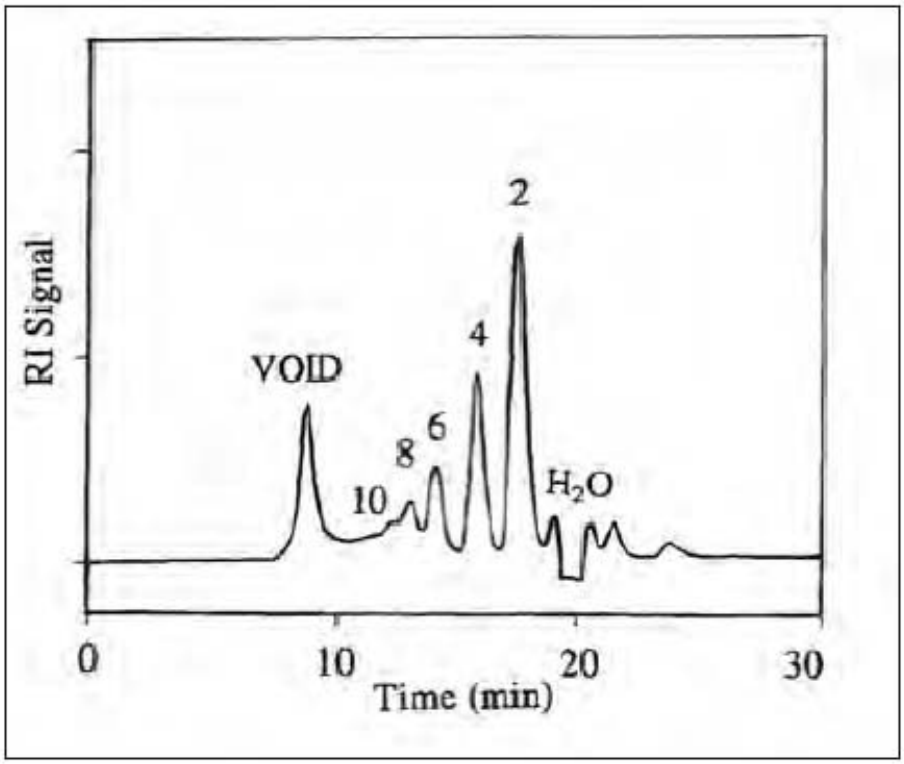


Figura 4.1. Cromatografía de Exclusión Molecular (SEC) de hidrolizados con kappa-carragenasa de kappa-Carragenanos. Void representa el polisacárido y los números el grado de polimerización de los oligosacáridos obtenidos. Tomado de Knutsen y col. (2001). Reproducido con permiso de Elsevier.

- Cromatografía de adsorción en carbón activo

El carbón ha sido utilizado desde principios del siglo XX para fraccionar carbohidratos. Ya en 1937, Hayashi describió el empleo de carbón activo para la separación de una mezcla de glucosa y sacarosa en ácido acético y agua; la sacarosa era adsorbida por el carbón mientras que la glucosa permanecía en disolución. Por otra parte, Whistler y Durso (Whistler y col., 1950) desarrollaron columnas rellenas con carbón activo y Celite™ para fraccionar mono-, di- y trisacáridos. Se han estudiado numerosos disolventes que provocan la desorción selectiva de los carbohidratos adsorbidos en el carbón, pero el más utilizado es la mezcla de etanol:agua en diferentes proporciones. Dicho eluyente fue propuesto por Whistler y Durso en 1950 y aún es el más empleado en la actualidad, e incluso recomendado por los métodos

oficiales de análisis de la AOAC para el fraccionamiento de carbohidratos en mieles . Para la separación de los azúcares en este alimento se emplea una solución de etanol al 1% v/v para eluir los monosacáridos, del 5% para los disacáridos y del 50% para los oligosacáridos. Se piensa que el mecanismo de retención se debe principalmente a la adsorción de los carbohidratos sobre la matriz de carbón, aunque también han sido descritas interacciones hidrofóbicas y de intercambio de electrones (Koizumi, 2002).

En los últimos años, el fraccionamiento de carbohidratos con carbón activo ha sido ampliamente utilizado. Morales y col. (2006) usaron cromatografía en columna y sistemas de agitación con carbón activo para fraccionar los carbohidratos presentes en muestras de miel, consiguiendo una purificación similar con ambas técnicas. Sanz y col. (2007) utilizaron este mismo procedimiento para eliminar los mono- y disacáridos de una mezcla de GOS obteniendo fracciones de alta pureza.

Actualmente, existen columnas de carbón grafitizado (GCC) para HPLC, tanto para aplicaciones preparativas como analíticas. Estas columnas se caracterizan por su alta estabilidad física y química, con la ventaja de permitir la resolución de isómeros de algunos carbohidratos utilizando disolventes que no contengan sales (Koizumi, 2002). En consecuencia las muestras eluidas pueden ser a su vez directamente analizadas por técnicas tales como espectrometría de masas.

Las GCC pueden ser utilizadas en un amplio rango de pH, pudiéndose eliminar los dobles picos de los anómeros del carbohidrato al aumentar el valor de pH del eluyente. De este modo, la **figura 4.2a** muestra la separación, con fines analíticos, de varias glucobiosas sin la presencia de dobles picos, caso contrario a lo mostrado en la **figura 4.2b**, donde el pH del eluyente no es alcalino, y en consecuencia los anómeros son detectables (Koizumi, 1996). Previamente, estos mismos autores (Koizumi y col., 1992) habían utilizado las GCC para la separación de glucobiosas pero con fines preparativos.

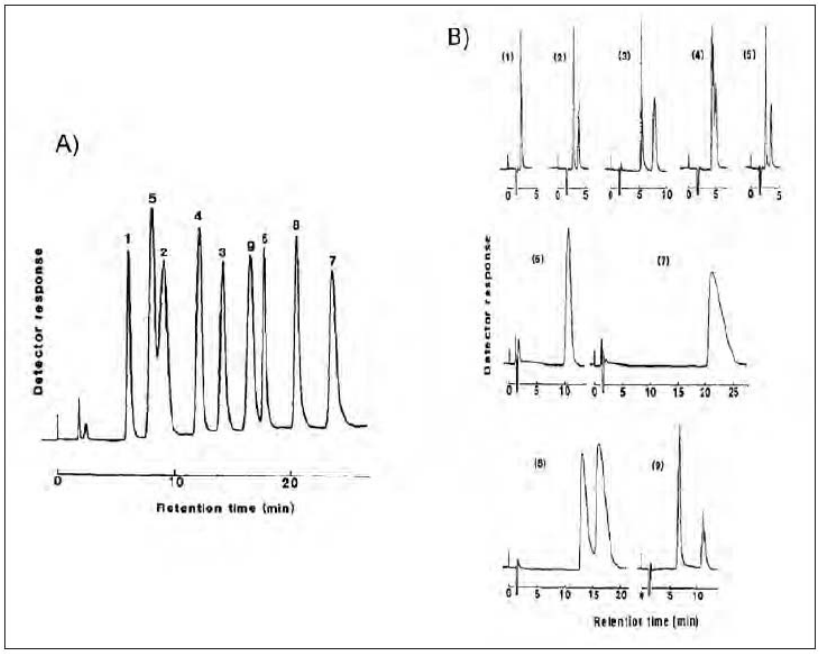


Figura 4.2. Cromatogramas de glucobiosas usando columnas de carbón grafitizado. (A) Separación simultánea con eluyente alcalino (NaOH 1 mM y Acetonitrilo 1,5-5%) (B) Análisis individual de cada glucobiosa con eluyente a base de acetonitrilo y agua (4:96). (1) Trehalosa, (2) kojibiosa, (3) nigerosa, (4) maltosa, (5) isomaltosa, (6) soforosa, (7) laminaribiosa, (8) celobiosa y (9) gentibiosa. Tomado de Koizumi (2002). Reproducida con permiso de Elsevier.

- Cromatografía de Intercambio Iónico (IEC)

La cromatografía de intercambio iónico, tanto aniónico como catiónico, es una técnica ampliamente usada en la actualidad para el fraccionamiento de carbohidratos; su utilización no solo se limita al aspecto analítico, sino también al preparativo.

En la cromatografía de intercambio catiónico intervienen una serie de efectos responsables de la separación del analito, tales como: intercambio de especies iónicas, exclusión por tamaño molecular (donde interviene tanto los radios de Stokes de la muestra como los tamaños de poro de la resina), exclusión iónica (siendo los ácidos débiles, como los azúcares, los compuestos con mayor tiempo de retención, en comparación con compuestos con menor valor de pKa), intercambio de ligandos e interacción del compuesto con la resina vía enlaces de hidrógeno (Huck y col. 2007;

Sanz y col., 2007). Panagiotopoulos y col. (2007) lograron separar oligosacáridos de monosacáridos procedentes de la hidrólisis ácida de muestras de materia orgánica marina empleando cromatografía de intercambio catiónico de alta resolución. La resolución se mejora generalmente cuando se usan resinas con cationes pesados como Ag^{+} y Pb^{+}, siendo el principio de separación la fuerza del complejo entre los grupos hidroxilo y estos cationes. De igual forma, mediante esta cromatografía, se han llevado a cabo separaciones de monosacáridos según la orientación axial o ecuatorial de sus grupos hidroxilos vecinales al complejo con el ión metálico. Por otro lado, Vente y col. (2005) encontraron que las resinas con K^{+} poseen más fuerza de adsorción con los azúcares que las resinas con Ca^{2+} o Na^{+}.

- Cromatografía de Interacción Hidrofílica

Esta técnica básicamente puede ser definida como una extracción líquido-líquido entre una fase acuosa formada entre la fase estacionaria y la fase móvil, aunque pueden también existir otros mecanismos más complejos que aún no han sido elucidados en su totalidad.

Troyer y col. (2001) usaron HILIC con columnas preparativas, para la purificación de glucosinolatos provenientes de extractos de plantas, que previamente habían sido parcialmente purificadas utilizando cromatografía de intercambio iónico. Por otro lado, Deguchi y col. (2008) realizaron HILIC en dos dimensiones con muestras de 2-piridilamino derivados de *N*-glucanos neutros y sialilados presentes en plasma humano. En este trabajo se obtuvo una alta eficiencia de separación de estos compuestos en función al número de ácidos siálicos, pudiendo ser también útil esta técnica para la separación de otros oligosacáridos.

- Cromatografía de lecho móvil simulado

La cromatografía de lecho móvil simulado (*Simulated moving bed*) ha sido aplicada desde 1960 en la purificación de hidrocarburos y, posteriormente, en azúcares de grado alimentario. Es un sistema de multicolumnas, conectadas en serie y rellenas con múltiples fases estacionarias, siendo las más comunes las de intercambioiónico y de exclusión molecular, formando así un sistema cerrado (Rodrigues y col., 2008; Toumi y

col., 2007). Su principio se basa en la simulación de un movimiento en contracorriente del eluyente y la fase estacionaria, lo cual maximiza la eficiencia del proceso (Mazzotti y col., 1997; Toumi y col., 2007). La **figura 4.3** muestra un esquema que representa el principio de separación de esta técnica. En líneas generales, el sistema se divide en cuatro zonas, cada una con la cantidad de columnas que sean necesarias para la separación de los compuestos de interés y a su vez cada columna con una válvula individual. En este sentido, Geisser y col. (2005) usaron 8 columnas para la separación de lactosa de oligosacáridos presentes en leche humana, mientras que Rodrigues y col. (2008) usaron únicamente dos columnas para la separación binaria de compuestos, aunque lo más común es encontrar al menos una columna en cada zona (Sanz y col., 2007). El eluyente y la muestra son introducidos en el sistema de manera continua, saliendo en diferentes puntos del sistema los compuestos más retenidos y los menos retenidos, controlando cada entrada y cada salida mediante una bomba externa. Existe también una quinta bomba, denominada bomba recicladora, capaz de mantener el flujo interno entre las columnas. Debido a los flujos externo e interno, el movimiento de la fase estacionaria es simulado y de allí el nombre de esta técnica (Schulte y col., 2001).

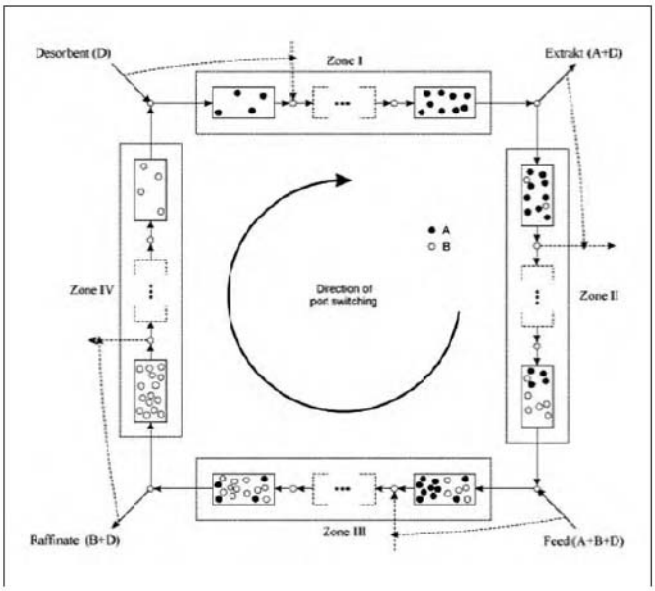


Figura 4.3. Principio de separación de la Cromatografía de Lecho Móvil Simulado (Simulated moving bed). Tomado de Toumi y col. 2007. Reproducida con permiso de Elsevier.

Técnicas de Separación por Membranas

Los procesos de separación con membranas han sido muy utilizados en un gran número de aplicaciones en la industria química debido a su simplicidad y eficiencia de operación, así como a su bajo consumo de energía (Hagg, 1998). Una membrana puede actuar como un filtro selectivo, a veces limitando la difusión entre dos soluciones o como una membrana activa en el que su estructura química determina la selectividad de transferencia de la muestra (Gilar y col., 2001).

Existen membranas de soporte líquido y poliméricas, siendo más estables estas últimas. A continuación se describen las principales técnicas que han sido utilizadas recientemente para el fraccionamiento de carbohidratos basados en diferentes tecnologías (ultrafiltración, nanofiltración, diálisis, extracción asistida con membranas).

- Ultrafiltración y Nanofiltración

Las técnicas de ultra- y nanofiltración han sido ampliamente usadas en el estudio de carbohidratos, no solo porque permiten el fraccionamiento de estos, sino también su purificación y concentración. En este sentido, Skorepova y Moresoli (2007) utilizaron ultrafiltración para eliminar carbohidratos en muestras de leche de soja y obtener un producto con alto contenido de proteínas. En este caso, el alto peso molecular de dichos polímeros, en comparación con el bajo peso de los carbohidratos (mono- y disacáridos), facilita la separación de ambos por ultrafiltración, lo cual también es válido para separar polisacáridos de mono-, di- y oligosacáridos.

Se han realizado numerosos estudios de purificación de oligosacáridos con técnicas de ultra- y nanofiltración (Goulas y col., 2003; Li y col., 2004; Vegas y col., 2008), siendo la diferencia más notable entre ambas técnicas el tamaño del poro de la membrana y las presiones utilizadas. Vegas y col. (2008) compararon varias membranas de ultra- y nanofiltración para purificar xilooligosacáridos provenientes de cáscaras de arroz, encontrando los mejores resultados en membranas poliméricas tubulares de 4 KDa de corte molecular y membranas de cerámica monolítica de 1 KDa, con porcentajes de recuperación del 70 y 78%, respectivamente. Igualmente, Goulas y col. (2003)

compararon membranas de nanofiltración de polietersulfona (150-300 Da de corte molecular) y de acetato de celulosa (1 KDa de corte molecular) en modo de diafiltración discontinua para purificar los oligosacáridos presentes en Panorich® (oligosacáridos derivados de panosa). Estos autores eliminaron el 80% de monosacáridos con pérdidas de di- y oligosacáridos del 12% en la membrana de menor corte de peso molecular, mientras que en la de 1 KDa observaron una alta pérdida de monosacáridos pero también de oligosacáridos.

Según los innumerables trabajos llevados a cabo sobre fraccionamiento de carbohidratos, los resultados de recuperación y pureza de los oligosacáridos de los di- y monosacáridos dependerán en gran medida del tipo de muestra, la membrana a utilizar y las condiciones en la que se lleve a cabo la ultra- o nanofiltración.

- Diálisis y microdiálisis

Los métodos de diálisis y microdiálisis se basan en el uso de membranas con una estructura de poro controlada que proporcionan una separación basada en el peso molecular mediante un proceso de difusión (Gilar y col., 2001). Los líquidos a ambos lados de la membrana se encuentran físicamente conectados a través de los poros y la fuerza que conduce el proceso de transferencia de masa se basa en una simple diferencia de concentración a lo largo de la membrana.

Aunque la mayoría de las aplicaciones de las técnicas de diálisis y microdiálisis a los carbohidratos han estado enfocados a su purificación y concentración (Gelders y col., 2003; Okatch y col., 2003, Okuda y col., 2001; Somboonpanyakul y col., 2006), también se han utilizado para la separación de carbohidratos de alto peso molecular (oligosacáridos y polisacáridos) de otros de menor tamaño (Gelders y col., 2003; Godshall y col., 2001).

Nilsson y col. (2001) utilizaron un sistema de microdiálisis-HPAEC-PAD para la caracterización de almidón hidrolizado. El uso de la microdiálisis permitió el estudio de las fracciones de cadena corta del almidón desramificado en presencia de amilosa sin un tratamiento de prefraccionamiento. El corte de peso molecular de la membrana

permitió la difusión de los oligosacáridos a través de ella, impidiendo el paso de moléculas más grandes (enzimas y polisacáridos de mayor peso molecular) al sistema cromatográfico. Por su parte, Charles-Bernard y col. (2005) utilizaron esta técnica para el fraccionamiento de los componentes no volátiles del café, obteniendo una fracción rica en polisacáridos y melanoidinas mediante el uso de una membrana de 3,5 kDa.

Otras técnicas empleadas para el fraccionamiento de carbohidratos

- Tratamientos microbiológicos

El uso de levaduras para eliminar algunos carbohidratos también puede ser considerado como una técnica de fraccionamiento. Baumgartner y col. (1986) usaron la levadura *Saccharomyces bayanus* para eliminar los carbohidratos de vainas de algarroba y poder así analizar sus ciclitoles sin interferencias. Después del tratamiento con levaduras, los carbohidratos restantes se eliminaron mediante cromatografía de intercambio aniónico.

Más recientemente, Yoon y col. (2003) han mostrado la utilidad de la levadura *Saccharomyces cerevisiae* para la separación selectiva de ciertos carbohidratos. Estos estudios han demostrado una alta especificidad de las enzimas de la levadura para eliminar algunos mono- y disacáridos como glucosa, fructosa, manosa, galactosa mientras que azúcares como la ramnosa, sorbosa no se vieron afectados. Esta técnica también ha sido utilizada por Sanz y col. (2005) para la obtención de una fracción de oligosacáridos de miel libre de los monosacáridos glucosa y fructosa con objeto de determinar su potencial prebiótico. Asimismo, el fraccionamiento con *S. cerevisiae* se ha aplicado con éxito a la detección de adulteraciones de la miel con distintos jarabes comerciales (Ruiz-Matute y col., 2007). La presencia de dianhídridos de fructosa (DFAs), compuestos marcadores de estos jarabes, fue puesta de manifiesto en las muestras adulteradas tras su fraccionamiento con estas levaduras. En la **Figura 4.4** se puede observar el cambio producido en el perfil de carbohidratos de la miel original (4A) tras ser incubada con *S. cerevisiae* (4B), donde los monosacáridos fueron prácticamente eliminados. En la **figura 4.4D** se observan los distintos DFAs que

presentaron las muestras adulteradas tratadas con levaduras, compuestos que no aparecen de forma natural en las mieles originales (4C).

Otros estudios han demostrado la utilidad de la *Zymomonas mobilis* para eliminar selectivamente glucosa, fructosa y sacarosa presentes en mezclas de oligosacáridos comerciales (de inulina, fructosa, maltosa, isomaltosa y gentiobiosa). Dichos carbohidratos se eliminaron completamente tras 12 horas de incubación sin ningún control de pH ni adición de nutrientes (Crittenden y col., 2002).

- Extracción con fluidos supercríticos (SFE)

Se puede definir como fluido supercrítico a aquel gas o líquido que se encuentra en condiciones de temperatura y presión por encima de su punto crítico. Sus propiedades son intermedias entre las de un líquido y un gas: en cuanto a solubilidad, su comportamiento es similar al de un líquido, mientras que presenta una alta difusividad (típica de los gases), que facilita su penetración en la matriz sólida. Como consecuencia, el proceso de extracción con fluidos supercríticos se realiza más rápidamente que con la extracción líquida convencional; además, las condiciones de extracción pueden ser controladas fácilmente.

El dióxido de carbono supercrítico ha sido muy utilizado como disolvente ya que combina una baja viscosidad, una alta capacidad de difusión y una elevada volatilidad; asimismo, permite trabajar bajo condiciones de extracción suaves evitando la degradación de productos biológicos. Sin embargo, la solubilidad de los carbohidratos en dióxido de carbono supercrítico es baja (Yau y col., 1994) pero aumenta notablemente cuando se añaden modificadores polares que aumenten la polaridad del disolvente (Dohr y col., 1993; Raynie, 2010). Son pocos los estudios que se han llevado a cabo para el fraccionamiento de carbohidratos mediante SFE. D´Souza y Teja (1988) optimizaron un método para la separación de glucosa y fructosa empleando dióxido de carbono supercrítico y etanol, mientras que Montañés y col. (2006 y 2008) han usado esta técnica para la separación de mezclas binarias de azúcares como galactosa tagatosa y lactosa lactulosa usando CO_2 supercrítico con un co-disolvente basado en isopropanol:agua y etanol:agua, respectivamente. Asimismo, estos mismos autores han aplicado la SFE a una muestra compleja de carbohidratos (Duphalac®)

consiguiendo una extracción selectiva de las cetosas frente a las aldosas presentes en la muestra (Montañés y col., 2008).

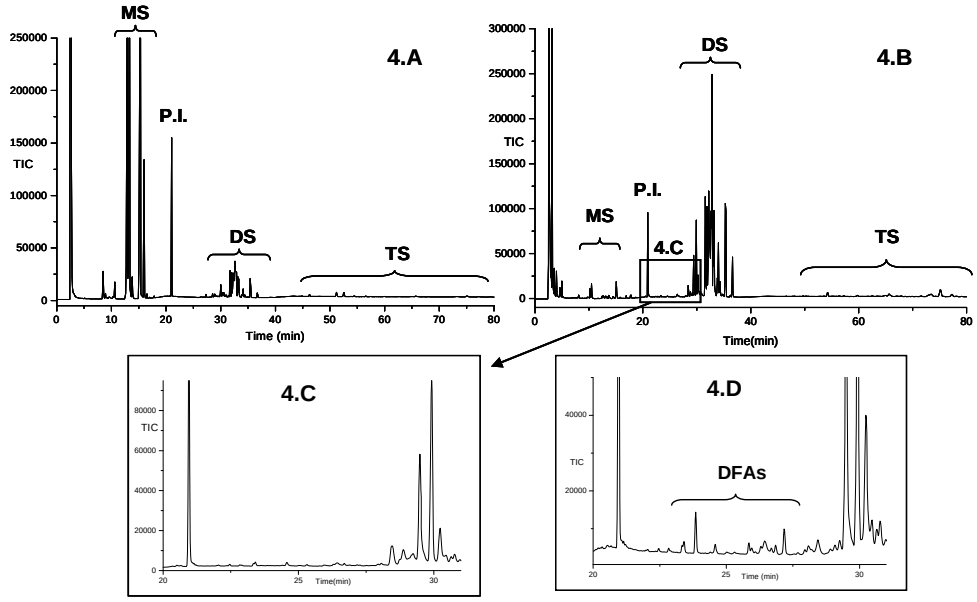


Figura 4.4. Perfil cromatográfico obtenido para los TMSO derivados de los carbohidratos de una miel de néctar antes (4A) y después de ser incubada con S*. cerevisiae* (4B), donde MS: monosacáridos, DS: disacáridos, TS: trisacáridos y P.I.: patrón interno. Ampliada, aparece la zona anterior a los disacáridos de la miel (4C) y la miel adulterada con un jarabe comercial (4D), ambas tratadas con levaduras. DFAs: anhídridos de difructosa. Modificado con permiso de American Chemical Society.

- Extracción presurizada con disolventes (PLE)

Esta técnica de extracción se basa en el empleo de disolventes a altas temperaturas de extracción y a altas presiones (siempre por debajo del punto crítico), de forma que el disolvente permanezca en estado líquido durante todo el proceso de extracción. Es significativamente más rápida que las técnicas de extracción tradicionales, proporcionando rendimientos de extracción mayores, y empleando además, menores volúmenes de disolvente (Smith, 2003). Al igual que la SFE, la PLE permite un fácil escalado por lo que es de gran interés para su uso en la industria. Esta ventaja se hace aún más interesante si se emplean como disolventes de extracción aquellos considerados “seguros”, también denominados disolventes GRAS, (Generally

Recognized As Safe), entre los cuales se encuentran el etanol y el agua. El empleo de estos disolventes está cobrando cada vez más importancia en la industria alimentaria (Burdock y col., 2006).

El equipo que se encuentra comercialmente disponible está registrado con el nombre de ASE ("*Accelerated Solvent Extraction*", Extracción Acelerada con Disolventes). Sin embargo, sobre este esquema básico de funcionamiento, han surgido diferentes variantes correspondientes a varios diseños de laboratorio incluyendo, por ejemplo, depósitos y bombas especificas para disolventes de lavado (Luthje y col., 2005; Ong y col., 2003), así como prototipos de célula con montaje de laboratorio (Ramos y col., 2006).

Esta técnica ha sido muy utilizada para el estudio de contaminantes en alimentos (Ashizuka y col., 2008; Liem, 1999; Obana y col., 1997) pero existen muy pocas aplicaciones para compuestos muy polares. Recientemente, se ha conseguido la extracción de lactulosa de una mezcla con lactosa utilizando como disolvente una disolución etanol:agua 70:30 a 40 ^{o}C y 1500 psi (Ruiz-Matute y col., 2007). Posteriormente, esta técnica se ha aplicado a la extracción selectiva de los azúcares de la miel mediante su combinación con la extracción con carbón activo, consiguiendo obtener fracciones ricas en oligosacáridos y con altos valores de recuperación. Estos resultados fueron comparados con los obtenidos mediante el fraccionamiento con carbón activo y con la utilización de *S. cerevisiae* mostrando ventajas en cuanto a reducción de tiempo de extracción, menores volúmenes de disolventes, menor manipulación de la muestra y mayor conservación del perfil de los di- y trisacáridos (Ruiz-Matute y col., 2008).

Cuando el disolvente empleado para la extracción es agua sometida a altas temperaturas (por encima de su punto de ebullición y por debajo de su temperatura crítica) y a presiones suficientes para que permanezca en estado líquido, a este proceso se le denomina Extracción con Agua Subcrítica (SWE), Extracción con Agua Sobrecalentada (SHWE) o Extracción con agua caliente Presurizada (PHWE). Se produce así un cambio de la constante dieléctrica del agua, permitiendo la extracción de compuestos que no podrían ser extraídos a presión atmosférica. Sin embargo, la

extracción con SWE ha sido poco utilizada para el fraccionamiento de carbohidratos debido a que el agua sobrecalentada puede actuar como un catalizador ácido-base muy efectivo (Jin y col., 2004), causando la degradación de los azúcares y la formación de hidroximetilfurfural (HMF) y otros compuestos asociados. A pesar de ello, se han intentado algunos fraccionamientos de azúcares procedentes de hidrolizados de hemicelulosa (Tiihonen y col., 2005) y de fibra de maíz (Allen y col., 2001). El empleo de mezclas de agua con disolventes orgánicos (metanol, etanol) a temperaturas controladas parece más prometedor para evitar degradaciones.

4.1.1.1.2. Conclusiones

Los carbohidratos están entre los compuestos más abundantes y diversos que se encuentran ampliamente distribuidos en la naturaleza por lo que presentan un interés especial para un gran número de áreas, tales como tecnología de alimentos, biotecnología, farmacia o medicina, entre otras. En consecuencia, su fraccionamiento permite una serie de ventajas para su análisis o posterior utilización.

La elección de la técnica de fraccionamiento de carbohidratos más adecuada depende de las características químicas y físicas de éstos, tales como: peso molecular, solubilidad, carga neta y capacidad de adsorción, además de la utilidad final de los mismos. En algunos casos, es importante que al fraccionar los carbohidratos se conserve su estructura inicial, ya que un simple cambio puede influir, drásticamente, en su uso final, por ejemplo cuando son utilizados en función de su bioactividad, como en el caso de oligosacáridos prebióticos, almidones resistentes que actúan como fibra dietética o carbohidratos que participan en la anti-adherencia de microorganismos patógenos en animales superiores. En otros casos es más importante obtener una pureza elevada de los carbohidratos de interés aunque el rendimiento obtenido sea relativamente bajo.

Por la alta complejidad de las matrices en las que pueden encontrarse los carbohidratos y la similitud estructural de los mismos, es difícil conseguir un fraccionamiento completo (alta pureza y alto rendimiento) empleando una sola

técnica, por lo que en ocasiones es necesario el uso combinado de varias de éstas con el fin de obtener los carbohidratos de interés.

La tendencia actual en este campo, al igual que para otras áreas, se basa en el desarrollo de técnicas que permitan fraccionamientos adecuados mediante procesos rápidos, económicos y seguros para el medio ambiente. A pesar de que se emplean técnicas novedosas tales como PLE o SFE, las técnicas tradicionales tales como SEC, IEC o el empleo de membranas son las que actualmente se imponen tanto en investigación como en la industria. En consecuencia, es indispensable un aumento de la investigación orientado al desarrollo de estas técnicas de fraccionamiento o en su defecto, la creación de otras que faciliten la separación y purificación de carbohidratos.

4.1.2. Comparison of fractionation techniques to obtain prebiotic galactooligosaccharides

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Abstract

The use of four different fractionation techniques for obtaining galactooligosaccharides (GOS) free from digestible carbohydrates was compared. Diafiltration did not show any selectivity among mono-, di- and oligosaccharides, whereas yeast treatment allowed a high recovery of GOS and disaccharides. Activated charcoal treatment showed a different selectivity in GOS recovery depending on the concentration of ethanolic water solutions used (1-15%). Ethanolic solutions of 8% led to a high recovery of GOS (~90%), but 20% of disaccharides were also recovered, whilst solutions with 10% ethanol allowed almost complete removal of disaccharides, but only ~53% of GOS trisaccharides were recovered. Finally, the purest GOS fractions (degree of polymerization up to 8) were obtained using size exclusion chromatography.

4.1.2.1. Introduction

Galactooligosaccharides (GOS) are non-digestible carbohydrates formed by galactose monomers and a unit of terminal glucose. GOS have been considered as prebiotics as they are “selectively fermented ingredients that allow specific changes, both in the composition and / or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” (Gibson, et al., 2004). GOS have been shown in both *in vivo* and *in vitro* studies to stimulate growth of species from *Bifidobacterium* and *Lactobacillus* genera (Macfarlane, et al., 2008, Sako, et al., 1999). These bacteria have the ability to metabolize prebiotic carbohydrates and produce short chain fatty acids (SCFA). It has been reported that some SCFA can be a source of energy for

different body tissues, diminish the cancer colon risk in animal models and have antagonistic properties to detrimental bacteria in the intestinal environment (Macfarlane, et al., 2006, Morishita, et al., 2002, Pool-Zobel, 2006). As a result, dietary GOS are being increasingly used in a wide range of functional foods such as infant foods, functional dairy products or fruit-based drinks.

GOS are industrially produced by transgalactosidation reactions using β-galactosidases (Kimura, et al., 1995, Sako, et al., 1999) from lactose, leading to a final product comprising oligosaccharides with a degree of polymerization (DP) of up to 8-9, and high amounts of mono- and disaccharides. In this sense, the removal of mono- and disaccharides fractions from GOS mixture is necessary to both evaluate their functional properties, e.g. *in vitro* prebiotic activity, and determine their structures. On the other hand, the removal of these carbohydrates could help to expand the applications of the purified GOS in the food and pharmaceutical industries. Among these applications could be the inclusion of purified GOS as ingredients in: *i*) diabetic foods, which could allow diabetic people to utilize the beneficial properties of GOS free from carbohydrates that increase the level of postprandial glucose; *ii*) specialized foods for individuals intolerant to lactose; and *iii*) low calorie foods with a reduction of some mono- and disaccharides.

Fractionation of oligosaccharides has been previously carried out following different techniques. Size exclusion chromatography (SEC) is a separation technique widely used for the fractionation of carbohydrates based on their DP. Fransen et al. (1998) used two connected SEC columns maintained at 60 ºC to fractionate a GOS mixture which did not initially contain digestible carbohydrates. Tzortzis et al. (2005) also used SEC with Bio-Gel P2 to fractionate GOS and to assay the effect of these carbohydrates over the adhesion of detrimental microorganism on HT29 cells. Shoaf et al. (2006) and Huebner et al. (2007) used SEC with Sephadex G-10 to purify GOS from a commercial product with 45% digestible carbohydrates to further study their anti-adhesive and prebiotic activities, respectively. However, no detailed analytical results were shown in these studies about GOS fractionation.

Cellooligosaccharides have also been fractionated based on their DP using different chromatographic techniques, mainly because of their efficacy and cost (Akpinar, et al., 2008). Waniska and Kinsella (1980) separated oligosaccharides from corn starch hydrolyzates using SEC, ultrafiltration and charcoal-celite chromatography, concluding that the charcoal–celite chromatography separated oligosaccharides more effectively than SEC or ultrafiltration. Recently, yeast treatment has been succesfully used by Goulas et al. (2007) to remove glucose from a mixture obtained by synthesis of GOS without any adverse effect on the GOS content. These authors observed that the glucose was almost completely converted to ethanol and CO_2, whereas the galactose present in the mixtures was only slightly reduced.

Although different methodologies have been proposed to fractionate GOS, to the best of our knowledge, a comparative study to determine which of these techniques can be considered the most appropriate to obtain a final product free from mono- and disaccharides has not been reported before. Therefore, the purpose of this study was to compare four fractionation techniques (diafiltration, yeast treatment, activated charcoal adsorption and SEC) to purify prebiotic GOS from a commercial source containing a high level of digestible carbohydrates.

4.1.2.2. Materials and methods

- *Standards and reagents*

All reagents were of analytical grade. Glucose, lactose, maltotriose, maltotetraose, raffinose, phenyl-β-D-glucoside, hexamethyldisilazane, hydroxylamine hydrochloride, acetic acid and trifluoroacetic acid were from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA). Sulphuric acid was acquired from Panreac (Barcelona, Spain), anthrone from Fluka (Madrid, Spain), ethanol and methanol from Prolabo (Paris, France) and pyridine from Merck (Darmstadt, Germany).

GOS were obtained from Vivinal-GOS®, kindly provided by Friesland Foods Domo (Zwolle, The Netherlands). This product has a 73 wt.% dry matter of which, 60 wt.% were GOS, 20 wt.% lactose, 19 wt.% glucose and 1 wt.% galactose.

- *Fractionation techniques*

All experiments were carried out in triplicate.

-Diafiltration

A laboratory scale diafiltration unit Model 8400 (Millipore Corp., Bedford, MA, USA) was used. Two cellulose acetate membranes (Millipore Corp.) with a molecular weight cut-off (MWCO) of 500 Da and 1,000 Da, were tested. The membranes were soaked for 12 hours in nanofiltered water in order to remove any interfering compounds such as sodium azide or glycerol. Each sample (300 mL, Vivinal-GOS® 5% w:v) was filtered at a pressure of 200 KPa. Samples of permeate and retentate (5 mL) were taken every 5 hours for further analysis.

-Yeast treatment

Vivinal-GOS® (5 g) was dissolved in 25 mL of nanofiltered water and incubated at 37 °C for 24 h with 250 mg *Saccharomyces cerevisiae* yeast (Maizena™, Unilever, Barcelona, Spain). Samples were taken at 2, 5, 10 and 24 hours, filtered through a 0.22 µm pore membrane (Millipore Corp., Bedford, MA, USA) and stored at -20 °C until further analysis.

-Activated charcoal treatment

The separation of mono- and disaccharides from GOS was carried out following the method of Morales et al. (Morales, et al., 2006) with some modifications. In brief, 500 mg of Vivinal-GOS® and 3 g of activated charcoal, Darco G60, 100 mesh (Sigma-Aldrich), were dissolved in 100 mL of different ethanolic solutions (1, 5, 8, 10 and 15 % v:v), in order to test the optimal concentration of adsorption of oligosaccharides and desorption of mono- and disaccharides, and stirred for 30 min. This mixture was filtered through Whatman N° 1 paper (Whatman International Ltd., Maidstone, UK) under vacuum and the activated charcoal was washed with 25 mL of corresponding ethanolic solution.

Desorption of oligosaccharides from the activated charcoal was carried out with 100 mL of ethanol 50% v:v. The mixture was stirred for 30 min and filtered as

previously described. The filtrates were evaporated under vacuum at 40 °C, dissolved in 5 mL of deionized water and filtered through 0.22 µm filters (Millipore Corp).

-Size exclusion chromatography

SEC was carried out using Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 x 1.6 cm), equilibrated with water and maintained at 4 °C. Each assay was run with 5 mL of Vivinal-GOS® (25 % w:v) and eluted with nanofiltered and degassed water at a flow of 0.25 mL min^{-1}. After the elution of the void volume (170 mL), 60 fractions of 5 mL were collected. The presence of carbohydrates in each fraction was assessed using the anthrone-sulfuric acid method (Roe, 1955). Qualitative determination of carbohydrate DP was carried out by electrospray ionization mass spectrometry (ESI-MS) as explained below. Fractions of the same DP were pooled and freeze dried for further analyses.

- *Analytical techniques*

-Gas chromatography-mass spectrometry

Mono-, di-, tri, and tetrasaccharides were quantified by gas chromatography-mass spectrometry (GC-MS) using a two-step derivatization: oximation and trimethylsilylation. Samples from SEC (500 µL) and 25 µL of yeast and active charcoal treated samples, respectively, were mixed with 350 µL of phenyl-β-D-glucoside (1 mg mL^{-1}) and raffinose (1 mg mL^{-1}), used as internal standards, and evaporated under vacuum. Sugar derivatives were formed using 350 µL of hydroxylamine hydrochloride in pyridine (2.5 % w:v) at 75 °C for 30 min; the oximes formed were silylated using 350 µL of hexamethyldisilazane and 35 µL of trifluoroacetic acid at 45 °C for 30 min. The samples were centrifuged at 5652 *xg* for 10 min and 1 µL of the supernatant was injected in the GC-MS.

GC-MS analyses were carried out using a HP-6890 chromatograph with an MD 5973 quadrupole mass detector (Hewlett-Packard, Palo Alto, CA, USA) and helium as carrier gas, operating at 1 mL min^{-1}. Injections were made in split mode, with a split ratio of 1:20. Injector and detector temperatures were 350 °C. Carbohydrates were

separated using an HT5 column (25 m x 0.22 µm id x 0.1 µm df) from SGE (SGE Europe, Milton Keynes, UK), coated with 5% phenyl polysiloxane-carborane. The temperature program used was proposed by Sanz et al. (Sanz, et al., 2004) with some modifications: the oven temperature was held at 200 °C for 15 min, then programmed to 270 °C at a heating rate of 15 °C min^{-1}, then programmed to 290 °C at 1 °C min^{-1} and finally programmed to 350 °C at 15 °C min^{-1} and held for 40 min. Chromatographic peaks were analyzed using a HPChem Station software (Hewlett-Packard). All analyses were carried out in triplicate.

Quantitative data for carbohydrates were calculated using internal standards. Standard solutions of glucose, lactose, maltotriose and maltotetraose over the expected concentration range in GOS were prepared to calculate the response factor relative to internal standards.

-Electrospray ionization mass spectrometry

ESI-MS was performed on an Agilent 1100 series (Hewlett-Packard) with a quadrupole mass detector equipped with an ESI source. Samples (20 µL) were injected by direct infusion and carried through in a 0.1% v:v acetic acid:methanol (50:50 v:v) eluent at a flow of 0.2 mL min^{-1}. The electrospray ionization was operated on positive mode, scanning from *m/z* 100 to 1400. Nebulizing gas (N_2) pressure was 276 KPa. Nitrogen drying gas was used at a flow rate of 12 L min^{-1} and 300 °C. The capillary voltage was 4000 V.

- *Statistical analysis*

Data were analyzed using a one-way analysis of variance (ANOVA) procedure followed by an LSD test as a post hoc comparison of means ($P < 0.05$). All data were expressed as means with standard deviations. The software Statistica for Windows version 6 (2002) by Stasoft Inc. (Tulsa, OK, USA) was used.

4.1.2.3. Results and discussion

The selection of the fractionation techniques used in this study (diafiltration, yeast treatment, activated charcoal adsorption and SEC) was based on the application of mild conditions with the aim of obtaining GOS with unaltered structure, as some processing conditions may affect prebiotic activity of carbohydrates (Huebner, et al., 2008). The GC-MS profile of the initial GOS mixture obtained under the conditions indicated in the materials and methods section allowed the detection and quantification of carbohydrates up to DP 4. The monosaccharides glucose and galactose, together with lactose and other minor disaccharides were identified in the sample (data not shown). High heterogeneity was observed for di-, tri- and tetrasaccharides, showing multiple peaks corresponding to the different links between galactose and glucose units.

- *Diafiltration*

Two membranes with different MWCO, 500 and 1,000 Da, were used. Neither of them were selective to retain GOS, and after five hours of diafiltration, the permeate contained 100% of mono-, di- and oligosaccharides from the initial GOS mixture (data not shown). Previous studies have reported on the fractionation of oligosaccharides using this technique, although ambiguous results were obtained. Martinez-Ferez et al. (2006) used two multi-channel tubular ceramic membranes with MWCO of 50 kDa and 1 kDa to isolate oligosaccharides from goat milk. A recovery of more than 80% of the original content of oligosaccharides was obtained using the 1 kDa membrane. Montilla et al. (2006) and van de Lagemaat et al. (2007) successfully separated fructooligosaccharides of DP ≥ 4 from fructose, glucose, sucrose and trisaccharides by diafiltration with a cellulose acetate membrane with MWCO of 1 kDa. Goulas et al. (2003) used three different membranes, two nanolfiltration membranes and another one of cellulose acetate with MWCO of 1 kDa. High proportions of oligosaccharides were lost in the permeate using the last membrane, which is in good agreement with our results, whilst the nanofiltration membranes allowed the recovery of 88% of oligosaccharides and 19% of monosaccharides. Overall, these results demonstrated that not only the molecular weight of the carbohydrate and the MWCO

of the membrane are fundamental parameters for diafiltration, but also the thin film membrane composition, and shape and polarities of the carbohydrates to be ultrafiltered are essential to their fractionation (Goulas, et al., 2007, Newcombe, et al., 1997). In none of these studies could a pure oligosaccharide fraction free from non-digestible carbohydrates be obtained using this technique.

- *Yeast treatment*

Figure 4.5 shows the results obtained during the incubation of the initial GOS mixture with *Saccharomyces cerevisiae* at 37 °C for 0, 2, 5, 10 and 24 hours. The concentration (mg carbohydrate g^{-1} initial GOS mixture) of monosaccharides (glucose and galactose) progressively decreased during the time of incubation, and they were completely fermented by the yeast after 10 hours of treatment due to their conversion into ethanol and CO_2 during the anaerobic glycolysis (Goulas, et al., 2007, Yoon, 2003). Yoon et al. (2003) reported similar results using carbohydrate solutions of glucose and galactose standards. However, Goulas et al. (2007) only found a slight reduction of galactose during the incubation of GOS with *Saccharomyces cerevisiae*, and this monosaccharide was not entirely fermented. These authors concluded that the level of ethanol produced could inhibit the assimilation of galactose by the yeast cells. The GOS concentration used in that work was higher than in our study and, consequently, the level of ethanol produced under our conditions would not be toxic to yeast, allowing it to completely ferment galactose.

No significant differences occurred in the disaccharide fraction during the first 10 hours, whereas an increment in the concentration and recovery of these carbohydrates at 24 hours of incubation was observed (**Figure 4.5**). This increase was due to the capacity of the yeast to synthesize trehalose (Jules, et al., 2004) as this disaccharide was identified by GC-MS analysis of the sample (data not shown). The incapacity of *S. cerevisiae* to remove the disaccharides present in the initial GOS mixture is a consequence of the absence of enzymes able to hydrolyze carbohydrates with β-linked galactose and glucose. For this reason, tri- and tetrasaccharides were also not hydrolyzed by yeast enzymes (**Figure 4.5**).

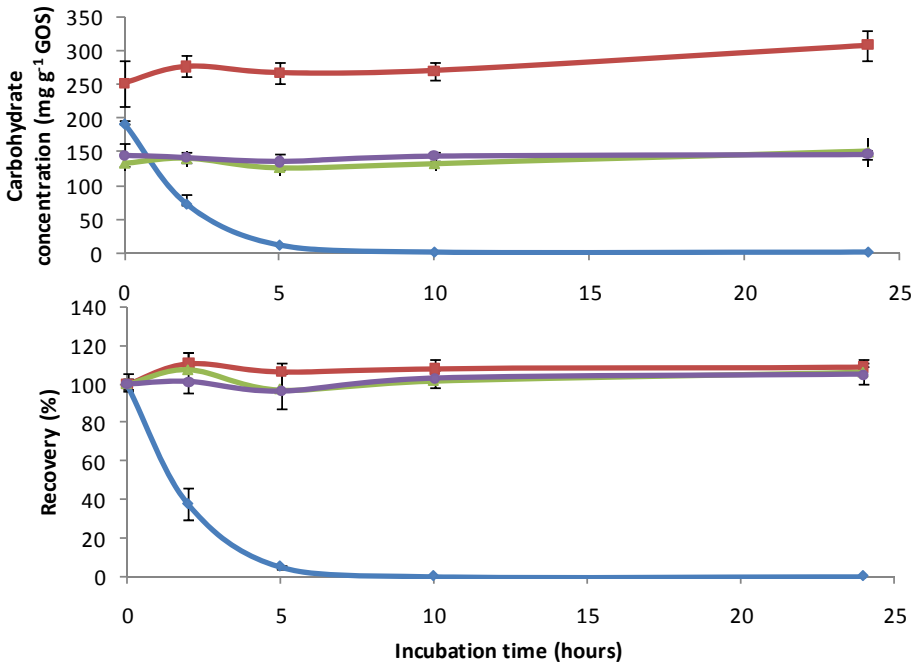

Figure 4.5. Concentration of carbohydrates (mg g^{-1} initial GOS mixture) determined by gas chromatography-mass spectrometry (a) and percentages recovered from each group (b) after yeast treatment. Monosaccharides, ♦; disaccharides, ■; trisaccharides ▲; tetrasaccharides, •; error bars represent standard deviation (n = 3).

- *Activated charcoal treatment*

In order to take a further step towards fractionation of GOS, different concentrations of ethanolic water solutions (1, 5, 8, 10 and 15 % v:v) were studied to remove mono- and disaccharides, whereas GOS of higher DP were recovered using ethanol 50% v:v. To the best of our knowledge, no data regarding the optimization of fractionation conditions of GOS using a simple and economical charcoal treatment, not requiring the use of graphitized cartridges or columns, have been published to date. **Figure 4.6** shows the concentration of carbohydrates and the recovered amounts from each carbohydrate group after charcoal treatment. Only minor amounts of monosaccharides were found after using 1% (v:v) ethanol followed by 50% (v:v) ethanol, whereas di-, tri- and tetrasaccharides were mostly recovered. Treatments

with 5%, 8%, 10% and 15% (v:v) ethanol allowed a recovery of disaccharides after desorption of 52.0, 21.4, 6.5 and 4.0%, of GOS, respectively. Regarding the recovery of GOS trisaccharides, the 10% and 15% (v:v) ethanol treatments were not selective enough and substantial amounts of trisaccharides were lost (**Figure 4.6**) in accordance with the results obtained by Morales et al. (2006) for maltodextrins. In contrast, the 5% and 8% (v:v) ethanol treatments allowed the recovery of ~90% of GOS trisaccharides. Finally, it is noteworthy to mention that GOS tetrasaccharides were mostly recovered regardless of the concentration of ethanol assayed (**Figure 4.6**).

In consequence, the selection of the most suitable treatment should be done depending on the finality of the fractionation, i.e. purity of the fractions or recovered amount. Although high amounts of trisaccharides are recovered with ethanol 8% v:v, the presence of disaccharides is also notable (21.4%). However, a 10% v:v ethanol treatment allowed a lower recovery of trisaccharides but also a higher degree of purity of the high molecular weight GOS fraction (DP 3-8). Therefore, to further evaluate GOS prebiotic activity in vitro, the 10 % v:v treatment was chosen as the optimum condition to obtain a good recovery and concentration of GOS with less disaccharides.

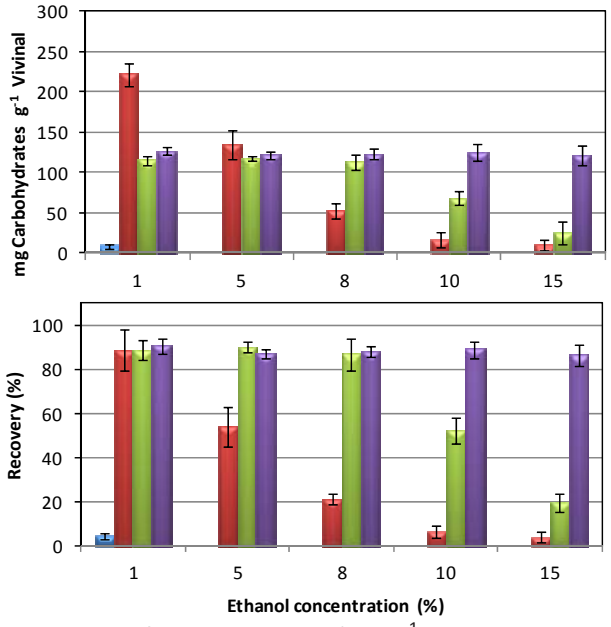

Figure 4.6. Concentration of carbohydrates (mg g^{-1} initial GOS mixture) determined by gas-chromatography-mass spectrometry (a) and percentage recovered from each group (b) after desorption from activated charcoal using ethanol (from 1 to 15 % v:v) to remove digestible carbohydrates. Monosaccharides, ■; disaccharides, ■; trisaccharides, ■; tetrasaccharides, ■; error bars represent standard deviation (n = 3).

- *Size exclusion chromatography*

The method described in this study was able to separate completely pure fractions of mono-, di-, tri-, tetra- and pentasaccharides, whereas hexa-, hepta- and octasaccharides were obtained with a high degree of purity (**Figure 4.7**). Furthermore, it also allowed us to obtain GOS with a high yield as the recovery values were between 81 and 92% (**Table 4.1**). Therefore, SEC fractionation could be especially useful when production and isolation of GOS with high purity and yield are required for further use. In this sense, the wide industrial and pharmaceutical application of carbohydrates and their polymers or derivatives may require exhaustive determination and separation, therefore, it may be necessary to initially partition sample components using SEC columns.

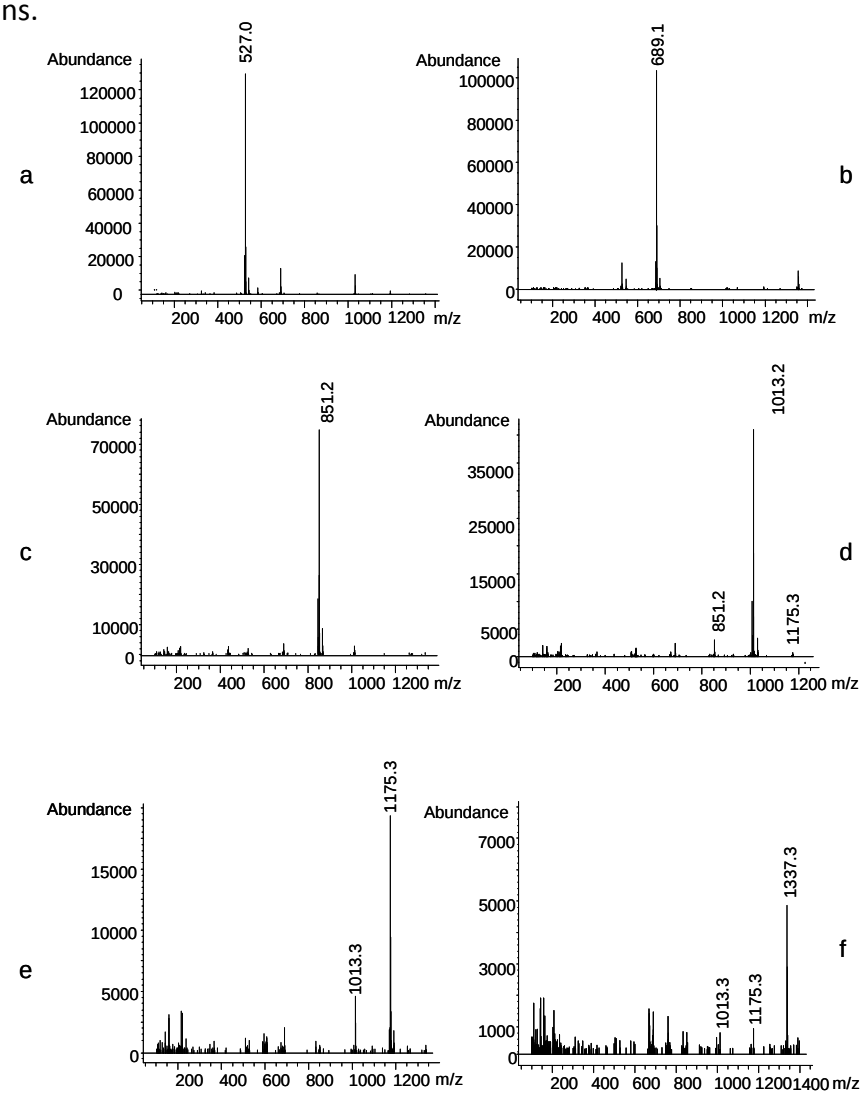

Figure 4.7. Electrospray ionization mass spectrometry profiles of galactooligosaccharides fractioned by size exclusion chromatography: a, trisaccharides; b, tetrasaccharides; c, pentasaccharides; d, hexasaccharides; e, heptasaccharides; f, octasaccharides.

- *Comparison of fractionation techniques*

Table 4.1 summarizes the best conditions chosen to obtain GOS with a high degree of purity and yield from the initial mixture. The highest concentration and recovery of tri- and tetrasaccharides was obtained with yeast treatment. This technique can be used to enrich the sample by elimination of monosaccharides for analytical purposes (Ruiz-Matute, et al., 2007). However, the disadvantages of the yeast treatment for the purification of GOS are the incapacity of the yeast cells to remove the disaccharides, the subsequent elimination of these cells from the sample and the metabolic products formed during the fermentation such as glycerol or trehalose. Nevertheless, Chen et al. (2007) indicated that prebiotic fructooligosaccharides and trehalose enhanced the growth and production of bacteriocins by lactic acid bacteria, suggesting a new potential application of this mixture of carbohydrates in food preservation. Consequently, it could be of interest to further evaluate this effect on the mixture of GOS and trehalose after treatment with *S. cerevisiae*.

The activated charcoal method represents a simple, economical and rapid procedure for the purification of GOS; the recovery of trisaccharides is lower than with yeast treatment but higher proportions of disaccharides (94%) were eliminated (**Table 4.1**). In addition, the activated charcoal method reported in this study is simpler, less time-consuming and less expensive than the traditional charcoal methods based on column or cartridge fractionation and, therefore, it could allow an easier scaling-up to the industry. The main disadvantages of activated charcoal treatments are incomplete removal of disaccharides, as well as the loss of trisaccharides.

SEC presents the best technique to obtain a good recovery of pure GOS fractions with a particular DP and with high yield (**Figure 4.7**). In addition, the permeation gel does not need to be regenerated and the mobile phase is water, which allows the direct use of these fractions for further analysis (e.g. ESI-MS, NMR, etc.). However, SEC is a time consuming technique, the permeation gel is expensive and the sample obtained is relatively dilute.

Table 4.1. Comparison of concentrations (mg g^{-1} initial GOS mixture) and percentage of recovery (in brackets) for each group of carbohydrates obtained at the optimum fractionation conditions for all the studied techniques. Errors are expressed as standard deviations (n=3).

	mg g^{-1} Vivinal-GOS® (% Recovery)			
	Initial GOS mixture[a]	**Yeast[b]**	**Charcoal activated[c]**	**SEC**
Monosaccharides	190.7 ± 4.7* (100)	0.0 (0.0)	0.0 (0.0)	175.4 ± 2.2 (92.3 ± 1.1)
Disaccharides	252.0 ± 34.3 (100)	270.1 ± 12.4 (108.1 ± 5.0)	16.4 ± 1.6 (6.6 ± 0.6)	202.5 ± 10.1 (81.0 ± 4.1)
Trisaccharides	132.8 ± 10.4 (100)	132.2 ± 8.3 (101.7 ± 6.4)	68.0 ± 8.5 (52.3 ± 6.0)	115.6 ± 10.0 (88.9 ± 7.7)
Tetrasaccharides	145.3 ± 16.8 (100)	144.1 ± 6.7 (102.9 ± 4.8)	124.6 ± 1.3 (89.2 ± 0.9)	121.3 ± 6.8 (86.6 ± 4.9)

[a]Original sample of Vivinal-GOS®; [b]Yeast treatment after 10 hours of incubation; [c]Second desorption with ethanol 50 % v:v for charcoal activated treatment after a first extraction with ethanol 10 % v:v; *Deviation standard

4.1.2.4. Conclusions

Our results show the feasibility of using yeast or activated charcoal (with 1% ethanol) treatments to selectively remove monosaccharides present in commercial GOS mixtures. Yeast treatment could be used when only the elimination of monosaccharides is required and disaccharides and metabolic products do not affect the purpose of the fractionation. In addition, the activated charcoal treatment with 10% ethanol was shown to be a rapid method to obtain considerable amounts of GOS (at gram levels) of high purity with no presence of mono- and disaccharides as lactose. Therefore, the mono- and disaccharides–free GOS obtained could have potential application as a prebiotic ingredient in diabetic or dietetic foods. Finally, SEC was the most appropriate method to obtain fractions with high purity and recovery, enabling the purification of GOS with different DP. This fact can be particularly important for further investigations focused on the study of the effect of the chain size on the GOS functionality.

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4.1.3. Characterization of galactooligosaccharides derived from lactulose

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Abstract

Galactooligosaccharides are non-digestible carbohydrates with potential ability to modulate selectively the intestinal microbiota. In this work, a detailed characterization of oligosaccharides obtained by transgalactosylation reactions of the prebiotic lactulose, by using β-galactosidases of different fungal origin (*Aspergillus oryzae*, *Aspergillus acuelatus* and *Kluyveromyces lactis*), is reported. Oligosaccharides of degree of polymerization (DP) up to 6 were characterized and quantified by HPLC-ESI MS from a complex mixture produced by transgalactosylation reaction with *A. oryzae* (GOSLuAo), whereas only carbohydrates up to DP4 and DP5 were detected for those obtained from the reaction with β-galactosidases from *K. lactis* (GOSLuKl) and *A. acuelatus* (GOSLuAa), respectively. Disaccharides (galactosyl-galactoses and galactosyl-fructoses) and trisaccharides were characterised in the three mixtures by GC-MS as their trimethylsilyl oximes. Galactosyl- and digalactosyl-glycerols were produced during the transgalactosylation reaction of lactulose with β-galactosidases from *A. acuelatus* and *K. lactis*, due to the presence of glycerol as enzyme stabiliser.

4.1.4.1. Introduction

Galactooligosaccharides (GOS) are considered non-digestible carbohydrates which are mainly constituted by galactose units and obtained from lactose by the action of β-galactosidases. These enzymes catalyse the hydrolysis of lactose into glucose and galactose, and also the transgalactosylation reactions with lactose as acceptor of galactose units giving rise to GOS of different glycosidic linkages and molecular weights (Otieno, 2010). A number of studies have demonstrated that hydrolysis rates and transgalactosylation pattern of GOS were different and related to

the enzyme source used, substrate concentration and reaction conditions (Cardelle-Cobas, et al., 2008, Cardelle-Cobas, et al., 2008, Chen, 2003, Maugard, et al., 2003, Tzortzis, et al., 2004, Tzortzis, et al., 2003). The beneficial effects of GOS on human gastrointestinal health have been extensively reported, being currently used as pharmaceutical as well as food ingredients (Ito, et al., 1990 , Tanaka, et al., 1983, Torres, et al., 2010).

Lactulose (Gal-β-(1→4)-Fru) is a synthetic disaccharide, produced by isomerization of lactose in basic media or enzyme-catalyzed synthesis, with a significant impact on human digestion (Olano, et al., 2009, Schuster-Wolff-Bühring, et al., 2010). Its physiological action on the colonic motility pattern (Schumann, 2002) and their ability to promote the selective growth of healthy intestinal bacteria, mainly bifidobacteria and lactobacilli populations, in human gut has been extensively reported (De Preter, et al., 2006, Tuohy, et al., 2002). However, its use can be limited due to its laxative effects at high doses and the fact that fermentation occurs mainly in the proximal colon which results in uncomfortable gas production (Tuohy, et al., 2005); as a result, only a reduced percentage of lactulose is likely to reach the distal colon, where most of the digestive disorders take place, and could limit its potential beneficial effects in gut health. It has been hypothesized that non-digestible oligosaccharides of longer degree of polymerization are more slowly fermented so that their metabolism take place more distally in the colon (Rumessen, et al., 1990). Such longer colonic persistence has been linked to enhanced beneficial effects within the gastrointestinal tract, being one of the main current targets in prebiotics development (Rastall, 2010). As a result, oligosaccharides derived from lactulose are currently attracting attention of the scientific community due to their prospective prebiotic applications (Cardelle-Cobas, et al., 2009, Cardelle-Cobas, et al., 2008, Martinez-Villaluenga, et al., 2008). Lactulose oligosaccharides have been obtained by transgalactosylation reactions catalysed by the action of β-galactosidases from different sources, including *Aspergillus acuelatus* (Cardelle-Cobas, et al., 2008) and *Kluyveromyces lactis* (Martinez-Villaluenga, et al., 2008). More recently, it has been reported that these oligosaccharides have the ability to promote the growth of

bifidobacteria in human faecal cultures in a similar way of recognised prebiotic GOS (Cardelle-Cobas, et al., 2009).

Although extensive characterization of GOS derived from lactose has been reported (Cardelle-Cobas, et al., 2009, Coulier, et al., 2009), data regarding GOS composition derived from lactulose are scarce. Up to date, only two trisaccharides [β-D-galactopyranosil-(1→6)-D-galactopyranosyl-(1→4)-fructopyranose and β-galactopyranosyl-(1→4)-[galactopyranosyl-(1→1)]-fructose have been previously identified in oligosaccharide mixtures from lactulose (Martinez-Villaluenga, et al., 2008).

High Performance Liquid Chromatography (HPLC) have been commonly used for the analysis of prebiotic oligosaccharides; different stationary phases such as alkyl-bonded silica, aminoalkyl-bonded silica, graphitized carbon, cation and anion exchange etc. are currently in the market (Sanz, et al., 2009). The use of mass spectrometric (MS) detectors coupled to HPLC systems has considerably enriched the field of carbohydrate analysis.

Gas chromatography-mass spectrometry (GC-MS) is also a suitable technique to determine di- and trisaccharide structures. Trimethylsilyl oximes (TMSO) are commonly used for oligosaccharide analyses considering their volatility, simplicity of preparation and easy data interpretation (Horvath, et al., 1997, Molnar-Perl, et al., 1997). Nevertheless, characterization of oligosaccharides of the same DP in complex mixtures is not an easy task mainly due to the non-availability of commercial standards and the similarity of their structures.

In this study, we report for the first time an extensive GC-MS and HPLC-MS characterization of oligosaccharides obtained from lactulose by transgalactosylation reactions catalysed by β-galactosidases from different fungal species (*Aspergillus oryzae*, *Aspergillus acuelatus* and *Kluyveromyces lactis*). Given that glycosidic linkages, monomeric composition and chain length could affect to their prebiotic properties, this study will reveal critical information to support the relationships between structure and potential prebiotic properties of these novel GOS.

4.1.4.2. Materials and methods

- *Standards*

Analytical standards of fructose, galactose, lactulose (Gal-β(1→4)-Fru), lactose (Gal-β(1→4)-Glc), maltose (Glc-α(1→4)-Glc), maltotriose (Glc-α(1→4)-Glc-α(1→4)-Glc), maltotetraose (Glc-α(1→4)-(Glc-α(1→4))$_2$-Glc), maltopentaose (Glc-α(1→4)-(Glc-α(1→4))$_3$-Glc), maltohexaose (Glc-α(1→4)-(Glc-α(1→4))$_4$-Glc), 1,6-galactobiose (Gal-β(1→6)-Gal), 1,4-galactobiose (Gal-β(1→4)-Gal), 1,3-galactobiose (Gal-β(1→3)-Gal), α,α-threhalose (Glc-α(1→1)-αGlc), α,β-threhalose (Glc-α(1→1)-βGlc) and β,β-threhalose (Glc-β (1→1)-βGlc) were obtained from Sigma (St. Louis, US); leucrose (Glc-α(1→5)-Fru), palatinose (Glc-α(1→6)-Fru), turanose (Glc-α(1→3)-Fru) were obtained from Fluka (Madrid, Spain), maltulose (Glc-α(1→4)-Fru) was from Aldrich Chem. Co. (Milwaukee, WI); trehalulose (Glc-α(1→1)-Fru) was a gift from Dr. W. Wach from Südzucker AG, Manheim; 6´-galactosyl-lactulose (Gal-β(1→6)-Gal-β(1→4)-Fru) was a gift from Dra. Corzo from CIAL-CSIC, Madrid, Spain.

- *Synthesis of galactooligosaccharides from lactulose*

The synthesis of GOS from lactulose was carried out by using optimal conditions previously reported for Lactozym 6500 L (*Kluyveromyces lactis*, GOSLuKl) (Martínez-Villaluenga, et al., 2008) , for Pectinex Ultra (*Aspergillus acuelatus*, GOSLuAa) (Cardelle-Cobas, et al., 2008) and for *Aspergillus oryzae* (GOSLuAO) (Cardelle-Cobas, 2009). Summarily, lactulose was incubated at 50-60 ^{o}C for 2, 7 and 20 h, depending on the enzymatic source. Mixtures were immediately immersed in boiling water for 5 min to inactivate the enzymes. Subsequently, GOS mixtures were treated with activated charcoal to remove monosaccharides following the method of Morales et al. (2006) with some modifications. Briefly, GOS mixtures (4 mL) were diluted with water (200 mL) and stirred with 2.4 g of Darco G-60 100 mesh activated charcoal (Sigma Chemical Co., St. Louis, MO) for 30 min. This mixture was filtered under vacuum and the activated charcoal was further washed with 50 mL of water. Oligosaccharides adsorbed onto the activated charcoal were then extracted by stirring for 30 min in 50

mL of 50:50 (v/v) ethanol:water. Activated charcoal was washed with 5 mL of this ethanol:water solution and subsequently eliminated by filtering through paper as previously described. The sample was evaporated under vacuum at 30ºC.

- *Analyses of galactooligosaccharides from lactulose*

-HPLC-ESI MS

Oligosaccharide analyses were performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard, Palo Alto, CA, USA) provided with an electrospray ionization (ESI) source. Samples (20 µL) were injected using a Rheodyne 7725 valve and separated in a porous graphitic carbon column (Hypercarb® 100 x 2.1 mm; 5 µm; Thermo Fisher Scientific, Barcelona, Spain) at a flow rate of 0.4 mL min^{-1} at 30 ºC. Elution gradient using Milli-Q water: methanol both having 0.1 % NH_4OH was changed from 70:30 (v:v) to 33:67 (v:v) in 27 min, then to 0:100 (v:v) in 7 min and kept for 6 min. Initial conditions were resumed in 2 min and were maintained for 15 min for conditioning. The electrospray ionization was operated under positive polarity using the following MS parameters: nebulizing gas (N_2) pressure 276 KPa, nitrogen drying gas at a flow rate of 12 L min^{-1} and 300 °C and capillary voltage of 4000 V. Mass spectra were acquired in SIM mode using a variable fragmentator voltage by registering the ions corresponding to sodium adducts of oligosaccharides under analysis: *m/z* 203 (monosaccharide), 365 (disaccharide), 527 (trisaccharides), 689 (tetrasaccharides), 851 (pentasaccharides), 1013 (hexasaccharides) and 1175 (heptasaccharides). Data were processed using HPChem Station software version 10.02 (Hewlett-Packard, Palo Alto, CA, USA).

Quantitative analysis was carried out using calibration curves of glucose and maltooligosaccharides (DP2-DP6) as standards in the range 0.001 – 0.01 mg mL^{-1}. Trace *m/z* $[M+Na]^+$ ions of mono-, di-, etc. were independently extracted for their quantification.

-GC-MS

GC analysis was carried out using a two-step derivatization procedure (oximation and trimethylsilylation). Oximes were obtained by addition of 350 µL of a solution 2.5% hydroxylamine chloride in pyridine after 30 min at 75 ºC. Oximes were then silylated with hexamethyldisilazane (350 µL) and trifluoroacetic acid (35 µL) at 45 ºC for 30 min. After reaction, samples were centrifuged at 10,000 rpm for 4 min, and 1 µL of supernatants was injected into the GC injection port.

GC-MS analyses were carried out in a Hewlett-Packard 6890 gas chromatograph coupled to a 5973 quadrupole mass detector (both from Agilent, Palo Alto, CA, USA), using helium as carrier gas. A 22 m x 0.25 mm i.d. x 0.25 µm film thickness fused silica column coated with SPB-1 (crosslinked methyl silicone) from Quadrex Corporation (Woodbridge,US) was used. Oven temperature was held at 200 ºC for 15 min, then programmed to 270 ºC at 15 ºC min^{-1} and programmed to 290 ºC at 1 ºC min^{-1}, and finally programmed to 300 ºC at 15 ºC min^{-1} and kept for 15 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 electronic impact (EI) mode at 70 eV, scanning the 35-700 *m/z* range. Interface and source temperature were 280 ºC and 230 ºC, respectively. Acquisition was done using a HPChem Station software (Hewlett-Packard, Palo Alto, CA, USA).

Identification of TMSO derivatives of carbohydrates was carried out by comparison of their retention indices (I^T) and mass spectra with those of standard compounds previously derivatized. Characteristic mass spectra and data reported in the literature (Cardelle-Cobas, et al., 2009, Ruiz-Matute, et al., 2010) were used for identification of those carbohydrates non available as commercial standards. These identifications were considered as tentative.

4.1.4.3. Results and discussion

- *LC-MS analysis*

HPLC-ESI MS profiles of oligosaccharides obtained by transgalactosylation reactions catalysed by β-galactosidases of *Aspergillus oryzae* (GOSLuAo), *Aspergillus acuelatus* (GOSLuAa) and *Kluyveromyces lactis* (GOSLuKl) are shown in **Figure 4.8**. Notable differences in the oligosaccharide pattern of the three mixtures can be observed. A more complex chromatogram was obtained for GOSLuAo, whereas two major peaks, corresponding to di- and trisaccharides respectively, can be mainly distinguished for GOSLuKl and GOSLuAa.

Table 4.2 shows the percentages of oligosaccharides of different DPs found in these complex mixtures. Oligosaccharides up to DP6 were detected for GOSLuAo, whereas oligosaccharides up to DP4 and DP5 were detected for GOSLuKl and GOSLuAa, respectively. After charcoal treatment, 1% of monosaccharides were still remaining in the three oligosaccharide mixtures. Disaccharides were the most abundant carbohydrates followed by tri- and tetrasaccharides.

Table 4.2. Percentage of oligosaccharides obtained by LC-MS of GOSLu mixtures.

		%		
	m/z	**GOSLuAa**	**GOSLuKl**	**GOSLuAo**
Monosaccharides	**203**	1	1	1
Disaccharides	**365**	78	71	78
Trisaccharides	**527**	15	26	14
Tetrasaccharides	**689**	6	2	5
Pentasaccharides	**851**	tr*	0	2
Hexasaccharides	**1013**	0	0	tr

* tr: traces

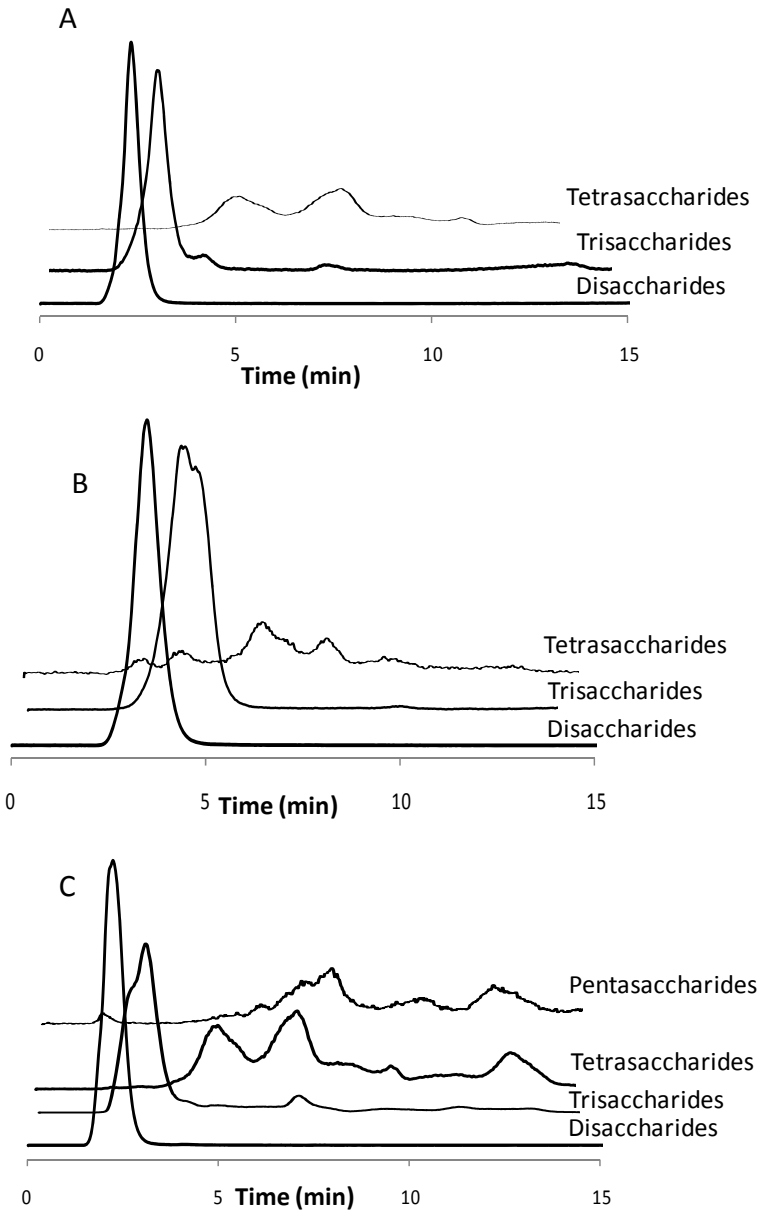

Figure 4.8. LC-MS profile of the GOS mixtures from GOSLuAa (A), GOSLuKl (B) and GOSLuAo (C).

- *GC-MS analysis*

GC-MS analyses (retention indices and characteristic *m/z* ratios) were used to determine the chemical structures of the main carbohydrates (di- and trisaccharides) present in the oligosaccharide reaction mixtures (**Table 4.3**). As an example, **Figure 4.9** shows the GC-MS profile of the TMS oximes of GOSLuAo. In contrast to HPLC method, mono-, di- and trisaccharides were clearly separated. Similar GC profiles were observed for GOSLuAa and GOSLuKl, although relative proportions of individual carbohydrates were different (**Table 4.4**). It is worth noting that reducing carbohydrates gave rise to two peaks corresponding to the TMS oximes of syn (*E*) and anti (*Z*) isomers after derivatization. As previously described, abundances of these peaks are generally found in a ratio close to 1 for those carbohydrates with a free ketose group, whereas this ratio varied from 3 to 7 for those carbohydrates with a free aldose group (Brokl, et al., 2009, Funcke, et al., 1979). Non-reducing carbohydrates showed a single peak corresponding to the octakis-TMS ether. It is also worthy to point out that all the carbohydrates produced in the reactions catalysed by β-galactosidases showed β-glycosidic linkages. The human gastrointestinal tract lacks enzymes with the ability to hydrolyse these glycosidic bonds, with the exception of that corresponding to lactose. Thus, the β-linked galactose is one of the key structural elements that protect the galactooligosaccharides from digestion, having them available for the beneficial bacteria of the human intestine (Moro, et al., 2005).

As previously observed by HPLC analyses, monosaccharides were still detected in the three samples after charcoal treatment. These peaks were identified as fructose (peaks 1 and 2) and galactose (peaks 3 and 4) (**Tables 4.3 and 4.4**).

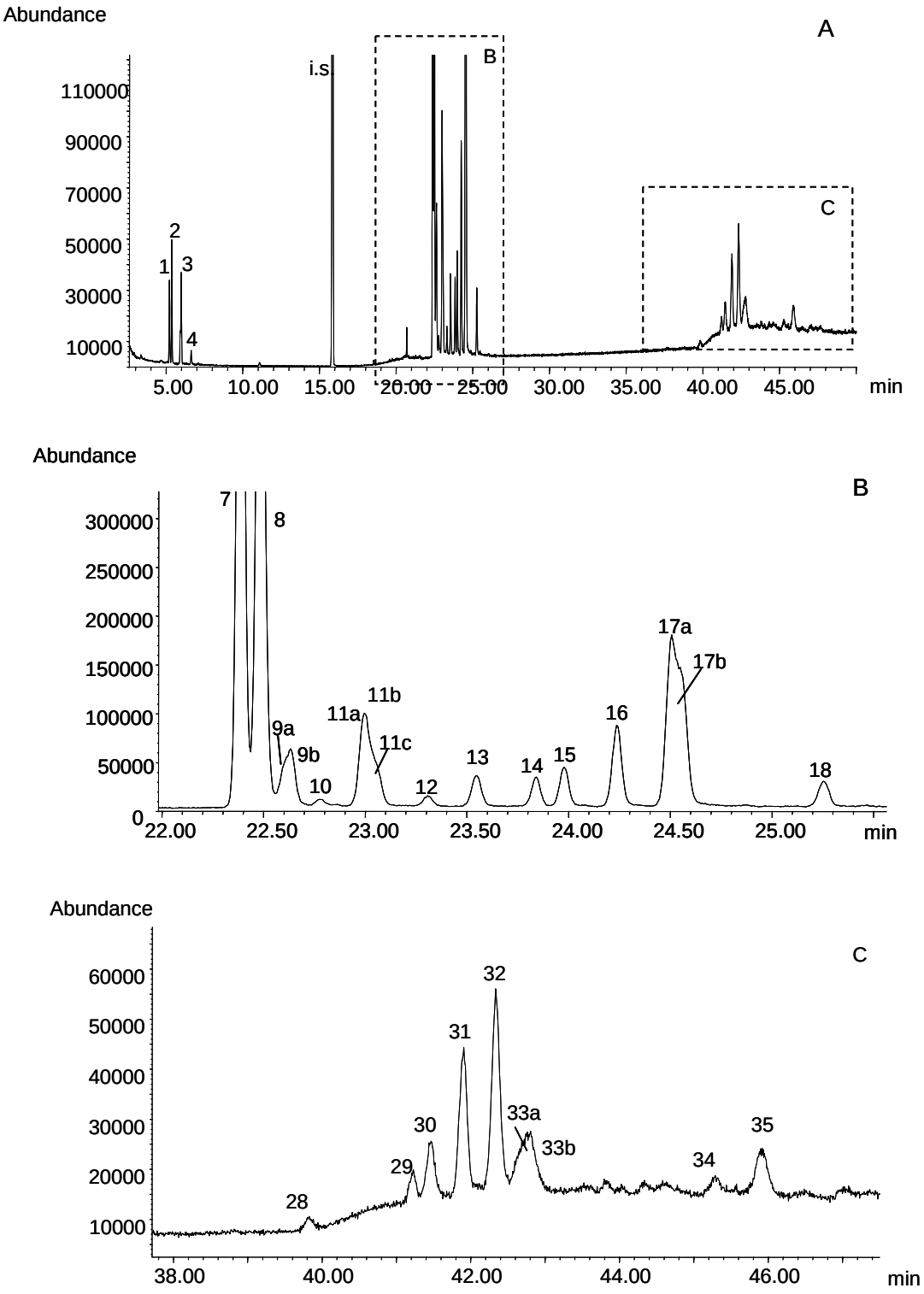

Figure 4.9. GC-MS profile of TMS oxime of mono- (A), di- (B) and trisaccharide (C) fractions of GOSLuAo (A). Peak numbers correspond to those carbohydrates indicated in **Table 4.3**.

Table 4.3. Retention times (t_R), retention indices (I^T), and relative abundance for characteristic *m/z* ratios of carbohydrates of GOSLu mixtures.

Peak number	Identification	t_R	I^T	*m/z ratios*														
				175	**191**	**204**	**205**	**217**	**244**	**305**	**307**	**319**	**334**	**337**	**361**	**422**	**451**	**538**
1+2	fructose	5.3	2002	1	9	4	5	37	-	1	28	1	-	-	-	5	-	-
3+4	galactose	6.03	2042	-	4	8	40	20	-	2	3	54	-	-	-	-	-	-
5	2-b-gal-glycerol	15.09	2347	1	5	100	20	18	-	2	-	1	-	8	3	-	-	-
6	1-b-gal-glycerol	16.89	2397	1	6	100	20	22	1	2	-	1	-	11	3		-	-
7+8	lactulose	22.49	2878-2887	1	22	76	24	37	2	4	1	15	1	-	41	-	1	1
9a	1,1-galactobiose	22.68	2903	2	66	37	14	46	3	3	-	6	-	-	50	-	-	-
9b	1,4-galactobiose *E*	22.71	2905	-	14	68	30	41	3	4	-	17	-	-	49	-	-	-
10	1,5-galactosyl-fructose 1	22.83	2915	-	-	81	32	32	-	10	-	17	-	-	43	-	-	-
11a	1,3-galactobiose *E*	23.03	2932	1	10	51	21	41	6	3	4	14	-	-	38	-	1	-
11b	1,2-galactobiose *E*	23.03	2932	1	22	50	25	44	5	5	13	38	1	-	25	-	1	2
11c	1,5-galactosyl-fructose 2	23.08	2937	-	20	59	17	36	3	4	2	11	-	-	40	-	-	5
12	1,4-galactobiose *Z* + unkown	23.34	2959	-	13	97	35	41	-	6	-	13	-	-	43	-	-	-
13	1,3-galactobiose *Z* – 1,2 galactobiose *Z*	23.58	2979	2	13	47	26	39	6	4	4	22	-	-	40	-	-	-
14	1,6-glucosyl-fructose 1	23.88	3003	-	11	86	22	59	2	4	-	5	2	-	34	3	2	8
15	1,6-glucosyl-fructose 2	24.01	3012	-	21	95	21	57	2	4	1	7	1	-	32	3	2	-
16	1,1-galactosyl-fructose 1	24.28	3029	1	13	56	20	49	1	3	35	7	-	-	9	-		2
17a	1,6-galactobiose *E*	24.55	3046	1	32	67	19	42	2	5	6	7	48	-		1	2	3
17b	1,1-galactosyl-fructose 2	24.59	3049	1	20	54	17	71	2	3	26	5	-	-	13	-		2
18	1,6-galactobiose *Z*	25.3	3094	-	30	65	20	41	2	5	8	8	-	-	53	-	2	-
19	digalactosyl-glycerol	25.93	3135	-	12	100	27	31	-	5	-	-	-	5	16	-		-
20	digalactosyl-glycerol	26.2	3152	-	9	100	22	18	-	3	-	-	-	3	10	-		-
21	digalactosyl-glycerol	27.91	3247	3	5	100	20	20	1	2	2	-	-	2	12	-	1	-
22	digalactosyl-glycerol	28.07	3255	6	5	100	21	23	1	3	1	2	-		14	-	1	-
23	digalactosyl-glycerol	28.41	3271	1	10	100	20	22	1	3	1	1	-	5	7	-	1	-
24	digalactosyl-glycerol	29.06	3303	-	22	100	27	65	5	-	-	5	-	15	36	-		-
25	digalactosyl-glycerol	29.6	3330	1	12	100	21	26	1	-	1	-	-	7	8	-	1	-
26	digalactosyl-glycerol	29.72	3336	1	12	100	20	26	1	3	1	2	-	7	9	-	1	-
27	digalactosyl-glycerol	29.96	3347	3	5	100	20	24	1	3	-	2	-		13	-	1	-
28	trisaccharide	39.92	3734	-	25	100	25	42	-	6	-	14	-	-	45	-	3	-
29	trisaccharide	41.32	3785	-	18	100	25	41	-	4	-	8	-	-	39	-	4	-
30	trisaccharide	41.58	3794	-	13	100	25	31	2	4	1	6	1	-	28	-	2	2
31	6' galactosyl-lactulose 1	42.03	3809	1	21	100	27	39	2	5	1	11	1	-	38	-	3	0
32	6' galactosyl-lactulose 2	42.51	3826	1	14	100	27	40	2	4	1	6	1	-	34	-	3	3
33a	trisaccharide	42.81	3835	1	19	100	31	52	2	3	6	2	1	-	39	-	2	5
33b	1,4-galactosyl-[1,1-galactosyl]-fructose	42.98	3841	1	18	100	28	46	2	3		8	-	-	30	-	2	1
34	trisaccharide	45.4	3923	-	22	100	43	70	-	-	29	-	-	-	35	-	-	-
35	trisaccharide	46.06	3945	-	39	100	33	57	-	-	11	-	-	-	50	1	-	-

- *Disaccharide fraction characterization*

Figure 4.9.B shows the disaccharide profile of GOSLuAo. Twelve peaks with I^T values from 2878 to 3094 were detected (**Table 4.3**). Lactulose (peaks 7 and 8) was identified by comparison of their I^T values and mass spectra with those of the corresponding standard.

Peak 9a showed a relationship of *m/z* 191: 204: 217 ions of 1.3: 1: 1.3 similar to that of trehaloses and characteristic of non-reducing sugars with 1→1 glycosidic linkages. Therefore, this peak could be assigned to 1,1-galactobiose. Peak 9b was identified as 1,4-galactobiose *E* by comparison with the commercial standard.

Peak 10 showed a small abundance and it could not be identified by its mass spectra. However, this peak was not present in the oligosaccharide mixtures of lactose with β-galactosidases (Cardelle-Cobas, et al., 2009) and it could correspond to an isomer of a galactosyl-fructofuranose with a 1→5 glycosidic linkage.

Peak 11 was constituted by a mixture of three carbohydrates, as it could be deduced from the traces corresponding to several characteristic ions. Peak 11a could be assigned to 1,3-galactobiose *E* previously identified in the transgalactosylation reaction products of lactose with β-galactosidases (Cardelle-Cobas, et al., 2009). This carbohydrate showed a relatively high ratio of *m/z* 205/204 ions and relative high abundance of *m/z* 244 ion. Presence of *m/z* 307 ion was also distinguished. This spectrum was also similar to that of α-D-galactopyranosyl-(1→3)-D-galactose standard (as well as to those of laminaribiose and nigerose) and can be considered characteristic of glycosidic linkage 1→3. Peak 11b was identified as 1,2-galactobiose *E* considering the high abundance of *m/z* 319 ion corresponding to the loss of a TMSOH group from the chain C3-C4-C5-C6 of a hexose residue and therefore, characteristic of 1→2 glycosidic linkages (Sanz, et al., 2002). Trailing edge (peak 11c) displayed a spectrum without characteristic ions which was compatible with the second isomer of the 1→5 galactosyl fructofuranose.

Peak 12 corresponded to the second peak of 1,4-galactobiose (isomer *Z*), whereas peak 13 was a mixture of 1,2-galactobiose Z and 1,3-galactobiose *Z* identified in a similar way to their *E* isomers.

Peaks 14 and 15 showed a similar mass spectrum and an abundance ratio of 0.81 characteristic of carbohydrates with a free ketose group. Therefore they could correspond to a galactosyl-fructose; *m/z* ion 422 indicate that these peaks could be assigned to 1,6-galactosyl-fructose. This is also supported by their high I^T values (Sanz, et al., 2002).

Peak 16 and 17b showed similar mass spectra characterised by a high *m/z* 307 ion. This fragment of relatively high intensity has been previously detected in di- and trisaccharides with a reducing fructose unit substituted in C1 or C3 (Brokl, et al., 2009, Sanz, et al., 2002) and correspond to the C4-C5-C6 chain. Considering the equivalent glucosyl-fructose standards (trehalulose and turanose), the disaccharide with 1→1 linkage was most retained than the 1→3, therefore these peaks with high I^T values (3029 and 3046) could correspond to 1,1-galactosyl-fructose. Moreover, *m/z* 334 ion characteristic of turanose did not appear in the mass spectrum of peaks 16 and 17b, whereas the *m/z* ratios 191/204/207 were closer to those showed by trehalulose.

Peaks 17a and 18 were assigned to 1,6-galactobiose by comparison with the commercial standard and characterised by its high I^T value and mass spectra with relatively high intensity of *m/z* 422 ion (peak 17a) which correspond to C1-C2-C3-C4 of the oxime chain [34] and typical of 1→6 linkages.

Traces of two peaks at retention times around 24.1 min with *m/z* 307 ions were only found for oligosaccharides from *Aspergillus oryzae* (GOSLuAo). These peaks could correspond to 1,3-galactosyl-fructose.

The non reducing compound β-galactopyranosyl (1-2) fructoside which should be formed in this reaction was not detected. A possible explanation for this may be the overlapping of this compound with the peaks of lactulose, which make very difficult to recognise its characteristic ion traces.

Table 4.4. Concentration (mg g^{-1}) of carbohydrates of GOS mixtures obtained by GC-MS. Peak numbers correspond to those carbohydrates indicated in **Table 4.3**.

Peak number	GOSLuKl	GOLuAa	GOSLuAo
1+2	8.1 (1.0)*	6.7 (1.1)	9.0 (2.0)
3+4	2.3 (0.3)	2.5 (0.7)	5.19 (0.8)
5	18.9 (1.0)	27.2 (3.6)	0.0
6	65.6 (0.4)	106.9 (0.7)	0.0
7+8	645.5 (22.0)	567.0 (69.1)	214.2 (2.7)
9	2.9 (1.1)	0.00	13.1 (0.8)
10	1.8 (0.5)	3.0 (0.3)	1.1 (0.2)
11	1.5 (0.3)	3.1 (0.3)	25.14 (1.4)
12	1.3 (0.0)	3.0 (0.2)	2.1 (0.0)
13	0.4 (0.0)	0.2 (0.3)	6.2 (1.0)
14	0.2 (0.0)	0.4 (0.1)	5.5 (1.0)
15	0.2 (0.0)	0.4 (0.0)	7.0 (0.7)
16	0.7 (0.0)	1.4 (0.0)	15.2 (2.2)
17	3.0 (0.0)	6.7 (0.2)	52.0 (0.1)
18	0.6 (0.0)	1.8 (0.1)	5.5 (0.7)
19	0.4 (0.0)	0.2 (0.0)	0.0
20	0.5 (0.1)	0.2 (0.0)	0.0
21	2.9 (0.0)	1.6 (0.2)	0.0
22	3.5 (0.1)	1.8 (0.2)	0.0
23	3.1 (0.0)	1.6 (0.2)	0.0
24	1.3 (0.1)	0.6 (0.1)	0.0
25	5.0 (0.4)	3.1 (0.4)	0.0
26	4.6 (0.1)	2.8 (0.5)	0.0
27	4.7 (0.3)	2.4 (0.3)	0.0
28	1.8 (0.2)	0.7 (0.2)	1.2 (0.2)
29	1.5 (0.2)	0.6 (0.1)	2.3 (0.1)
30	36.8 (3.5)	15.1 (1.4)	5.2 (0.1)
31	75.3 (6.2)	37.2 (0.7)	12.7 (0.3)
32	100.2 (2.9)	48.4 (0.8)	17.0 (0.9)
33	25.5 (1.4)	12.3 (1.3)	9.6 (0.4)
34	0.0	0.0	2.3 (0.2)
35	0.0	0.0	6.0 (0.5)

* Standard deviation

- *Trisaccharide fraction characterization*

Eight peaks corresponding to trisaccharides with I^T values between 3734-3945 were detected in GOSLuAo (**Figure 4.9 C**), six of them also appeared in GOSLuAa and GOSLuKl (**Table 4.4**). Peaks 31 and 32 were assigned to 6´-galactosyl-lactulose by comparison with the standard purified by Martínez-Villaluenga et al. (Martinez-Villaluenga, et al., 2008).

Peak 33b was assigned to the reducing trisaccharide β-galactopyranosyl-(1→4)-[β-galactopyranosyl-(1→1)]-fructose by comparison with GOS from lactulose using Lactozym 3000 L HP where it was identified as a major trisaccharide by Cardelle-Cobas et al. (2008).

A *m/z* relatively low 307 ion was detected in peaks 34 and 35. As mentioned above, this ion is indicative of a carbohydrate with a reducing ketose substituted in position C1 or C3, although in this case its relative abundance makes this assignation doubtful. Moreover, *m/z* 422 ion was also distinguished at very low a level which is characteristic of 1→6 linkages. Peaks 28 and 29 did not show significant fragments and could not be characterised.

- *Galactosyl- and digalactosyl-glycerols characterization*

GOSLuAa and GOSLuKl also showed two peaks eluting before and after internal standard (peaks 5 and 6 with I^T values of 2347 and 2397, respectively, **Figure 4.10 A**) corresponded to galactosyl-glycerols (1-O-β-galactosyl-glycerol and 2-O-β-galactosyl-glycerol), previously detected in transgalactosylation reactions of lactose using β-galactosidase from Pectinex (*A. acuelatus*) (Cardelle-Cobas, et al., 2008) . In the present study, both peaks were detected in transgalactosylation reactions of lactulose with both Pectinex and Lactozym (*K. lactis*). Both enzymatic preparations use glycerol as stabiliser which can act as acceptor of galactosyl groups. These compounds showed a mass spectrum with *m/z* 204, 217 and 337 as characteristic ions. It should be noted that 1-β-galactosyl-glycerol was one of the most abundant compounds of these mixtures (**Table 4.3**).

Chromatographic peaks with IT values between 3135 and 3347 were identified as digalactosyl-glycerols (**Figure 4.10 B**). Peaks 19-21, 23-26 showed the *m/z* 337 characteristic ion which corresponds to the fragment TMSiO-C^+HOCH_2-CHOTMSi-CH_2OTMSi or to the equivalent TMSiO-C^+HOCH-$(CH2OTMSi)_2$ and include the glycosidic carbon and an OTMSi from the sugar ring. Thus, all these compounds contained the glycerol chain with two free hydroxyls, being the most probable structure

galactopyranosyl-galactopyranosyl-glycerol, formed by addition of a galactosyl group to both 1-O-β-galactosyl-glycerol and 2-O-β-galactosyl-glycerol at different positions.

However, peaks 22 and 27 showed a similar mass spectrum, but *m/z* 337 ion was lacking and a fragment at *m/z* 175 was clearly visible. Thus, these products could contain two units of galactose linked to different hydroxyl groups of glycerol, i.e 1,2-digalactosyl-glycerol and 1,3-digalactosyl-glycerol. However, this last compound should be present in higher concentration that the 1,2 isomer and that was not detected in both GOSLuAa and GOSLuKl products.

Interestingly, the formation of galactosyl-glycerols and digalactosyl-glycerols could be the cause of the lower yields of oligosaccharides (DP4, DP5 and DP6) obtained from trangalactosylation reactions from lactulose using *A. acuelatus* and *K. lactis* compared to those of *A. oryzae* (**Figure 4.8**). These findings may reveal the drawback of using glycerol as a stabilizer in β-galactosidase preparations addressed to produce oligosaccharides of DP above 3.

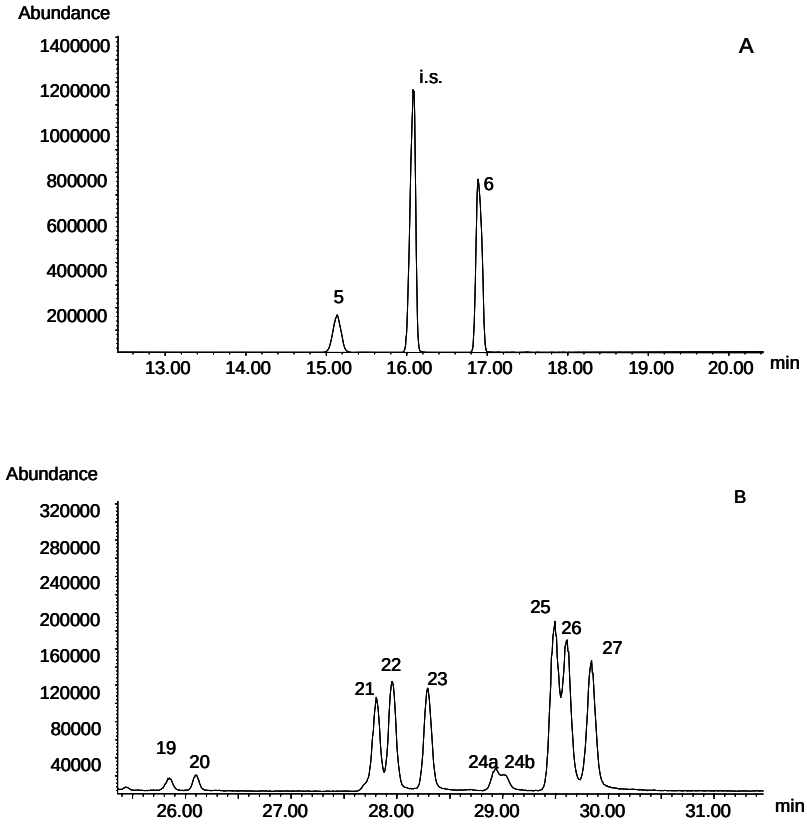

Figure 4.10. Galactosyl- (A) and digalactosyl-glycerol (B) profiles of GOSLuAa obtained by GC-MS. Peak numbers correspond to those carbohydrates indicated in **Table 4.3**.

- *Conclusions*

Oligosaccharides obtained by transgalactosylation reactions from lactulose using β-galactosidases from different sources (*A. acuelatus*, *A. oryze* and *K. lactis*) has been characterised by first time. Oligosaccharides of DP up to 6 were quantified in GOSLuAo by HPLC-ESI MS, whereas only carbohydrates up to DP4 and DP5 were detected for GOSLuKl and GOSLuAa, respectively. Disaccharides, either galactosyl-galactoses or galactosyl-fructoses, and trisaccharides were characterised in the three mixtures by GC-MS. Galactosyl- and digalactosyl-glycerols were also formed during the transgalactosylation reaction of lactulose with Pectinex and Lactozym.

Considering the substantial influence of the chemical structure (type of linkage, nature of the monosaccharide, and degree of polymerization) on the prebiotic activity of oligosaccharides (Sanz, et al., 2005, Sanz, et al., 2005, Sanz, et al., 2006, Sanz, et al., 2006), the knowledge of the carbohydrate composition of these novel oligosaccharides is crucial for providing new evidences for their potential prebiotic activity.

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4.1.3. Hydrophilic interaction liquid chromatography coupled to mass spectrometry for the characterization of prebiotic galactooligosaccharides

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Abstract

Three different stationary phases (sulfoalkylbetaine zwitterionic, polyhydroxyethyl aspartamide and ethylene bridge hybrid (BEH) with trifunctionally-bonded amide), operating at hydrophilic interaction liquid chromatographic (HILIC) mode, have been assayed and compared for the analysis of complex mixtures of galactooligosaccharides (GOS). Chromatographic methods have been optimized to obtain the best separation between two consecutive galactose containing standards and maltodextrins, measured on the basis of resolution. Influence of several factors such as chemical modifiers (formic acid, ammonium acetate and ammonium hydroxide), organic solvent and gradients of the mobile phases in the separation of oligosaccharides have been studied. The best results were achieved on the BEH amide stationary phase, using acetonitrile: water with 0.1% ammonium hydroxide as mobile phase, where the most of oligosaccharides were successfully resolved.

Characteristic MS^2 fragmentation profiles of disaccharides containing galactose, glucose and/or fructose units with different linkages were evaluated and used for the characterization of di-, tri- and tetrasaccharides of three commercial prebiotic GOS mixtures (GOS-1, GOS-2 and GOS-3) by HILIC-MS^n. Similar qualitative and quantitative composition was observed for GOS-1 and GOS-3, whereas different linkages and abundances were detected for GOS-2. In general, (1→4) and (1→6) glycosidic linkages were the main structures found in GOS, although (1→2) and (1→3) linkages were also identified. Regarding molecular weight, up to pentasaccharides were detected in these samples, disaccharides being the most abundant carbohydrates.

4.1.3.1. Introduction

Galactooligosaccharides (GOS) are non-digestible neutral carbohydrates with the ability to manipulate the composition of colonic microflora in order to improve the gastrointestinal health (Gibson, et al., 1995, Torres, et al., 2010). These carbohydrates are enzymatically produced by transgalactosylation reactions of lactose catalized by β-galactosidases to give rise galactose oligomers with a terminal glucose, with different glycosidic linkages and degrees of polymerization (DP). Depending on the enzymatic source used for their synthesis, the chemical structure of these oligosaccharides varies (Cardelle-Cobas, et al., 2008, Gosling, et al., 2010, Martinez-Villaluenga, et al., 2008) and, consequently, their effect on gut microflora can change (Sanz, et al., 2005).

The characterization of GOS structures is a required and important task to understand their mechanism of action on human gut. However, structural analysis of GOS, that involves the determination of linkage position, monomeric composition and anomericity, is not straightforward considering the resulting complex mixtures, high number of isomers and scarce availability of standards.

In general, the analysis of oligosaccharides can be carried out either by spectroscopic, chromatographic, electrophoretic or spectrometric techniques depending on the required level of detail and the type of carbohydrate product (Coulier, et al., 2009). Nuclear magnetic resonance (NMR) is a very useful technique for structural determination; however, a tedious purification step for each compound is required (Lu, et al., 2010). Chromatographic techniques such as gas chromatography (GC) and high performance liquid chromatography (HPLC) usually coupled to mass spectrometry (MS), which provides qualitative and quantitative information of independent oligosaccharides, are the most widely used.

GC-MS is useful for the characterization and quantitation of low molecular weight carbohydrates (mono-, di- and trisaccharides) although a previous derivatization step is mandatory for their analysis (Cardelle-Cobas, et al., 2009, Sanz, et al., 2002).

Different operation modes of HPLC have been applied to the analysis of oligosaccharides. Low retention of underivatized carbohydrates is usually attained

using reverse phase columns, whereas better separation can be achieved by high performance anion exchange chromatography (HPAEC) although complex profiles are obtained when families of oligosaccharides with different linkage variants are present (Morales, et al., 2006).

Hydrophilic interaction liquid chromatography (HILIC) is gaining a great importance in the last years for the separation of polar compounds such as carbohydrates (Alpert, 1990, Alpert, et al., 1994). Partitioning of polar analytes between the bulk eluent and a water-rich layer partially immobilized on the stationary phase is the main retention mechanism described for HILIC (Alpert, 1990), however, different functional groups can be present on the stationary phase giving rise to secondary interactions such as electrostatic (Guo, et al., 2005, McCalley, 2010). Different stationary phases are currently used for this separation mode; silica particles or monolithic supports (Ikegami et al., 2008) either modified with aminopropyl, diol, zwitterionic or amide groups and polymer based packing, among others, can be found (Jandera, 2011).

Sensitive detection of oligosaccharides after HPLC analysis represents an additional difficulty for their analysis. The absence of chromophore and fluorophore groups avoids their direct detection by UV or fluorescence detectors, whereas pulse amperometric detection (PAD), when coupled to HPAEC, is a suitable tool for oligosaccharide analysis (Rocklin, et al., 1983) and has been applied for several applications. Nevertheless, the use of mass spectrometric (MS) detectors coupled to HPLC systems has considerably enriched the field of oligosaccharide analysis, allowing the determination of their molecular weight (Stahl, et al., 2002). Multi-stage mass spectrometry (MS^n) can also provide structural information; however, scarce studies have been still carried out about its utility for the characterization of neutral oligosaccharides (Neri, et al., 2011, Zhang, et al., 2008). Moreover, the addition of appropriate metals to HPLC mobile phases to form complexes with carbohydrates or their previous derivatization (peracetylation or permethylation) is usually required to facilitate the sequential identification of residues by MS (Zaia, 2004).

Characterization of different GOS has been generally carried out by the combination of a great variety of analytical methodologies (methylation analysis

followed by GC-MS, NMR, HPAEC-PAD-MS, ESI-MS) with previous fractionation of the oligosaccharides (yeast treatment, SEC, HILIC) (Coulier, et al., 2009, Lu, et al., 2010, Neri, et al., 2011). HILIC-MS has been used for the analysis of GOS previously fractionated by cation exchange chromatography to determine their molecular weights (Sinclair, et al., 2009). On the other hand, Fu et al. 2010 used a "click" maltose column made in their laboratory to separate GOS. A good resolution among the different degrees of polymerization was obtained, however, no separation of isomers was observed.

In this manuscript three different HILIC stationary phases have been assayed to obtain the best separation of oligosaccharides. HILIC-MS methods have been optimized and applied to the analysis of different and complex commercial GOS mixtures. Characterization of their structures has been accomplished by MS^n without any previous modification of carbohydrate structure.

4.1.3.2. Materials and methods

- *Standards*

1,3-galactobiose (Gal-α(1→3)-Gal), 1,4-galactobiose (Gal-β(1→4)-Gal), 1,6-galactobiose (Gal-β(1→6)-Gal), galactotriose (Gal-α(1→3)-Gal-β(1→4)-Gal), galactotetraose (Gal-α(1→3)-Gal-β(1→4)-Gal-α(1→3)-Gal) were acquired from Dextra Laboratories (Reading, UK), whereas lactose (Gal-β(1→4)-Glc), maltose (Glc-α(1→4)-Glc), maltotriose ((Glc-α(1→4))$_2$-Glc), maltotetraose ((Glc-α(1→4))$_3$-Glc), maltopentaose ((Glc-α(1→4))$_4$-Glc), nigerose (Glc-α(1→3)-Glc), raffinose (Gal-α(1→6)-Glc-α(1→2)-β-Fru) and stachyose (Gal-α(1→6))$_2$-Glc-α(1→2)-β-Fru) were obtained from Sigma (St. Louis, US), and lactulose (Gal-β(1→4)-Fru), melibiose (Gal-α(1→6)-Glc), and verbascose ((Gal-α(1→6))$_3$-Glc-α(1→2)-β-Fru) from Fluka (Madrid, Spain).

- *Samples*

Vivinal-GOS® (GOS-1) was kindly provided by Friesland Foods Domo (Zwolle, The Netherlands), BiMuno (Clasado, Reading, UK) (GOS-2) and Yum-Yum GOS™ (Jarrow Formula, USA) (GOS-3) were acquired in local markets.

- *HILIC-MS*

GOS analyses were performed on an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) equipped with an oven (Kariba Instruments, UK) and coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard, Palo Alto, CA, USA) provided with an electrospray ionization (ESI) source. Samples (20 µL) were injected using a Rheodyne 7725 valve. Three columns and different conditions were used for the analyses: (i) Sulfoalkylbetaine zwitterionic stationary phase (ZIC®-HILIC column; 150 x 2.1 mm, 3.5 µm particle size, 200 Å pore size, SeQuant™, Umea, Sweden) at a flow rate of 0.2 mL min^{-1}; (ii) Polyhydroxyethyl aspartamide stationary phase (PolyHydroxyethyl-A column; 100 x 2.1 mm; 3 µm particle size, 300 Å pore size, The Nest Group, Inc., Southborough, MA) at a flow rate of 0.4 mL min^{-1} and (iii) Ethylene bridge hybrid (BEH) with trifunctionally-bonded amide phase (XBridge column; 150 x 4.6 mm; 3.5 µm particle size, 135 Å pore size, Waters, Hertfordshire, UK) at a flow rate of 0.4 mL min^{-1}. Different binary gradients consisting of acetonitrile (MeCN) : water or methanol (MeOH) : water, with addition of different modifiers as indicated in **Table 4.5**, were assayed for the three columns and optimized. The temperature of elution was kept at 35 ºC for all cases.

The electrospray ionization source was operated under positive polarity using the following MS parameters: nebulizing gas (N_2) pressure 276 KPa, nitrogen drying gas at a flow rate of 12 L min^{-1} and 300 °C and capillary voltage of 4000 V. Ions corresponding to mono-sodiated adducts $[M+Na]^+$ of the oligosaccharides under analysis were monitored in SIM mode using default variable fragmentor voltages at the following *m/z* values: 365.0 (disaccharides), 527.0 (trisaccharides), 689.0 (tetrasaccharides) and 851.0 (pentasaccharides). Data were processed using HPChem Station software version 10.02 (Hewlett-Packard, Palo Alto, CA, USA).

Quantitative analysis was performed in triplicate by the external standard method, using calibration curves in the range 9.6-400 ng for maltose, maltotriose, maltotetraose, maltopentaose, and maltohexaose. Correlation coefficients were obtained from these calibration curves. Reproducibility of the method was estimated on the basis of the intra-day and inter-day precision, calculated as the relative standard deviation (*RSD*) of retention times and concentrations of oligosaccharide standards obtained in n = 5 independent measurements. Limit of detection (*LOD*) and limit of quantitation (*LOQ*) were calculated as three and ten times, respectively, the signal to noise ratio (*S/N*), where *N* is five times the standard deviation of the noise (Foley, et al., 1984).

Table 4.5. Mobile phases used to optimize the chromatographic methods on a zwitterionic, a polyhydroxyethyl aspartamide and a BEH amide column for the separation of oligosaccharides.

Solvents	Modifiers	Concentration
MeOH : H_2O	Ammonium acetate	5 mM
MeCN : H_2O	Ammonium acetate*	0.1; 3.5; 5; 6.5; 20 mM
	Ammonium hydroxide	0.1%
	Formic acid	0.1%

*Present in aqueous phase with the exception of 5 mM, where the salt was contained in both solvent.

- *HILIC-MS^n*

These experiments were carried out on a Finnigan Surveyor pump with quaternary gradient system coupled to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections (20 µL) were carried out by a Finnigan Surveyor autosampler. All instruments (Thermo Fisher Scientific, San José, CA, USA), and data acquisition were managed by Xcalibur software (1.2 version; Thermo Fisher Scientific).

The mass spectrometer spray voltage was set at 4.5 kV and the heated capillary temperature at 290 °C. Nitrogen (99.5% purity) was used as sheath (0.9 L min^{-1}) and auxiliary (9 L min^{-1}) gas, and helium (99.9990% purity) as the collision gas in the

collision induced dissociation (CID) experiments. Mass spectra were acquired in the positive ion mode.

Fragmentation behaviour of the oligosaccharides was studied by infusing a solution of each oligosaccharide (10 µg mL^{-1} in MeCN : water, 60:40, v/v) at a flow rate of 10 µL min^{-1} using the syringe pump included in the instrument and mixing it with 100 µL min^{-1} of MeCN : water (60:40, v/v) both with 0.1% ammonium hydroxide by means a zero-dead volume T-piece. Sheath and auxiliary gases were set at 0.6 and 6 L min^{-1}, respectively. CID experiments were carried out by isolating each $[M+Na]^+$ ion in the ion trap (isolation width 1.0 *m/z*), and subjecting them to a normalized collision energy (NCE%) selected to preserve a signal of the precursor ion in the order of 5%. The process was repeated up to two times by successive isolation (isolation width 1.0 m/z) of the generated ions corresponding to the loss of a monosaccharide unit (loss of 162 u).

Separation of GOS samples were performed on the BEH column following the elution gradient optimized in Section 3.1 that uses MeCN (solvent A) : water (solvent B) both with 0.1% ammonium hydroxide at 35 ºC. Optimal separation of isomeric oligosaccharides was obtained by changing solvent A from 80% to 50% in 31 min and, then, kept for 5 min. Initial conditions were recovered after 0.1 min and were kept for 15 min before the following injection.

Considering that two different LC systems were used, slight differences in oligosaccharide separations were only observed in two chromatographic peaks. Bearing in mind the fragmentation study realized with standards by infusion in Section 3.2, the following *m/z* (and NCE%) were used in the HILIC-MS^n analysis of the samples: 365.1 (29%) for disaccharides, 527.2 (31%) > 365.1 (29%) for trisaccharides, 689.2 (32%) > 527.2 (31%) > 365.1 (29%) for tetrasaccharides.

Identifications of GOS mixtures were tentative in all cases considering the absence of commercial standards.

4.1.3.3. Results and discussion

- *Optimization of HILIC methods*

Optimization of HILIC methods was based on the chromatographic behaviour of (i) a homologous series of maltodextrins (DP2-DP7) and (ii) oligosaccharide standards containing galactose units, to assess the separation among carbohydrates of both different molecular weights and/or isomeric composition.

Evaluation of the methods was carried out on the basis of the shortest retention times (t_R), the best peak symmetry, calculated as the ratio of the front to back widths (at 50% of the peak height) and the highest resolution (R_s, calculated as $2(t_{R2}-t_{R1})/(w_{b1}+w_{b2})$, where 1 and 2 refer to two consecutive eluting carbohydrates and w_b is the peak width at base); R_s values should be higher than 1.0 to get an appropriate separation and peak symmetry close to 1 to get a good symmetry of the peaks. In those cases where α and β isomers appeared as unresolved peaks, PeakFit software (v4.12; SeaSolve Software Inc.) was used for peak deconvolution.

First of all, the effect of different modifiers and organic solvents were assayed in the three HILIC columns using a gradient based on the method proposed by Sinclair et al. 2009 with some modifications (the organic solvent (solvent A) changed from 80% to 50% in 40 min) unless otherwise stated.

- *Effect of formic acid*

The effect of 0.1% formic acid added to both solvents (MeCN and water) as mobile phase for separation of oligosaccharides on the three HILIC stationary phases was firstly assessed. In all cases, reducing carbohydrates showed split peaks corresponding to α and β isomers. This effect has been described by different authors who suggested the use of basic pH to avoid the mutarotation of carbohydrates (Koizumi, 2002, Schumacher, et al., 1995). The homologous series of maltodextrins were well resolved under these conditions in polyhydroxyethyl aspartamide, BEH amide and zwitterionic columns (R_s>1). However, broad peaks with poor symmetry (higher than 1) were obtained in the three columns tested; as an example, in polyhydroxyethyl aspartamide column the maltotriose eluted having a w_b of 0.91 min

and a symmetry of 1.57. However, the appearance of two peaks per reducing carbohydrate impaired the separation of isomers showing, thus, a bad resolution among galactose containing oligosaccharides in the three columns (data not shown). Therefore, formic acid was discarded for further analyses.

- *Effect of ammonium acetate*

Ammonium acetate is a widely used salt for operation with HILIC columns due to its solubility at high percentages of organic solvents (Antonio, et al., 2008, Cubbon, et al., 2007). Separation of standard oligosaccharides using ammonium acetate 5 mM present in aqueous and organic mobile phase (H_2O and MeCN) was evaluated in the three columns with dissimilar results.

All tested oligosaccharides were very poorly resolved under these conditions ($R_s \leq 0.6$) in the zwitterionic column with retention times varying from 3.97 min of lactulose to 4.84 min of verbascose. Moreover, split peaks corresponding to α and β isomers were obtained for reducing carbohydrates, probably because the pH (4.75 in the aqueous phase) was not basic enough to avoid mutarotation of carbohydrates.

Separation of maltodextrins using the polyhydroxyethyl aspartamide column showed better resolution than the ZIC-HILIC column. However, broad peaks and low symmetry values were found in the former (i.e. w_b=1.38 min and the symmetry 0.63 for maltose).

On the other hand, good resolution was achieved for the homologous series of maltodextrins using the BEH amide column with resolution values higher than 1.0 and t_R of 20.1 min for maltose and 34.2 min for maltoheptaose. However, similarly to the results obtained for the zwitterionic column, split peaks were found for reducing carbohydrates.

Effect of methanol as solvent A instead of acetonitrile was also evaluated under these conditions as suggested by Sinclair et al. (2009) for the three columns. Although t_R of oligosaccharides were substantially shorter than those obtained with acetonitrile, (i.e t_R of maltose using methanol in BEH amide column was 7.5 min and 20.1 min using MeCN), resolution values among all tested carbohydrates were very low for BEH amide

(R_s<0.85) and zwitterionic columns (R_s < 0.14). Coelution of all carbohydrates in a single broad peak was observed for the polyhydroxyethyl aspartamide column. This behaviour can be due to the protic nature of both methanol and water, which compete to solvate the stationary phase and provide strong hydrogen bonding interactions with each other (Jandera, 2011). Therefore, the use of acetonitrile as mobile phase was selected for further studies.

As it was previously described by Alpert (1990), HILIC retention is inversely proportional to the increase of salt concentration in the mobile phase. Therefore, four different concentrations (0.1, 3.5, 6.5 and 20 mM) of ammonium acetate only present in the aqueous phase were evaluated and, in consequence, the concentration of this salt increased as the water content rose. No substantial differences were detected among the different concentrations of salt for both zwitterionic and polyhydroxyethyl aspartamide columns. **Figure 4.11** shows the HILIC profile of maltodextrins obtained using the polyhydroxyethyl aspartamide column under these conditions. These profiles indicated that the order of elution of carbohydrates on these columns was not related to the salt content in the mobile phase. Likewise, no suppression of the MS signal was observed by increasing the salt concentration which could be explained by the high volatility of ammonium acetate. Therefore, an intermediate concentration of ammonium acetate (6.5 mM) in water mobile phase was selected. Similar results were observed by Strege (1998) for the HILIC separation of polar compounds for drug discovery processes where only slight changes were detected between 0 and 3.3 mM buffer salt concentrations. Tolstikov and Fiehn (2002) also used similar mobile phases for the analysis of polar compounds of plant origin in the polyhydroxyethyl aspartamide column; however, to the best of our knowledge, there are not data about the separation of different isomeric carbohydrates under these conditions in this stationary phase. Moreover, coelution of sucrose (DP2) and raffinose (DP3) was reported in the previous work, whereas oligosaccharides of different molecular weight could be separated under our optimised conditions (**Figure 4.11**).

On the other hand, better resolution was obtained using a salt gradient than the elution method containing 5 mM ammonium acetate in both solvents, acetonitrile

and water. Thus, resolution values were much higher using a salt gradient (R_s ≥1.6) than those obtained using 5mM ammonium acetate in both solvents (R_s≤1.0).

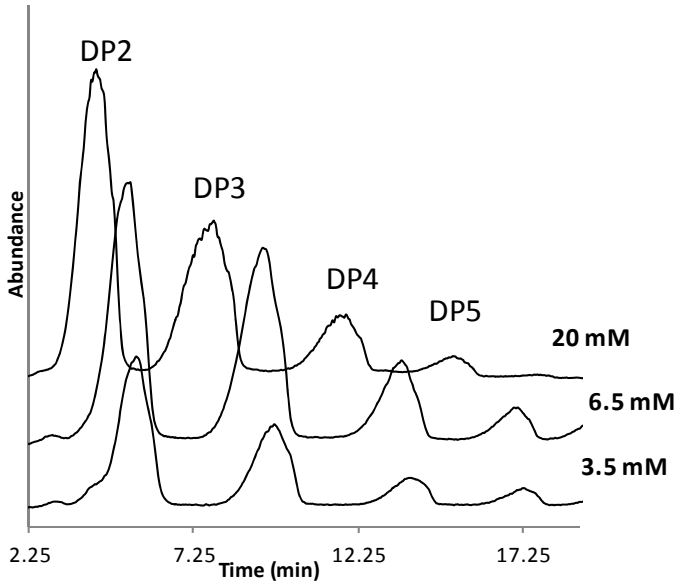

Figure 4.11. HILIC profiles of maltodextrins (DP2–DP5) separated on a polyhydroxyethyl A column, using 3.5, 6.5 and 20 mM of ammonium acetate in the aqueous phase.

Different binary gradients using these mobile phases were assayed to optimise the separation of both maltodextrins and galactose containing oligosaccharides. For the zwitterionic column, the best results were obtained varying MeCN from 80% to 50% in 50 min. Although split peaks were obtained for reducing carbohydrates their resolution (**Table 4.6**) was worse than that found using formic acid 0.1%. Carbohydrates without anomeric carbon (lactulose, raffinose, stachyose and verbascose) showed a single peak and a good resolution among them; however some of these peaks were not symmetric (**Table 4.6**). In general, separation of the standard oligosaccharides was carried out in function of increasing carbohydrate molecular weights, whereas the most retained isomeric carbohydrates were the oligosaccharides with 1→6 linkages.

Elution gradient was also optimized for polyhydroxyethyl aspartamide column and selected conditions were: solvent A kept at 80% for 3 min and changed to 50% for 40 min; under these experimental conditions, this stationary phase was unable to separate anomeric compounds and single peaks were detected in reducing

carbohydrates. Similarly to the previous column, maltodextrins were eluted in the order of increasing molecular weight, with R_s values from 1.4 to 2.1 (**Table 4.6**), whereas among disaccharides, those with 1→3 and 1→4 glycosidic linkages were the first to elute followed by (1→6)-linked carbohydrates. Although elution times ranged from 5.1 min of 1,4-galactobiose to 20.1 min of verbascose, broad peaks were obtained (i.e. 1,6-galactobiose: w_b= 1.6 min; galactotriose: w_b= 1.8 min; and so on) and resolution among them was poor (**Table 4.6**). Only peaks corresponding to i) galactotriose and stachyose and ii) galactotetraose and verbascose were well resolved, although only verbascose presented an acceptable symmetry (0.9).

Separation of oligosaccharide standards using BEH amide column using linear gradients of ammonium acetate at different concentrations was similar to that obtained under 5mM ammonium acetate in both mobile phases (acetonitrile and water), contrary to the results obtained with polyhydroxyethyl aspartamide and zwitterionic columns where the salt gradient improved the separation of maltodextrins and galactose containing oligosaccharides as explained above. Moreover, splits peaks for reducing carbohydrates due to the separation of anomers were also detected using the BEH amide column (**Table 4.6**), showing a similar behaviour than that found in the zwiterionic column.

Table 4.6. Retention time (t_R; min), resolution (R_s) and symmetry of standard carbohydrates analyzed using a zwitterionic, a polyhydroxyethyl aspartamide and a BEH amide column using acetonitrile and water containing 6.5 mM of ammonium acetate.

Column	Maltodextrins	t_R	R_s	Symmetry	Galactose containing oligosaccharides	t_R	R_s	Symmetry
Zwitterionic	Maltose 1	11.1		1.1	Lactulose	10.8		0.7
	Maltose 2	12	0.1	1.1	Galactobiose (α 1-3) 1	11.7	0.2	2.2
	Maltotriose 1	15.1	0.2	1.1	Galactobiose (β 1-4) 1	11.8	0.1	1
	Maltotriose 2	16.1	0	1.1	Lactose 1	12	0	2.1
	Maltotetraose 1	19	0.6	0.9	Lactose 2	12.9	0.6	1.4
	Maltotetraose 2	20	0.3	0.9	Galactobiose (β 1-4) 2	13	0.1	0.8
	Maltopentaose 1	22.5	0.2	1	Melibiose 1	13.4	0.3	1.6
	Maltopentaose 2	23.5	0.5	1.1	Galactobiose (α 1-3) 2	13.7	0.1	0.5
	Maltohexaose 1	25	0.8	1.1	Melibiose 2	14.3	0.3	1
	Maltohexaose 2	26.1	0.5	1.1	Galactobiose (β 1-6) 1	14.5	0.1	1.1
	Maltoheptaose 1	27.3	0.5	1.2	Galactobiose (β 1-6) 2	16	0.5	1.1
	Maltoheptaose 2	28.3	0.4	1.1	Raffinose	16.1	0.1	0.9
					Galactotriose 1	16.2	0	0.9
					Galactotriose 2	17.4	0.3	0.8
					Galactotetraose 1	20.5	0.1	2.7
					Stachyose	22	0.7	0.8
					Galactotetraose 2	22	0	0.8
					Verbascose	26.5	0.3	0.8
Polyhydroxyethyl aspartamide	Maltose	4.3		1.1	Galactobiose (β 1-4)	5.1		0.9
	Maltotriose	7.8	1.6	1.9	Galactobiose (α 1-3)	5.3	0.1	0.6
	Maltotetraose	11.9	1.7	2.1	Lactose	5.9	0.3	1.6
	Maltopentaose	15.1	1.6	1.1	Lactulose	6.5	0.3	1
	Maltohexaose	18.6	2.1	1.3	Galactobiose (β 1-6)	7.6	0.3	2
	Maltoheptaose	20.6	1.4	1.7	Melibiose	7.8	0.3	0.9
					Raffinose	11	0.7	1.3
					Galactotriose	11.5	0.2	1.2
					Stachyose	14.9	1.2	1.2
					Galactotetraose	14.9	0.0	0.8
					Verbascose	20.1	2.8	0.9
BEH amide	Maltose 1	21.4		1.3	Galactobiose (α 1-3) 1	20.8		0.6
	Maltose 2	21.8	0.6	0.6	Galactobiose (β 1-4) 1	21.4	0.5	0.4
	Maltotriose 1	25.7	6.2	2.0	Lactulose	21.5	0.1	1.0
	Maltotriose 2	26	0.5	0.9	Galactobiose (α 1-3) 2	22.0	0.6	0.7
	Maltotetraose 1	29.1	4.7	1.8	Galactobiose (β 1-4) 2	22.3	0.4	1.2
	Maltotetraose 2	29.4	0.4	0.3	Lactose 1	22.5	0.2	0.9
	Maltopentaose 1	31.8	5.3	0.0	Lactose 2	22.5	0.0	1.0
	Maltopentaose 2	31.9	0.2	0.3	Melibiose 1	23.4	1.8	0.9
	Maltohexaose 1	33.8	3.2	10.5	Melibiose 2	23.8	0.6	0.8
	Maltohexaose 2	33.9	0.3	0.6	Galactobiose (β 1-6) 1	24.4	0.7	1.1
					Galactobiose (β 1-6) 2	25.3	0.9	1.3
					Galactotriose 1	25.7	0.4	0.9
					Galactotriose 2	25.8	0.2	0.7
					Raffinose	26.5	1.0	1.0
					Galactotetraose 1	29.3	2.6	1.0
					Galactotetraose 2	30.0	0.6	0.8
					Stachyose	30.1	0.1	0.7
					Verbascose	33.7	3.5	1.3

- *Effect of ammonium hydroxyde*

To avoid the appearance of split peaks, 0.1% ammonium hydroxide was used in both mobile phases (MeCN and water). Although one single peak was obtained for each oligosaccharide, no satisfactory resolution was achieved under these conditions for the zwitterionic and polyhydroxyethyl aspartamide columns either for the separation of the maltodextrins or the galactose containing oligosaccharides (data not shown). However, these conditions resulted in a good resolution of maltodextrins on BEH amide column (R_s~ 4.8). In this column, different binary gradients using MeCN and water as mobile phases containing both 0.1% ammonium hydroxide were assayed to optimise the separation of both maltodextrins and galactose containing oligosaccharides; the best results were obtained varying MeCN from 80% to 50% in 31 min, as previously reported by Brokl et al. (2011) for the separation of fructooligosaccharides, gentiooligosaccharides and oligosaccharides from dextransucrase cellobiose acceptor reactions. Maltodextrins eluted within 34 min; t_R increasing with their molecular weight as consequence of the increase in hydrophilicity due to the increased number of hydroxyl groups. Wuhrer et al. (2004) and Melmer et al. (2011) reported a similar behaviour of *N*-glycans in amide-based ligand columns. The galactose containing oligosaccharides eluted from 19.8 min of lactulose to 32.4 min of verbascose. Disaccharides with 1→3 and 1→4 linkages were the first to elute followed by carbohydrates with 1→6 glycosidic linkages. In general, resolution values were higher than 1, except for those between galactobiose 1→4 and 1→3; galactobiose 1→3 and lactose; and galactotriose and raffinose (**Table 4.7**). Therefore, BEH column under these elution conditions was selected for the analysis of commercial GOS mixtures.

Overall, the three tested columns provided substantial differences in selectivity, peak shape and, especially, in retention efficiency. This fact can be expected according to the different nature of the surface chemistry of the assayed stationary phases. In general terms, the best separation of GOS standards and maltodextrins was achieved using the BEH amide column which was selected for further analyses. Successful separations of monosaccharide and other small polar compounds have been previously performed on amide-silica HILIC columns (Brokl, et

al., 2011, Guo, et al., 2005, Karlsson, et al., 2005). The great retention efficiency observed for the GOS eluted on the BEH amide column can be due to the contribution of strong hydrogen-bonding effects between the amide group of the stationary phase and polar compounds containing hydroxyl groups (Guo, et al., 2003), such as GOS. A similar behaviour has recently been reported for the separation of estrogen metabolites on an amide-silica HILIC column (Nguyen, et al., 2010). Likewise, differences of properties in terms of column dimension and, especially, of particle properties (particle size, pore size and surface area) could also have an effect on retention of the GOS. Thus, the BEH amide column has the biggest surface area (185 m^2/g with a particle size of 3.5 µm and a pore size of 135 Å), whilst the sulfoalkylbetaine zwitterionic has a surface area of 135 m^2/g (3.5 µm particle size and 200 Å pore size) and the polyhydroxyethyl aspartamide has the lowest surface area (100 m^2/g with 3 µm particle size and 300 Å pore size). Therefore, the increased retention of the GOS on the BEH amide column might be also due to the increased surface area for analyte binding in addition to the functionality of the stationary phase (Nguyen, et al., 2010).

Table 4.7. Retention time (t_R; min), resolution (R_s) and symmetry of standard carbohydrates analyzed with a BEH amide column using acetonitrile: water with 0.1% ammonium hydroxide as mobile phase.

Maltodextrins	t_R	R_s	Symmetry	Galactose containing oligosaccharides	t_R	R_s	Symmetry
Maltose	20.3		1	Lactulose	19.8		1.1
Maltotriose	24.7	6.1	1	Galactobiose (β 1-4)	20.6	1.2	1
Maltotetraose	28.2	6.0	1	Galactobiose (α 1-3)	21	0.5	1.1
Maltopentaose	30.9	5.1	0.9	Lactose	21.1	0.1	0.9
Maltohexaose	32.9	3.8	1.1	Melibiose	22.4	2.6	1
Maltoheptaose	34.5	3.3	1.2	Galactobiose (β 1-6)	23.2	1.4	1
				Raffinose	24.7	2.9	0.8
				Galactotriose	24.8	0.2	0.9
				Galactotetraose	28.5	6.5	1
				Stachyose	29.1	1.1	0.9
				Verbascose	32.4	9.5	0.9

- *Fragmentation of disaccharides by MS^n*

Previous to the structural characterization of GOS samples, MS^2 fragmentation behaviour of several standard disaccharides containing galactose, glucose and/or fructose units was evaluated (**Table 4.8**). The ion at *m/z* 365 corresponds to the sodium adduct of disaccharides and it was the precursor ion considered for MS^2 analyses. 1,3-galactobiose spectrum was characterized by the high abundance of the *m/z* fragment 347 (corresponding to the loss of a molecule of water) followed by the loss of the monosaccharide unit (ion at *m/z* 203). Low intensities relative to the base peak were also detected for the ions at *m/z* 275 and 305 corresponding to the losses of $C_3H_6O_3$ and $C_2H_4O_2$, respectively. However, higher abundances of ion at *m/z* 275 were observed for nigerose, which could be attributed to the differences in the monosaccharide composition. Similar fragmentation profiles, but different relative ratios of the fragment ions had been previously observed by Zhang et al. (2008) for disaccharides with the same linkage but different monosaccharide residues.

Analogous MS^2 fragmentation (prevalent fragments at *m/z* 305, 347 and 203 corresponding to the neutral losses of $C_2H_4O_2$, H_2O and the monosaccharide unit, respectively), was observed for lactose and 1,4-galactobiose. In contrast, lactulose (galactosyl-(1→4)-fructose) fragmentation showed different abundances for these characteristic ions.

1,6-galactobiose and melibiose (both with 1→6 glycosidic linkage) showed a similar fragmentation characterized by abundances in decreasing order of ions at *m/z* 305, 275, 245 (corresponding to the loss of $C_4H_8O_4$) and 335 (corresponding to the loss of CH_2O). The main difference between fragmentations of these disaccharides was the higher abundance of the ion at *m/z* 203 corresponding to the monosaccharide for the melibiose and the abundance of the *m/z* ion 347 for 1,6-galactobiose. These results are in agreement with those found by Zhang et al. (2008), who showed the characteristic fragmentation pattern of five different disaccharides, among them 1,3-galactobiose, maltose, and isomaltose, with 1→3, 1→4 and 1→6 linkages, respectively.

1,1 and 1,2-linked disaccharides with galactose units could not be acquired, but considering the similar fragmentation of 1→4 and 1→6 linkages with those shown by

Zhang et al. (2008), the reported fragmentation patterns of trehalose and 1,2-mannobiose were also used for the characterization of commercial GOS. In that work, Zhang et al. (2008) described that the MS^2 fragmentation of 1,1-linked disaccharide was dominated by the *m/z* ion at 203, although it was also detected the very minor presence of the *m/z* ion at 305. Nevertheless, the characteristic fragmentation pattern of 1,2-linked disaccharides gave rise to the main neutral loss of $C_4H_8O_4$ (*m/z* ion at 245), followed by the ions in decreasing order of abundance at *m/z* 203, 347, 275 and 305.

Table 4.8. Relative abundances of characteristic *m/z* ratios of neutral losses from MS^2 of standard disaccharides.

Standard	Glycosidic linkage	Monomeric units	Neutral losses (m/z ion)						
			$C_6H_{10}O_5$ -203	$C_4H_8O_4$ -245	$C_3H_6O_3$ -275	$C_2H_4O_2$ -305	CH_4O_2 -317	CH_2O -335	H_2O -347
α,α,-Trehalose	1→1	Glc, Glc	100	-	-	-	-	-	-
Kojibiose	1→2	Glc, Glc	-	93.2	-	-	-	-	-
1,3- Galactobiose	1→3	Gal, Gal	27	-	3.1	1.1	-	-	100
Nigerose	1→3	Glc, Glc	38.2	-	33.2	-	-	-	100
Lactose	1→4	Gal, Glc	27.1	2.2	3.2	100	1.7	1.6	72
1,4- Galactobiose	1→4	Gal, Gal	42.9	0.8	1.7	100	1.1	9.1	46.6
Lactulose	1→4	Gal, Fru	2.3	-	0.1	7.2	14.7	1.3	100
1,6- Galactobiose	1→6	Gal, Gal	9.6	11	46.2	100	-	1.1	17
Melibiose	1→6	Gal, Glc	39.7	2.4	16	100	-	1.4	-

Gal: galactose; Glc: glucose; Fru: fructose

- *Characterization of commercial GOS by HILIC-MS and HILIC-MS^n*

Figure 4.12 shows the SIM profiles of the three commercial GOS mixtures by HILIC-MS using the BEH column. Di-, tri-, tetra- and pentasaccharides were observed in all samples, whereas traces of hexasaccharides where detected in GOS-1 and GOS-3 (data not shown).

Three main peaks were clearly distinguished for disaccharides of GOS-1. HILIC-MS^2 analyses (**Table 4.9**) using *m/z* 365 as precursor ion, showed relative high intensities of fragments at *m/z* 347, 275, 203 for peak 1 which could correspond to a disaccharide

with 1→3 linkage. However, relative abundances of these *m/z* fragments are different to those observed for 1,3-galactobiose which could be attributed to a different monomeric composition, more similar to that of nigerose (**Table 4.8**). It has been reported that galactosyl-(1→3)-glucose (26% wt) is more abundant than the 1,3-galactobiose (1% wt) in Vivinal-GOS® (Coulier, et al., 2009). Therefore, this peak could be attributed to the first compound or a mixture of both. Peak 2 was the most abundant disaccharide of GOS-1 and showed a MS^2 fragmentation pattern different to those of commercial standards, probably due to the co-elution of different compounds. The most abundant fragments were *m/z* 305, 203 and 347 characteristic of 1→4 linked disaccharides and could correspond to 1,4-galactobiose. However, high relative abundances of ion *m/z* 245 distinctive of 1→2 linkages can be also observed. Therefore, this peak could be a mixture of (1→4)- and (1→2)- linked disaccharides. Coulier et al. (2009) reported the presence of lactose, 1,4-galactobiose and galactosyl-(1→2)-glucose in Vivinal-GOS®. Therefore, peak 2 could be a mixture of these three disaccharides. Peak 3 could clearly correspond to a (1→6)- linked disaccharide considering the relative abundances of *m/z* ions at 305, 275 and 245 and could be assigned to allolactose (galactosyl-(1→6)-glucose) which was previously identified by Coulier et al. (2009) following isolation, methylation and NMR analyses in Vivinal-GOS®.

Regarding trisaccharides of GOS-1, five peaks were observed (**Figure 4.12**), however, resolution among them was not completely achieved which could difficult mass interpretation. MS^2 and MS^3 fragmentations were carried out using the ions *m/z* 527 and 365 as precursor ions, respectively. HILIC-MS^2 and HILIC-MS^3 analyses of peak 4 revealed a characteristic fragmentation of 1→3 linkages, similar to that observed for peak 1, as the main ion fragments corresponded to the neutral losses of $C_3H_6O_3$ (*m/z* fragments 437 and 275, in MS^2 and MS^3 spectra, respectively) and H_2O (*m/z* fragments 509 and 347, in MS^2 and MS^3 spectra, respectively) (**Table 4.10**). Therefore, this peak could tentatively be assigned to Gal-(1→3)-Gal-(1→3)-Glc, although mixtures with other trisaccharides with different monosaccharide composition could not be discarded. Two compounds can be clearly distinguished by HILIC-MS^2 of peak 5. First of them, peak 5a, showed a *m/z* fragmentation pattern characteristic of (1→2)-linked carbohydrates (Zhang, et al., 2008) differing from 2α-mannobiose in the relative

abundance of the neutral loss of monomeric units: *m/z* 365 for the MS^2 fragmentation of the trisaccharide, and *m/z* 203 for the MS^3 fragmentation of the disaccharide, being this loss more abundant in the first case (**Table 4.10**). HILIC-MS^3 of this peak revealed a similar fragmentation profile to peak 2 which could indicate the presence of a mixture of two compounds with 1→2 and 1→4 glycosidic linkages. Gal-(1→4)-Gal-(1→2)-Glc has been previously identified in Vivinal-GOS® (Coulier, et al., 2009), however, the presence of x-(1→2)-Gal-(1→2)-Glc has not been previously reported. HILIC-MS^2 and HILIC-MS^3 analyses of peak 5b seem to indicate the presence of 1→4 glycosidic linkages with the characteristic MS^2 losses of $C_2H_4O_2$, $C_6H_{10}O_5$, H_2O and $C_6H_{12}O_6$, and MS^3 losses of $C_2H_4O_2$, H_2O, $C_6H_{10}O_5$ and CH_2O, which is indicative of the presence of Gal-(1→4)-Gal-(1→4)-Glc. Peak 6 could be tentatively assigned to Gal-(1→6)-Gal-(1→4)-Glc considering the MS^2 (losses of $C_6H_{12}O_6$, $C_2H_4O_2$ and H_2O) and MS^3 (losses of $C_2H_4O_2$, $C_3H_6O_3$ and $C_4H_8O_4$) fragmentations although contribution of Gal-(1→4)- cannot be discarded taking into account the relative ratios of the fragment ions in MS^3. Peak 7 showed the characteristic patter of 1→6 glycosidic linkages for both MS^2 and MS^3 fragmentations and could correspond to Gal-(1→6)-Gal-(1→6)-Glc. Finally, MS^3 of peak 8 clearly revealed the presence of 1→6 glycosidic linkage (losses of $C_2H_4O_2$, $C_3H_6O_3$ and $C_4H_8O_4$), however MS^2 was more confusing, considering the fragment at *m/z* 467, the low abundance of *m/z* 437 and the relatively high intensity of *m/z* 407. This profile is similar to that detected for peak 2 and could be assigned to a mixture of 1→2 and 1→4 linkages.

Five peaks corresponding to tetrasaccharides were observed in GOS-1 by HILIC-MS (**Figure 4.12**). Fragments at *m/z* 689 and 527 were used as precursor ions of MS^2 and MS^3, respectively. Fragment at *m/z* 365 was also used as a precursor ion of MS^4, although detected ions had much lower abundances (data not shown). Characterization of these peaks was more complex considering the low abundances and the existence of multiple coelutions. Only some linkages could be tentatively assigned as indicated in **Table 4.11**.

A similar reasoning was followed for the characterization of di-, tri- and tetrasaccharides of GOS-2 and GOS-3. These data are shown in **Tables 4.9**, **4.10** and **4.11**. In general, GOS-3 showed a similar qualitative composition to GOS-1, however,

notable differences were observed for GOS-2 which exhibited a lower diversity of glycosidic linkages. This fact is supported by the high similarity of the chromatographic profiles of GOS-1 and GOS-3 in oligosaccharide retention times and peak shapes, whilst the HILIC profile of GOS-2 exhibited some differences in terms of retention times and, especially, in peak abundances (**Figure 4.12**).

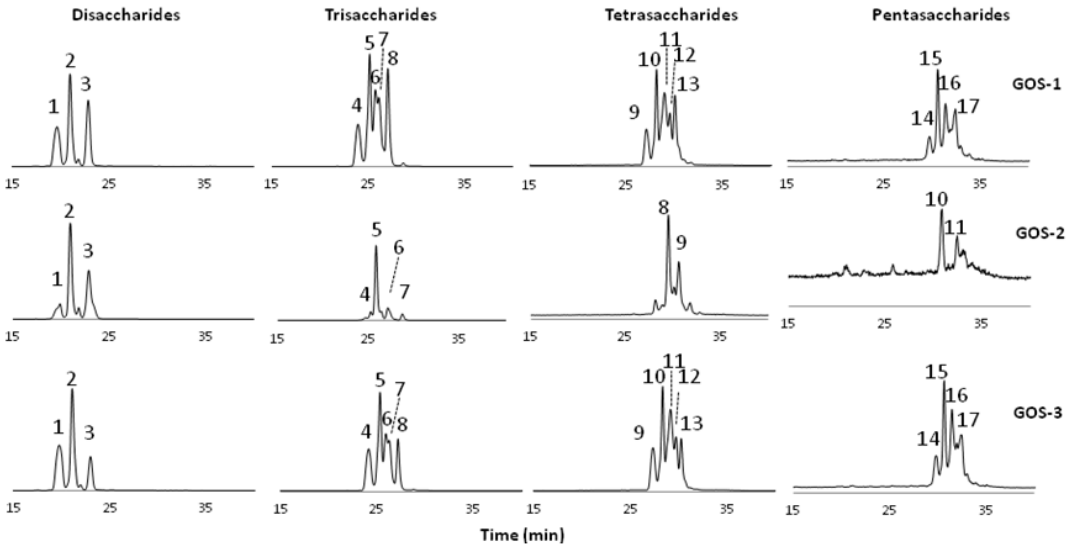

Figure 4.12. HILIC profiles of commercial GOS separated on an ethylene bridge hybrid with trifunctionally-bonded amide column.

Table 4.9. Relative abundance for characteristic *m/z* ratios of specific losses from MS^2 of disaccharides from commercial GOS separated in a BEH amide stationary phase.

		Disaccharides							
		MS^2							
	Neutral loss	$C_6H_{10}O_5$	$C_4H_8O_4$	$C_3H_6O_3$	$C_2H_4O_2$	CH_4O_2	CH_2O	H_2O	Tentative identification
Sample	Peak / m/z	203	245	275	305	317	335	347	
GOS-1	1	6.8	0.3	45.1	0.3	-	-	100	Gal-(1→3)-Glc
	2	67.4	42.8	0.9	100	-	4.1	50.6	Gal-(1→4)-Glc and Gal-(1→2)-Glc
	3	3.4	6.1	25.3	100	0.6	1.9	3.7	Gal-(1→6)-Glc
GOS-2	1	4	-	12.8	3.8	4.3	-	100	Gal-(1→3)- Glc + lactulose
	2	16.7	7.4	0.4	100	2.7	5.9	52.3	Gal-(1→4)-
	3	2.1	6.7	28	100	0.2	1.9	4.7	Gal-(1→6)-Glc
GOS-3	1	7.7	-	38.9	0.8	1.6	0.4	100	Gal-(1→3)-Glc
	2	51.7	30.8	0.6	100	-	4.2	43.6	Gal-(1→4)- and Gal-(1→2)- Glc
	3	2.1	5.7	21.5	100	0.2	1.8	5.3	Gal-(1→6)-Glc

Table 4.10. Relative abundance for characteristic *m/z* ratios of specific losses from MS^2 and MS^3 of trisaccharides from commercial GOS separated on a BEH amide stationary phase.

Trisaccharides									
					MS^2				
	Neutral loss	**$C_6H_{12}O_6$**	**$C_6H_{10}O_5$**	**$C_4H_8O_4$**	**$C_3H_6O_3$**	**$C_2H_4O_2$**	**CH_2O**	**H_2O**	*Identification*
Sample	**Peak / *m/z***	**347**	**365**	**407**	**437**	**467**	**497**	**509**	
GOS-1	4	18.2	100.0	-	70.4	4.0	-	99.7	-Gal-(1→3)-Glc
	5a	3.2	100.0	66.2	5.3	3.2	-	5.3	-Gal-(1→2)-Glc
	5b	12.5	86.4	7.2	5.9	100.0	0.4	43.4	-Gal-(1→4)-Glc
	6	46.0	96.0	7.4	1.0	100.0	1.9	30.7	-Gal-(1→4)-Glc
	7	1.7	60.4	6.1	23.8	100.0	1.3	17.8	-Gal-(1→6)-Glc
	8	2.9	76.0	13.6	5.0	100.0	2.5	14.1	-Gal-(1→4)-Glc + -Gal-(1→2)-Glc
GOS-2	4	9.6	89.9	3.8	12.5	100	-	88.3	-Gal-(1→6)-x
	5	32.1	22.7	4.0	1.7	100	3.0	45.7	-Gal-(1→4)-x
	6	10.1	33.6	2.6	12.2	100	0.5	7.8	-Gal-(1→6)-x
	7	0.4	4.7	2.4	9.8	100	-	1.0	-Gal-(1→6)-x
GOS-3	4	12.8	77.7	-	62.9	5.1	0.6	100.0	-Gal-(1→3)-Glc
	5a	6.0	90.8	100.0	0.8	1.9	-	8.8	-Gal-(1→2)-Glc
	5b	8.8	99.1	6.0	5.0	100.0	2.2	37.3	-Gal-(1→4)-Glc
	6	34.8	100.0	2.2	2.2	58.9	-	35.3	-Gal-(1→4)-Glc + -Gal-(1→6)-Glc
	7	3.4	62.4	5.9	27.2	100.0	1.2	25.2	-Gal-(1→6)-Glc
	8	2.0	72.4	5.8	7.4	100.0	2.6	21.6	-Gal-(1→4)-Glc + -Gal-(1→6)-Glc
					MS^3				
	Neutral loss	**$C_6H_{10}O_5$**	**$C_4H_8O_4$**	**$C_3H_6O_3$**	**$C_2H_4O_2$**	**CH_4O_2**	**CH_2O**	**H_2O**	***Identification***
Sample	**Peak / *m/z***	**203**	**245**	**275**	**305**	**317**	**335**	**347**	
GOS-1	4	4.5	-	100.0	-	-	-	88.1	Gal-(1→3)-
	5a	50.5	88.3	-	100.0	-	24.6	-	Gal-(1→4)- + x*-(1→2)-
	5b	17.2	-	-	100.0	1.0	9.4	26.0	Gal-(1→4)-
	6	-	14.6	15.2	100.0	-	6.5	22.6	Gal-(1→4)- + Gal-(1→6)-
	7	-	3.8	31.2	100.0	-	2.4	18.1	Gal-(1→6)-
	8	-	5.7	21.4	100.0	-	1.3	5.9	Gal-(1→6)-
GOS-2	4	-	10.7	-	100.0	11.8	5.8	20	Gal-(1→4)- + Gal-(1→2)-
	5	6.8	2.6	23.8	100.0	2.2	15.9	33.9	Gal-(1→6)-
	6	27.3	43.1	53.6	100.0	-	16.0	8.2	Gal-(1→6)-
	7	-	-	96.9	-	-	-	-	Gal-(1→3)-
GOS-3	4	-	-	100.0	7.4	-	-	50.4	Gal-(1→3)-
	5a	56.2	59.2	-	100.0	-	4.5	17.4	Gal-(1→4)- + x*-(1→2)-
	5b	5.4	9.0	-	100.0	1.2	5.5	29.7	Gal-(1→4)-
	6	23.1	30.9	6.8	100.0	14.1	-	18.5	Gal-(1→4)- + Gal-(1→2)-
	7	-	-	53.1	100.0	-	6.7	21.5	Gal-(1→6)-
	8	12.4	8.5	7.6	100.0	-	6.3	23.2	Gal-(1→6)-

* x: correspond to an unknown monosaccharide unit not previously described in the literature.

Regarding GOS-2 disaccharides (**Table 4.9**), in peak 1 co-eluted two different carbohydrates, probably Gal-(1→3)-Glc characterized by the fragment at *m/z* 275 and lactulose which showed high contribution of *m/z* 347 and low of *m/z* 305 and 317 (**Table 4.9**). Presence of 1→4 glycosidic linkage could be easily detected in peak 2 of GOS-2, whereas the contribution of 1→2 linkage (fragment at *m/z* 245) was smaller than those of GOS-1 and GOS-3. Peak 3 was identified as Gal-(1→6)-Glc, likewise in the other two samples. The main trisaccharide (peak 5, **Table 4.10**) was assigned to Gal-(1→6)-Gal-(1→4)-x, whereas peak 4 could be characterized by a mixture of two compounds (Gal-(1→4)-Gal-(1→6)-x and Gal-(1→2)-Gal-(1→6)-x). Peaks 6 and 7 showed the typical MS^2 fragmentation of (1→6) linkages, MS^3 spectra being characteristic of (1→6) and (1→3), respectively. Tetrasaccharides showed very low abundances and mainly presence of -(1→4)- and -(1→6)- could be hypothesized (**Table 4.11**).

Table 4.11. Relative abundance for characteristic *m/z* ratios of specific losses from MS^2, and MS^3 of tetrasaccharides from commercial GOS separated on a BEH amide stationary phase.

Tetrasaccharides								
MS^2								
	Neutral loss	$C_6H_{12}O_6$	$C_6H_{10}O_5$	$C_4H_8O_4$	$C_3H_6O_3$	$C_2H_4O_2$	H_2O	Identification
Sample	Peak / m/z	509	527	569	599	629	671	
GOS-1	9	13.6	100	-	61.7	1.2	17.8	-(1→3)-
	10a	9	100	78.3	3.4	11.8	8.1	-(1→2)- + -(1→6)-
	10b	5.4	100	14.2	4	49.2	19.2	-(1→6)- + -(1→2)-
	11	5.1	100	3.7	6.2	20.5	6.2	-(1→6)-
	12	10	100	12.3	7	94.8	8.5	-(1→4)- + unkown
	13	6.4	84.8	4.1	-	100	12.3	-(1→4)-
GOS-2	8	70.5	42.9	2.7	3.5	100	24.3	-(1→4)- + -(1→6)-
	9	19.8	23	4.3	3.6	100	26.9	-(1→4)- + -(1→6)- +-(1→2)-
GOS-3	9	9.3	100	1.2	71.1	5.2	26.1	-(1→3)-
	10a	3.2	100	-	1	9.4	8.6	-(1→6)-
	10b	6.5	100	-	2.2	53.2	19.6	-(1→6)-
	11	6.8	100	-	-	38	6	-(1→4)-
	12	5.6	100	7.8	3.3	92.6	4.9	-(1→4)- + unkown
	13	3	98.1	3.3	1.9	100	13.7	-(1→4)- + unkown

Continuation table 4.11.

	Tetrasaccharides							
		MS^3						
	Neutral loss	$C_6H_{12}O_6$	$C_6H_{10}O_5$	$C_4H_8O_4$	$C_3H_6O_3$	$C_2H_4O_2$	H_2O	Identification
Sample	Peak / m/z	347	365	407	437	467	509	
GOS-1	9	4.5	35.9	-	100	10	31	-(1→3)-
	10a	-	100	37.3	-	45.3	-	-(1→4)- + -(1→2)-
	10b	8.2	77.4	11	4.1	100	19	-(1→6)- + -(1→4)-
	11	22.4	100	16	-	52.3	44.8	-(1→4)- + -(1→2)-
	12	5.4	48.5	-	17	100	10	-(1→4)- or -(1→6)-
	13	6.8	32.1	19.5	9.9	100	27.3	-(1→6)- + unknown
GOS-2	8	-	62.2	-	-	100	-	-(1→4)-
	9	-	20	-	-	100	-	-(1→4)-
GOS-3	9	6.7	50.2	-	100	1.2	26	-(1→3)-
	10a	-	100	30.3	-	75.8	18	unknown
	10b	9.6	91.2	18	7.4	100	21.4	-(1→6)- + -(1→4)- +-(1→2)-
	11	-	100	4.4	15.9	63	32.5	-(1→4)- + -(1→6)-
	12	-	24.9	12	-	100	13.9	-(1→4)-
	13	-	100	14.2	-	11	11.7	unknown

- *Quantitation of GOS by HILIC-MS*

Quantitative analysis was carried out following the external standard method using the homologous series of maltodextrins. Limit of detection (*LOD*) showed values of 0.04-0.08 ng injected; whereas limit of quantitation (*LOQ*) was 0.14-0.28 ng injected. Intra- and inter-day reproducibility was also evaluated, relative standard deviation being lower than 10 % for the different standards analyzed.

Table 4.12 shows quantitative data for GOS mixtures. Disaccharides were the main carbohydrates present in GOS samples (54, 76 and 53% for GOS-1, GOS-2 and GOS-3, respectively); lactose (quantified together with Gal-(1→2)-Glc in GOS-1 and GOS-3) being the most abundant. Regarding trisaccharides, similar percentages were observed for GOS-1 and GOS-3 (~29 %), whilst GOS-2 had lower percentages (22.5%). Likewise, tetrasaccharides of GOS-2 only constituted the 1.5 % of its composition, whereas levels of 11-12% were found in GOS-1 and GOS-3. Only traces of pentasaccharides could be detected in GOS-2. Therefore, yields of oligosaccharides in

GOS-1 and GOS-3 were higher than those found in GOS-2, probably due to the manufacturing conditions used to obtain these products (Gosling, et al., 2010).

Table 4.12. Relative percentages of quantified and identified oligosaccharides using a BEH amide stationary phase in commercial GOS.

Sample	DP	Peak number	%	Identification
GOS-1	DP2	1	15.11 (0.07)[§]	Gal-(1→3)-Glc
		2	22.20 (0.18)	Gal-(1→4)-Glc + Gal-(1→2)-Glc
		3	17.07 (0.16)	Gal-(1→6)-Glc
	DP3	4	3.86 (0.02)	Gal-(1→3)-Gal-(1→3)-Glc
		5a+5b	8.55 (0.07)	Gal-(1→4)-Gal-(1→2)-Glc + x*-(1→2)-Gal-(1→2)-Glc + Gal-(1→4)-Gal-(1→4)-Glc
		6	5.05 (0.12)	Gal-(1→4)-Gal-(1→4)-Glc + Gal-(1→6)-Gal-(1→4)-Glc
		7	5.00 (0.11)	Gal-(1→6)-Gal-(1→6)-Glc
		8	6.38 (0.04)	Gal-(1→6)-Gal-(1→4)-Glc + Gal-(1→6)-Gal-(1→2)-Glc
	DP4	9	1.25 (0.02)	x-(1→3)-x-(1→3)-x-(1→3)-x
		10a+10b	2.58 (0.04)	x-(1→6)-x-(1→4)-x-(1→y)-x + x-(1→6)-x-(1→2)-x-(1→y)-x + x-(1→6)-x-(1→6)-x-(1→4)-x + x-(1→6)-x-(1→4)-x-(1→4)-x
		11	3.66 (0.04)	x-(1→6)-x-(1→4)-x-(1→y)-x + x-(1→6)-x-(1→2)-x-(1→y)-x
		12	1.44 (0.03)	x-(1→4)-x-(1→4)-x-(1→6)-x or x-(1→y)-x-(1→6)-x-(1→6)-x
		13	2.24 (0.07)	x-(1→4)-x-(1→6)-x-(1→y)-x + x-(1→4)-x-(1→y)-x-(1→y)-x
	DP5	14	1.13 (0.02)	Unknown
		15	1.82 (0.04)	Unknown
		16	1.11 (0.03)	Unknown
		17	1.56 (0.02)	Unknown
GOS-2	DP2	1	9.26 (0.18)	Gal-(1→3)- Glc + lactulose
		2	37.89 (1.65)	Gal-(1→4)-Glc
		3	29.17 (0.39)	Gal-(1→6)-Glc
	DP3	4	1.53 (0.35)	Gal-(1→4)-Gal-(1→6)-x + Gal-(1→2)-Gal-(1→6)-x
		5	17.62 (0.24)	Gal-(1→6)-Gal-(1→4)-x
		6	2.99 (0.11)	Gal-(1→6)-Gal-(1→6)-x
		7	0.34 (0.18)	Gal-(1→3)-Gal-(1→6)-x
	DP4	8	1.02 (0.06)	x-(1→4)-x-(1→4)-x-(1→y*)-x + x-(1→6)-x-(1→4)-x-(1→y*)-x
		9	0.50 (0.02)	x-(1→4)-x-(1→4)-x-(1→y*)-x + x-(1→6)-x-(1→4)-x-(1→y*)-x + x-(1→6)-x-(1→4)-x-(1→y*)-x
	DP5	10	tr	Unknown
		11	tr	Unknown

Continuation table 4.12.

Sample	DP	Peak number	%	Identification
GOS-3	DP2	1	18.88 (0.25)	Gal-(1→3)-Glc
		2	26.11 (0.48)	Gal-(1→4)- + Gal-(1→2)- Glc
		3	8.34 (0.04)	Gal-(1→6)-Glc
	DP3	4	5.61 (0.05)	Gal-(1→3)-Gal-(1→3)-Glc
		5a + 5b	10.20 (0.08)	Gal-(1→4)-Gal-(1→2)-Glc + x*-(1→2)-Gal-(1→2)-Glc + Gal-(1→4)-Gal-(1→4)-Glc
		6	4.96 (0.15)	Gal-(1→4)-Gal-(1→4)-Glc + Gal-(1→4)-Gal-(1→2)-Glc + Gal-(1→4) -Gal-(1→6)-Glc + Gal-(1→2) -Gal-(1→6)-Glc
		7	4.30 (0.09)	Gal-(1→6)-Gal-(1→6)-Glc
		8	3.80 (0.05)	Gal-(1→6)-Gal-(1→4)-Glc + Gal-(1→6)-Gal-(1→6)-Glc
	DP4	9	1.66 (0.09)	x-(1→3)-x-(1→3)-x-(1→4)-x
		10a + 10b	3.09 (0.19)	x-(1→6)-x-(1→y)-x-(1→y)-x + x-(1→6)-x-(1→6)-x-(1→y)-x + x-(1→6)-x-(1→4)-x-(1→y)-x + x-(1→6)-x-(1→2)-x-(1→y)-x
		11	4.18 (0.17)	x-(1→4)-x-(1→4)-x-(1→6)-x + x-(1→4)-x-(1→6)-x-(1→6)-x
		12	1.34 (0.12)	x-(1→4)-x-(1→4)-x-(1→y)-x + x-(1→y)-x-(1→4)-x-(1→y)-x
		13	1.38 (0.12)	x-(1→4)-x-(1→y)-x-(1→6)-x + x-(1→y)-x-(1→y)-x-(1→6)-x + x-(1→4)-x-(1→y)-x-(1→3)-x + x-(1→y)-x-(1→y)-x-(1→3)-x
	DP5	14	1.13 (0.04)	Unknown
		15	1.83 (0.09)	Unknown
		16	1.73 (0.06)	Unknown
		17	1.45 (0.02)	Unknown

*x: unknown monosaccharide unit. *y: an unknown bond. [§]Standard deviation (n = 3). tr: traces.

4.1.3.4. Conclusions

The results presented in this work show the usefulness of HILIC-MS^n to separate and tentatively characterize complex mixtures of GOS without a previous fractionation, enrichment or derivatization step. The three studied silica-based HILIC columns exhibited substantial differences in peak shape, retention and selectivity which could be mainly attributed to the nature of the surface chemistry of the assayed stationary phases (sulfoalkylbetaine zwitterionic, polyhydroxyethyl aspartamide and ethylene bridge hybrid (BEH) with trifunctionally-bonded amide). Likewise, differences in the dimension of columns and, especially, particle properties (particle size, pore size and surface area) might also contribute to the retention of GOS. In this context, polar compounds possessing a high number of hydroxyl groups such as GOS were efficiently retained and separated on the BEH amide stationary phase using acetonitrile: water with 0.1% ammonium hydroxide as mobile phase.

The characterization of prebiotic GOS is of paramount importance for the elucidation of the structure-bioactivity relationship with respect to the effect of these carbohydrates on the human gastrointestinal health. MS^n characterization of GOS (in terms of monosaccharide composition, degree of polymerization and glycosidic linkages) should be considered tentative, taking into account the lack of standards. However, it requires much less handling, is less tedious and time consuming than the combination of complex techniques (isolation of each compound by fractionation methods and the subsequent analysis by NMR and methylation procedures) traditionally proposed in the literature.

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4.2. Desarrollo de métodos analíticos para el análisis de péptidos procedentes del CMP

4.2.1. Prefacio

Una de las técnicas analíticas más usadas para la caracterización de péptidos es la cromatografía líquida de alta resolución (HPLC), siendo la fase inversa (RP) el modo de operación más empleado. Sin embargo, este modo de operación se basa, principalmente, en interacciones hidrofóbicas entre el analito y la fase estacionaria y en consecuencia los compuestos altamente polares pueden ser eluidos con una resolución baja o nula.

El interés del CMP reside en que, por un lado, es uno de los componentes principales presentes en uno de los subproductos más importantes de la industria láctea como es el suero de quesería. Por otro lado, está formado por un grupo heterogéneo de péptidos de carácter multifuncional debido a sus múltiples modificaciones post-traduccionales, lo que le hace especialmente atractivo para su inclusión como ingrediente en la formulación y/o diseño de nuevos alimentos funcionales. El CMP es una secuencia altamente hidrofílica, por lo que es de esperar que los péptidos generados a partir de su hidrólisis enzimática presenten un carácter principalmente polar. En este sentido, la HILIC puede ser una excelente alternativa para obtener una mayor resolución en este tipo de compuestos. Además, el acoplamiento a la espectrometría de masas en tándem mejora la versatilidad del análisis, permitiendo una caracterización exhaustiva de péptidos, incluyendo sus posibles modificaciones post-traduccionales, como glicosilaciones o fosforilaciones que, también, contribuyen a la alta hidrofilicidad del CMP.

En esta sección se discutirá el análisis de *O*-sialoglicopéptidos, fosfopéptidos y péptidos sin modificaciones post-traduccionales, derivados de la hidrólisis enzimática del CMP, usando para ello cromatografía líquida de alta resolución acoplada a espectrometría de masas. De este modo, se optimizaron, aplicaron y compararon distintos métodos analíticos empleando dos modos de operación, HILIC y RPLC, mientras que la detección se llevo a cabo empleando dos analizadores de IT y Q-TOF.

La **sección 4.2.2** está basada en el trabajo titulado "**Development of a new method using hydrophilic interaction liquid chromatography (HILIC) - tandem mass spectrometry for the characterization of *O*-sialoglycopeptides from proteolytically**

digested caseinomacropeptide" de Hernández-Hernández y col. publicado en *Proteomics* 10 (2010) 3699-3711. En este trabajo se logró desarrollar por primera vez, un método basado en ZIC-HILIC para la caracterización de *O*-sialoglicopéptidos procedentes de hidrolizados de CMP, y que podría ser aplicable a otros hidrolizados proteicos que contengan *O*-glicosilaciones en su estructura. Se pudo además elucidar la estructura peptídica de estos hidrolizados mediante espectrometría de masas en tándem, todo ello en una sola inyección y sin necesidad de aplicar modificaciones como derivatizaciones o hidrólisis del enlace glicosídico. Asimismo, el método desarrollado permitió la separación de los isómeros de glicanos sialilados tanto en su forma libre como formando parte de los *O*-glicopéptidos debido a su distinto carácter ácido.

Por otro lado, en la **sección 4.2.3** se presenta el trabajo titulado "**Detection of two minor phosphorylation sites for bovine k-casein-macropeptide by reversed phase liquid chromatography–tandem mass spectrometry**" de Hernández-Hernández y col. publicado en *Journal of Agricultural and Food Chemistry* 59 (2011) 10848-10853, en dicho trabajo se logró identificar, por primera vez, un nuevo sitio de fosforilación presente en el CMP y, en consecuencia, en la k-caseína bovina. Además, se comparó la capacidad de un analizador de trampa iónica con la de un cuadrupolo acoplado a un tiempo de vuelo, para el análisis de fosfopéptidos. Al igual que en la sección anterior podría ser aplicable a cualquier otra muestra proteica con fosforilaciones presentes en su estructura. Los resultados obtenidos indicaron que el cuadrupolo acoplado a un tiempo de vuelo es un analizador más adecuado que la trampa iónica para la caracterización de fosfopéptidos que contengan en su secuencia más de un residuo posible de fosforilación.

4.2.2. Development of a new method using hydrophilic interaction liquid chromatography (HILIC) - tandem mass spectrometry for the characterization of *O*-sialoglycopeptides from proteolytically digested caseinomacropeptide

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Proteomics (**2010**) 10: 3699–3711.

Abstract

This work addresses the optimization of HILIC-ESI-MS^n conditions for the comprehensive characterization of *O*-glycopeptides from proteolytically digested caseinomacropeptide (CMP). *O*-Glycopeptides were satisfactorily analysed on a zwitterionic HILIC column based on their glycan structure and amino acid sequence. The contribution of ionic interactions to the retention of charged glycopeptides was found to be substantial. Thus, *O*-glycopeptides carrying neutral glycans were more retained than *O*-sialoglycopeptides due to that negatively charged sialic acid residues were electrostatically repelled by the stationary phase. In addition, glycopeptides differing only in the position of the linkage of the sialic acid moiety could be separated. The same chromatographic behaviour was observed for model systems constituted by a synthetic tetrapeptide covalently conjugated to neutral and sialylated carbohydrates. Subsequent detection of CMP *O*-glycopeptides was carried out on an electrospray ion trap tandem mass spectrometer at both positive and negative ionization modes. MS^2 fragmentation at positive ionization mode was valid for determining the glycan structure as the resulting main fragments corresponded to Y_n-type ions derived from sequential glycosidic bond fragmentation, whilst the fragmentation of the peptide structure was preferably obtained through the formation of b_n-type ions at the MS^3 stage, allowing the complete structure elucidation of the peptidic chain. Overall, the developed method allowed the identification and characterization of 41 *O*-glycopeptides covering all the known glycosylation sites without any previous enrichment step. These results point out that HILIC coupled to multistage MS procedures can be a powerful technique for future glycoproteomic applications.

4.2.2.1. Introduction

Protein glycosylation is one of the most usual post-translational modifications with important functions in all biological systems. Carbohydrates can be attached to proteins in two major ways resulting in the so-called *N*-glycans and *O*-glycans. *N*-glycans consist in a carbohydrate moiety *N*-linked to the amide nitrogen of asparagine side chains within an asparagine-x-serine/threonine motif where x corresponds to any amino acid except for proline, although there is also the option of having cysteine instead of serine/theronine. However, no unique sequence motif for *O*-glycosylation is known (Vance, et al., 1997). Moreover, the typical microheterogeneity of peptide (genetic variance) and oligosaccharide structures described for *O*-glycoproteins makes their characterization a challenging task and, hence, advanced analytical techniques are required (Seipert, et al., 2009).

Caseinomacropeptide (CMP) is the soluble C-terminal fragment derived from the action of chymosin or pepsin cleavage on α-casein, during the primary stage of cheesemaking or during digestion in the stomach. CMP is a heterogeneous group of acidic peptides due to extensive post-translational modifications such as *O*-glycosylation, phosphorylation and genetic variance. CMP is also considered as a multifunctional peptide with many possible biological applications (Abd-El-Salam, et al., 1996, Manso, et al., 2004, Thomae-Worringer, et al., 2006). Structure-activity studies have particularly pointed out the importance of the sugar residues for the different biological functions (Dziuba, et al., 1996). Glycosylated forms represent about 60% of the total bovine CMP (Vreeman, et al., 1986), and five different mucin-type carbohydrate chains, composed of the sialic acid *N*-acetylneuraminic acid (Neu5Ac), galactose (Gal) and *N*-acetylgalactosamine (GalNAc), have been identified in bovine CMP (Saito, et al., 1992): *i*) monosaccharide (GalNAc-O-R), *ii*) disaccharides (Gal-β(1→3)-GalNAc-O-R) *iii*) trisaccharide (Neu5Ac-α(2→3)-Gal-β(1→3)-GalNAc-O-R and Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc-O-R) and tetrasaccharide (Neu5Ac-α(2→3)-Gal-β(1→3)(Neu5Ac-α(2→6))-GalNAc-O-R). In the last twenty years, hydrophilic interaction liquid chromatography (HILIC) has proved to be a powerful tool in separating polar compounds such as carbohydrates (Alpert, 1990, Alpert, et al., 1994), pharmaceutical compounds (Guo, et al., 2003), metabolites and other small polar molecules (Guo, et

al., 2005, Godejohann, 2007). Whilst the main retention mechanism described for HILIC is the partitioning of polar analytes between the bulk eluent and a water-rich layer partially immobilized on the stationary phase (Alpert, 1990), functional groups on the stationary phases might also have an important influence on selectivity through secondary interactions such as ionic interactions (Guo, et al., 2005, McCalley, 2010), specific adsorption (Hemström, et al., 2006), hydrogen bonding (Berthod, et al., 1998), and even hydrophobic interactions (Hao, et al., 2008).

HILIC has been also successfully used in the analysis of *N*-glycopeptides (Wuhrer, et al., 2005, Takegawa, et al., 2006, Takegawa, et al., 2006, Takegawa, et al., 2008, Picariello, et al., 2008), but very little information is found about the separation of *O*-glycopeptides by this technique (Wuhrer, et al., 2005, Takegawa, et al., 2006). *O*-Linked glycosylation consists of attaching the glycans to the hydroxyl oxygen of serine or threonine residues in sequence regions of high hydroxyamino acid density imparting, thus, a polar character to the peptidic chain. Therefore, HILIC coupled to mass spectrometry (MS) by an electrospray interface (ESI) could be a useful technique to separate and characterize *O*-glycopeptides.

In this work a HILIC multi-stage mass spectrometric method (HILIC-ESI-MS^n) has been developed to characterize *O*-sialoglycopeptides in a tryptic/chymotryptic hydrolyzate of bovine CMP. Likewise, a synthetic tetrapeptide, covalently conjugated to carbohydrates of different molecular mass and monosaccharide composition (i.e., a couple of sialylated trisaccharide isomers, one sialopentasaccharide, and three neutral carbohydrates with different degree of polymerization) were also analysed in order to gain a better understanding on the mechanism of the separation of glycopeptides by HILIC on a zwitterionic column. Furthermore, in order to confirm the amino acidic sequence of the target CMP glycopeptides, MS^3 spectra obtained from the collisionally generated ion corresponding to the unglycosylated peptide were successfully performed.

4.2.2.2. Materials and methods

- *Materials*

All chemicals were purchased from Sigma-Aldrich and were of analytical grade unless otherwise stated. CMP was kindly provided by Davisco Foods International, Inc (Le Sueur, Minnesota, USA).

- *Proteolysis of CMP*

CMP was subjected to a combined trypsin/chymotrypsin proteolysis (overnight at 37 °C and pH 7) at 1 : 0.05 : 0.025 CMP : trypsin : chymotrypsin ratios (w : w : w). Activities of porcine pancreas trypsin (EC 3.4.21.4, type IX-S) and bovine pancreas α-chymotrypsin (EC 3.4.21.1, Type I-S) were 13000 - 20000 U and ≥40 U per mg of protein, respectively.

- *Preparation of model glycated tetrapeptides*

Aliquots of a solution consisting of 0.25 mg mL^{-1} acetylS-D-K-P and 0.25 mg mL^{-1} of the following and individual carbohydrates: i) galactose, ii) lactose, iii) maltopentaose, iv) Neu5Acα2-3Galβ1-4Glc, v) Neu5Acα2-6Galβ1-4Glc, and vi) Neu5Acα2-6Galβ1-4GlcNAcβ1-3Galβ1-4Glc in 10 mM ammonium acetate pH 6.8, were lyophilized. These were kept under vacuum in a desiccator at 40 °C for 1 day (for galactose) and 3 days (for the rest of carbohydrates) at water activity of 0.44 achieved with a saturated K_2CO_3 solution. The glycation occurred by condensation between the carbonyl group of carbohydrates (glucose moiety) and the ε-amino group of the lysine residue, leading to an *N*-glycosylamine that rearranges into the so-called Amadori product. Although MS^2 fragmentation would be different for glycated and *O*-glycosylated peptides, model systems were analysed by HILIC-ESI-MS as described below and identified on the basis of their expected $[M+H]^+$ and $[M-H]^-$ ions for the neutral and sialylated compounds, respectively.

- *HILIC-ESI-MS^n analysis*

All experiments were carried out on a Finnigan Surveyor pump with quaternary gradient system coupled to a Finnigan LCQ Deca ion trap mass spectrometer using an

ESI interface. Sample injections (10 µL) were carried out by a Finnigan Surveyor autosampler. All instruments (Thermo Fisher Scientific, San José, CA, USA), and data acquisition were managed by Xcalibur software (1.2 version; Thermo Fisher Scientific).

- *Chromatographic conditions*

The proteolytically digested CMP (1.5 µg) and the model glycated tetrapeptides (0.05 µg) were injected, and their separation performed at 25 ºC on a sulfoalkylbetaine zwitterionic stationary phase ZIC®-HILIC column (150 mm x 2.1 mm, 3.5 µm particle size, 200 Å pore size, SeQuant™) at a flow rate of 100 µL min^{-1}. The separation of the CMP hydrolysate was optimized by using different linear binary gradient programmes of Milli-Q water and acetonitrile with formic acid (final content of 0.05%, v:v) as an organic modifier. Once the gradient was optimized, the total concentration of formic acid was also adjusted (from 0.005% to 1.5%, v:v) in order to obtain the best resolution for the separation of CMP glycopeptides and the model glycated tetrapeptides.

- *Mass spectrometric conditions*

The mass spectrometer spray voltage was set at 4.5 kV, heated capillary temperature at 200 °C, nitrogen (99.5% purity) was used as sheath (0.6 L min^{-1}) and auxiliary (6 L min^{-1}) gas, and helium (99.999% purity) as the collision gas. Full scan mass spectra were recorded in the positive and negative ion modes between *m/z* 100 and 2,000. MS^2 data were acquired in the automatic data-dependent mode with a normalized collision energy of 35%, using a total cycle time of approximately 5 s, and with an ion selection threshold of 5 10^5 counts. MS^3 experiments were also carried out with a normalized collision energy of 35% but selecting the appropriated target ion transitions. Automatic gain control was used to maintain constant ion populations into the ion trap analyzer at 5 10^7 and 2 10^7 for full scan MS and MS^n spectra, respectively.

- *Data treatment*

Data processing was managed by Xcalibur software (2.0 version, Thermo Fisher Scientific). The initial assignment of observed ions to the corresponding amino acid sequences was based on the known sequence of CMP by using the protein database Swiss-Prot and TrEMBL and the tools Peptide Mass and FindPept available at

www.expasy.org. Parameters for the search were the following: i) monoisotopic peptide masses were indicated as $[M+H]^+$ with cysteines treated with nothing; ii) as enzymes, trypsin/chymotrypsin were chosen; iii) four missed cleavages were allowed; iv) peptides with a mass larger than 350 u were displayed; v) the mass tolerance was kept at 0.5 u.

To confirm the sequence of the glycopeptides, the MS^3 spectra were compared with the theoretical fragmentation of the putative peptides, obtained from the MS-Product software program (Protein Prospector, http://prospector.ucsf.edu/). (*Supporting information*)

- *pH measurement and pKa estimation*

The pH determination was carried out using a pH meter (S40 Seven Multi, Mettler-Toledo International Inc, USA) equipped with a combined glass Ag/AgCl electrode (Mettler-Toledo International Inc, USA). The pH values ($^{w}_{w}pK_a$) were taken in hydro-organic solutions after calibration of the instrument with pH 4.01 and 7.00 aqueous buffer solutions. The notation $^{s}_{w}pK_a$ means that the pH is directly measured in the solution (s) after the electrode was calibrated in pure water (w).

The estimation of $^{w}_{w}pK_a$ values was done by using the ADME suite 5.0 (Advanced Chemistry Development, Inc., Toronto, ON, Canada; www.acdlabs.com). The $^{s}_{w}pK_a$ values for each compound were calculated at different ratios of acetonitrile:water, according to the following equation (Espinosa, et al., 2000):

$$^{s}_{w}pK_a = a_s \, ^{w}_{w}pK_a + b_s + \delta \qquad (1)$$

where a_s and b_s are the slope and the intercept, respectively, of the linear relationship between $^{s}_{w}pK_a$ values in acetonitrile:water and the $^{w}_{w}pK_a$ values in pure water for aliphatic carboxylic acids, and δ is a parameter that allows conversion between both pK_a scales [24]. The three parameters depend on the hydro-organic composition.

4.2.2.3. Results and discussion

- *Optimization of HILIC separation of the proteolytically digested CMP*

It is generally accepted that retention in HILIC is mainly driven by 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 (Alpert, 1990, Hemström, et al., 2006, Yang, et al., 2009). Thus, seven different binary gradients of acetonitrile (solvent A) and Milli-Q water (solvent B) both with 0.05% formic acid (v/v) were assayed. Finally, the best conditions for the separation of the hydrolysate of CMP were as follows: from 0 to 5 min a constant 30% B; from 5 to 40 min a linear gradient from 30 to 45% B; from 40 to 45 min a linear gradient from 45 to 90% B; from 45 to 75 min at 90% B isocratic; ramped to original composition in 1 min; then equilibrated for 15 min. The resulting base peak chromatogram for the proteolytically digested CMP revealed the presence of a complex mixture of peptides eluting between 4 and 30 min (**Figure 4.13 B**) indicating a high rate of proteolysis of the CMP.

Since other secondary mechanisms, in addition to partitioning, such as electrostatic interactions and hydrogen bonding may also be involved in the HILIC retention process (Hemström, et al., 2006, Yoshida, 2004), the effect of the final concentration of formic acid, from 0.005 to 1.5% (v/v) leading to a mobile phase $^{s}_{w}\mathrm{pH}$ range from 3.6 to 2.1, on the separation of the CMP hydrolysate was also investigated. Changing pH should have an effect mainly on the net charge of the peptides as it has been described that the surface charge of the sulfoalkylbetaine zwitterionic stationary phase ($-CH_2N^+(CH_3)_2-CH_2-CH_2-CH_2-SO_3^-$) is not largely affected by changes in pH (Hemström, et al., 2006), specially between pH 3 and 8 (Boersema, et al., 2007). Consequently, different separation selectivity was observed depending on the concentration of formic acid, indicating that the interactions between the peptides or the terminal unit of the glycans and the stationary phase changed. Figure 1 shows the base peak chromatograms of the CMP hydrolysate obtained under different concentrations of formic acid. In general, a trend of longer retention with increasing pH was obtained and, consequently, addition of the lowest concentration of formic

acid, 0.005% v/v (**Figure 4.13 A**), seemed to provide the best chromatographic separation conditions among all assayed concentrations.

- *HILIC separation of the glycated tetrapeptide model systems*

In order to rationalize the retention behaviour of glycosylated peptides, several model systems of the tetrapeptide, acetylS-D-K-P, covalently conjugated to different carbohydrates (neutral and sialylated) indicated in the experimental section, were analysed under different concentrations of formic acid (**Figures 4.14 and 4.15**).

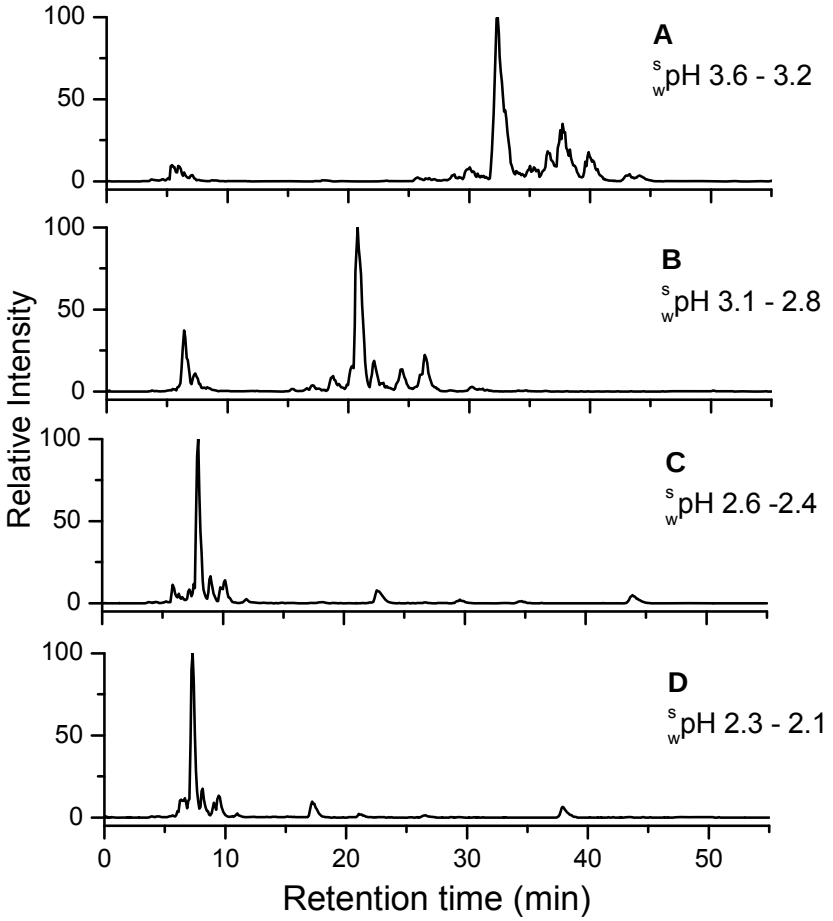

Figure 4.13. HILIC-ESI-MS base peak profiles monitorized at positive ionization mode of proteolytically digested CMP using different formic acid concentrations (% v/v). A) 0.005%; B) 0.05%; C) 0.5%; D) 1.5%. $^{s}_{w}\mathrm{pH}$ values at 70:30 and 10:90 (v:v) acetonitrile:water of mobile phase are shown in the figure.

The retention of the unglycated peptide increased steadily in the studied pH range, especially between 2.6 and 3.6 (**Figure 4.14**, peak 1). At the lowest concentration of formic acid, 0.005% v/v, the unglycated peptide exhibited a long retention time (38.7 min) probably due to the high hydrophilicity of the positively charged peptidic chain, with three of the four residues being polar amino acids. The presence of a lysine residue, which is a basic amino acid, largely contributed to the HILIC retention as the zwitterionic stationary phase acts, in addition to the water-retaining property, as weak-cation exchange LC column between the basic amino acids and the negatively charged terminal sulfonate group (Hemström, et al., 2006). The decrease in the retention time of the positively charged peptide as the concentration of formic acid increased could be attributed to a gradual suppression of the electrostatic (attraction) interactions with the zwitterionic stationary phase. In good agreement with this behaviour, a decrease in the capacity factor of peptides such as bradykinin or angiotensin II separated in a ZIC-HILIC column was observed as the formic acid concentration increased (http://www.nestgrp.com/pdf/Zp1/ZIC_02poster.pdf).

Regardless the formic acid concentration, a remarkable increase of the retention was detected following glycation of the tetrapeptide with neutral carbohydrates (**Figure 4.14**, peaks 2, 3 and 4), this increase being proportional to the length of the attached carbohydrate. This behaviour can be attributed to the incorporation of galactose or glucose residues into the peptidic chain that leads to an increase in peptide hydrophilicity and, therefore, to a higher partitioning (hydrogen bonding) with the water-enriched layer covering the polar hydrophilic stationary phase.

When this model peptide was glycated with sialylated oligosaccharides, a dramatic decrease in the retention time was found at 0.005% (v/v) of formic acid, this effect even being more considerable for the sialylated trisaccharide isomers than for the pentasaccharide (**Figure 4.15 A**, peaks 8, 9 and 10). Nevertheless, increasing the formic acid concentration affected selectivity in the ZIC-HILIC column (**Figures 4.15 B, C and D**).

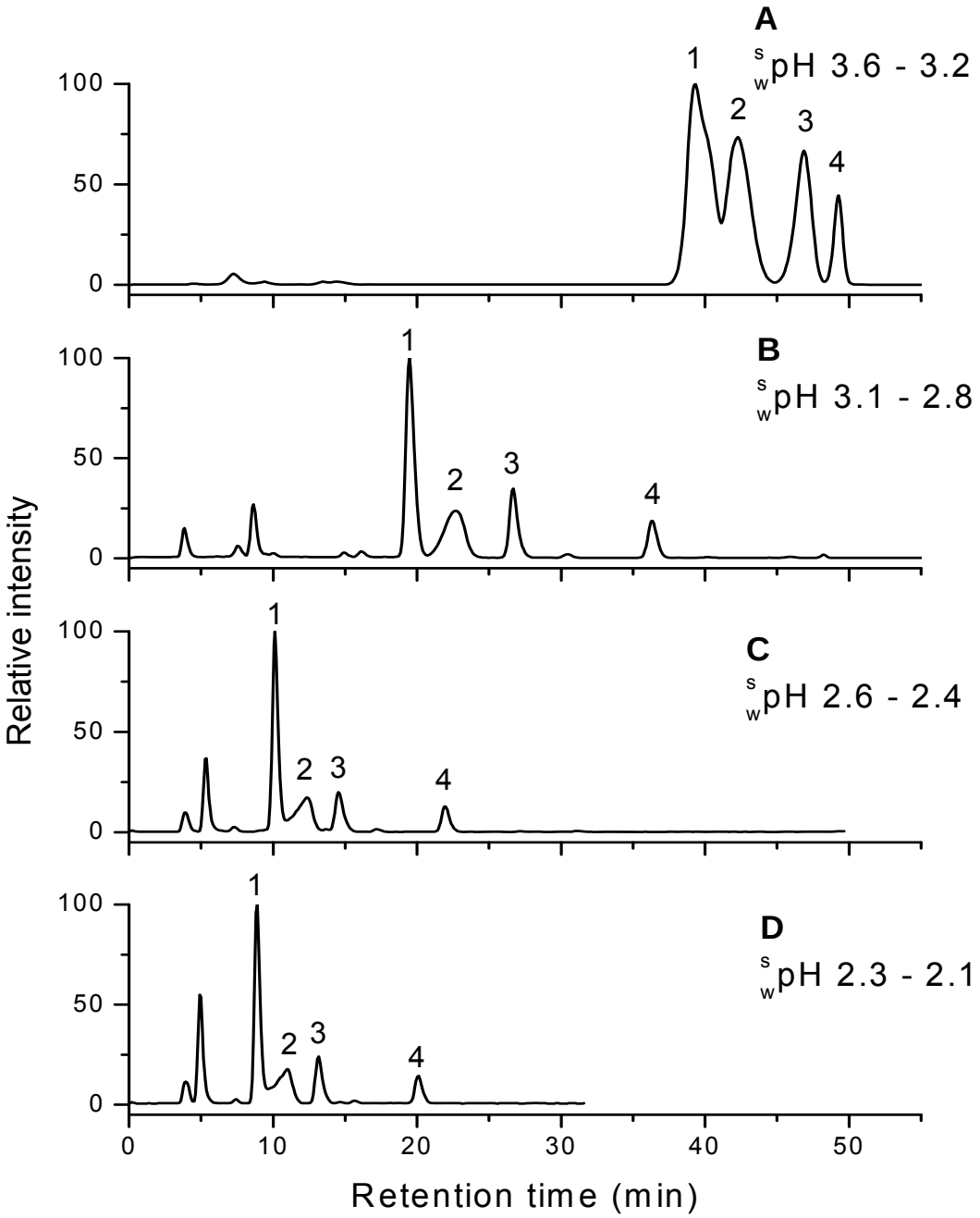

Figure 4.15. HILIC-ESI-MS base peak profiles monitorized at positive ionization mode of the tetrapeptide (acetylS-D-K-P) model system conjugated with neutral carbohydrates using different formic acid concentrations (% v/v). A) 0.005%; B) 0.05%; C) 0.5%; D) 1.5%. Labelled peaks are as follows: **(1)** Tetrapeptide; **(2)** Tetrapeptide conjugated with Galactose; **(3)** Tetrapeptide conjugated with Lactose; **(4)** Tetrapeptide conjugated with Maltopentaose. $^{s}_{w}\mathrm{pH}$ values at 70:30 and 10:90 (v:v) acetonitrile:water of mobile phase are shown in the figure.

Thus, the retention of the unglycated peptide (peak 1) gradually decreased with the increased formic acid concentration as explained above; whilst an increase in the retention of the glycated peptides (peaks 8, 9 and 10) was observed when 0.05% of formic acid was assayed. Higher concentrations of formic acid (0.5 and 1.5%) have hardly any effect on the retention of the glycated peptides with both trisaccharides and the pentasaccharide (**Figures 4.15 C and D**, peaks 8, 9 and 10). It is well know, that sialic acids are strongly acidic, e. g. the pK_a of Neu5Ac is between 2.6 and 2.9 (Scheinth.Bm, et al., 1968), and the carboxylic acid group at the C-1 position is easily ionized providing, thus, a negative charge to the molecule. Therefore, it is very plausible that, in addition to hydrophilic interactions, some electrostatic repulsion between the localized negative charge on the sialylated oligosaccharides and that on the stationary phase are involved in the retention of the glycated model tetrapeptide. These electrostatic repulsion interactions could also explain the low retention of the free sialylated carbohydrates, non-attached to the peptide, particularly when 0.005% formic acid was used (**Figure 4.15**, peaks 5, 6 and 7). The ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ values of the sialylated oligosaccharides were calculated by using the ADME suite programme, and their respective ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ values at 70:30 and 10:90 of acetonitrile:water (v:v) through Equation 1 (**Table 4.13**). From the calculated ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ values, it can be inferred that at 0.005% of formic acid concentration (pH of the mobile phase 3.6-3.2), all sialylated oligosaccharides can be negatively charged due to the deprotonation of the carboxylic acid group at the C-1 of the sialic acid. Whereas by increasing the amount of formic acid (i.e., lowering the pH of the mobile phase), the retention of the free sialylated oligosaccharides was also increased because of the partial protonation of the sialic acids and, thereby, minimizing the electrostatic repulsions with the negatively charged terminal sulfonate group (**Figure 4.15**, peaks 5, 6 and 7).

Finally, it should be remarkable that both sialylated trisaccharide isomers could be partially separated either in their unconjugated form (**Figure 4.15**, peaks 5 and 6) or conjugated with the tetrapeptide (**Figure 4.15**, peaks 8 and 9), the trisaccharide with the α2-3 linked sialic acid being less retained than the isomer with the α 2-6 linked sialic acid. This fact reveals that the ZIC-HILIC column has the capacity for separation of isomeric sialylated glycans with small oligosaccharide and peptide chains.

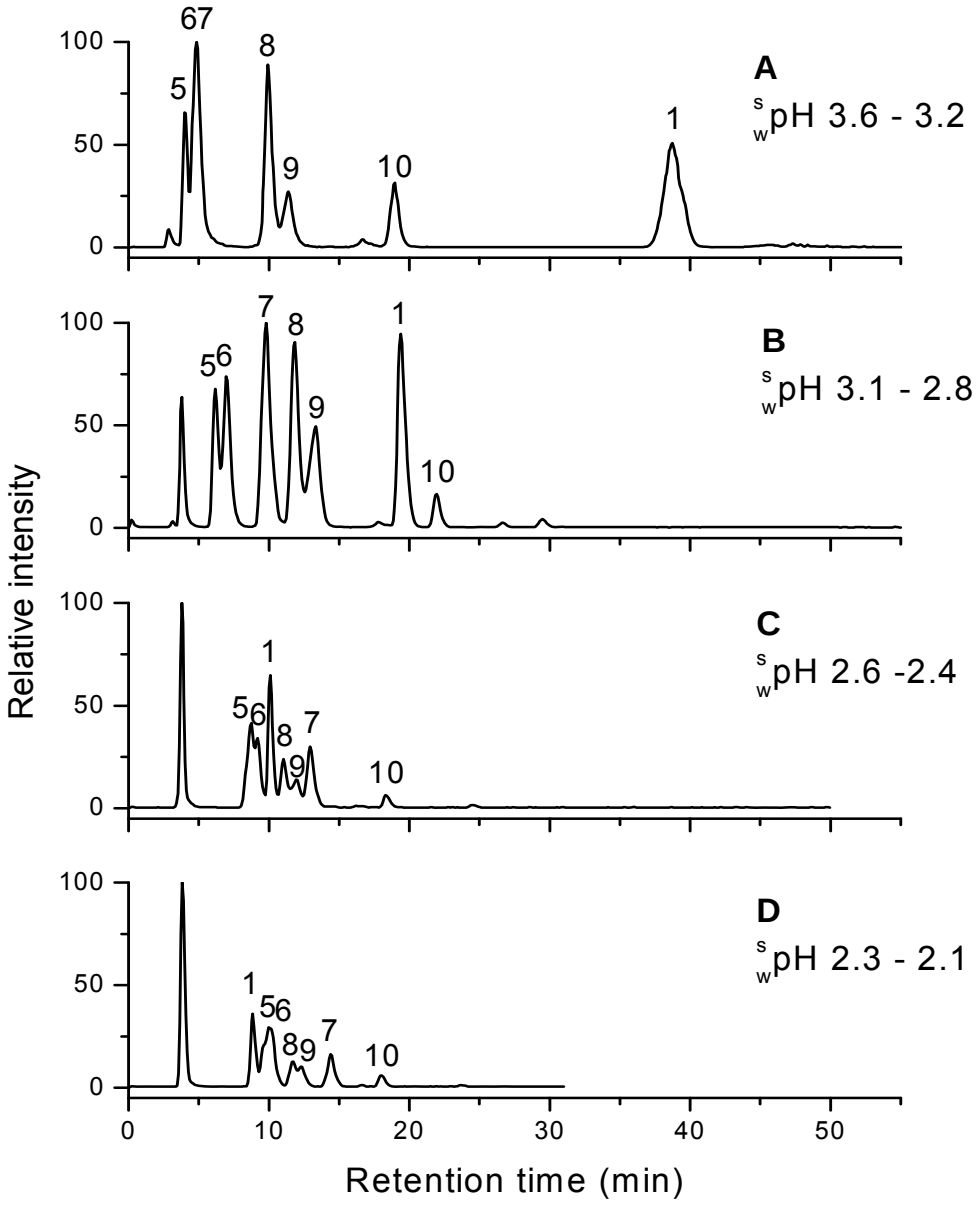

Figure 4.15. HILIC-ESI-MS base peak profiles monitorized at negative ionization mode of the tetrapeptide (acetylS-D-K-P) model system conjugated with sialylated carbohydrates using different formic acid concentrations (% v/v). A) 0.005%; B) 0.05%; C) 0.5%; D) 1.5%. Labelled peaks are as follows: (1) Tetrapeptide; (5) Neu5Ac-α(2→3)-Gal-β(1→3)-GalNAc; (6) Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc; (7) Neu5Ac-α(2→3)-Gal-β(1→3)(Neu5Ac-α(2→6))-GalNAc; (8) Tetrapeptide conjugated with Neu5Ac-α(2→3)-Gal-β(1→3)-GalNAc; (9) Tetrapeptide conjugated with Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc; (10)Tetrapeptide conjugated with Neu5Ac-α(2→3)-Gal-β(1→3)(Neu5Ac-α(2→6))-GalNAc. values at 70:30 and 10:90 (v:v) acetonitrile:water of mobile phase are shown in the figure.

Takegawa et al. (2006) described a similar behaviour, although they studied the separation of large isomeric tri- and tetra-antennary *N*-glycan structures. These authors indicated that the higher retention of the α(2-6) sialylated isomers could be due to the higher flexibility in changing its conformation/orientation as compared to the relatively rigid α(2-3) sialylated isomer so, thus, the electrostatic repulsion interaction might be minimized. In our case, according to their ${}^{s}_{w}\mathrm{p}K_{a}$ values (**Table 4.13**), we also postulate that the α(2-6) sialylated isomer is slightly less acidic and, therefore, less negatively charged than the trisaccharide with the α(2-3) linked sialic acid, resulting in a diminishing of the electrostatic repulsion and leading to a higher retention. In addition, free or conjugated sialylated pentasaccharide (**Figure 4.15**, peaks 7 and 10) had a higher retention than the corresponding sialylated trisaccharide isomers probably due to the partitioning effect as explained above under all assayed conditions.

Overall, it can be concluded that the best separation between the neutral and the sialylated model peptides was obtained with the lowest concentration of formic acid assayed (0.005%, v:v). This behaviour agrees with the separation of CMP, which contains either neutral or sialylated glycans, at different concentrations of formic acid shown in **Figure 4.13**. Furthermore, it should be noted that the presence of low concentrations of formic acid in the mobile phase are favoured for MS detection, as these tend to improve the signal intensity. Consequently, the addition of 0.005% of formic acid was used for the characterization of the *O*-sialoglycopeptides from proteolytically digested CMP.

- *Characterization of O-sialoglycopeptides derived from CMP by HILIC-ESI(+)-MS^n*

HILIC-ESI(+)-MS^2 analyses of *O*-glycopeptides mainly resulted in glycosidic bond fragmentation, providing Y_n-type ions, according to the nomenclature of Domon and Costello [29] for glycan cleavages, corresponding to intact peptidic chain fragments formed by sequential losses of carbohydrate residues from the $[M+H]^+$ ion (Supporting information). While only the minor sugar oxonium ion (B_n-type) corresponding to the intact glycan could be detected (i.e: *m/z* 948; **Figure 4.16**).

Table 4.13. ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ and ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ values of the sialylated oligosaccharides identified in bovine

Sialyloligosaccharide structure	${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ [1]	${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ [2]	
		70:30	***10:90***
Neu5Ac-α(2→3)-Gal-β(1→4)-Glc	2.3	3.7	2.4
Neu5Ac-α(2→6)-Gal-β(1→4)-Glc	2.4	3.9	2.6
Neu5Ac-α(2→6)-Gal-β(1→4)-GlcNAc-β(1→3)-Gal-β(1→4)-Glc	2.5	3.9	2.6
Neu5Ac-α(2→3)-Gal-β(1→3)-GalNAc	2.3	3.7	2.4
Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc	2.5	3.9	2.6
Neu5Ac-α(2→3)-Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc	2.5 and 2.3	3.9 and 3.7	2.6 and 2.4

CMP and of those employed for the glycation of the model tetrapeptide acetylS-D-K-P.

[1] Calculated by ADME suite software (see materials and methods section).

[2] Calculated by Equation 1 at 70:30 and 10:90 acetonitrile:water (v/v) mixtures.

Therefore, based on the detected Y-ions, the carbohydrate sequence of the glycan could be completely elucidated. Thus, the analysis by HILIC-ESI-MS^2 on a three-dimensional ion trap monitoring neutral losses of 291 u (singly charged ion, supporting information) or 145.5 u (doubly charged ion, **Figure 4.17**), corresponding to the loss of one molecule of Neu5Ac, was a suitable and direct method to detect *O*-sialoglycopeptides. Furthermore, a wide range of retention times (from 3 to 50 min) of *O*-sialoglycopeptides was observed under these conditions, which is indicative of the importance of the nature of the amino acidic sequence (*i.e*., hydrophilicity and surface net charge), as well as the charge state and size of the attached glycan. Likewise, monitorization of neutral losses of 162 / 203 u (singly charged ions) and 81 / 101.5 u (doubly charged ions) corresponding to Hex / HexNAc residues, respectively, were also successfully performed in order to detect *O*-glycopeptides not containing sialic acid residues (supporting information).

Whilst the most abundant ion present in all MS^2 spectra corresponded to the unglycosylated (intact) peptide, the sequence informative *b*- and *y*-ions, following the nomenclature of Roepstorff y Fohlman (1984), resulting from peptide backbone cleavage were normally very weak or undetected. This fact impaired the accurate and direct identification of the amino acidic sequence of target glycopeptides by HILIC-

ESI(+)-MS^2 analyses. Nevertheless, taking advantage of multi-stage fragmentation capabilities of ion traps, analyses of the corresponding unglycosylated peptide ion with a subsequent stage of MS were successfully performed. Thus, MS^3 spectra obtained from the collisionally generated ion corresponding to the unmodified peptide were preferentially characterized by the *b*-serial ions owing to the free *N*-terminus (**Figure 4.18**), allowing an accurate identification of the peptide backbone (Supporting information).

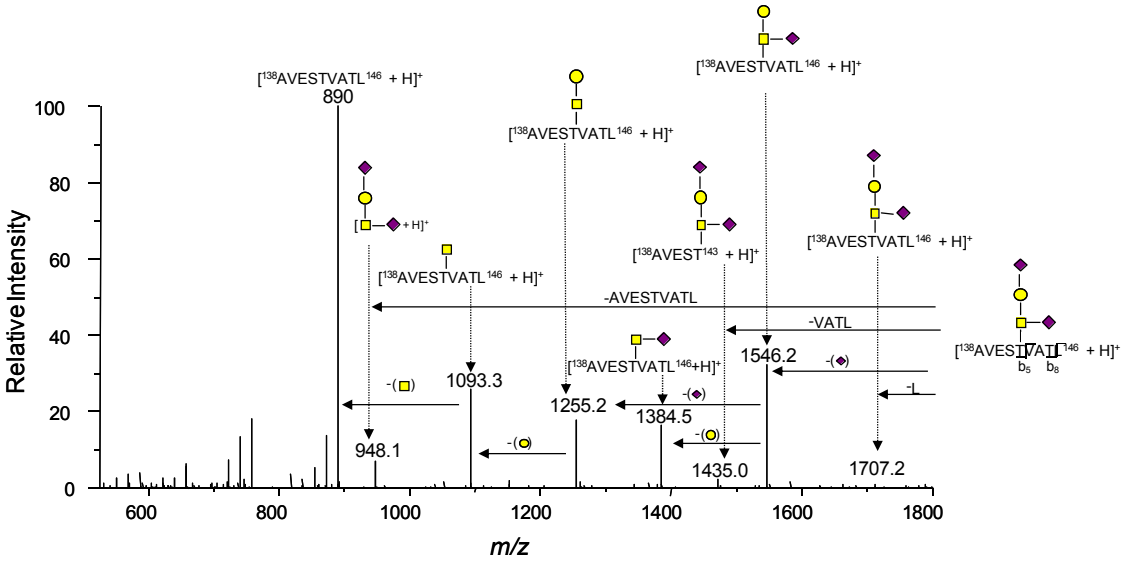

Figure 4.16. MS^2 spectrum of a singly charged ion at *m/z* 1837.5 corresponding to the protonated CMP peptide ^{138}A-L^{146} glycosylated with the sialyl-tetrasaccharide (Neu5Ac-α(2→3)-Gal-β(1→3)(Neu5Ac-α(2→6))-GalNAc) at the T^{142} residue. Symbols: ■ *N*-Acetyl-Galactosamine; ● Galactose; ♦ Sialic acid.

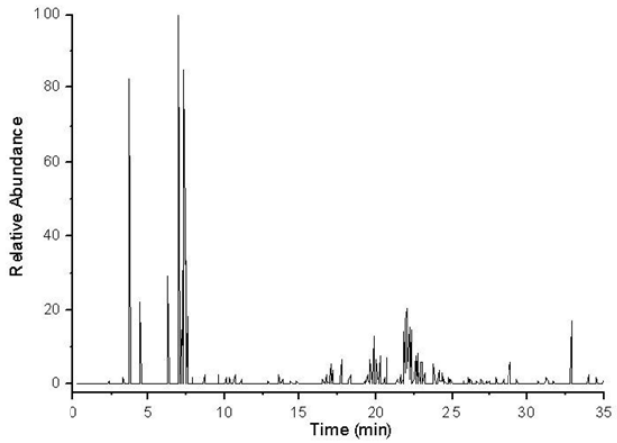

Figure 4.17. Monitorization profile at positive ionization mode of neutral loss of 145.5 (doubly charged ion) corresponding to one molecule of Neu5Ac in the MS^2 spectrum of proteolytically digested CMP.

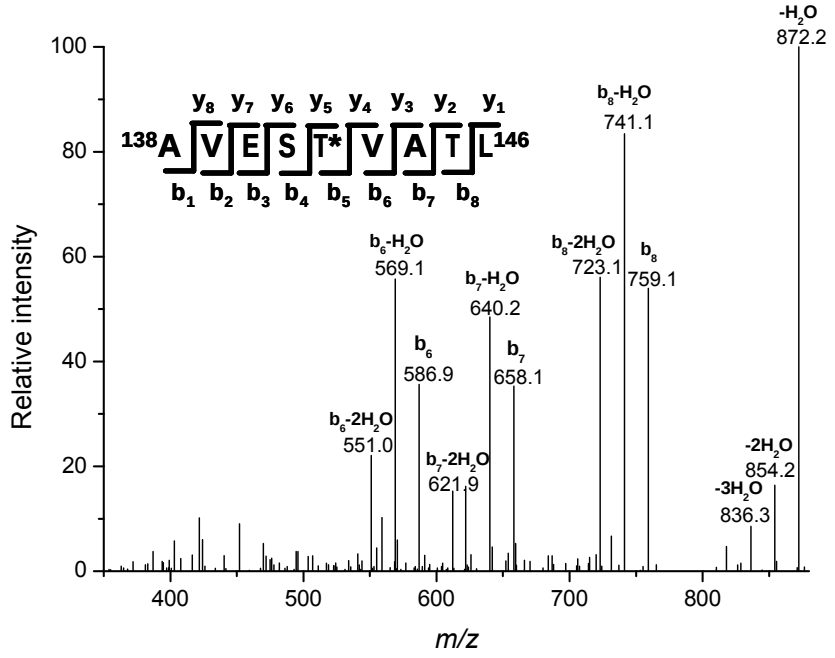

Figure 4.18. MS^3 spectrum of a singly charged ion corresponding to the CMP glycopeptide ^{138}A-L^{146} from collisionally generated unglycosylated peptide ion ($[M+H]^+$ = 890.2) derived from the MS^2 analysis at positive ionization mode.

Table 4.14 summarizes the 41 *O*-glycopeptides belonging to CMP genetic variants A and B identified by HILIC-ESI(+)-MS^n. Three glycopeptides, ^{113}N-N^{123}, ^{138}A-L^{146}, and ^{161}T-V^{169}, could be characterized containing either the sialyltetra- or the sialyltrisaccharide glycans. This implies an additional advantage of using HILIC separation with a low concentration of formic acid, as it is very usual that at least one sialic acid residue can be lost during sample processing for proteomics or reverse phase LC analysis due to its lability under acidic conditions; thus, impairing the detection of CMP glycopeptides containing the attached tetrasaccharide (Holland, et al., 2004, Holland, et al., 2005).

According to their order of elution, it was observed that those glycopeptides containing the tetrasaccharide (comprised of two Neu5Ac residues) had shorter retention times than the counterpart glycopeptide containing the trisaccharides (one Neu5Ac), which in turn also had a shorter retention time than the corresponding glycopeptide modified with the (neutral) di- or monosaccharide. These data were in very good agreement with the behaviour observed above for the model tetrapeptide

glycated with sialylated and neutral oligosaccharides. This behaviour was also observed for those *O*-sialoglycopeptides, such as ^{113}N-N^{123}, largely retained in the column (t_R of 21 min; **Table 4.14**). In this case this high retention could be predominantly attributed to the presence of one lysine residue in the peptidic chain which may largely contribute to the HILIC retention as it was observed for the model tetrapeptide.

On the other hand, the detection of isobaric sialoglycopeptides at different t_R values was mostly observed when the attached glycan was a trisaccharide (**Table 4.14**). This fact was attributed to the occurrence of two different isomeric trisaccharides, linear (α(2-3)) or branched (α(2-6)), depending on the attachment of the sialic acid. In a similar way to the couple of sialylated trisaccharide isomers employed for the glycation of the model tetrapeptide, the branched CMP *O*-linked trisaccharide had higher ${}^{\mathrm{w}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ and ${}^{\mathrm{s}}_{\mathrm{w}}\mathrm{p}K_{\mathrm{a}}$ values than the *O*-linked trisaccharide with the α(2-3) linked sialic (**Table 4.13**). Thereby, it could be expected that the CMP glycopeptides containing the branched trisaccharide had a higher HILIC retention due to a low degree of electrostatic repulsion interactions than their counterparts carrying the linear isomeric trisaccharide, as it occurred for the model peptide glycated with the sialylated trisaccharide isomers (**Figure 4.15**, peaks 8 and 9). This behaviour was confirmed by the MS^2 detection of the fragment $Y_{1\beta}$-type ion corresponding to the initial neutral loss of Gal residue ($[M+H]^+$ = 1399.2 in **Figure 4.19 B**), denoting the presence of this carbohydrate at the terminal position of the glycan structure and, therefore, revealing the presence of the branched trisaccharide (**Figure 4.19**). Consequently, as it is shown in **Table 4.14**, peptides having the same amino acid sequence but glycosylated with the sialyl α(2-3) trisaccharide eluted earlier than those conjugated with the sialyl α(2-6) isomer.

Furthermore, glycopeptides ^{124}T-E^{137} and ^{125}I-E^{137} modified with the single monosaccharide, and ^{124}T-T^{135} that contained the disaccharide also showed two different t_R which could be due to the attachment of the glycan to a different glycosylation site (T^{131} or T^{133}) in the peptide chain. A similar behaviour was previously described in the characterization by RPLC of intact glycoforms of bovine (Molle, et al., 1995), ovine (Moreno, et al., 2000) and caprine (Moreno, et al., 2001) CMP.

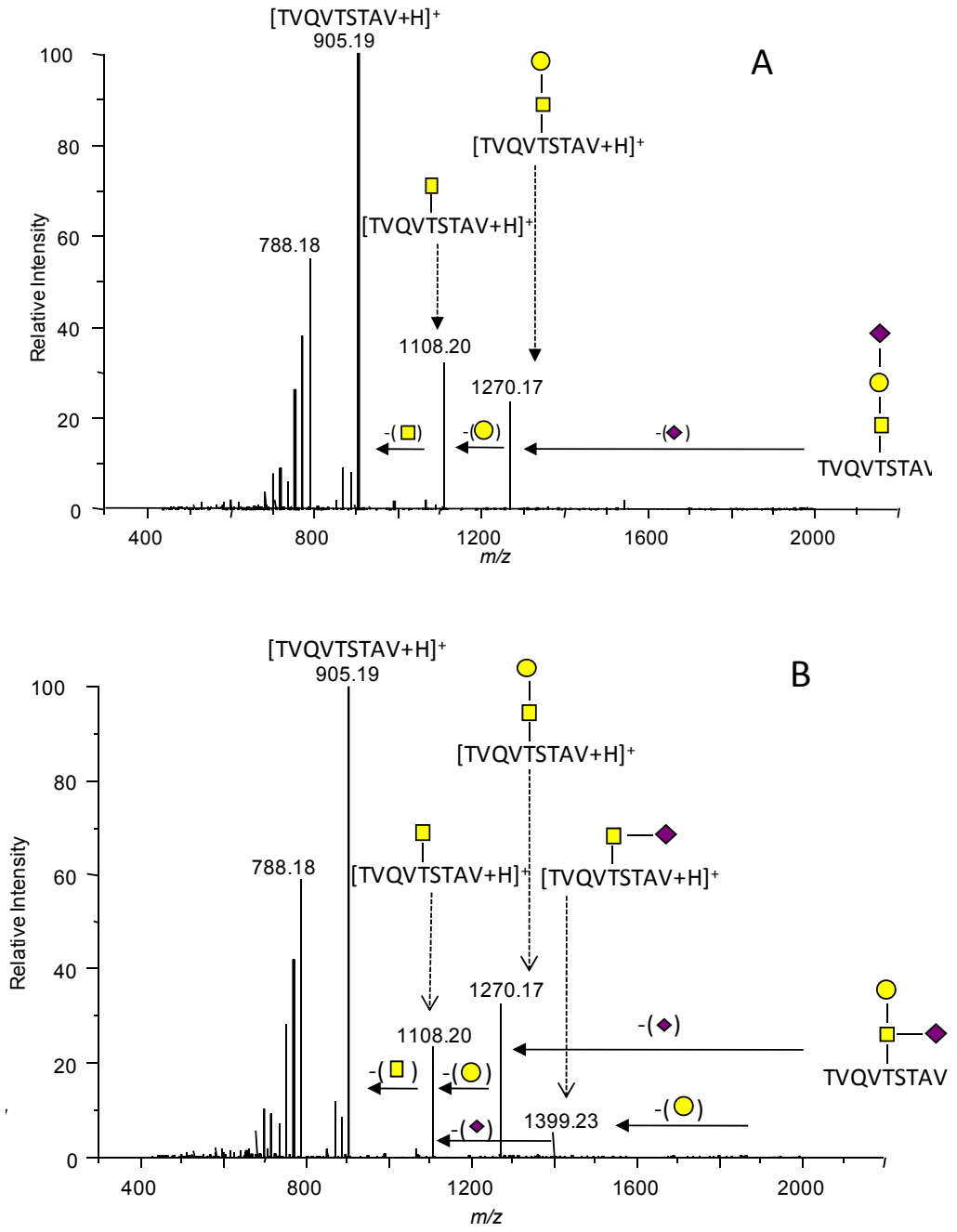

Figure 4.19. MS^2 spectra at positive ionization mode of a singly charged ion ($[M+H]^+$ = 1561.40) corresponding to the CMP peptide ^{161}T-V^{169} glycosylated with A) the sialyl-trisaccharide Neu5Ac-α(2→3)-Gal-β(1→3)-GalNAc and B) the sialyl-trisaccharide Gal-α(1→3)-(NeuAc-α(2→6))-GalNAc at the ^{165}T residue. Symbols: ■ *N*-Acetyl-Galactosamine; ● Galactose; ♦ Sialic acid.

Table 4.14. *O*-Glycopeptides identified in proteolytically digested CMP by HILIC-ESI-MS^n.

t_R (min)	Glycopeptide sequence	Position[a)]	Attached glycan[b)]	Exp. Mass $[M+H]^{+}$[c)]	Genetic variant	T glycosylated residue number
4.6	AVESTVATL	138-146	I	1837.4	A & B	142
7.9	AVESTVATL	138-146	II	1546.4	A & B	142
10.2	AVESTVATL	138-146	III	1546.4	A & B	142
9.9	TIASGEPTSTPTTE	124-137	II	2048.6	A	131 or 133
12.7	TIASGEPTSTPTTE	124-137	III	2048.6	A	131 or 133
46.5	TIASGEPTSTPTTE	124-137	IV	1756.4	A	131 or 133
41.1 and 42.5	TIASGEPTSTPTTE	124-137	V	1594.4	A	131 or 133
4.6	TVQVTSTAV	161-169	I	1852.4	A & B	165
9.5	TVQVTSTAV	161-169	II	1561.4	A & B	165
12.5	TVQVTSTAV	161-169	III	1561.4	A & B	165
49.2	TVQVTSTAV	161-169	IV	1270.3	A & B	165
46.5	TVQVTSTAV	161-169	V	1108.4	A & B	165
9.7	VTSTAV	164-169	II	1233.3	A & B	165
14.1	VTSTAV	164-169	III	1233.3	A & B	165
51.1	VTSTAV	164-169	IV	942.2	A & B	165
49.9	VTSTAV	164-169	V	780.2	A & B	165
10.2	IASGEPTSTPTTE	125-137	II	1946.7	A	131 or 133
12.8	IASGEPTSTPTTE	125-137	III	1946.7	A	131 or 133
48.9	IASGEPTSTPTTE	125-137	IV	1655.7	A	131 or 133
45.4 and 46.7	IASGEPTSTPTTE	125-137	V	1493.6	A	131 or 133
9.4	TIASGEPTSTPTIE	124-137	II	2059.4	B	131 or 133
45.4	TIASGEPTSTPTIE	124-137	IV	1768.4	B	131 or 133
41.1	TIASGEPTSTPTIE	124-137	V	1606.5	B	131 or 133
8.6	TEIPTIN	117-123	II	1443.4	A & B	121
45.4	TEIPTIN	117-123	IV	1152.3	A & B	121
40.9	TEIPTIN	117-123	V	990.3	A & B	121
10.7	IEAVESTVATL	136-146	II	1788.5	B	142
10.4	STVA	141-144	II	1033.2	A & B	142
51.0	STVA	141-144	IV	742.4	A & B	142
50.1	STVA	141-144	V	580.3	A & B	142
10.3	TIASGEPTSTPT	124-135	II	1817.3	A & B	131 or 133
13.1	TIASGEPTSTPT	124-135	III	1817.3	A & B	131 or 133
47.9 and 48.6	TIASGEPTSTPT	124-135	IV	1526.4	A & B	131 or 133
45.7	TIASGEPTSTPT	124-135	V	1364.3	A & B	131 or 133
15.5	STPTIE	132-137	III	1303.4	B	133
45.9	STPTIE	132-137	V	850.3	B	133
21.1	NQDKTEIPTIN	113-123	I	2220.0	A & B	121
48.9	NQDKTEIPTIN	113-123	II	1928.4	A & B	121
50.3	NQDKTEIPTIN	113-123	III	1928.4	A & B	121
50.2	PTTEAVEST	134-142	IV	1299.3	A	136
48.7	PTTEAVEST	134-142	V	1137.3	A	136

[a)] Residue numbering is based on the Swiss-Prot entry for the mature form of bovine k-casein (accession number P02668).

[b)] Structure of the attached glycans are as follows: **I**, Tetrasaccharide, Neu5Ac-α(2→3)-Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc-O-R; **II**, Trisaccharide, Neu5Ac-α(2→3)-Gal-β(1→3)-GalNAc-O-R; **III**, Trisaccharide, Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc-O-R; **IV**, Disaccharide, Gal-β(1→3)-GalNAc-O-R; **V**, Monosaccharide, GalNAc-O-R.

[c)] Monoisotopic mass values.

- *Identification of the specific glycosylation-site*

Bovine CMP has up to six well-defined *O*-glycosylation sites located at T^{121}, T^{131}, T^{133}, T^{136}, T^{142} and T^{165} (residue numbering is based on the Swiss-Prot entry for the mature form of bovine α-casein, accession number P02668) as it was determined by solid-phase Edman degradation (Pisano, et al., 1994). A more recent proteomic identification of the modified sites, which involved the previous separation of the glycoforms by two-dimensional electrophoresis, revealed a distinct hierarchy in the *O*-glycosylation of α-casein that implies an ordered addition of glycans to T^{131}, T^{142} and T^{133} (Holland, et al., 2005). Our work has permitted a comprehensive characterization of the *O*-glycopeptides of CMP because the identified structures covered all threonine residues described as potential *O*-glycosylation sites (**Figure 4.20**). Thus, in 24 of 41 glycopeptides an unambiguous characterization of the *O*-glycosylation site could be obtained based on the previously described glycosylation sites (**Table 4.14**). Moreover, as glycosylation confers protease resistance to nearby peptide bonds, it is very plausible that the peptide ^{134}P-T^{142} is glycosylated at T^{136} rather than T^{142}.

Nevertheless, the specific glycosylation site of a few peptides located at the region ^{124}T-E^{137}, which contains three potential glycosylation-sites (T^{131}, T^{133} and T^{136}), could not be straightforward assigned (**Table 4.14**). In order to overcome this drawback, several strategies were carried out. The first approach was based on the use of the fragment containing the peptidic chain attached to the GalNAc residue (which was the second most abundant ion in the positive-ion MS^2 spectra) as a precursor ion to trigger the MS^3 spectra. A similar experiment was successfully carried out for the identification of *N*-glycosylation sites (Deguchi, et al., 2006) by using electrospray ionization on a linear ion trap time-of-flight mass spectrometer. Nevertheless, in the case of CMP *O*-glycopeptides analysed on an ion trap, this approach resulted in the rapid cleavage of the glycosidic bond (according to the high abundance of the whole peptide fragment without the GalNAc residue attached). The only detected peptide fragment which still carried the GalNAc residue attached was the *b*-ion containing all the sequence except the C-terminal amino acid residue, thus, providing no further evidence of the specific glycosylation site (supporting information).

Secondly, despite MS^2 spectra of glycopeptides showed abundant ions from glycosidic bond and very low abundant ions derived from peptide-bond fragmentation,

useful data could be obtained from an extended search for the low-abundant peptide-backbone ions with minimal fragmentations of glycans. Using this approach, the T^{136} could be ruled out as a glycosylation site in the peptides $^{124}T\text{-}E^{137}$ (**Figure 4.21 A**) and $^{125}I\text{-}E^{137}$ (supporting information). For example, this was possible due to the detection of the minor b_{10} ion, i. e. the fragment $^{124}T\text{-}T^{133}$ that contains the T^{131} and T^{133} residues, glycosylated either with the complete trisaccharide (*m/z* 1601) or the GalNAc residue (*m/z* 1148) in the MS^2 spectrum of the glycopeptide $^{124}T\text{-}E^{137}$ (**Figure 4.22 A**). However, it was not feasible to distinguish between T^{131} and T^{133} as glycosylation sites, since b_9 and b_8 or y_6 and y_5 ions, all of them containing only one of the two potential glycosylation sites, could not be detected. This strategy was also used to verify the glycosylation sites which were unambiguously assigned according to the described potential glycosylation sites in glycopeptides containing several threonine residues. As examples, this was the case for $^{138}A\text{-}L^{146}$ (**Figure 4.16**) or $^{117}T\text{-}N^{123}$ (**Figure 4.21 B**). In the latter case, the fragment y_4 ($^{120}P\text{-}N^{123}$), indicative of the T^{121} as the specific glycosylation site, was reasonably abundant due to the termed “proline effect” resulting from cleavages at the N-terminal side of proline (Hunt, et al., 1986, Vaisar, et al., 1996). In this sense, it has been described that *y* ions that have a proline residue as their N-terminus are normally over-represented in the tandem mass spectra due to their relatively higher stability (Schaaff, et al., 2000). Similarly, Medzihradszky et al. (1996) reported a direct mass-spectrometric approach to structurally elucidate *O*-linked glycopeptides and their glycosylation sites using high-energy tandem mass spectra obtained on four sector instrument (EBEB), equipped with a liquid secondary ion mass spectrometry source. In that work, glycosylated peptide fragment ions were also scarce and of low abundance, but sufficiently relevant for the determination of the glycosylation site.

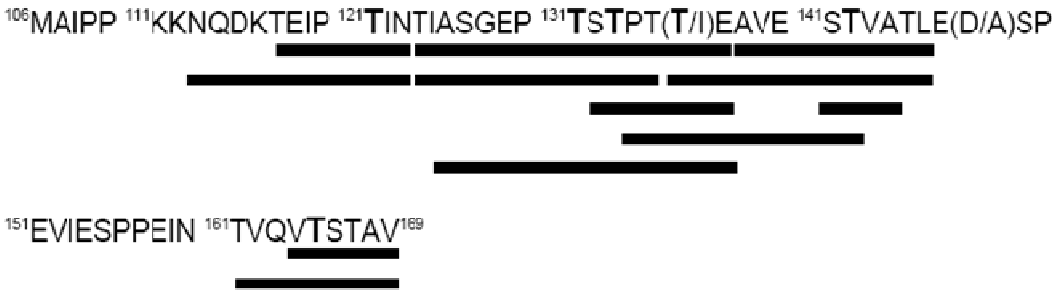

Figure 4.20. Amino acid sequence of CMP (genetic variants A and B). Potential glycosylation sites are in bold and identified glycopeptides are underlined. Residue numbering is based on the Swiss-Prot entry for the mature form of bovine α-casein (accession number P02668).

- *Characterization of O-sialoglycopeptides derived from CMP by HILIC-ESI(–)-MS^n*

HILIC-ESI(–)-MS^2 analyses were mostly dominated by the Y_n-type fragmentation derived exclusively from the losses of Neu5Ac residues. This fact is due to sialic acid is the only carbohydrate found in CMP that can provide negative charge to the glycopeptides at acidic or neutral pH because its acidic nature. Furthermore, MS^2 analyses at negative ionization mode also provided additional information on non-glycosylated threonine residues due to the loss of C_2H_4O (44 u) from their side chains. As an example, **Figure 4.22** shows the MS^2 spectrum at negative ionization mode of the glycopeptide ^{161}T-V^{169} containing the tetrasaccharide linked to T^{165} with two additional non-glycosylated threonine residues (T^{161} and T^{167}). Additionally, neutral losses of 98 u were also detected and attributed to serine dephosphorylation (supporting information). In this sense, it has been described that S^{149} is fully phosphorylated, being S^{127} the second most important phosphorylation site in bovine CMP (Vreeman, et al., 1986, Rasmussen, et al., 1997).

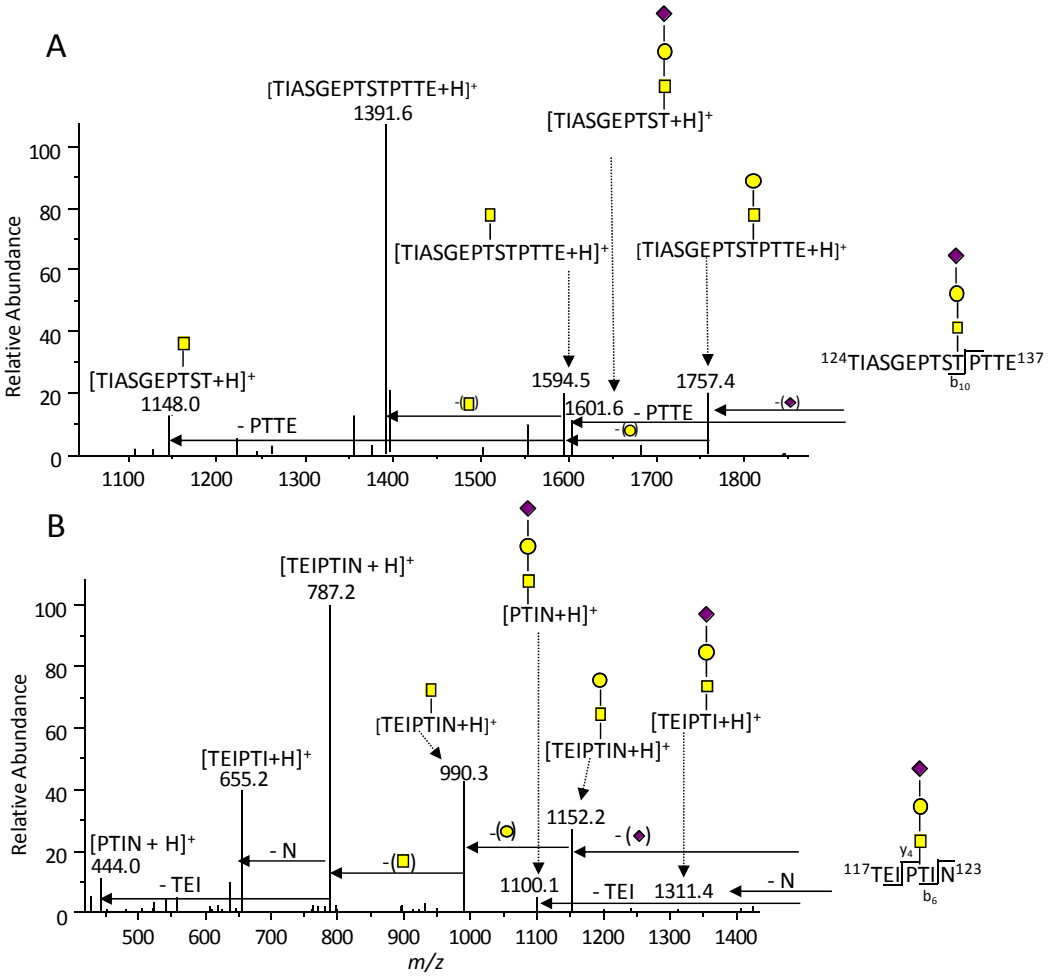

Figure 4.21. MS^2 spectra at positive ionization mode of: A) a doubly charged ion ($[M+H]^{2+}$ = 1025.0) corresponding to the CMP peptide ^{124}Thr-Glu^{137}; and B) a singly charged ion ($[M+H]^{+}$ = 1443.5) corresponding to the CMP peptide ^{117}T-N^{123}, both glycosylated with the sialyl-trisaccharide Neu5Acα2-3Galβ1-3GalNAc.

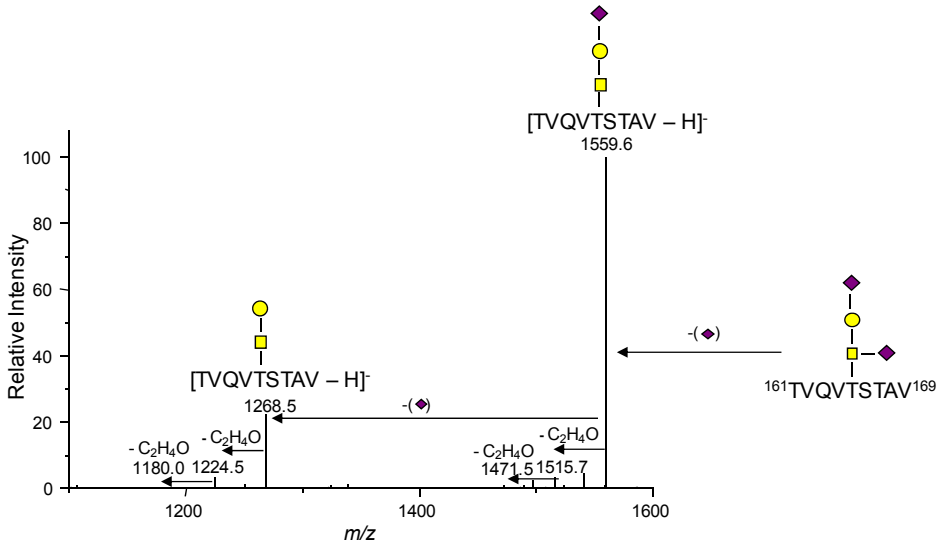

Figure 4.22. MS^2 spectrum at negative ionization mode of a singly charged ion ($[M-H]^{-}$ = 1850.6) corresponding to the CMP peptide ^{161}T-V^{169} glycosylated with the sialyl-tetrasaccharide (Neu5Ac-α(2→3)-Gal-β(1→3)-(Neu5Ac-α(2→6))-GalNAc) at T^{165} residue.

4.2.2.4. Conclusions

Overall, the developed HILIC-ESI(+)-MS^n method allowed the characterization of 41 *O*-glycopeptides from CMP genetic variants A and B without the need of a previous enrichment step. This method was suitable for i) the satisfactory separation of the glycopeptides according to their glycan structure and amino acidic sequence; and ii) for the subsequent determination of the amino acid and carbohydrate sequences of glycopeptides. Furthermore, complementary information about the degree of sialylation and the number of non-glycosylated threonine and phosphorylated serine residues was also obtained by performing analysis at negative ionization mode under the same chromatographic conditions.

To conclude, our study points out the potential of HILIC coupled to multistage MS procedures for future glycoproteomic applications. In the glycobiology field, combined advances in chromatographic and mass spectrometric techniques are being of great utility for gaining a better understanding of the molecular basis of the function of glycoproteins in biological processes.

Acknowledgements

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4.2.3. Detection of two minor phosphorylation sites for bovine k-casein macropeptide by reversed phase liquid chromatography–tandem mass spectrometry

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Journal of Agricultural and Food Chemistry (**2011**) 59: 10848-10853**.**

Abstract

This work addresses the characterization of phosphopeptides in bovine κ-casein macropeptide by RPLC-ESI-MS^2. Two different mass spectrometers, equipped with an ion trap (IT) or a quadrupole time-of-flight (Q-TOF) analyzer, were used to perform an accurate phosphorylation site assignment. Eight phosphopeptides from a total of identified peptides were characterized. MS^2 spectra of phosphopeptides were dominated by the neutral loss of a phosphoric acid molecule (H_3PO_4), and by sufficient informative fragment ions resulting from peptide backbone cleavages enabling the elucidation of the phosphopeptide sequence. A higher number of sequence informative b- and y-ions were detected by using a Q-TOF instead of an IT analyzer. In addition to the well-established phosphorylation sites at Ser^{149} and Ser^{127}, this study also revealed the presence of two minor phosphorylation sites at Thr^{145} and Ser^{166}. These findings indicate that RPLC-ESI-MS^2 on a Q-TOF analyzer is a useful technique for identifying low abundance phosphorylation sites in caseins.

4.2.3.1. Introduction

Phosphorylation is one of the most important post-translational modifications occurring in proteins and it can play a crucial role in their structure and biological properties (Hunter, 1987). Phosphoproteins are particularly abundant in milk, and caseins are the main phosphoproteins found in milk of most mammalian species (Jenness, et al., 1970). Basically, bovine caseins are a mixture of four different phosphoproteins, α_{s1}-, α_{s2}-, β- and K-casein, in the approximate proportions 4:1:4:1,

respectively (Davies, et al., 1977). Phosphorylation is a key event for the primary function of the caseins, i.e. the formation of large micelles upon binding of Ca^{2+} (Fiat, et al., 1989). Likewise, it has been described that caseinphosphopeptides may possess anticariogenic properties and they are also important in the bioavailability of Ca^{2+} from milk and dairy products by increasing its passive absorption and other trace elements in the distal small intestine (FitzGerald, 1998).

The most common type of casein phosphorylation involves the formation of phosphate ester bonds with the hydroxyl side chains of serine and, although much less frequently, threonine. Thus, bovine caseins are phosphorylated at different levels, and the common genetic variants of α_{s1}-, α_{s2}-, β- and K-caseins normally contain respectively 8-9, 11-13, 5 and 1-2 phosphate groups (Mercier, 1981). In the case of K-casein, several posterior studies revealed the presence of up to three phosphate residues per molecule, being the estimated abundance of the triphosphorylated form of CMP around 1.2-2% (Vreeman, et al., 1986, Molle, et al., 1995). This low abundance can probably explain that only two phosphorylation sites have clearly been determined, i.e. the residue Ser^{149} which is fully phosphorylated and Ser^{127} as the second most important phosphorylation site. However, significant advances of mass spectrometry-based proteomics techniques, especially in terms of sensitivity and dynamic range, are enabling the detection and fragmentation of phosphorylated peptides at lower and lower levels and, consequently, a wide number of novel *in vivo* phosphorylation sites is being characterized (Kjeldsen, et al., 2007, Via, et al., 2011). In this context, evidence for a third phosphorylation site being occupied at Thr^{145} has recently been published following a study based on the characterization of bovine K-casein by two-dimensional gel electrophoresis after a cysteine-tagging enrichment strategy (Holland, et al., 2006).

The main aim of this work was the identification of phosphopeptides and the characterization of phosphorylation sites in bovine K-casein by RPLC-ESI-MS^2 using two different mass analyzers (ion trap (IT) and quadrupole time-of-flight (Q-TOF)) in order to make an accurate phosphorylation site assignment. For that purpose, the 64 C-terminal amino acids of K-casein, also called caseinomacropetide (CMP), was selected because phosphorylations, as well as all the other post-translational modifications, are found exclusively in this C-terminal portion of K-casein.

4.2.3.2. Materials and Methods

- *Hydrolysis of bovine CMP*

CMP (mixture of A and B variants) was kindly provided by Davisco Foods International (Le Sueur, MN, USA). CMP was subjected to a combined trypsin/chymotrypsin proteolysis (overnight at 37 ºC and pH 7) at 1: 0.05 : 0.025 CMP : trypsin : chymotrypsin ratios (w : w : w) (Section 4.2.2). Enzymatic activities of porcine pancreas trypsin (EC 3.4.21.4, type IX-S) and bovine pancreas α-chymotrypsin (EC 3.4.21.1, Type I-S) were 13,000–20,000U and ≥40U per mg of protein, respectively.

- *RPLC-ESI-MS^2 analyses*

Two different LC-MS^2 instruments were used in this study to analyze the hydrolyzed CMP. The first one was a Finnigan Surveyor pump with quaternary gradient system coupled to a Finnigan LCQ Deca ion trap mass spectrometer using an ESI interface. Sample injections (5 µL) were carried out by a Finnigan Surveyor autosampler. All instruments were from Thermo Fisher Scientific (San José, CA, USA). The electrospray voltage was set at 4.5 kV, heated capillary temperature at 200 ºC, nitrogen (99.5% purity) was used as sheath (0.6 L min^{-1}) and auxiliary (6 L min^{-1}) gas, and helium (99.999% purity) as the collision gas. Mass spectra were recorded in the positive ion mode between *m/z* 450 and 2000. MS^2 data were obtained by collision induced dissociation (CID) of the $[M+H]^+$ ion and acquired in the automatic data-dependent mode with a relative collision energy of 35%, using a total cycle time of approximately 5 s. Ion selection threshold was set at 5×10^5 counts. Data acquisition and processing were managed by Xcalibur software (1.2 version, Thermo Fisher Scientific).

The second LC-MS^2 instrument was an Agilent 1200 Series LC system (equipped with a binary pump, an autosampler and an column oven) coupled to an 6520 quadrupole time-of-flight mass spectrometer (Q-TOF) using an ESI interface working in the positive ion mode. Sample injections (20 µL) were carried out by the autosampler. All instruments were from Agilent Technologies (Santa Clara, CA). The electrospray source parameters were adjusted as follows: spray voltage 4.5 kV, drying gas temperature 300 °C, drying gas flow rate 6 L min^{-1}, nebulizer pressure 30 psi, and

fragmentor voltage 150 V. Nitrogen (99.5% purity) was used as drying and nebulizer gases, while nitrogen of a higher purity (99.999%) was used as the collision gas. MS^2 spectra were obtained by CID, selecting the target masses (see Results section) and applying collision energies ranging between 30 and 80 eV. Data acquisition and processing were done using Agilent Mass Hunter Workstation Acquisition v. B.02.00 software.

In both cases, the hydrolyzed CMP was dissolved in acetonitrile 5% (v/v) at a concentration of 2.0 mg mL^{-1}, and their separation performed at 25 ºC on a Hypersil HyPurity C_{18} (100 mm × 2.1 mm, 3 µm) column (Thermo Fisher Scientific) at a flow rate of 100 µL min^{-1}. A gradient of two eluents was used: eluent A consisted in 0.1% (v/v) of formic acid (analytical grade, Merck, Darmstadt, Germany) in water, and eluent B in acetonitrile (LC-MS Chromasolv® grade, Sigma-Aldrich, St. Louis, MO, USA) containing 0.1% of formic acid (v/v). The elution program was as described by Moreno et al. (Moreno, et al., 2008).

- *Data treatment*

Full scan mass spectra obtained in the ion trap LC-MS system were used for an initial assignment of observed ions to the corresponding amino acid sequences. It was based on the known sequence of K-casein (Dumas, et al., 1972) by using the protein database Swiss-Prot and TrEMBL and the tools Peptide Mass and FindPept available at www.expasy.org. Parameters for the search were the following: (i) monoisotopic peptide masses were indicated as $[M+H]^+$ with cysteines treated with nothing; (ii) as enzymes, trypsin/chymotrypsin were chosen; (iii) two missed cleavages were allowed; (iv) peptides with a mass larger than 500 u were displayed; (v) the mass tolerance was kept at 0.5 u; (vi) potential phosphorylation on serine and threonine residues was considered. To confirm the sequence of these assigned peptides, their experimental MS^2 spectra from the ion trap LC-MS^2 system were compared with the theoretical fragmentation of the putative peptides, obtained from the MS-Product software (Protein Prospector, http://prospector.ucsf.edu/). This program was also used for the identification of the phosphopeptides from the MS^2 spectra obtained by the Q-TOF LC-MS^2 system.

4.2.3.3. Results

- *Phosphopeptide characterization by RPLC-ESI-MS^2 on an ion trap mass spectrometer*

The initial use of an ion trap mass spectrometer was based on its robustness, ease of use, high sensitivity in scan, and ability to perform multi-stage fragmentation. The resulting RPLC-ESI-MS base peak chromatogram for bovine CMP after hydrolysis revealed the presence of a complex mixture of peptides eluting between 3 and 33 min, with no traces of the intact CMP (**Figure 4.23**). This profile indicated that bovine CMP was efficiently hydrolyzed at neutral pH by trypsin and chymotrypsin, which is in good agreement with previous findings reported by Shammet et al. (1992). The presence of a large number of hydrophobic amino acids (6 Val, 7 Ile, and 6 Ala) together with 3 Lys residues in the CMP sequence explains the extensive hydrolysis of CMP with trypsin and chymotrypsin (Shammet, et al., 1992).

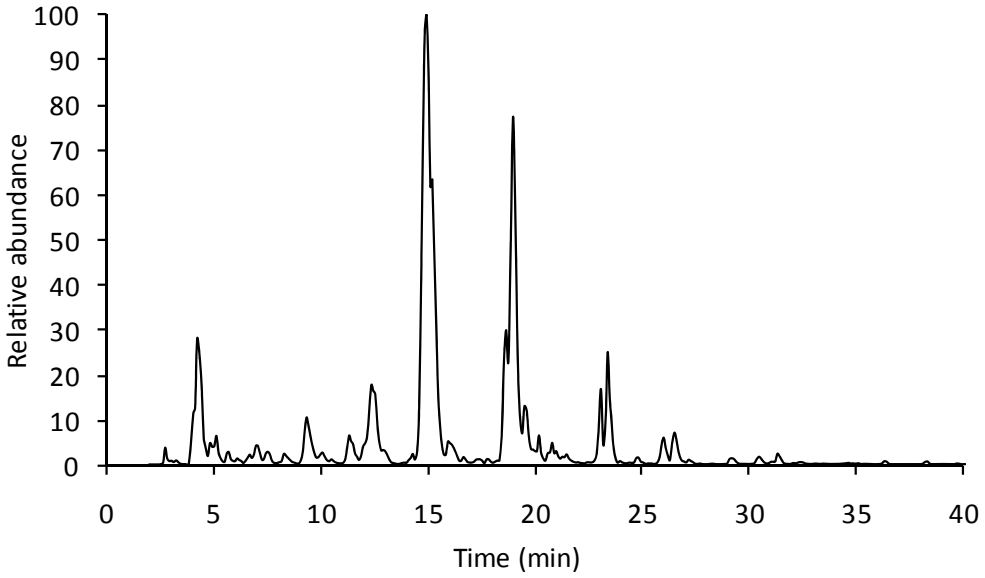

Figure 4.23. RPLC-ESI-MS base peak profile monitorized on an ion trap at positive ionization mode of hydrolyzed (trypsin/chymotrypsin) bovine CMP.

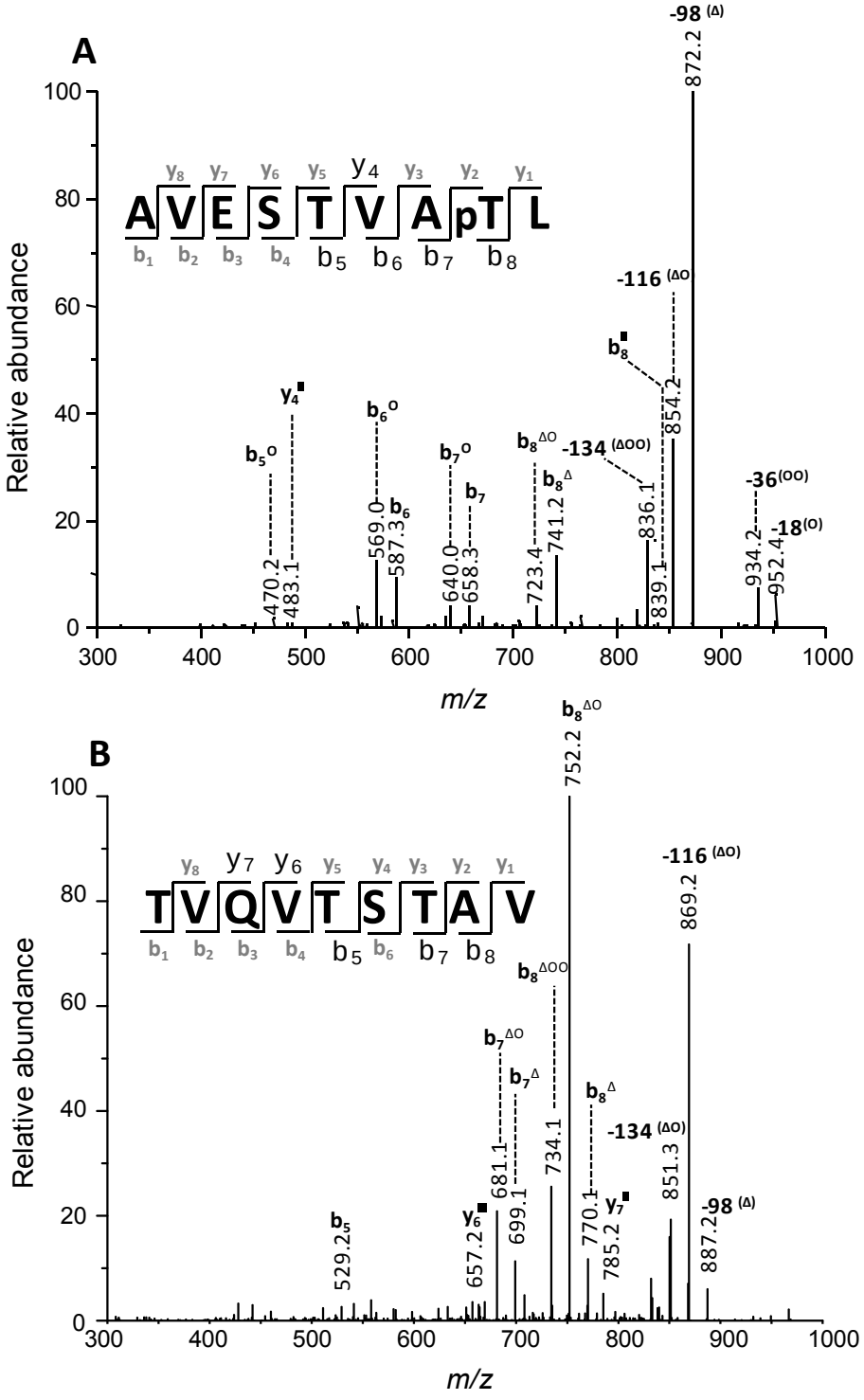

Figure 4.24. MS^2 spectra of singly charged ions corresponding to K-casein mono-phoshphorylated peptides (A) ^{138}AVESTVApTL^{146}, and (B) ^{161}TVQVTSTAV^{169} acquired using an ion trap mass spectrometer. Phosphorylation of ^{161}TVQVTSTAV^{169} could be at S^{166} or at T^{167}. ■ = + 80 u (+HPO_3); Δ = - 98 u (-H_3PO_4 or -(H_2O + HPO_3)); o = -18 u (-H_2O).

The RPLC-ESI-MS^2 characterization of the non-glycosylated fraction of CMP confirmed the presence of 26 peptides with molecular masses ranging between 504 and 1,848 u (**Table 4.15**). The identified peptides covered the whole CMP sequence, with the exception of the NH_2-terminal methionine. Collision-induced dissociation (CID) MS^2 spectra of phosphothreonine- and, especially, phosphoserine-containing peptides normally show an intense neutral loss of H_3PO_4 (-98 u) from the precursor ion due to the lability of the phosphate group (Boersema, et al., 2009). In addition to this neutral loss, it is necessary to detect sufficient informative fragment ions resulting from peptide backbone cleavages in order to identify the phosphopeptide sequence. Thus, six of these 26 peptides (^{124}Thr-Thr^{135}, ^{124}Thr-Glu^{137}, ^{149}Ser-Asn^{160}, ^{147}Glu-Asn^{160} genetic variant A and B, and ^{145}Thr-Asn^{160}) resulted to be mono-phosphorylated at Ser residues located at positions 149 or 127 (**Table 4.15**). This is in good agreement with the well-established determination of the phosphorylation sites of bovine K-casein, where Ser^{149} is fully phosphorylated and Ser^{127} is the second most important phosphorylated site (Mercier, 1981).

Nonetheless, there were two additional MS^2 spectra that unambiguously indicated the presence of two mono-phosphorylated peptides matching sequences, ^{138}Ala-Leu^{146} and ^{161}Thr-Val^{169}, which did not contain any of the two described phosphorylation sites (**Figure 4.24**). Furthermore, peptide ^{138}Ala-Leu^{146} could have three potential phosphorylation sites at Ser^{141}, Thr^{142} and Thr^{145}, whilst peptide ^{161}Thr-Val^{169} contains Thr^{161}, Thr^{165}, Ser^{166} and Thr^{167} as potential phosphorylation sites. Thus, the MS^2 spectrum of the precursor ion of $[M + H]^+$ at *m/z* 970.2 (^{138}Ala-Leu^{146}) was characterized by the presence of fragments derived from the neutral loss of H_3PO_4 at *m/z* 872.2 with one and two subsequent dehydrations, as well as the detection of abundant fragment ions b_6 and b_7 and minor ion b_5 and their corresponding singly and doubly dehydrations. The mass values corresponding to the fragment ions b_5 to b_7 were unmodified indicating that these peptide fragments are not phosphorylated (**Figure 4.24 A**). This fact indicated that Ser^{141} and Thr^{142} could be ruled out as phosphorylation sites, leaving Thr^{145} as the only residue susceptible to be phosphorylated. This was further confirmed by the detection of the abundant ion at *m/z* 741.2 derived from the loss of a phosphoric acid molecule (H_3PO_4) of b_8 ion that contains the residue Thr^{145}. Likewise, detection of b_8 and y_4 minor ions containing the

phosphate group (corresponding to +80 u compared to the unmodified peptide) further corroborated the residue Thr^{145} as the phosphorylation site.

MS^2 spectrum of the precursor ion of $[M + H]^+$ at *m/z* 985.2 corresponding to peptide ^{161}Thr-Val^{169} was dominated by the presence of fragment ions at *m/z* 869.2, 851.3 and 887.2 corresponding to the neutral loss of a phosphoric acid molecule (H_3PO_4) and singly and doubly dehydrations, and by the detection of b_8 and b_7 ions after losing H_3PO_4. These main fragments ions were not sufficient to exclude any potential phosphorylation site. Nevertheless, detection of less abundant fragments corresponding to unmodified b_5, and phosphorylated y_7 and y_6 ions left residues Ser^{166} and Thr^{167} as possible phosphorylation sites (**Figure 4.24 B**). Unfortunately, no additional ions that could resolve this ambiguity were detected.

Finally, and considering the capacity of ion trap analyzers to perform multiple stages of fragmentation, MS^3 spectra obtained from collisionally generated ion derived from the loss of H_3PO_4 were performed with the aim of detecting additional peptide backbone fragmentation. However, the effect of another fragmentation stage on phosphopeptide characterization was not significant in identifying additional peptide fragments (data not shown).

- *Phosphopeptide characterization by RPLC-ESI-MS^2 on a quadrupole time-of-flight mass spectrometer*

The use of a second type of mass spectrometer was carried out in order to unambiguously assign the correct phosphosite localization in peptides containing multiple potential sites of phosphorylation and, thus, to confirm the determination of new and/or minor phosphorylation sites in the bovine K-casein macropeptide. In this context, it is noteworthy to mention that a quadrupole time-of-flight mass spectrometer may represent advantages such as a high sensitivity in MS^2 for parent product scans, as well as its ability to give accurate mass measurements for fragment identification.

Table 4.15. Non-glycosylated peptides identified in hydrolyzed bovine CMP by RPLC-ESI-MS^2 using either an ion trap or a quadrupole time-of-flight mass spectrometers.

t_R (min)	Peptide sequence	Position[a]	Exp. Mass $[M+H]^{+b}$	Genetic variant
3.3	NQDK	113-116	504.1	A & B
4.0	AIPPK	107-111	525.1	A & B
4.0	IASGEPTSTPTTE	125-137	1290.5	A
4.1	TVQVT	161-165	547.0	A & B
4.2	AVESTVA	138-144	676.2	A & B
4.6	TIASGEPTSTPTTE	124-137	1391.4	A
4.9	STPTIE	132-137	647.1	B
5.0	TIAS[c]GEPTSTPT	124-135	1241.3	A & B
5.2	TIAS[c]GEPTSTPTTE	124-137	1471.3	A
7.0	TVQVTSTAV	161-169	905.1	A & B
7.1	TVQVTS[c]TAV	161-169	985.3	A & B
7.8	IEAVESTVA	136-144	918.2	B
8.4	IASGEPTSTPTIE	125-137	1302.6	B
9.4	STVATL	141-146	591.1	A & B
11.9	TIASGEPTSTPTIE	124-137	1403.4	B
12.5	VIESPPEIN	152-160	997.3	A & B
13.2	AVESTVAT[c]L	138-146	970.4	A & B
15.0	TEIPTIN	117-123	787.2	A & B
16.0	AVESTVATL	138-146	890.2	A & B
16.6	IEAVESTVATL	136-146	1132.3	B
16.7	VIESPPEINTVQ	152-163	1325.5	A & B
18.2	S[c]PEVIESPPEIN	149-160	1390.3	A & B
18.4	EAS[c]PEVIESPPEIN	147-160	1590.4	B
19.0	EDS[c]PEVIESPPEIN	147-160	1634.4	A
20.2	VIESPPEINTVQVT	152-165	1525.5	A & B
21.5	TLEDS[c]PEVIESPPEIN	145-160	1848.4	A

[a] Residue numbering is based on the Swiss-Prot entry for the mature form of bovine κ-casein (accession number P02668).
[b] Monoisotopic mass values.
[c] Phosphorylated residue.

On the one hand, Palumbo and Reid (2008) indicated that the application of multistage mass spectrometry (MS^2 and MS^3) in an ion trap is valid for routine phosphopeptide identification but not for unambiguous phosphorylation site localization in peptides containing multiple potential phosphorylation sites. These authors reported that, in linear and conventional 3D ion traps under typical CID-MS^2 conditions, the phosphate group might be gas-phase rearranged and transferred prior to fragmentation to another serine, threonine or tyrosine residue present in the precursor peptide and not originally phosphorylated, due to the relatively long (millisecond) time scales associated with ion activation in these instrument platforms. In contrast, by using tandem-in-space instruments such as triple quadrupole

(microsecond activation time scale), no product ions resulting from the gas-phase phosphate group rearrangement reactions were observed. Conversely, recent works carried out using CID on ion-trap analyzers have indicated that this phenomenon is negligible (Aguiar, et al., 2010, Mischerikow, et al., 2010). Thus, evidences for the gas-phase phosphate group rearrangements were observed specifically for doubly charged species, although the extent of the transfer reaction was insufficient to mislead the phosphorylation site assignment (Aguiar, et al., 2010).

On the other hand, it has been also described that ion trap mass spectrometers generate a higher level of neutral loss than tandem-in-space instruments (Lehmann, et al., 2007) which is attributed to difference in collision energy used and the timeframe allowed for fragmentation (Boersema, et al., 2009). This fact implies that the fragment corresponding to the neutral loss of H_3PO_4 (-98 u) should be less abundant in triple quadrupole or Q-TOF mass spectrometers, allowing the detection of more fragments ions derived from peptide backbone cleavages and, consequently, enabling a more accurate phosphopeptide sequencing. For this reason, the eight phosphorylated peptides previously identified on the ion trap were also analyzed in the Q-TOF mass spectrometer (a tandem-in-space instrument) at three different collision energy values. MS^2 spectra confirmed the phosphorylation at Ser^{149} or Ser^{127} of the six peptides previously identified on the ion trap mass spectrometer (data not shown). Q-TOF MS^2 spectra corresponding to peptides ^{138}Ala-Leu^{146} and ^{161}Thr-Val^{169} are shown in **Figures 4.25** and **4.26**, respectively. MS^2 spectra of phosphopeptide ^{138}Ala-Leu^{146} clearly confirmed that Thr^{145} was phosphorylated. Thus, fragment ions b_5 to b_7 and their corresponding singly and doubly dehydrations were detected in their unmodified form, whilst b_8 ion was detected after losing H_3PO_4. Furthermore, fragment ions y_7, y_6, y_4 and y_3 were also detected in their phosphorylated form, being the two latter ones indicative of the phosphorylation at Thr^{145} (**Figure 4.25 A**).

Q-TOF MS^2 spectra corresponding to peptide ^{161}Thr-Val^{169} showed the detection of fragment ions b_3 to b_5 in their unmodified form excluding residues Thr^{161} and Thr^{165} as phosphorylation sites. In addition, ions b_6 to b_8 were detected after losing H_3PO_4, and they were also detected with a much less abundance containing the phosphate group. The detection of the fragment ion b_6 in its phosphorylated form and after losing H_3PO_4 could allow the unambiguous localization of the phosphorylation at

Ser[166]. In addition, fragment ions y_5, y_6 and y_7 with and without the phosphate group could also be detected (**Figure 4.26**).

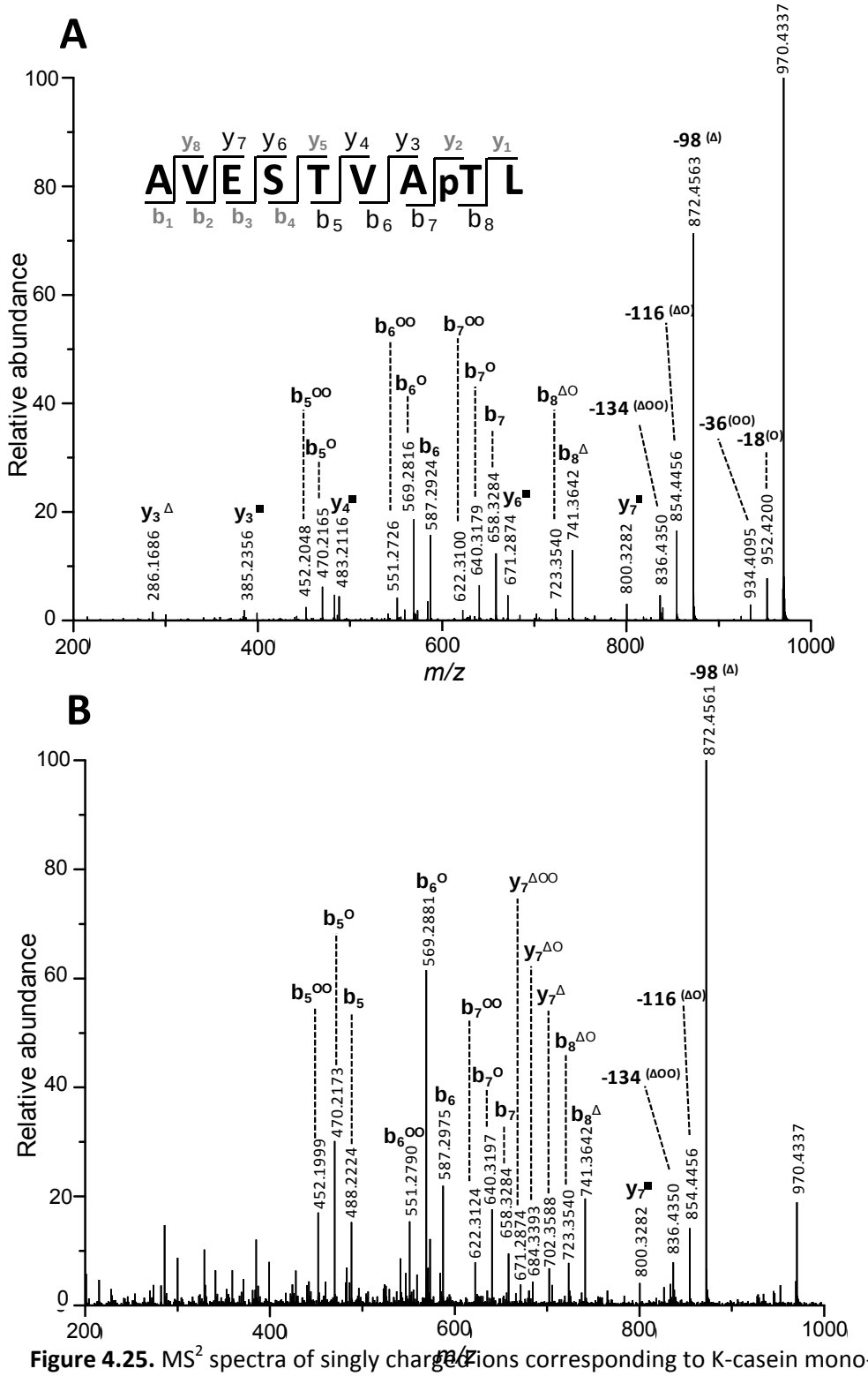

Figure 4.25. MS^2 spectra of singly charged ions corresponding to K-casein mono-phoshphorylated peptide [138]AVESTVApTL[146] acquired using a quadrupole time-of-flight (Q-TOF) mass spectrometer. **(A)** CID: 30 eV and **(B)** CID = 40 eV. ■ = + 80 u (+HPO_3); Δ = - 98 u (-H_3PO_4 or -(H_2O + HPO_3)); o = -18 u (-H_2O).

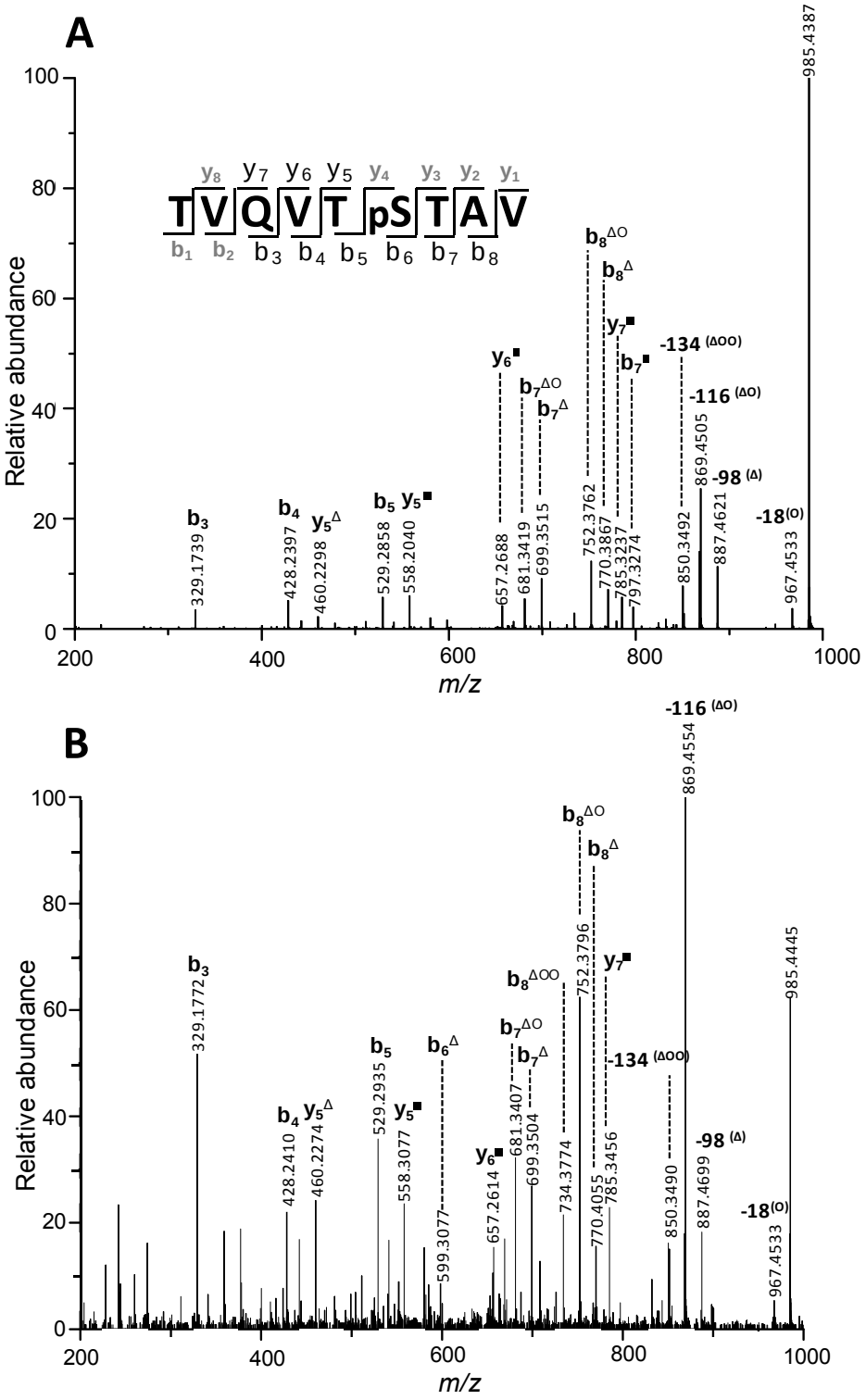

Figure 4.26. MS^2 spectra of singly charged ions corresponding to β-casein mono-phoshphorylated peptide ^{161}TVQVTpSTAV^{169} acquired using a quadrupole time-of-flight (Q-TOF) mass spectrometer. **(A)** CID: 30 eV and **(B)** CID = 40 eV. ■ = + 80 u (+HPO_3); Δ = - 98 u (-H_3PO_4 or -(H_2O + HPO_3)); o = -18 u (-H_2O).

4.2.3.4. Discussion

The determination of the minor phosphorylation site at Thr^{145} conforms to the sequence motif, Ser/Thr-X-Glu/Ser (phosphorylated), recognized by the casein kinase from lactating bovine mammary gland (also called Golgi apparatus casein kinase or G-CK). Thus, our data support the finding of Holland et al. (Holland, et al., 2006) who pointed out Thr^{145} as the third phosphorylation site. Nevertheless, the phosphorylation of Thr residues instead of Ser residues in caseins is much more uncommon and this might reflect alternative kinase activities (Holland, et al., 2006).

Regarding the determination of the previously unrecognized phosphorylation site, Ser^{166}, this is localized within a sequence that apparently does not fulfill any phosphorylation site motifs recognised by G-CK or even other casein kinases such as CKI or CKII (Kemp, et al., 1990). Therefore, the determination of Ser^{166} as a new phosphorylation site might be explained by either a possible implication of a novel or uncharacterized protein kinase in which the sequence motif is fulfilled by the sequence surrounding Ser^{166} or that G-CK is also able to recognize consensus sequences different from the well-established, Ser/Thr-X-Glu/Ser(phosphorylated). In this sense, Brunati et al. (Brunati, et al., 2000) reported a novel consensus sequence in a proline-rich protein-1 (PRP1), Ser-X-Gln-X-X-$(Asp/Glu)^3$, which is phosphorylated by G-CK with an efficiency similar to the canonical one. Another example could be the human osteopontin which is a milk integrin-binding highly phosphorylated glycoprotein that also contains a phosphorylated serine residue located within a sequence atypical of those normally recognized by G-CK or CKII (Christensen, et al., 2005). Furthermore, to the best of our knowledge, it is striking that, despite its importance, the genes encoding for the G-CK are still unknown and their activity has to be ascribed to the category of "orphan" enzymes (Lespinet, et al., 2006, Salvi, et al., 2010). Likewise, the determination of a fourth phosphorylation site in bovine K-casein might lead to the occurrence of a four-phosphorylated form in a very low abundance, since the three-phosphorylated form was estimated to be present as low as 1.2-2% (Vreeman, et al., 1986, Molle, et al., 1995). Another possibility could be that the three-phosphorylated form of bovine K-casein could contain either Thr^{145} or Ser^{166} as the third phosphorylation site.

On the other hand, Ser^{168} in the caprine and ovine K-casein sequences, which is equivalent to Ser^{166} in the bovine sequence, have previously been described as a major phosphorylation site (Rasmussen, et al., 1997, Mercier, et al., 1977, Mercier, et al., 1976), and this behavior was attributed to the fact that the C-terminal sequences of bovine and caprine/ovine K-caseins differ by the substitution of Ala (bovine) by Glu (caprine/ovine), leading, thus, to the occurrence of the consensus sequence for G-CK (^{168}Ser-Thr-Glu^{170}) in the caprine and ovine species (Mercier, 1981). However, our data point out that this residue is also a minor phosphorylation site in the bovine counterpart.

Finally, these results highlight the complex heterogeneity of milk caseins despite the large number of studies performed on milk proteins. Likewise, although the biological or structural role of these minor phosphorylation sites, if any, remain to be determined, data presented in this work indicate that RPLC-ESI-MS^2 on a quadrupole time-of-flight mass spectrometer is a useful technique for the identification of low abundance phosphorylation sites in caseins. This is particularly important if it is considered that even a single phosphorylation event can have a significant impact on protein activity (Joung, et al., 1995).

Acknowledgement

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4.3. Bioactividad *in vitro* e *in vivo* de GOS, de péptidos procedentes del CMP y de sus respectivos glicoconjugados

4.3.1. Prefacio

Una vez llevado a cabo el análisis estructural tanto de los GOS como de los péptidos derivados de la hidrólisis enzimática del CMP, en esta sección se investigarán y discutirán algunas de sus propiedades bioactivas, determinadas tanto en sistemas *in vitro* (**sección 4.3.2**) como *in vivo* (**sección 4.3.3**). De igual forma, dichos estudios se llevaron a cabo no sólo con estos compuestos por separado sino también a través de la conjugación de los mismos, vía reacción de Maillard. Dicha formación de glicoconjugados se realizó con el objetivo de estudiar un posible efecto sinérgico entre ambos tipos de compuestos y, en consecuencia, intentar elucidar cómo afecta la glicación a las propiedades de los compuestos independientes, consiguiendo así ingredientes multifuncionales. La síntesis de dichos conjugados se encuentra detallada en la sección **4.3.2.2 y 4.3.2.3**.

Cabe destacar que debido al alto rendimiento y variabilidad estructural que presentaron los GOS-Lu sintetizados con la β-galactosidasa de *A. oryzae* (**sección 4.1.3**), fueron estos oligosacáridos los elegidos para realizar los ensayos tanto *in vivo* como *in vitro*, así como para la formación de los glicoconjugados correspondientes. Además, y con fines comparativos, en la mayor parte de los estudios se realizaron ensayos paralelos con GOS comerciales derivados de lactosa (GOS-La) y lactulosa dado su reconocido carácter prebiótico.

Previo a la determinación de su actividad prebiótica, se evaluó la influencia de los GOS sobre el crecimiento de diferentes especies de *Lactobacillus*, conocidos probióticos, así como su efecto sobre la resistencia de dichas bacterias a diferentes condiciones gastrointestinales que podrían reducir su supervivencia. Este estudio se recoge en el trabajo de Hernández-Hernández y col. titulado "**Effect of prebiotic carbohydrates on the growth and tolerance of lactobacillus**" publicado *en Food Microbiology*, 2012, (aceptado, en prensa) (**sección 4.3.2.1**). En este trabajo se emplearon GOS-Lu y GOS-La previamente fraccionados según se indica en la **sección 4.1.3**, con el fin de eliminar carbohidratos digeribles, como la lactosa, glucosa y galactosa que podrían dar lugar a falsos positivos. En este estudio también se usó lactulosa como control positivo. Los ensayos se realizaron incubando dichas muestras

con cultivos iniciadores puros. Como resultado de este estudio se observó que los GOS-Lu y GOS-La son capaces de ser asimilados por las diferentes especies de *Lactobacillus,* además de mantener el crecimiento de las mismas por un mayor período de tiempo comparado con la lactulosa. Por otro lado, se observó un efecto cepa-dependiente en la resistencia que presentaron las especies de *Lactobacillus* bajo diferentes condiciones gastrointestinales extremas y en la hidrofobicidad de las células de *Lactobacillus*, siendo mayor en las cepas cuya fuente de carbono eran GOS. Son pocos los estudios realizados hasta el momento sobre el efecto de los prebióticos en la modulación de la resistencia de las bacterias a diferentes condiciones gastrointestinales. Dicha resistencia, es necesaria para que las bacterias probióticas puedan colonizar el intestino grueso y de esta manera, ejercer sus efectos positivos sobre la salud.

De manera similar, los glicoconjugados obtenidos a partir de hidrolizados de CMP y GOS-Lu, GOS-La o lactulosa fueron usados como fuentes de carbono con el objetivo de estudiar su efecto sobre el crecimiento de diferentes especies de lactobacilos, así como sobre su resistencia a la presencia de sales biliares, una de las condiciones gastrointestinales más extremas por las que debe pasar un probiótico con el fin de colonizar el intestino grueso. Este estudio se detalla en la **sección 4.3.2.2** y está basado en el manuscrito titulado "**Effect of hydrolyzed caseinomacropeptide conjugated galactooligosaccharides on the growth and bile resistance of *Lactobacillus* strains**" enviado al *Journal of Food Science*, 2011, (En revisión). En este estudio, al igual que en la **sección 4.3.2.1**, se observó un efecto cepa-dependiente tanto en el crecimiento de las bacterias, como en la resistencia a la presencia de sales biliares. Por otro lado, las diferentes cepas de lactobacilo estudiadas, mostraron ser capaces de usar los glicoconjugados como fuente de carbono, además de mejorar, en la mayoría de los casos, la resistencia a sales biliares.

Posteriormente, se llevaron a cabo estudios sobre el efecto *in vitro* de los carbohidratos de interés en la microflora intestinal. En primer lugar se estudió las capacidades prebióticas de los carbohidratos en cuestión (GOS-La, GOS-Lu y lactulosa) y del CMP hidrolizado por separado, así como de sus respectivos conjugados. Este estudio está desarrollado en el trabajo titulado "***In vitro* fermentation by human gut bacteria of proteolytically digested caseinomacropeptide non-enzymatically**

glycosylated with prebiotic carbohydrates" publicado en *Journal of Agricultural and Food Chemistry*, 2011, 59, 11949-11955 (**sección 4.3.2.3.**). Los resultados obtenidos indicaron que la glicación no afectó a las propiedades prebióticas propias de GOS-La, GOS-Lu y lactulosa sin conjugar. En consecuencia, la glicación de carbohidratos prebióticos, vía Reacción de Maillard, podría representar una nueva alternativa para la obtención de ingredientes multifuncionales al combinar la bioactividades de los GOS y del CMP y podría permitir la llegada de los prebióticos a zonas más distales del intestino teniendo en cuenta el metabolismo descrito para el compuesto de Amadori.

Por otro lado, se estudió la influencia *in vitro* de los GOS-Lu y GOS-La, tanto en su forma libre como conjugada con los hidrolizados de CMP, en la adhesión y en la respuesta inflamatoria intestinal de dos patógenos intestinales (*Listeria monocytogenes* CECT 935 y *Salmonella enterica* CECT 443) empleando mucina y Caco-2, respectivamente, como cultivos de células humanas de origen intestinal. Los resultados obtenidos y su discusión aparecen reflejados en la **sección 4.3.2.4** en el manuscrito de Laparra y col., enviado al *Journal of Agricultural and Food Chemistry,* 2011, (En revisión) y que lleva por título: "**Neoglycoconjugates of caseinomacropeptide and galactooligosaccharides from lactose and lactulose protect against the inflammatory response(s) in intestinal (Caco-2) cells to intestinal pathogens**". Los resultados más relevantes indicaron que los GOS-La y GOS-Lu tras su conjugación con el hidrolizado de CMP redujeron significativamente la adhesión a mucina de los dos patógenos intestinales estudiados. En cuanto a la respuesta inflamatoria intestinal, se pudo comprobar que los GOS-Lu y su respectivo conjugado con hidrolizados de CMP inhibieron la producción de citoquinas pro-inflamatorias, como el factor de necrosis tumoral (TNF)-α e interleucina (IL)-1β, en cultivos de células humanas de origen intestinal (línea Caco-2) colonizados por el patógeno intestinal *Listeria monocytogenes* CECT 935. Sin embargo, los GOS-La y su respectivo glicoconjugado no ejercieron dicho efecto positivo.

Una vez evaluadas las propiedades *in vitro* tanto de los compuestos de interés libres como conjugados, se llevaron a cabo estudios *in vivo* (**sección 4.3.3**) llevados a cabo, usando como modelo biológico ratas Wistar macho en crecimiento. En dichos estudios se determinó, en primer lugar, el grado de digestibilidad ileal de los GOS-Lu,

GOS-La y lactulosa descrito en la **sección 4.3.3.1** e incluido en el trabajo de Hernández-Hernández y col. titulado: "**The estructure and composition of galacto-oligosaccharides affects their resistance to ileal digestión and prebiotic properties in rats**" y enviado al *The Journal of Nutrition*, 2012 (En revisión). En este caso se observó una considerable digestibilidad ileal en los GOS-La, mientras que los GOS-Lu y la lactulosa presentaron un grado de digestibilidad muy reducido. Por otro lado no se observó la presencia de estos carbohidratos en muestras fecales, indicando su fermentación en el instetino grueso. Estos resultados indicaron que tanto los GOS-Lu como la lactulosa son capaces de llegar en mayor concentración que los GOS-La y en cantidades fisiológicamente relevantes al intestino grueso donde serán selectivamente metabolizados por la microbiota, evidenciado por el efecto bifidogénico observado en muestras fecales y como se muestra en las secciones posteriores. Cabe destacar que los antecedentes sobre la digestibilidad *in vivo* de los GOS-Lu eran inexistentes y muy escasos en el caso de los GOS-La, a pesar de ser estos últimos mucho más comunes y estar comercialmente disponibles. Además, la mayoría de los estudios disponibles sobre la digestibilidad de los GOS-La no se realizaron con métodos *in vivo* directos como los presentados en esta memoria, sino con métodos *in vivo* indirectos en humanos, como la determinación de la excreción de hidrógeno, que describen la resistencia a la digestión gastrointestinal de estos oligosacáridos con datos incongruentes y poco concluyentes. Por otro lado, en este trabajo se estudió el efecto prebiótico de estos carbohidratos (GOS-Lu, GOS-La y lactulosa) en las heces de las ratas, observándose un marcado efecto bifidogénico en el grupo de animales que ingirió GOS-Lu y GOS-La respecto al control; sin embargo, dicho efecto fue mayor en el grupo de animales alimentados con GOS-Lu.

Por último, la **sección 4.3.3.2** incluye los resultados más relevantes recogidos en la patente titulada "**Galacto-oligosacáridos derivados de lactulosa multifuncionales con actividad inmunomoduladora y prebiótica**" [*N. de solicitud: 201130784; Fecha de prioridad: 16 de mayo de 2011*] y que no se han detallado en las secciones anteriores. De este modo, se estudió la actividad inmunomoduladora *in vivo* de los GOS-Lu, empleando el mismo modelo animal que el utilizado para el estudio de la dugestibilidad ileal (**sección 4.3.3.1**). Los resultados obtenidos mostraron que la

administración de los GOS-Lu indujeron la expresión de las interleucinas (IL) pleiotrópicas IL-6 e IL-10, lo que puede favorecer la regulación de los procesos inflamatorios a nivel intestinal. Además, la inclusión de los GOS-Lu en la dieta de los animales causó una mayor expresión del factor de transcripción nuclear NFκB, implicado en diversos procesos de señalización intracelular, crecimiento y supervivencia celular. Por otro lado, la actividad prebiótca tanto de GOS-Lu como de GOS-La se determinó en el colón de los animales modelos, observándose un mayor efecto bifidogénico usando GOS-Lu además de una mayor biodiversidad de este género bacteriano.

En resumen, los datos *in vivo* aportados en la **sección 4.3** demuestran que los GOS-Lu en comparación con los GOS comerciales derivados de la lactosa presentan un valor añadido a su efecto prebiótico (mayor efecto estimulador del crecimiento de bifidobacterias) con un efecto adicional por modular la función inmunológica.

4.3.2. Estudios *In vitro*

4.3.2.1. Effect of Prebiotic Carbohydrates on the Growth and Tolerance of *Lactobacillus*

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Abstract

Resistance to gastrointestinal conditions is a requirement for bacteria to be considered probiotics. In this work, we tested the resistance of six different *Lactobacillus* strains and the effect of carbon source to four different gastrointestinal conditions: presence of α-amylase, pancreatin, bile extract and low pH. Novel galactooligosaccharides synthesized from lactulose (GOS-Lu) as well as commercial galactooligosaccharides synthesized from lactose (GOS-La) and lactulose were used as carbon source and compared with glucose. In general, all strains grew in all carbon sources, although after 24 h of fermentation the population of all *Lactobacillus* strains was higher for both types of GOS than for glucose and lactulose. No differences were found among GOS-Lu and GOS-La. α-amylase and pancreatin resistance was retained at all times for all strains. However, a dependence on carbon source and *Lactobacillus* strain was observed for bile extract and low pH resistance. High hydrophobicity was found for all strains with GOS-Lu when compared with other carbon sources. However, concentrations of lactic and acetic acid were higher in glucose and lactulose than GOS-Lu and GOS-La. These results show that the resistance to gastrointestinal conditions and hydrophobicity is directly related with the carbon source and *Lactobacillus* strains. In this sense, the use of prebiotics as GOS and lactulose could be an excellent alternative to monosaccharides to support growth of probiotic *Lactobacillus* strains and improve their survival through the gastrointestinal tract.

4.3.2.1.1. Introduction

Probiotics are live microorganisms (mainly lactobacillus and bifidobacteria) which administered in adequate amounts confer a health benefit to the host (FAO/WHO, 2003). The *Lactobacillus* genus is distributed in various ecological niches and is an important constituent of the human and animal gut microbiota (Charteris, et al., 1997).

Lactobacilli are currently added to a variety of functional foods and several studies have demonstrated their beneficial properties in human health (Reid, et al., 2011). However, an important requirement is that these bacteria should be able to survive gastrointestinal conditions (amylases in the oral cavity, low pH in the stomach, bile secretions and pancreatic juice in the duodenal section of the small intestine). Several *in vivo* (Jain, et al., 2004, Park, et al., 2008, Reid, 2008) and *in vitro* (Charteris, et al., 1998, Fernandez, et al., 2003, Pitino, et al., 2010) studies have indicated that some *Lactobacillus* strains only partially survive the passage through gastrointestinal tract and it is said that generally a population of 10^7 – 10^9 CFU per mL of bacterial cells should be present in foods in order to colonize, at least temporally, the intestine (Lee, et al., 1995). Nevertheless, it has been observed that only specific strains can survive these conditions. In this sense, Fernández et al. (2003) reported that *L. acidophilus* and *L. gasseri* strains were resistant to low pH and to the presence of different gastrointestinal enzymes. Similarly, Pitino et al. (2010) observed that six different strains of *L. rhamnosus* were resistant to a simulated human digestion process and Charteris et al. (1998) studied the survival of seven different *Lactobacillus* species where *L. fermentum* KLD was considered intrinsically resistant; additionally, these authors found that the addition of milk protein improved the tolerance of the probiotics to gastrointestinal conditions. Similar results have been found by Chavarri et al. (2010) and Madureira et al. (2010) using microencapsulation with alginate-chitosan and whey cheese matrix, respectively.

Kimoto-Nira et al. (2010) have recently studied the resistance of *Lactococcus lactis* G50 grown in six different non-prebiotic carbohydrates (fructose, glucose, galactose, xylose, lactose and sucrose) under simulated gastrointestinal stress. The survival behaviour of G50 strain was found to be dependent on the carbon source

where they were grown. However, to the best of our knowledge the resistance to gastrointestinal conditions of *Lactobacillus* strains grown in prebiotic carbohydrates has rarely been considered. Valerio et al. (2006) reported the protective effect of artichokes on different probiotics strains in the gastrointestinal tract could be hypothetically attributed to the presence of prebiotic carbohydrates and to the physical structure of the vegetable matrix.

Prebiotics are defined as "nondigestible food ingredients that beneficially affects host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon" (Gibson, et al., 2004). Some prebiotic carbohydrates are currently available in the market, such as fructooligosaccharides, lactulose, inulin and galactooligosaccharides from lactose (GOS-La) (Rastall, 2010). However, currently there is considerable interest in the discovery of new carbohydrates with potential prebiotic properties. Among them, galactooligosaccharides from lactulose (GOS-Lu) have recently been studied (Cardelle-Cobas, et al., 2008, Martinez-Villaluenga, et al., 2008). GOS-Lu can be obtained by transgalactosylation reaction of the lactulose by the action of β-galactosidases from different bacterial sources (Cardelle-Cobas, et al., 2008, Martinez-Villaluenga, et al., 2008). Recently, it has been reported that GOS-Lu have the ability to promote the growth of bifidobacteria using *in vitro* fermentation systems with human fecal cultures in a similar manner as the more highly recognised prebiotic GOS-La (Cardelle-Cobas, et al., 2009).

Therefore, the aim of this study was to investigate the growth of six *Lactobacillus* strains, normally used in fermented food, with different prebiotics (lactulose, GOS from lactose and GOS from lactulose) as carbon sources and to determine their resistance to different gastrointestinal conditions (amylases, low pH, bile extract and pancreatin). Hydrophobicity as a measure of potential adhesion of lactobacillus, as well as lactic and acetic acid concentrations produced during incubation were also evaluated.

4.3.2.1.2. Materials and Methods

- *Chemicals*

Glucose, lactulose, bile extract, pancreatin and α-amylase (1440 units/mg protein) from porcine pancreas, β-galactosidase from *Aspargillus oryzae* (8.0 units/mg protein) and *n*-hexadecane was purchased from Sigma-Aldrich (St. Louis, USA). The bacteriological growth media supplements were obtained from EMD Chemicals, Gibbstown, NJ. The galactooligosaccharide from lactose (GOS-La) was obtained from Vivinal-GOS®, kindly provided by Friesland Foods Domo (Zwolle, The Netherlands). This product has a 73 wt% dry matter, the composition of which was 60 wt% GOS, 20 wt% lactose, 19 wt% glucose and 1 wt% galactose, as stated by the supplier. Duphalac® (Solvay Pharma, Brussels, Belgium) was used to obtain the galactooligosaccharides from lactulose (GOS-Lu).

- *Preparation of galactooligosaccharides*

In order to purify the GOS-La, the industrial product Vivinal-GOS® was fractionated using size exclusion chromatography, following the method reported by Hernandez et al. (2009) (**Section 4.1.2**) with some modifications. In brief, 80 mL of Vivinal-GOS® (25 % w/v) were injected in a Bio-Gel P2 (Bio-Rad Hercules, CA, USA) column (90 x 5 cm) using water as mobile phases, at 1.5 mL min^{-1}. Sixty fractions of 10 mL were collected, after the elution of void volume. The fractions degree polymerization (DP) was determined by electrospray ionization mass spectrometry (ESI-MS) at positive mode, ranging from monosaccharides to octasaccharides. Fractions with DP ≥ 3 were pooled and freeze dried.

GOS from lactulose were obtained following the method previously described (**Section 4.1.4**). A solution (450 g L^{-1}) of lactulose (Duphalac®) was dissolved in 50 mM sodium phosphate buffer and 1 mM $MgCl_2$, pH 6.5, after addition of 8 U mL^{-1} of β-galactosidase from *Aspergillus oryzae* (Sigma, St. Louis, MO USA), and incubation at 50 ^oC for 20 h under continuous agitation at 300 rpm. After incubation, the mixtures were immediately immersed in boiling water for 5 min to inactivate the enzymes. The DP of initial GOS-Lu mixture contained from monosaccharides to octasaccharides.

Subsequently, GOS-Lu mixture was fractionated using size exclusion chromatography in order to remove mono- and disaccharides, following the previous methodology applied to Vivinal-GOS®.

- *Bacterial Strains*

Lactobacillus bulgaricus ATCC7517 (LB), *Lactobacillus casei* ATCC11578 (LC), *Lactobacillus delbrueckii* subsp. *lactis* ATCC4797 (LD), *Lactobacillus plantarum* ATCC8014 (LP1), *Lactobacillus plantarum* WCFS1 (LP2) and *Lactobacillus sakei* 23K (LS) were purchased in lyophilized form and maintained at -80 °C for long-term storage. All these strains are considered as probiotics as previously reported in different works (Jain, et al., 2004, Park, et al., 2008, Reid, 2008).

Freeze-dried strains were grown in Lactobacilli MRS broth or in Lactobacilli MRS agar (EMD Chemicals, Gibbstown, NJ) at 37 °C in an anaerobic chamber (10% CO_2: 5% H_2: 85% N_2) (Coy Laboratory Products, Ann Arbor, MI) after transfer through an airlock with two exchanges of N_2 gas followed by one exchange of the oxygen-free mixed gas of the same composition as within the chamber.

- *Growth conditions*

Bacteria were grown in MRS basal media carbohydrate free containing: 10 g L^{-1} protease peptone, 10 g L^{-1} beef extract, 5 g L^{-1} yeast extract, 1 g L^{-1} Tween 80, 2 g L^{-1} ammonium citrate, 5 g L^{-1} sodium acetate, 0.1 g L^{-1} magnesium sulphate, 0.05 g L^{-1} manganese sulphate, 2 g L^{-1} dipotassium sulphate and 0.5 g L^{-1} cysteine-HCl. Glucose, lactulose, GOS-La and GOS-Lu were dissolved in water (10 % w/v) and sterilized by filtration, this solution was added to MRS basal media to a final concentration of 1% w/v. The incubation was carried out under anaerobic conditions at 37 °C. Inoculum was prepared from 48 h MRS grown *Lactobacillus* cells and approximately 1 x 10^7 CFU per mL of each *Lactobacillus* strain (individually) was added to the MRS basal media containing 1% w/v of glucose, lactulose, GOS-La or GOS-Lu and incubated under anaerobic conditions, at 37 °C during 24, 48, 72 and 120 hours. Viable count was carried out by plating on MRS agar in duplicate. All experiments were carried out in triplicate.

- *Lactic and acetic acid analyses*

The incubated samples at 24, 48, and 72 h were centrifuged at 13,000 *g* for 10 min to remove all insoluble particles and the lactic and acetic acid fermentation products were quantified using a BioRad HPX-87H HPLC column (Watford, UK) at 50 ºC, with a 0.005 mM H_2SO_4 as mobile phase, in isocratic mode, at a flow rate of 0.6 mL min^{-1} (Sanz et al., 2005). The analyses were carried out in triplicate.

Since minor levels of acetic acid were initially present in the MRS broth, this value was quantified and subtracted from the amounts calculated for the samples subjected to incubation.

- *Tolerance to different gastrointestinal conditions*

One mL aliquots of cultures was taken after 48 h of fermentation as outlined previously and then centrifuged for 15 min, at 4 ºC and 8,000 rpm. The cells were washed twice using PBS buffer. The cell pellet was re-suspended in 1 mL of PBS pH 7.0 with: (i) bile extract (0.3 % w/v), or (ii) α-amylase (100 U mL^{-1}) or (iii) pancreatin (0.2 % w/v; a mixture of digestive enzyme secreted by the pancreas and commonly used to simulate the pancreatic juice present in the intestinal digestion), or (iv) 1 mL of saline solution adjusting the pH with HCl 0.1 M (0.85 % w/v; pH 2.5) for low pH studies. The percentage of survival was calculated from triplicate experiments using the following formula:

$$\% \text{ survival} = (\beta / \alpha) * 100$$

Where α is the CFU per mL of the assayed strain at 48 h and β the CFU per mL of the same strain after incubation with the different gastrointestinal conditions.

- *Hydrophobicity of bacteria*

Hydrophobicity was determined following the method proposed by Kimoto-Nira et al. (2010) with somes modifications. After 48 h of incubation the bacteria grown on the different substrates (glucose, lactulose, GOS-La and GOS-Lu) were washed and suspended in PBS in order to obtain an OD_{620} of 1.0. One millilitre of *n*-

hexadecane was added to 1.0 mL of cell suspensions. The solution was incubated during 10 min at 30 ºC, mixed during 60 s and then left to stand for 15 min. The aqueous phase was removed and the OD_{620} determined. The percentage of hydrophobicity was calculated using the following equation: 100 x [1-(Initial OD_{620}/OD_{620} after incubation with *n*-hexadecane]. The analyses were carried out by triplicate.

- *Statistical analyses*

Statistical analyses were performed using Statistica for Windows version 6 (2002) by Statsoft Inc. (Tulsa, USA). Differences between bacterial survival, % of hydrophobicity and lactic and acetic acid concentrations were tested using one-way ANOVA test, followed by a least significant difference (LSD) test as a post hoc comparison of means ($P<0.05$).

4.3.2.1.3. Results and Discussion

- *Growth of Lactobacillus strains with prebiotic sources*

The growth profiles of six different *Lactobacillus* strains in the presence of lactulose, GOS-La, GOS-Lu are shown in **Figure 4.27**. Glucose was also included in this study for comparative purposes. All *Lactobacillus* strains grew during the first 24 h for all the substrates. Higher growth rates were observed for LC and LD with glucose and lactulose than with GOS-La and GOS-Lu substrates, whereas for LP1, LP2 and LS the initial growth rates were similar for all carbohydrates tested, and for LB the lowest initial growth was obtained with glucose. However, after this time, growth rates of all *Lactobacillus* strains decreased quickly when they were grown with glucose and lactulose, whilst all strains kept constant or were slightly modified with GOS-Lu and GOS-La. This response could be attributed to different reasons. It is known that carbohydrates with longer chain lengths are fermented more slowly (Cummings, et al., 2001) which is in agreement with the fermentation kinetics of lactobacillus strains exhibited in presence of GOS-La and GOS-Lu (**Figure 4.27**). Likewise, this could also

explain the initial higher growth observed for LC and LD with glucose and lactulose at 24 hours of incubation. However, no notable differences were detected between GOS-La and GOS-Lu for all fermentation times and strains. Similar behaviour has previously been reported in some bifidobacteria species, using fructooligosaccharides and inulin as the carbon sources, where the oligomers with high molecular weight promoted a higher bacterial growth than other substrates with lower molecular weight (Vernazza, et al., 2006).

Conversely the metabolism of large carbohydrate molecules requires the use of glycosidases and specific transport mechanisms for the hydrolysis products (Vernazza, et al., 2006). In *Lactobacillus* genus, the β-galactosidases are specifically located in the cytoplasm (Fortina, et al., 2003) which implies that for the metabolization of GOS, *Lactobacillus* strains need a transport system in order to hydrolyze these oligosaccharides into the cell by β-galactosidases. This could explain the slower growth of LC and LD strains at 24 h with GOS-Lu and GOS-La compared with glucose and lactulose; however, for LP1, LP2 and LS, the similar values for initial growth provide evidence for a strain-dependence on the assimilation of carbon source.

Furthermore, it has been previously observed that the monomeric composition, polymerization degree and type of glycosidic linkages can affect the growth of probiotic strains (Rastall, et al., 2005). GOS-La obtained from Vivinal-GOS® primarily consist of β-(1-4) linkages (Coulier, et al., 2009, Rastall, 2010) and GOS-Lu consist of β-(1-6), being the most abundant trisaccharide 6´-galactosyl-lactulose (**Section 4.1.4**). Cardelle-Cobas et al. (2011) when studying the effect of different trisaccharides isolated from GOS-Lu and GOS-La mixtures on different bacteria strains, including *Lactobacillus*, reported a preference for linkages β-(1-6) instead of β-(1-4); however, the results obtained in our work showed no differences in growth responses of *Lactobacillus* strains using GOS-Lu or GOS-La.

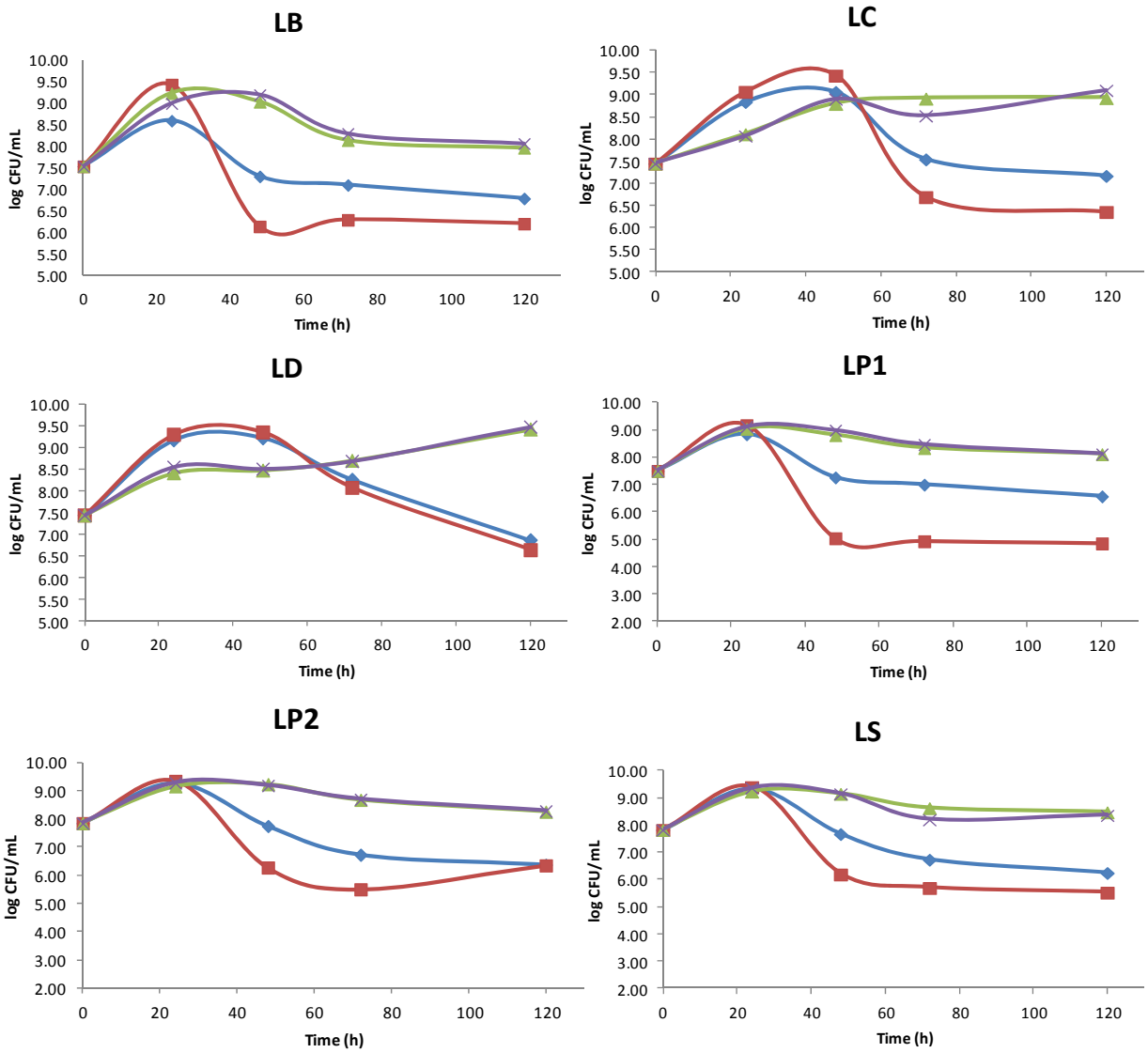

Figure 4.27. Growth of lactobacillus strains in MRS containing different carbohydrates carbon source. (♦) Glucose, (■) Lactulose, (▲) GOS from lactulose, (X) GOS from lactose. LB (*L. bulgaricus* ATCC7517), LC (*L. casei* ATCC11578), LD (*L. delbrueckii* subsp. Lactis ATCC4797), LP1 (*L. plantarum* ATCC8014), LP2 (*L. plantarum* WCFS1), LS (*L. sakei* 23K).

- *Lactic and acetic acid production*

In general, for all strains and carbon sources tested, concentrations of lactic acid were higher than that of acetic acid (**Table 4.16**). *Lactobacillus* strains grown in glucose and lactulose generated higher concentrations of lactic acid than GOS-La and GOS-Lu, whilst similar levels of acetic acid were found for all assayed carbohydrates. The low amount of lactic acid produced in GOS grown culture could be due to the slower and prolonged fermentation by the bacterial strains. This could also have an influence on the higher survival rate of *Lactobacillus* strains grown in GOS substrates (**Figure 4.27**), as a lower acid production leads to less acidic pH values. No significant differences were, in general, detected among the different incubation times either for each carbohydrate or between GOS-La and GOS-Lu. Lactic and acetic acids are fermentation products of lactic acid bacteria (Lindgren, et al., 1990). These acids decrease the pH and consequently can prevent the over growth of pathogenic bacteria in the intestine (Roy, et al., 2006). Short chain fatty acids (SCFA) such as acetic and lactic acids are involved in multiple beneficial effects on the host. Acetic acid is metabolised by different human tissues representing a route to obtain energy from non-digestible carbohydrates (Roberfroid, et al., 2010, Roy, et al., 2006); however, lactic and acetic acids are assimilated by different species present in the gut microbiota, producing butyric acid which can be involved in multiple positive effects such as the reduction of colon cancer risk (Falony, et al., 2009, Roberfroid, et al., 2010, Roy, et al., 2006).

These results support that *Lactobacillus* strains are able to hydrolyze GOS synthesized from lactose and lactulose, as well as lactulose to produce beneficial metabolites as final products.

Table 4.16. Lactic acid and acetic acid concentrations (mM) after 24, 48 and 72 h of fermentation using glucose, lactulose, GOS from lactulose (GOS-Lu) and from lactose (GOS-La). LB (*L. bulgaricus* ATCC7517), LC (*L. casei* ATCC11578), LD (*L. delbrueckii* subsp. Lactis ATCC4797), LP1 (*L. plantarum* ATCC8014), LP2 (*L. plantarum* WCFS1), LS (*L. sakei* 23K).

			Strain					
Carbon Source	**Acid**	**Time (h)**	**LB**	**LC**	**LD**	**LP1**	**LP2**	**LS**
Glucose	**Lactic**	24	209.70 (0.20)*^{efg}	178.54 (14.71)^{efg}	203.52 (1.92)^{efg}	184.33 (23.85)^{efg}	203.18 (5.57)^{efg}	189.12 (6.32)^{efg}
		48	228.67 (27.53)^{g}	197.93 (29.10)^{efg}	203.09 (0.44)^{efg}	184.55 (29.74)^{efg}	208.12 (0.40)^{efg}	192.89 (16.52)^{efg}
		72	214.89 (5.69)^{fg}	189.86 (23.11)^{efg}	199.74 (0.40)^{efg}	230.39 (41.87)^{g}	199.79 (3.62)^{efg}	198.20 (9.37)^{efg}
	Acetic	24	36.76 (2.89)^{abcde}	34.34 (2.17)^{abc}	33.61 (1.15)^{abc}	32.68 (4.37)^{ab}	32.82 (0.49)^{ab}	37.37 (4.84)^{abcde}
		48	42.65 (0.91)^{abcde}	31.71 (1.34)^{a}	31.97 (1.13)^{a}	33.67 (3.10)^{abc}	34.87 (0.22)^{abc}	35.42 (0.44)^{abcd}
		72	38.52 (3.94)^{abcde}	31.04 (0.14)^{a}	39.16 (14.93)^{abcde}	40.65 (8.53)^{abcde}	31.35 (0.89)^{a}	37.11 (2.68)^{abcde}
Lactulose	**Lactic**	24	180.67 (25.27)^{efg}	154.68 (3.47)^{de}	165.38 (3.50)^{efg}	199.46 (1.36)^{efg}	201.05 (6.86)^{efg}	158.91 (1.39)^{def}
		48	215.54 (26.31)^{fg}	208.51 (13.35)^{efg}	203.18 (7.44)^{efg}	204.92 (3.37)^{efg}	206.51 (1.45)^{efg}	195.12 (7.39)^{efg}
		72	202.73 (10.70)^{efg}	200.28 (3.02)^{efg}	204.26 (0.51)^{efg}	227.11 (36.28)^{g}	203.36 (6.14)^{efg}	202.85 (2.10)^{efg}
	Acetic	24	42.38 (0.90)^{abcde}	38.39 (1.57)^{abcde}	37.95 (1.93)^{abcde}	49.11 (16.90)^{abcde}	46.66 (1.25)^{abcde}	48.70 (5.52)^{abcde}
		48	49.46 (3.45)^{abcde}	37.95 (4.93)^{abcde}	32.41 (5.85)^{ab}	42.19 (1.08)^{abcde}	37.99 (6.26)^{abcde}	45.07 (3.18)^{abcde}
		72	42.96 (6.43)^{abcde}	35.03 (2.19)^{abc}	31.84 (0.56)^{a}	48.42 (6.61)^{abcde}	35.33 (3.20)^{abcd}	44.12 (7.82)^{abcde}
GOS-Lu	**Lactic**	24	69.20 (1.52)^{abc}	183.87 (29.23)^{efg}	42.90 (6.90)^{ab}	63.61 (0.94)^{abc}	66.72 (6.73)^{abc}	67.42 (4.53)^{abc}
		48	80.73 (4.40)^{abc}	40.42 (2.05)^{ab}	35.56 (9.37)^{ab}	75.06 (5.23)^{abc}	66.87 (6.21)^{abc}	65.58 (6.10)^{abc}
		72	77.09 (2.50)^{abc}	43.80 (0.77)^{ab}	44.51 (0.99)^{ab}	87.71 (12.00)^{abc}	67.30 (6.55)^{abc}	71.26 (8.22)^{abc}
	Acetic	24	43.02 (5.75)^{abcde}	42.49 (6.04)^{abcde}	42.04 (0.94)^{abcde}	63.07 (2.84)^{e}	31.61 (2.12)^{a}	58.52 (2.72)^{bcde}
		48	54.13 (2.07)^{abcde}	44.37 (2.05)^{abcde}	43.64 (3.40)^{abcde}	51.95 (0.09)^{abcde}	44.42 (13.69)^{abcde}	53.11 (0.12)^{abcde}
		72	48.56 (10.26)^{abcde}	42.32 (5.80)^{abcde}	52.51 (13.54)^{abcde}	59.21 (11.69)^{cde}	42.42 (14.22)^{abcde}	54.94 (2.83)^{abcde}
GOS-La	**Lactic**	24	65.66 (2.90)^{abc}	33.06 (11.05)^{a}	54.12 (1.89)^{abc}	46.91 (1.55)^{abc}	76.81 (2.04)^{abc}	77.96 (3.09)^{abc}
		48	83.71 (9.03)^{abc}	33.20 (10.09)^{a}	36.14 (12.14)^{ab}	102.97 (22.16)^{cd}	82.53 (0.36)^{abc}	80.25 (0.28)^{abc}
		72	76.73 (7.55)^{abc}	32.87 (9.92)^{a}	33.52 (10.84)^{a}	91.42 (16.87)^{bc}	85.21 (2.95)^{abc}	84.91 (1.25)^{abc}
	Acetic	24	45.22 (0.25)^{abcde}	40.21 (12.37)^{abcde}	46.47 (2.11)^{abcde}	57.36 (1.97)^{abcde}	61.59 (10.40)^{de}	64.06 (3.85)^{e}
		48	52.99 (2.45)^{abcde}	47.05 (1.43)^{abcde}	42.46 (1.66)^{abcde}	59.38 (9.52)^{cde}	52.35 (0.34)^{abcde}	52.84 (1.89)^{abcde}
		72	51.69 (0.32)^{abcde}	47.69 (1.31)^{abcde}	46.59 (2.88)^{abcde}	57.10 (10.38)^{abcde}	50.01 (1.82)^{abcde}	53.46 (1.79)^{abcde}

*Standard deviation in parentheses

Different letters indicate significant differences ($P \leq 0.05$) for each acid

Table 4.17. Survival (%) of strains grown in glucose, lactulose, GOS from lactulose (GOS-Lu) and from lactose (GOS-La) in the presence of α-amylase and pancreatin after 1 and 3 hours of fermentation.

	% Survival			
Carbon source / Strain	**α-amylase 1h**	**α-amylase 3h**	**Pancreatin 1h**	**Pancreatin 3h**
LB				
Glucose	95.74 (2.02)[§a]	99.53 (2.59)[a]	107.86 (2.95)[b*]	115.95 (2.95)[c*]
Lactulose	109.45 (1.97)[b*]	99.31 (3.54)[a]	119.96 (0.22)[cd*]	125.06 (14.91)[d*]
GOS-Lu	89.72 (0.84)[c]	98.23 (11.03)[a]	95.20 (2.82)[a]	114.45 (1.46)[c*]
GOS-La	105.55 (1.43)[b*]	104.90 (3.74)[b]	106.73 (3.82)[b*]	108.00 (3.41)[b*]
LC				
Glucose	100.78 (6.59)[ab]	101.66 (6.48)[a]	108.66 (5.95)[b*]	104.60 (1.59)[abc]
Lactulose	96.58 (0.23)[a]	99.06 (2.12)[a]	106.53 (4.72)[b*]	105.58 (3.26)[bc*]
GOS-Lu	108.20 (4.61)[b*]	110.45 (2.46)[*b]	120.02 (6.26)[d*]	107.27 (6.98)[b*]
GOS-La	105.22 (12.03)[b*]	106.81 (10.80)[a]	103.56 (0.21)[abc]	100.54 (0.85)[ab]
LD				
Glucose	105.66 (1.55)[c*]	107.70 (3.15)[c]	106.60 (2.23)[b*]	111.23 (0.28)[cd*]
Lactulose	103.99 (1.08)[b]	106.48 (2.56)[b]	106.71 (3.48)[bc*]	107.65 (0.68)[bc*]
GOS-Lu	93.91 (10.28)[a]	93.05 (10.62)[a]	105.81 (9.42)[bc*]	113.19 (2.63)[d*]
GOS-La	95.66 (5.18)[a]	93.77 (3.93)[a]	97.27 (3.54)[a]	108.46 (0.56)[bc*]
LP1				
Glucose	99.00 (3.13)[a]	96.87 (2.47)[a]	91.33 (5.80)[b*]	91.28 (3.48)[a*]
Lactulose	106.43 (0.17)[bc*]	101.92 (0.22)[a]	97.99 (13.74)[ab]	99.73 (0.48)[a]
GOS-Lu	105.09 (0.00)[cd*]	97.80 (0.46)[a]	100.75 (6.74)[a]	101.05 (9.82)[a]
GOS-La	106.98 (4.30)[d*]	100.77 (1.35)[a]	99.27 (4.05)[ab]	97.63 (6.64)[a]
LP2				
Glucose	98.09 (4.42)[b]	92.63 (6.64)[a*]	97.01 (2.52)[a*]	95.82 (5.24)[c*]
Lactulose	102.64 (3.15)[c]	101.56 (3.08)[bc]	98.60 (1.06)[abcd]	98.00 (0.66)[acd]
GOS-Lu	100.15 (3.20)[bc]	100.72 (0.93)[bc]	99.00 (1.67)[abd]	101.02 (0.68)[b]
GOS-La	101.34 (4.10)[bc]	100.06 (3.09)[bc]	99.87 (2.55)[ab]	100.49 (1.53)[ab]
LS				
Glucose	96.80 (0.98)[b]	99.86 (5.95)[b]	101.55 (3.13)[ab]	104.64 (0.57)[ab]
Lactulose	76.71 (0.87)[a*]	87.68 (4.92)[a]	100.16 (0.28)[a]	101.85 (0.40)[a]
GOS-Lu	108.21 (4.59)[c*]	101.90 (2.18)[b]	106.31 (4.40)[b*]	108.58 (5.16)[b*]
GOS-La	104.23 (6.56)[c]	100.18 (2.23)[b]	101.67 (7.82)[ab]	103.05 (6.00)[a]

[§]Standard deviation in parentheses

Different letters indicate significant differences ($P \leq 0.05$) for each strain and treatment

[*]Significant differences with 0 hours for each strain and treatment

Table 4.18. Survival (%) of strains grown in glucose, lactulose, GOS from lactulose (GOS-Lu) and from lactose (GOS-La) in the presence of bile extract and low pH after 1 and 3 hours of fermentation.

Carbon source / Strain	% Survival			
	Bile extract 1h	Bile extract 3h	Low pH 1h	Low pH 3h
LB				
Glucose	100.50 (1.41)[§a]	96.94 (1.16)[a]	28.87 (1.16)[b*]	ND
Lactulose	98.36 (0.02)[a]	98.45 (2.83)[a]	72.90 (12.23)[a*]	ND
GOS-Lu	78.41 (4.96)[d*]	43.19 (6.04)[b*]	74.61 (1.61)[a*]	ND
GOS-La	87.95 (1.36)[e*§]	62.01 (0.82)[*c]	72.34 (0.86)[a*]	ND
LC				
Glucose	29.84 (5.77)[a*]	ND	29.85 (8.73)[a*]	ND
Lactulose	30.97 (4.83)[a*]	ND	21.91 (3.44)[b*]	ND
GOS-Lu	45.08 (6.69)[c*]	38.15 (4.27)[b*]	ND	ND
GOS-La	44.60 (4.57)[c*]	33.31 (0.85)[ab*]	ND	ND
LD				
Glucose	55.84 (0.38)[b*]	38.78 (11.65)[a*]	60.63 (0.41)[d*]	28.03 (3.00)[e*]
Lactulose	72.93 (6.77)[d*]	63.45 (7.77)[c*]	42.05 (0.29)[b*]	ND
GOS-Lu	37.63 (1.26)[a*]	35.88 (1.40)[a*]	55.21 (6.35)[c*]	ND
GOS-La	52.02 (0.13)[b*]	33.22 (1.41)[a*]	37.94 (1.08)[a*]	ND
LP1				
Glucose	95.51 (3.69)[de*]	90.48 (2.30)[bc*]	77.58 (2.48)[a*]	ND
Lactulose	99.69 (0.36)[a]	99.57 (0.36)[ae]	73.82 (3.41)[a*]	ND
GOS-Lu	92.68 (6.73)[cd*]	90.07 (4.30)[bc*]	22.08 (0.01)[c*]	ND
GOS-La	89.93 (4.46)[bc*]	87.66 (1.92)[b*]	25.76 (0.12)[b*]	ND
LP2				
Glucose	84.45 (2.04)[ab*]	77.81 (0.99)[c*]	69.56 (7.01)[f*]	ND
Lactulose	85.29 (0.73)[ab*]	81.13 (1.04)[de*]	77.39 (5.92)[f*]	29.81 (2.42)[cd*]
GOS-Lu	85.96 (2.51)[b*]	79.14 (5.15)[cd*]	52.87 (2.36)[e*]	19.58 (0.13)[ab*]
GOS-La	82.45 (1.18)[ae*]	73.51 (4.04)[f*]	42.75 (13.00)[de*]	33.92 (0.79)[bc*]
LS				
Glucose	80.36 (3.32)[a*]	81.58 (7.00)[a*]	ND	ND
Lactulose	82.30 (1.37)[a*]	82.08 (2.82)[a*]	ND	ND
GOS-Lu	87.09 (3.56)[b*]	87.54 (1.38)[b*]	42.04 (0.22)[b*]	23.68 (0.03)[a*]
GOS-La	83.66 (0.23)[a*]	83.31 (1.69)[a*]	46.05 (5.46)[b*]	ND

[§]Standard deviation in parentheses

Different letters indicate significant differences ($P \leq 0.05$) for each strain and treatment

[*]Significant differences with 0 hours for each strain and treatment

ND no detected

- *Tolerance to different gastrointestinal conditions*

The survival responses of the *Lactobacillus* strains, previously grown in the different carbohydrates tested, after 1 and 3 hours of being exposed to different gastrointestinal conditions are shown in **Tables 4.17** and **4.18**.

All the strains survived after 1 and 3 h of exposure to α-amylase and pancreatin treatments (**Table 4.17**), although a significant decrease in survival of LS incubated with lactulose in the presence of amylase was observed. Survival rate values were greater than 100 % in some cases which could be due to the presence of low molecular weight carbohydrates in the commercial enzymatic preparations. Pitino et al. (2010) reported an increase on the survival of some strains of *L. rhamnosus* during simulation of duodenal digestion, due to the presence of a carbon source in the MRS broth used as the vehicle for digestion of the cells. Similar data were found by Kimoto-Nira et al. (2009) for *Lactococcus lactis* in media containing bile salts and lactose as carbon source.

Survival to bile extract appeared to be dependent on the carbon source and the *Lactobacillus* strain at both tested times (**Table 4.18**). After 1 hour a general decline in bacteria numbers was detected for all strains and carbon sources, with the exception of LB grown on glucose and lactulose and LP1 on lactulose. This decrease was greater at 3 hours of treatment. LC and LD exhibited the lowest survival rates for all carbohydrate sources, whereas LP1 was the most resistant strain.

Regarding LB, its survival after bile treatment was higher when it was incubated with glucose and lactulose, whereas LC survived better when it was incubated with GOS-Lu and GOS-La as compared to non-survival in the presence of glucose and lactulose after 3 hours of fermentation. LD grown on lactulose exhibited its highest survival rate in the presence of the bile extract. Lower significant differences in bile tolerance were detected for LP1, LP2 and LS grown on the different carbohydrate sources.

Charteris et al. (1998) reported that a level of survival higher than 30% would be considered intrinsically tolerant to gastric transit by when using simulated gastric and pancreatic juices. Although the results presented here are based on resistance to

bile extracts, this value could be considered to classify the *Lactobacillus* strains, tested for the different gastrointestinal conditions, as being as tolerant or not tolerant. Following this premise, most of the strains grown in the different carbohydrates used could be considered as bile tolerant, with the exception of LC using glucose and lactulose. Similarly, Fernandez et al. (2003) and Koll et al. (2010) reported tolerance to bile salts at 0.15 and 2% w/v, respectively, of different *Lactobacillus* strains grown in MRS agar.

Tolerance to gastric pH (2.5) expressed as % survival is shown in **Table 4.18**. In general, after 1 h of exposition, significant survival decreases were observed for all assayed strains. LB and LS grown on prebiotic carbohydrates exhibited a higher resistance to pH conditions than the strains grown on glucose, whereas LC and LD grown on glucose were more tolerant. LP1 and LP2 grown on lactulose or glucose exhibited higher resistance to low pH values. Although gastric emptying is strongly influenced by volume and composition of gastric contents, type of meal and/or gastrointestinal disorders (Bolondi, et al., 1985), the average time for 50% of gastric emptying has been estimated to be approximately 1.2 hours (Read, et al., 1986). This means that physiologically relevant levels of most of the studied *Lactobacillus* strains could be able to reach further down the gastrointestinal tract. Finally, at extreme exposure times to treatment (3 h), only LP2 grown on lactulose, GOS-La or GOS-Lu, LD grown on glucose and LS grown on GOS-Lu could be detected.

- *Hydrophobicity of bacteria*

The percentage of hydrophobicity of all strains after 48h of fermentation is shown in **Table 4.19**. It is worth noting that LB, LC and LD grown with GOS-Lu exhibited the highest values of hydrophobicity, whereas hydrophobicity of LP1 and LS was higher when they were grown on GOS-La. Both prebiotic carbohydrates also contributed to the higher hydrophobicity values of LP2. Hydrophobic index of bacteria is related to their adhesion capacity to intestinal cells (Wadstrom, et al., 1987). This capacity is necessary for the bacteria to colonize, at least temporally, the intestine and consequently, they may be considered as probiotics. Therefore, LB, LC and LD strains grown on GOS-Lu and LC, LP1 and LS strains grown on GOS-La could exhibit the higher

adhesion capacity. It has also been reported that hydrophobicity index varies depending on the strain and the carbon source used (Kimoto-Nira, et al., 2010) which is in good agreement with our results.

Table 4.19. Hydrophobicity (%) of strains grown in glucose, lactulose, GOS from lactulose (GOS-Lu) and from lactose (GOS-La).

	% Hydrophobicity					
Carbon source	**LB**	**LC**	**LD**	**LP1**	**LP2**	**LS**
Glucose	46.76 (8.40)^{h§}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	13.35 (1.29)^{cd}
Lactulose	0.00 (0.00)^{a}	6.65 (1.44)^{abc}	0.00 (0.00)^{a}	0.00 (0.00)^{a}	21.55 (8.91)^{e}	29.76 (11.97)^{f}
GOS-Lu	64.05 (14.11)^{i}	79.47 (6.47)^{j}	80.09 (0.73)^{j}	0.00 (0.00)^{a}	28.75 (5.19)^{f}	15.40 (5.29)^{d}
GOS-La	3.73 (0.17)^{ab}	62.72 (1.50)^{i}	0.00 (0.00)^{a}	66.38 (4.45)^{i}	27.90 (2.38)^{f}	48.57 (2.76)^{h}

*Standard deviation

§ Different letters indicate significant differences ($P \leq 0.05$) for each acid

VL: Vivinal-GOS purified and GOS: galactooligosaccharides from lactulose purified.

In conclusion, resistance to gastrointestinal conditions (mainly to bile extracts and gastric pH values) and bacterial hydrophobicity depend highly on carbohydrates used as carbon source and the *Lactobacillus* strain. Growth of some *Lactobacillus* strains on different prebiotics could help to increase their resistance to gastrointestinal conditions, thus, enhancing their survival through the gastrointestinal tract, as well as to promote their adhesion capacity. Additionally, food matrix effects may also contribute to the ability of a probiotic to survive through the gastrointestinal tract (Sanders, et al., 2010). Thus, several studies have previously shown that the inclusion of milk-based products improved the resistance to gastrointestinal conditions of different probiotics including some *Lactobacillus* strains (Charteris, et al., 1998, Fernandez, et al., 2003, Madureira, et al., 2011, Martinez, et al., 2011). A possible explanation for this response is that milk proteins could act as buffering agents and/or inhibitors of digestive proteases (Charteris, et al., 1998). On the basis of these studies, it could be expected that the combined use of milk-based products and GOS-La or

GOS-Lu might increase the survival of the assayed *Lactobacillus* strains. These findings may help to expand the applications of lactulose, and galactooligosaccharides derived from lactulose and lactose in synbiotic products with important applications in the design of new functional food ingredients.

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4.3.2.2. Effect of hydrolyzed caseinmacropeptide conjugated galactooligosaccharides on the growth and bile resistance of *Lactobacillus* strains

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Abstract

In recent decades the concept of functional foods has attracted the general public and gained importance in industrial and academic research towards the product development. Despite the availability of range of commercial functional food ingredients additional research and development efforts are still needed to enhance the efficacy of existing prebiotic compounds or to identify new prebiotic targets. In an effort to enhance the health benefits of functional food component in this present study we synthesized the conjugates of hydrolyzed caseino-macropeptide conjugated with prebiotic galactooligosaccharides (hCMP:GOS) and demonstrated their effect on growth and bile tolerance of six *Lactobacillus* strains. Growth study showed the ability of hCMP:GOS to serve as a sole carbon source to *Lactobacillus* strains. In bile resistance study hCMP:GOS grown *Lactobacillus* cells exhibited enhanced bile tolerance and retained 90% viability up to 3 h. Significant quantity of acetate and lactate at maximum level of 80 mM was detected in HPLC analysis of cell free culture supernatant. It demonstrated the ability of *Lactobacillus* strains to ferment the hCMP:GOS as a carbon source. Overall results of this study indicate the potential of hCMP:GOS conjugates to use as a potential prebiotic substrate to enhance the growth and bile tolerance in *Lactobacillus* strains and serve as a fermentable substrate to produce beneficial metabolites to the host.

4.3.2.2.1. Introduction

In recent years products claiming to be healthier and to have functional and health properties have gained importance in industrial and academic research and have become a speciality food in the public supermarkets (De Sousa, et al., 2011, Holzman, 2011).

One of the promising research areas in the functional food and nutraceutical products is the development of synbiotics (Thammarutwasik, et al., 2009). Synbiotics are defined as a combination of a probiotics and a prebiotics and there has been growing interest in this concept (Rastall, et al., 2005, Rastall, et al., 2002). The specific health effects of selected probiotic bacterial strains used in synbiotics are becoming increasingly accepted. Several beneficial health effects have been claimed to be based on the presence of probiotic bacteria in the colon. Reports on role of probiotics in combat diarrhea, relief of lactose intolerance, resistance to microbial infections, colorectal cancer prevention, treatment of inflammatory bowel disease, alleviation of constipation, increased immune function, ability to synthesize neuroactive compounds have been reported and reviewed extensively (Loo, et al., 1999, Lyte, 2011, Parracho, et al., 2007, Roberfroid, 2000, Salminen, et al., 1999, Sleator, 2010, Sleator, et al., 2008, Soccol, et al., 2010, Thomas, et al., 2010, Van Loo, 2004, Wohlgemuth, et al., 2010). Indeed, the use of probiotic therapy has attracted research interest in human infectious, inflammatory and allergic disease (Ghishan, et al., 2011, Quigley, 2010, Sleator, 2010).

The metabolic activity of probiotic strains and their effect on human health depends upon the strain specific properties and the dose administered. However, the availability and type of prebiotic compounds in the gastrointestinal tract plays a significant role in determining the viability and beneficial metabolic activity of the probiotic strains (Cummings, et al., 2001, Langlands, et al., 2004). Prebiotics are substances that selectively improve the activity, survival and colonization of probiotics by providing a fermentable carbon source particularly in the large intestine. A variety of prebiotics including β-glucans, inulin, and fructo-oligosaccharides, galacto-oligosaccharides, xylo-oligosaccharides have been used to improve the growth and

metabolic activity of probiotic *Lactobacillus* and *Bifidobacterium* spp. (De Sousa, et al., 2011). The effect of prebiotics on cell proliferation, differentiation, apoptosis, mucin production, immune function, mineral absorption, lipid metabolism, and gastrointestinal peptides has been well documented (Roy, et al., 2006).

Similarly, biologically active peptides have interesting applications as supplement in food and pharmaceutical preparations (Fitzgerald, et al., 2003, Fitzgerald, et al., 2006, Hartmann, et al., 2007). The field of bioactive peptides has intensified during the past two decades and numerous peptides of plant and animal origin with relevant bioactive potential have been discovered (Hartmann, et al., 2007). Milk proteins are one of the known sources of biofunctional peptides that may impart improved health benefits when ingested (Fitzgerald, et al., 2003). Caseinomacropeptides (CMP) obtained by enzymatic hydrolysisof κ-casein during the manufacture of cheese has been receiving more interest for the supplementation of functional foods. CMP and its hydrolyzed fragments have been shown to have various biological activities as physiological regulators, and modulating secretion of intestinal hormones (Hartmann, et al., 2007, Meisel, 1997, Meisel, 1998, Meisel, et al., 1990, Thomae-Worringer, et al., 2006). CMP has been correlated with a wide variety of biological activities including immunosuppression capacity, inhibition of pathogen invasion (LaClair, et al., 2009, Mikkelsen, et al., 2006, Requena, et al., 2009), and nutritional management of metabolic diseases (Abdel-Salam, et al., 2010, Ney, et al., 2008). Exploiting the potential of bioactive peptides is an important task in the discovery of novel functional foods with enhanced bioavailability through the addition and fortification of enriched bioactive peptides with other health promoting compounds. Conjugation of hydrolysates of hCMP with prebiotic galactooligosaccharides by the glycation could be one of the product development methods to make a value added prebiotics for human nutrition and could be a potential novel ingredient for functional foods. Sugita-Konishi et al. (2004) conjugated the bioactive sialoglycopeptides (SGP) with carboxymethyl cellulose and carboxymethyl dextran to improve the SGPs retention time in the gut to prevent the *Salmonella* infection *in vivo* (Sugita-Konishi, et al., 2004). In their study they have

demonstrated the strong protective effect of these glycoconjugates against *Salmonella* infection in Caco-2 cells and suggested the potential of glycoconjugates as food ingredient with a preventive effect on *Salmonella* infection. However, in this study effect of SGP conjugates on the probiotic bacteria was not reported.

In the present study we used Maillard reaction to prepare glycoconjugates of hydrolysed CMP with lactose (GOS-La) and lactulose (GOS-Lu) derived galactooligosaccharides and demonstrated their potential use to support the growth of six *Lactobacillus* strains. Acetate and lactate produced by the *Lactobacillus* strains were quantified to confirm the fermentability of hCMP-conjugated GOS (hCMP:GOS). In addition, the protective role of hCMP:GOS-La and hCMP:GOS-Lu towards the *Lactobacillus* cells in the bile extract was investigated.

4.3.2.2.2. Materials and Methods

- *Galactooligosaccharides and glycoconjugates*

Lactose derived commercial galactooligosaccharide (GOS-La) Vivinal-GOS® was a kind gift from Friesland Foods Domo (Zwolle, The Netherlands). Commercial prebiotic lactulose Duphalac® (Solvay Pharma, Brussels, Belgium) was used to synthesize the lactulose derived galactooligosaccharides (GOS-Lu) using the method of Clemente et al. (2011). GOS-La and GOS-Lu were purified by size exclusion chromatography (**Section 4.1.2**). Caseinomacropeptide (CMP) was kindly provided by Davisco Foods International, Inc., Eden Prairie, MN, and hydrolyzed following the method described previously (**Section 4.2.2**). The glycoconjugates were prepared by mixing aliquots of hydrolyzed CMP with GOS-La or GOS-Lu (2:1 w/w) in 0.1 M sodium phosphate buffer (pH 7.0). The mixture was lyophilized and stored in a vacuum desiccator at 40 °C for 16 days and the water activity was maintained at 0.44 with a saturated K_2CO_3 solution.

- *Bacterial strains and media*

Lactobacillus bulgaricus ATCC 7517 (LB), *L. casei* ATCC11578 (LC), *L. delbrueckii* subsp. *lactis* ATCC4797 (LD), *L. plantarum* ATCC8014 (LP1), *L. plantarum* WCFS1 (LP2),

L. sakei 23K (LS) strains were grown in Lactobacilli MRS broth or on Lactobacilli MRS agar (EMD Chemicals, Gibbstown, NJ) at 37 °C in anaerobic chamber (10% CO_2: 5% H_2: 85% N_2) (Coy Laboratory Products, Ann Arbor, MI). For the prebiotics preference study modified defined MRS medium (MRS without carbohydrate) containing 1% protease peptone, 1% beef extract, 0.5% yeast extract, 0.1% Tween-80, 0.2% ammonium citrate, 0.5% sodium acetate, 0.01% magnesium sulfate, 0.005% manganese sulfate, 0.2% dipotassium phosphate, 0.05% Cysteine-HCl was used (Man, 1960). GOS, hydrolyzed hCMP, and hCMP:GOS-La, hCMP:GOS-Lu (glycoconjugates) were dissolved in sterile distilled water and filter sterilized through 0.2µ filter (VWR International, Sugar Land, TX) and stored at 4 °C until used.

- *Growth study*

For the growth study filter sterilized GOS, hCMP and glycoconjugates solutions were separately added as a sole carbon source in the defined MRS broth to a final concentration of 1%. Inoculum was prepared from 48 h MRS grown *Lactobacillus* cells and approximately 1 x 10^7 CFU/mL of each *Lactobacillus* strain (individually) was added to the medium and incubated at 37 °C under anaerobic atmospheric conditions. MRS media with unconjugated GOS-La, GOS-Lu, and hCMP were used as controls. Growth and viability of each strain in each test carbon source was monitored at regular intervals by serial dilution and plating on MRS agar plates.

- *Bile resistance study*

Bile tolerance of *Lactobacillus* cells grown in GOS, hCMP, and glycoconjugates as carbon source was measured by survival study as described by Kimoto-Nira et al. (2009 and 2010). Briefly, bacterial cells grown in test carbohydrates and glycoconjugates for 48 h were collected and washed twice with 1X PBS and the cell pellets were subsequently mixed with 0.3% (w/v) porcine bile extract (Sigma Chemicals, St. Louis, MO). The treatment mixtures were incubated for 3 h at 37 °C in anaerobic condition as described above. Samples were taken at 0, 1, and 3 h and plated onto MRS agar for viable colony count.

- *Acetate and lactate analysis*

The concentration of acetate and lactate in the cell free culture supernatant (1.5 mL) collected at 24, 48, and 72 h was determined by the method of Sanz et al. (2005) using HPLC system with a UV detector (210 nm) (Agilent Technologies 1200 series, USA). The organic acid resin column HPX-87H HPLC (BioRad, Hercules, CA) was operated with a flow rate of 0.6 mL/min of 0.005 mM H_2SO_4 as a mobile phase at 50°C in isocratic mode.

- *Data analysis*

Data obtained from three independent biological experiments each performed in triplicate were used for statistical analysis. Statistical analyses were performed using Statistica for Windows version 6 (Statsoft Inc., Tulsa, OK). Differences between bacterial survival, acetate and lactate concentrations were tested using one-way ANOVA test, followed by a least significant difference (LSD) test as a post-hoc comparison of means ($P<0.05$).

4.3.2.2.3. Results

- *Growth of Lactobacillus strains in glygoconjugates*

To examine the potential prebiotic effect of glycoconjugates, *Lactobacillus bulgaricus* ATCC 7517 (LB), *L. casei* ATCC11578 (LC), *L. delbrueckii* subsp. *lactis* ATCC4797 (LD), *L. plantarum* ATCC8014 (LP1), *L. plantarum* WCFS1 (LP2), *L. sakei* 23K (LS) were grown in defined MRS medium containing GOS, hCMP, and glycoconjugates as a sole carbon source. In the growth study variable growth pattern was observed depends on the bacterial strain and the carbon source used in the media (**Figure 4.28**). Overall all the six *Lactobacillus* strains exhibited significant growth in GOS-La and GOS-Lu, and their glycoconjugates (hCMP:GOS-La and hCMP:GOS-Lu). Unlike GOS or glycoconjugates, cells grown in hCMP as a sole carbon source did not show increase in the number of viable cells, however, maintained their viability up to 120 h of incubation. Strains LB, LC, and LD showed a higher growth in all the test carbon

sources and did not show any difference in the growth pattern between the carbon sources. Strains LP1, LP2, and LS exhibited better growth in GOS-La, GOS-Lu, and glycoconjugates for up to 120 h, whereas, the hCMP did not support the growth after 24 h of incubation. Strains LP2 and LS were able to utilize hCMP:GOS-La and hCMP:GOS-Lu similar to unconjugated prebiotic GOS and were able to maintain their viability up to 120 h.

- *Bile tolerance of glycoconjugates grown Lactobacills cells*

To determine the bile tolerance and relative survival percentage of viable cells, the presence of surviving colony forming units (CFU/mL) at 0, 1, and 3 h of bile treatment were monitored. The bile tolerance and survival patterns of all six *Lactobacillus* strains are shown in **Figure 4.29.** After 1 and 3 h of treatment *L. bulgaricus* exhibited better bile tolerance in hCMP:GOS than the unconjugated GOS. After 1 h of treatment hCMP: GOS-La and hCMP:GOS-Lu grown cells remained 100 and 93% viable, respectively, whereas, unconjugated GOS-La, GOS-Lu grown LB cells elicited decreased viability of 87 and 78%, respectively. After 3 h of bile treatment hCMP:GOS-La and hCMP:GOS-Lu grown LB cells retained 91 and 67% viable cells. Glycoconjugates grown *L. casei* cells showed better viability after 1 h of bile treatment. However, after 3 h of treatment number of viable LC cells was decreased. When compared to glycoconjugates grown LC cells, viability of unconjugated GOS-La and GOS-Lu grown cells decreased to less than 50% at both 1and 3 h of treatment. *L. delbrueckii* (LD) cells grown in hCMP:GOS-Lu showed only 67% of viable cells after 1 h exposure to bile. hCMP:GOS-La and GOS grown LD cells retained less than 50% of viable cells after 1 and 3 h of bile treatment. hCMP grown LD cells were only 23% viable after 1 h of bile treatment and after 3 h reached nondetectable levels. For strain *L. plantarum* ATCC8014 (LP1), unconjugated GOS-La and GOS-Lu grown cells showed better viability in 1 and 3 h of bile treatment than the hCMP conjugated GOS. Conversely, unconjugated hCMP alone did not support the bile tolerance and the LP1 cells were nondetectable within 1 h of bile treatment. *L. plantarum* WCFS1 (LP2), *L. sakei* 23K (LS) cells grown in all the test substrates showed significant number of viable

cells even after 3 h of bile treatment. However, even after 3 h of bile treatment hCMP and glycoconjugates supported the enhanced bile tolerance and survival (above 90%) than unconjugated GOS.

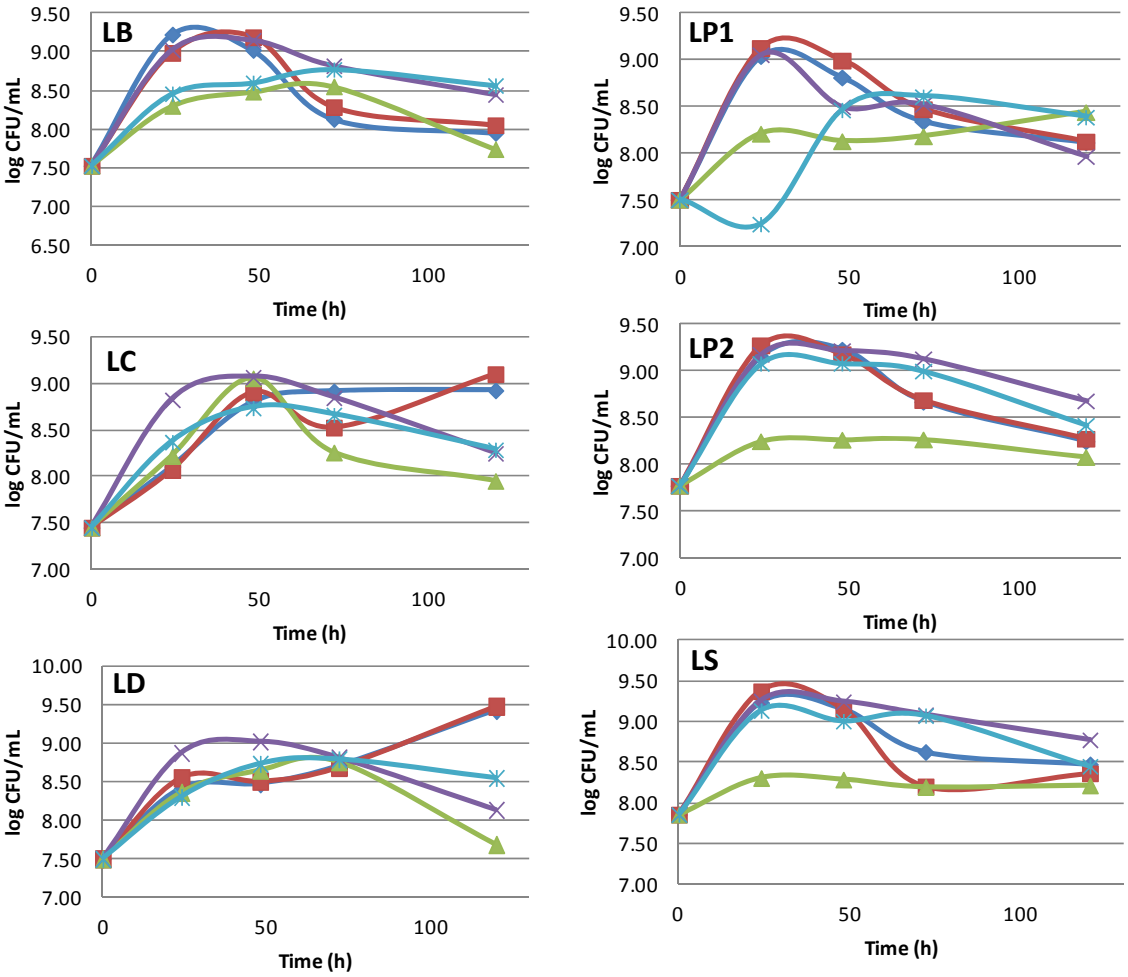

Figure 4.28. Growth of *Lactobacillus bulgaricus* ATCC 7517 (LB), *L. casei* ATCC11578 (LC), *L. delbrueckii* subsp. *lactis* ATCC4797 (LD), *L. plantarum* ATCC8014 (LP1), *L. plantarum* WCFS1 (LP2), *L. sakei* 23K (LS) in MRS broth containing GOS-La (◊); GOS-Lu (□); hCMP(Δ); hCMP:GOS-La (×); hCMP:GOS-Lu (*). Colony forming units per mL (CFU/mL) were calculated as an average with standard deviation from data obtained from three independent studies.

- *Fermentability of glyconjugates by Lactobacillus strains*

Since the prebiotics are one of the fermentable substrates for the probiotics to produce beneficial metabolites, we measured the quantity of the lactate and acetate produced by the *Lactobacillus* strains grown in glycoconjugates and their corresponding unconjugated GOS and hCMP. All the six *Lactobacillus* strains were able to ferment the glycoconjugates hCMP:GOS-La and hCMP:GOS-Lu similar to unconjugated prebiotic GOS-La, GOS-Lu and produced significant quantity of lactate and acetate as fermentation products in the culture media. However, compare to lactate high concentrations of acetate were detected in the HPLC analysis (**Table 4.20**).

All six *Lactobacillus* strains were able to ferment the glyconjugates similar to unconjugated GOS-La and GOS-Lu and produced significant amount of lactate in the culture medium (**Table 4.20**). However, GOS-La and GOS-Lu grown cells produced high levels of lactate in entire incubation period studied . Except strain LD, all other strains produced greater than 50 mM lactate up to 72 h of incubation in GOS-La and GOS-Lu containing culture media. Among the glycoconjugates, hCMP:GOS-Lu grown cells produced maximum of 40 mM lactate up to 72 h of incubation. Compared to LC, LD, LP1, and LS, strains LB and LP2 produced 40 mM lactate in hCMP:GOS-Lu containing growth media. Compare to GOS and hCMP:GOS, less than 25 mM lactate was detected in the cell free supernatant obtained from the hCMP grown culture.

On the other hand, strain and substrate dependent variations were observed in the amount of acetate produced in the cell free culture supernatant (**Table 4.20**). LB strain grown in GOS and hCMP:GOS-Lu produced almost 40 and 50 mM acetate at 24 and 48 h incubation, respectively. However, at 72 h of incubation higher concentration (62 mM) of acetate was detected only in hCMP:GOS-Lu supernatant. hCMP:GOS-La grown LB cells produced significant levels (40 mM) of acetate but lesser than the amount detected in GOS and hCMP:GOS-Lu supernatant**.** All the growth substrates were able to metabolize by the LC cells and almost equal amount of acetate was detected at 24 h of incubation. Afterwards, at 48 h only GOS-Lu and GOS-La grown cells were able to produce high concentrations (44 and 47 mM) of acetate. However,

after 72 h of incubation high levels of acetate (47 mM) concentration was detected in GOS-La, hCMP:GOS-Lu, and hCMP:GOS-La grown LC culture supernatants. Strain LD produced between 35 to 45 mM acetate after 24 h of incubation in all the test substrates. Subsequently, gradual increase in the acetate concentration was observed up to 52 and 47 mM in culture supernatant collected at 48 and at 72 h from GOS-Lu and GOS-La grown LD culture**.** Glycoconjugate hCMP:GOS-Lu grown LD cells produced 28 and 41 mM acetate at 48 and 72 h, respectively. Whereas, hCMP:GOS-La grown cells produced 37 and 39 mM acetate at 48 and 72 h of incubation, respectively**.** GOS-Lu and GOS-La grown LP1 culture supernatant exhibited high concentrations (51 and 59 mM) of acetate at 24 and 48 h of incubation (**Table 4.20**)**.** Initially at the 24 h sample less acetate concentration was recorded in the glyconjugates supernatants. Later at 48 and 72 h, gradual increase in the acetate concentrations was detected in the culture supernatants obtained from hCMP:GOS-Lu and hCMP:GOS-La grown LP1 cultures. After 72 h comparable acetate concentration (55 mM) was detected in hCMP:GOS-Lu grown LP1 culture supernatant**.** In strain LP2, high levels of acetate concentration was detected in culture supernatant obtained from GOS-La grown culture**.** However, the acetate concentration was gradually decreased from 61 to 50 mM during the incubation period. Conversely, regardless of incubation time in hCMP:GOS-Lu and hCMP:GOS-La grown LP2 culture supernatants the level of acetate concentrations was stable (40 and 38 mM, respectively) in all three time points. During the 72 h incubation period gradual increase from 40 to 52 mM acetate production was observed in hCMP:GOS-Lu grown LS cells, while GOS-Lu and GOS-La grown culture showed gradual decrease in the acetate production. hCMP:GOS-La culture supernatant did not show significant changes in the acetate concentrations and remained between 37 to 39 mM in all three time points. It did not show any time dependent variations in the levels acetate concentration as observed in GOS-Lu, GOS-La and hCMP:GOS-Lu.

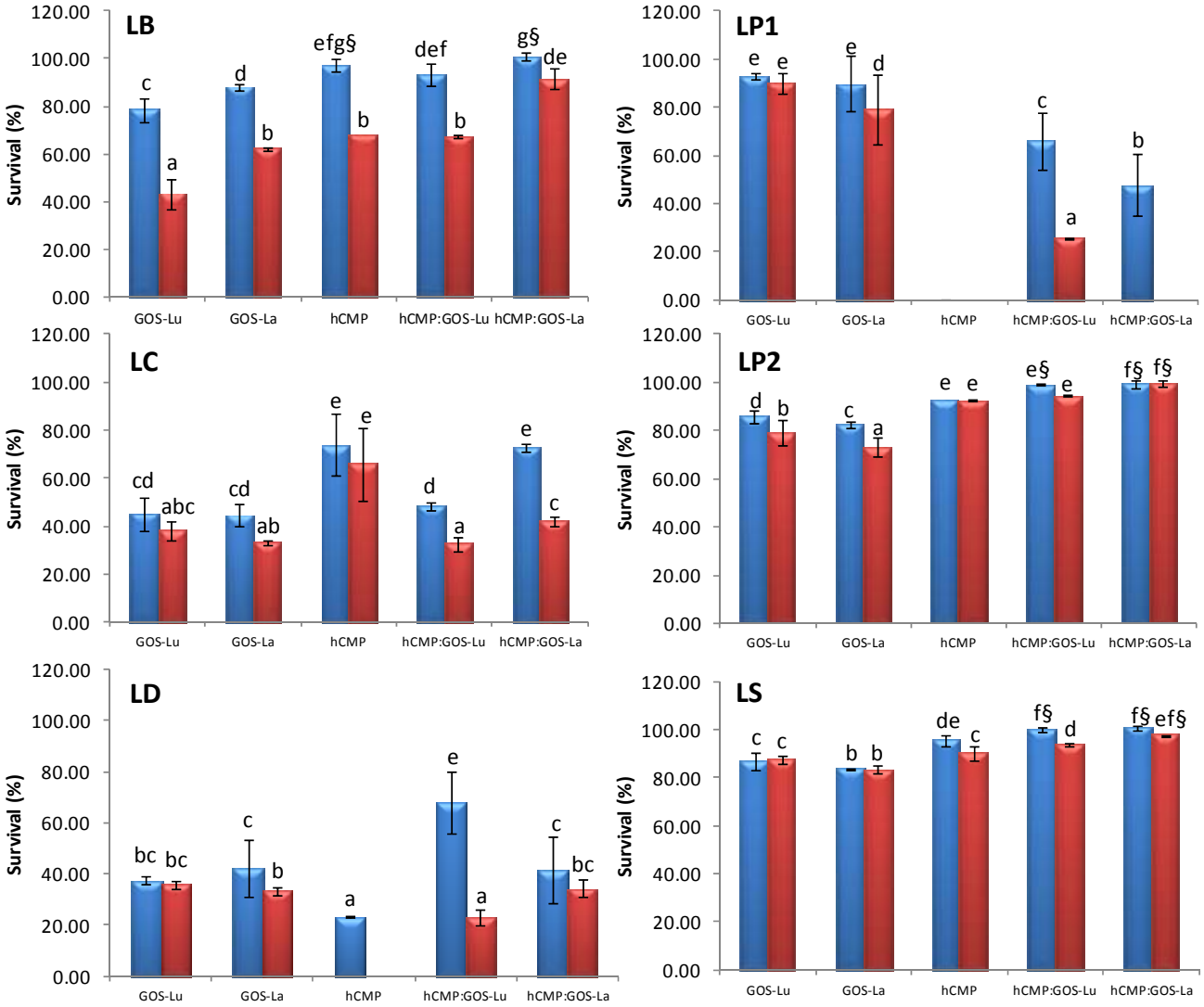

Figure 4.29. Bile tolerance and survival of oligosaccharides (GOS-Lu and GOS-La), hydrolyzed casein macropeptide (hCMP), and hydrolyzed CMP conjugated glycoconjugates (hCMP:GOS-Lu, hCMP:GOS-La) grown **(LB)** *Lactobacillus bulgaricus* ATCC 7517; **(LC)** *L. casei* ATCC11578; **(LD)** *L. delbrueckii* subsp. *lactis* ATCC4797; **(LP1)** *L. plantarum* ATCC8014; **(LP2)** *L. plantarum* WCFS1; **(LS)** *L. sakei* 23K after 1h (■) and 3h (■) bile treatment. Survival percentage was calculated as an average with standard deviation from data obtained from three independent studies. Different letters indicate significant differences ($P \leq 0.05$) for each strain and carbon source. §Significant differences with 0 h for each strain and carbon source.

Table 4.20. HPLC analysis of lactate and acetate produced in the cell free culture supernatant of **(LB)** *Lactobacillus bulgaricus* ATCC 7517; **(LC)** *L. casei* ATCC11578; **(LD)** *L. delbrueckii* subsp. *lactis* ATCC4797; **(LP1)** *L. plantarum* ATCC8014; **(LP2)** *L. plantarum* WCFS1; **(LS)** *L. sakei* 23K grown in GOS-Lu; GOS-La; hCMP; hCMP:GOS-Lu and hCMP:GOS-La at different incubation time period.

Carbon Source	Acid	Time (h)	LB	LC	LD	LP1	LP2	LS
GOS-Lu	Lactic	24	69.20 (1.52)^{b}	183.87 (29.23)^{b}	42.90 (6.90)^{cde}	63.61 (0.94) ^{bcd}	66.72 (6.73)^{b}	67.42 (4.53)^{c}
		48	80.73 (4.40)^{b}	40.42 (2.05)^{a}	35.56 (9.37)^{bcd}	75.06 (5.23)^{cde}	66.87 (6.21)^{b}	65.58 (6.10)^{c}
		72	77.09 (2.50)^{b}	43.80 (0.77)^{a}	44.51 (0.99)^{de}	87.71 (12.00)^{de}	67.30 (6.55)^{b}	71.26 (8.22)^{cd}
	Acetic	24	43.02 (5.75)^{cdefg}	42.49 (6.04)^{bcd}	42.04 (0.94)^{abc}	63.07 (2.84)^{f}	31.61 (2.12) ^{ab}	58.52 (2.72)^{fg}
		48	54.13 (2.07)^{hi}	44.37 (2.05)^{bcd}	43.64 (3.40)^{bc}	51.95 (0.09)^{bcdef}	44.42 (13.69)^{bcd}	53.11 (0.12)^{efg}
		72	48.56 (10.26) ^{defgh}	42.32 (5.80)^{bcd}	52.51 (13.54)^{c}	59.21 (11.69) ^{def}	42.42 (14.22) ^{abcd}	54.94 (2.83)^{fg}
GOS-La	Lactic	24	65.66 (2.90) ^{b}	33.06 (11.05)^{a}	54.12 (1.89)^{e}	46.91 (1.55)^{abc}	76.81 (2.04)^{bc}	77.96 (3.09)^{cde}
		48	83.71 (9.03)^{b}	33.20 (10.09)^{a}	36.14 (12.14)^{bcd}	102.97 (22.16)^{e}	82.53 (0.36)^{c}	80.25 (0.28)^{de}
		72	76.73 (7.55)^{b}	32.87 (9.92)^{a}	33.52 (10.84)^{abcd}	91.42 (16.87)^{de}	85.21 (2.95)^{c}	84.91 (1.25)^{e}
	Acetic	24	45.22 (0.25)^{defgh}	40.21 (12.37)^{bcd}	46.47 (2.11)^{bc}	57.36 (1.97) ^{def}	61.59 (10.40)^{e}	64.06 (3.85)^{g}
		48	52.99 (2.45)^{ghi}	47.05 (1.43)^{d}	42.46 (1.66) ^{abc}	59.38 (9.52)^{ef}	52.35 (0.34)^{de}	52.84 (1.89)^{defg}
		72	51.69 (0.32)^{fgh}	47.69 (1.31)^{d}	46.59 (2.88)^{bc}	57.10 (10.38)^{def}	50.01 (1.82)^{cde}	53.46 (1.79)^{efg}
hCMP	Lactic	24	26.22 (4.07)^{a}	24.56 (2.82)^{a}	26.58 (3.15) ^{bc}	23.84 (1.91)^{a}	23.37 (0.20)^{a}	24.86 (0.79)^{ab}
		48	19.85 (0.14)^{a}	23.22 (1.73)^{a}	23.92 (0.07)^{a}	24.39 (0.42)^{a}	23.63 (1.50)^{a}	24.52 (0.48)^{ab}
		72	28.42 (2.44)^{a}	23.06 (4.42)^{a}	26.07 (4.50) ^{bc}	23.59 (0.68)^{a}	20.91 (2.59)^{a}	21.99 (2.63)^{ab}
	Acetic	24	30.74 (1.41)^{a}	41.02 (5.85) ^{bcd}	38.73 (4.00) ^{abc}	28.32 (3.49)	31.90 (5.57)^{ab}	31.72 (2.84)^{ab}
		48	32.76 (1.11)^{ab}	39.72 (2.71)^{bcd}	33.88 (1.21) ^{ab}	36.52 (2.67)^{abc}	30.67 (3.80) ^{ab}	30.93 (1.49)^{a}
		72	40.67 (1.18)^{abcde}	41.31 (8.04) ^{bcd}	35.58 (9.66) ^{ab}	37.49 (3.99) ^{abc}	29.12 (3.16)^{a}	45.19 (17.86)^{bcdef}
hCMP:GOS-Lu	Lactic	24	38.44 (2.73)^{a}	27.32 (6.82)^{a}	33.83 (2.42) ^{abcd}	30.26 (2.29)^{a}	28.97 (7.51)^{a}	31.95 (1.06)^{b}
		48	36.81 (6.41)^{a}	25.25 (5.15)^{a}	23.43 (0.84)^{a}	28.30 (2.65)^{a}	25.45 (0.11)^{a}	28.22 (0.42)^{b}
		72	41.85 (0.37)^{a}	32.91 (4.07)^{a}	32.60 (0.83)^{abc}	37.66 (10.01)^{ab}	28.32 (6.38)^{a}	31.25 (1.18)^{b}
	Acetic	24	39.45 (6.41)^{abcd}	40.49 (10.58) ^{bcd}	38.93 (6.49) ^{abc}	45.55 (3.28)^{bcdef}	42.10 (9.24) ^{abcd}	40.16 (7.22)^{abcde}
		48	50.58 (2.58)^{efgh}	24.98 (11.98)^{a}	27.83 (1.94)^{a}	42.00 (12.61)^{abcde}	35.31 (2.29)^{abc}	47.94 (8.83)^{cdef}
		72	62.01 (12.37)^{i}	45.84 (5.21)^{cd}	41.00 (6.17) ^{abc}	54.95 (8.94)^{cdef}	41.77 (10.27) ^{abcd}	52.21 (4.05)^{defg}
hCMP:GOS-La	Lactic	24	27.50 (7.74)^{a}	26.29 (9.45)^{a}	28.54 (3.23) ^{bc}	20.01 (0.12)^{a}	23.60 (1.05)^{a}	23.77 (1.33)^{a}
		48	26.35 (7.42)^{a}	23.82 (3.84)^{a}	25.10 (8.57)^{bc}	18.53 (1.97)^{a}	20.39 (9.22)^{a}	15.32 (4.19) ^{ab}
		72	30.52 (0.94)^{a}	24.96 (3.82)^{a}	26.24 (4.74) ^{bc}	19.74 (5.80)^{a}	20.21 (7.67)^{a}	20.21 (7.67) ^{ab}
	Acetic	24	31.90 (9.00)^{a}	36.63 (2.30) ^{abcd}	39.58 (7.27) ^{abc}	30.30 (2.79)	37.95 (2.19)^{abcd}	36.81 (6.06)^{abc}
		48	33.94 (6.36)^{abc}	37.97 (12.05) ^{abcd}	37.71 (10.61)^{abc}	43.60 (1.59) ^{abcde}	38.43 (6.24)^{abcd}	35.35 (7.24)^{abc}
		72	45.34 (3.42)^{defgh}	42.14 (7.54) ^{bcd}	39.00 (4.50) ^{abc}	43.59 (14.76) ^{abcde}	39.34 (10.63)^{abcd}	39.34 (10.63)^{abcd}

* Standard deviation in parentheses

Different letters indicate significant differences ($P \leq 0.05$) for each strain and acid

4.3.2.2.3. Discussion

Although there are several prebiotic compounds available in the market, in recent years the concept of functional food has moved towards food ingredients exerting a positive effect on the probiotics as well as beneficial to the human host. Generally, prebiotics are compounds that positively affect the metabolism of the probiotic bacteria present in the gut. In the same way, the bioactive peptides such as caseinomacropeptide also known as glycomacropeptides (GMP) act as regulatory compound in the human host (Hartmann, et al., 2007, Meisel, 1997). CMP has been reportedly associated with a variety of beneficial biological activities in human. It plays important role in the modulation of immune responses, promotion of bifidobacterial growth, binding of cholera toxin, and inhibition of bacterial and viral adhesions (Mikkelsen, et al., 2006, Phelan, et al., 2009, Requena, et al., 2009). Thus, these bioactive peptides represent potential health enhancing nutraceuticals for food and pharmaceutical applications.

In an effort to study the potential prebiotic effect of hCMP conjugated with galactooligosaccharides in probiotic bacteria, hCMP was conjugated with lactose- and lactulose-derived galactooligosaccharides via Maillard reaction and used in this study. It is well known concept that carbohydrate metabolism by lactic acid bacteria is species and even strain dependent (Poolman, 1993). In this study we have investigated the potential of hCMP:GOS to support the growth of probiotic bacteria similar to unconjugated prebiotic GOS. We used six *Lactobacillus* strains namely *L. bulgaricus* ATCC 7517, *L. casei* ATCC11578, *L. delbrueckii* subsp. *lactis* ATCC4797, *L. plantarum* ATCC8014, *L. plantarum* WCFS1, *L. sakei* 23K as a test probiotic strains. Results of the growth study demonstrated the ability of *Lactobacillus* strains to utilize the glycoconjugates as their sole carbon source to retain their viability up to120 h with a significant increase in the number of viable cells. The growth pattern and viability of all *Lactobacillus* strains grown in hCMP:GOS were comparable to the cells grown in unconjugated GOS-La, GOS-Lu. Unlike the glyconjugates or GOS, cells grown in unconjugated hCMP did not exhibit comparable growth pattern. Strains LB, LC, LD showed significant growth up to 72 h, but for strains LP1, LP2, and LS hCMP did not support the growth. As reported in earlier growth studies on probiotics we observed

variable growth pattern for each bacterial strain and each carbon source used in this study. In previous studies there has been considerable species and strain differences among probiotic strains in carbohydrate utilization abilities and kinetics have been reported (Poolman, 1993). In a recent study, Cardelle-Cobas et al. (2011) evaluated the growth of *Streptococcus salivarius, Bifidobacterium breve, Lactobacillus reuteri, L. fermentum, L. plantarum* strains CLB7 and CLC17, *L. salivarius, L. animalis,* and *L. murinus* in purified fractions of trisaccharides synthesized from lactose and lactulose. In their study they found probiotic strains were able to utilize the purified GOS, however, certain trend in the GOS utilization pattern was observed for each bacterial strain (Cardelle-Cobas, et al., 2011). Interestingly, in our study hCMP conjugated GOS supported the growth of LB, LP2 and LS for longer time period up to120 h of incubation and exhibited a significant increase in the number of viable CFUs. Generally, the prebiotic oligosaccharides must be hydrolyzed by the bacterial glycosidases prior to uptake of the resultant hydrolyzed monosaccharides. It is, therefore, reasonable to believe that while maintaining the viability LB, LP2 and LS cells retained a longer time to utilize the hCMP conjugated oligosaccharides. Since, many chronic gut disorders, however, such as colon cancer and ulcerative colitis originate in the distal colon and progress towards the proximal region, this prolonged utilization of glycoconjugates by the probiotic bacteria could be an advantageous property to improve the prebiotic effect more effectively throughout the colon. Consequently, significant health benefits of prebiotics could be achieved if the availability of prebiotic substrates and prolonged saccharolytic fermentation are extended to the distal region of the colon (Rastall, et al., 2005). One suggested method to achieve this extended prebiotic effect is to increase the molecular weight of the prebiotic oligosaccharides (Rastall, et al., 2005), which has been achieved in our study by conjugating the beneficial bioactive peptide to the prebiotic galactooligosaccharides. Similarly, in earlier studies the selective benefit of the sugar composition and molecular size of the prebiotic substrate to some type of *Bifidobacteria* and lactobacilli have been demonstrated (Rycroft, et al., 2001, Van Laere, et al., 2000).

After evaluating the growth supporting property of hCMP:GOS conjugates we tested the role of these glycoconjugates in the bile tolerance of *Lactobacillus* strains.

The ability to withstand in bile is one of the essential and preferred characteristics for probiotics to use in the food applications. Once bacteria reach the duodenal section of the intestinal tract they are exposed to deleterious bile compounds and subsequently their viability is reduced (Begley, et al., 2005, Begley, et al., 2006). Results of our bile resistance study showed the tolerance and survival of the *Lactobacillus* strains in the presence of bile extract was significantly changed based on the carbon source (hCMP:GOS or GOS) used for their growth. Interestingly, we observed enhanced bile tolerance in glycoconjugates grown LB, LP2, and LS cells than their GOS grown cells. The toxic effects of bile could be alleviated partly by adding specific prebiotic substrates that can be metabolized by probiotic strains. In an earlier study, altered bile tolerance in *Lactococcus lactis* subsp. c*remoris* and *L. lactis* subsp. *lactis* strains grown in different types of carbohydrates used as a carbon source in the growth medium was reported (Kimoto-Nira, et al., 2009, Kimoto-Nira, et al., 2010). Similarly, Perrin et al. observed certain level of increased bile salts resistance in bifidobacterial cells grown in fructooligosaccharides than glucose and fructose and this resistance pattern was reportedly depended on the carbon sources used for the bacterial growth (Perrin, et al., 2000). It has previously been reported that exclusion of bile from a lactococcal cells requires energy, and the ability to acquire energy from the growth substrate may relate to the bile resistance in lactococcal strains (Kimoto-Nira, et al., 2009, Yokota, et al., 2000). From our results, therefore, we speculate fermentation of hCMP:GOS conjugates might provide required energy comparable to unconjugated prebiotic carbon sources to *Lactobacillus* strains to withstand and survive in the bile environment. Lactobacilli are the genus most commonly used as probiotics in food production (Turpin, et al., 2010). After ingestion, these bacterial cells must be able to survive in the deleterious conditions of bile acids in the small intestine (Whitehead, et al., 2008). In our study after 48 h of growth in hCMP:GOS, *Lactobacillus* strains gained certain level of bile tolerance and showed significant percentage of viable cells even after 3 h of bile treatment. This interesting result suggests that hCMP:GOS conjugates could be potentially used as a carbon source to culture the probiotic *Lactobacillus* strains used to prepare probiotics supplemented food products. This approach could be used to protect the probiotic bacterial cells from the toxic effects of bile during the consumption.

Once the undigested prebiotic substrates reach the intestine they are fermented by the gut microbes. The anaerobic breakdown of carbohydrate and protein by bacteria is known conventionally as fermentation. The end-products of fermentation reactions are important because they are absorbed from the gut and influence various aspects of gut physiology beyond functioning solely as a crude caloric source (Cummings, et al., 1991, Macfarlane, et al., 2003, Neish, 2009). The products of carbohydrate fermentation by gut bacteria, primarily organic acids such as acetate and lactate are beneficial to host health. Acetate and lactate formation are reportedly consistent in the metabolic activities in the species of *Bifidobacterium* and *Lactobacillus.* Acetate is one of the most important short chain fatty acids (SCFAs) in the colon and readily absorbed and transported to the liver and subsequently used for lipogenesis (Hijova, et al., 2007). Previous reports show that the acetate and lactate produced by the *Bifidobacterium* sp. and *Lactobacillus* sp. from the fermentation of prebiotic carbohydrates are directly utilized by other intestinal bacterial species to produce different types of SCFAs (Belenguer, et al., 2006, Edwards, et al., 2002, Falony, et al., 2006). Research study of Bourriau et al. (2005) on the lactate metabolism by human colonic microflora clearly showed that the selective group of human fecal microflora rapidly and entirely utilize the lactate to synthesize the other types of beneficial SCFAs (Bourriaud, et al., 2005). In our study the fermentability of hCMP:GOS conjugates by the *Lactobacillus* strains was confirmed by detecting the levels of acetate and lactate concentrations in the culture supernatant. Results of HPLC analysis confirmed the presence of lactate and acetate as a fermentation product in the cell free culture supernatants obtained from the *Lactobacillus* cells grown in glycoconjugates. All the strains were able to metabolize the glyconjugates hCMP:GOS-La and hCMP:GOS-Lu similar to prebiotic GOS-La, GOS-Lu. However, significant variations in the quantity of acetate and lactate were observed in each *Lactobacillus* strains and the carbon sources used for their growth. The amount and proportion of metabolites including organic acids and SCFA produced during fermentation are determined by many factors including the availability and type of growth substrate (Marx, et al., 2000). Similar to our present study the strain and growth substrate dependent variations in the organic acids production was reported in

earlier studies (Cardelle-Cobas, et al., 2009, Marx, et al., 2000, Sanz, et al., 2005, Wichienchot, et al., 2006). The increased acetate to lactate ratio was reported in bifidobacterial strains grown in β-(2,6)-fructose-oligosaccharides compared to fructose grown culture (Marx, et al., 2000). Similarly, Sanz et al.(2005) observed the high level lactate and acetate production in lactulose and sucrose containing culture media fermented by the fecal slurry (Sanz, et al., 2005). Recently, Cardelle-Cobas et al. (2009) observed increased acetic acid concentration when the fecal slurry culture was grown in lactose- and lactulose-derived oligosaccharides containing media.

4.3.2.2.5. Conclusions

The interest of the novel prebiotics use in food and nutraceutical compounds has strongly increased in the last few years and has stimulated the research efforts related to the discovery and development of new multipurpose prebiotics (Mussatto, et al., 2007). In this present study we showed the ability of glycoconjugates of hydrolyzed caseinomacropeptides conjugated prebiotic galactooligosaccharides as a carbon source to *Lactobacillus* strains. Results of our study showed the potential of these glycoconjugates to support the growth and enhance the bile tolerance of probiotic *Lactobacillus* strains in vitro. In addition, HPLC detection of lactate and acetate in the cell free culture supernatant obtained from the glycoconjugates grown *Lactobacillus* culture media demonstrate the fermentability of these glycoconjugates to produce beneficial metabolites. In this same aspect further follow up in vivo studies would be carried out to improve these peptide conjugated prebiotics to serve as a multipurpose beneficial functional food component.

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4.3.2.3. *In vitro* fermentation by human gut bacteria of proteolytically digested caseinomacropeptide non-enzymatically glycosylated with prebiotic carbohydrates

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Abstract

The *in vitro* fermentation selectivity of hydrolyzed caseinomacropeptide (CMP) glycosylated, via Maillard reaction (MR), with lactulose, galactooligosaccharides from lactose (GOSLa) and galactooligosaccharides from lactulose (GOSLu) was evaluated, using pH-controlled small scale batch cultures at 37 ^{o}C under anaerobic conditions with human faeces. After 10 and 24 hour of fermentation, neoglyconjugates exerted a bifidogenic activity, similar to those of the corresponding prebiotic carbohydrates. No significant differences were found in *Bacteroides*, *Lactobacillus-Enterococcus*, *Clostridium histolyticum* subgroup, *Atopobium* and *Clostridium coccoides* – *Eubacterium rectale* populations. Concentrations of lactic acid and SCFA produced during the fermentation prebiotic carbohydrates were similar to those produced for their respective neoglycoconjugates at both fermentation times. These findings, joined with the functional properties attributed to CMP, could open up new applications of MR products involving prebiotics as novel multiple functional ingredients with potential beneficial effects on human health.

4.3.2.3.1. Introduction

Caseinomacropeptide (CMP) is the 64 C-terminal amino acids of k-casein released by chymosin or pepsin cleavage during the manufacture of cheese or digestion in the stomach. CMP comprises a heterogeneous group of polypeptides which differ, mainly, in the degree of glycosylation and phosphorylation (Delfour, et al., 1965). Many biological properties have been attributed to CMP either in its intact form or after

enzymatic hydrolysis (Manso, et al., 2004, Recio, et al., 2009). Several works have reported the capacity of bovine CMP to interact with pathogens, viruses, bacteria and toxins, depending on the content and structure of the glycans present in the amino acidic sequence (Dziuba, et al., 1996, Stromqvist, et al., 1995). Initially, the *in vitro* bifidogenic effect of intact CMP was reported by using pure cultures (Azuma, et al., 1984, Idota, et al., 1994, Janer, et al., 2004). However, this effect was not later supported by Brück et al. (Brück, 2002) using breast milk supplemented with CMP and fermented with infant faecal slurries, although a significant decrease in *C. histolyticum*, *Bacteroides* and *E. coli* was reported. Similarly, the prebiotic effect of intact CMP-supplemented infant formulae could not be demonstrated after ingestion by healthy term infants initially breast-fed (Bruck, et al., 2006).

Currently, the Maillard reaction is used to improve functionality of food proteins without requiring the addition of chemical reagents (Oliver, et al., 2006). Different technological applications based on emulsifying, textural, and solubility properties of proteins have been considered, although the effect on biological properties such as the selective fermentation of gut microflora, is also gaining a great importance. However, there is currently very little and controversial information on this topic. Ames et al. (Ames, et al., 1999) reported the non-specific increase of anaerobic bacteria after fermentation of melanoidins produced from an aqueous glucose-lysine model system; whereas Borrelli & Fogliano (Borrelli, et al., 2005) observed that bread crust melanoidins stimulated the growth of bifidobacteria. Later on, an increase of some detrimental species of bacteria (sulphate-reducing bacteria and clostridia) and a decrease of bifidobacteria and lactobacilli was found after using bovine serum albumin (BSA) glycated with glucose as substrate of fermentation with human fecal bacteria compared to native BSA (Tuohy, et al., 2006). Maillard reaction (MR) products derived from roasted cocoa bean reduced the growth of pathogenic bacteria *(E. coli* spp. and *Enterobacter cloacae*) and bifidobacteria (Summa, et al., 2008).

Nevertheless, to the best of our knowledge, there is no information on the fermentation selectivity of proteins or peptides conjugated with prebiotic carbohydrates. Huebner et al. (Huebner, et al., 2008) studied the effect of MR conditions on the prebiotic activity of different commercial fructooligosaccharides

conjugated with glycine using pure culture assays. However, these prebiotics were not previously fractionated and the formation of the MR products could be attributed to the presence of minor reducing sugars such as glucose and fructose.

It should be taken into consideration that *in vitro* and *in vivo* studies have shown that non-digested Amadori compounds, first stable products of the MR, could reach the colon where they can be fermented by microorganisms (Finot, 2005, Gibson, et al., 2004, Hernández, et al., 2009). In consequence, the conjugation between food proteins or peptides and prebiotic carbohydrates could potentially allow carbohydrates to reach the distal parts of the colon, where many chronic gut disorders originate (Clemente, et al., 2011). Furthermore, increasing attention is being focused on the production of ingredients with multiple functional properties. Therefore, the conjugation of prebiotic carbohydrates with proteins or peptides possessing widely recognized functional properties can be of great interest. In this work, we have investigated the effect of a fraction rich in Amadori compounds derived from the glycation under controlled conditions of previously proteolytically digested CMP with three different carbohydrates (lactulose, GOS from lactose and novel GOS from lactulose) on the human intestinal microbiota as represented by faeces, using small scale *in vitro* batch culture.

4.3.2.3.2. Materials and Methods

- *Chemicals*

Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (Dorset, UK) and the bacteriological growth media supplements were obtained from Oxoid (Basingstoke, UK). Bovine caseinomacropeptide (CMP) was kindly provided by Davisco Foods International, Inc (Le Sueur, MN). Galactooligosaccharides from lactose (GOS-La) were obtained from Vivinal-GOS®, kindly provided by Friesland Foods Domo (Zwolle, The Netherlands). This product had a 73 wt% dry matter, the composition of which was 60 wt% GOS, 20 wt% lactose, 19 wt% glucose and 1 wt% galactose, as stated by the supplier. Duphalac® (Solvay Pharma, Brussels, Belgium) was

used to obtain the galactooligosaccharides from lactulose (GOS-Lu). Duphalac® has 67% lactulose (w:v), < 11% galactose (w:v), < 6% lactose (w:v), < 4.7% epilactose (w:v), < 2% tagatose (w:v), as stated by the supplier.

- *Preparation of galactooligosaccharides*

In order to remove digestible and non-prebiotic mono- and disaccharides and obtain oligosaccharides (GOSLa), the commercial product Vivinal-GOS® was fractionated using size exclusion chromatography, following the method previously reported (**Section 4.1.2**) with some modifications. In brief, 80 mL of Vivinal-GOS® (25 % w/v) were injected in a preparative Bio-Gel P2 (Bio-Rad, Hercules, CA) column (90 x 5 cm) using water as mobile phase, at 1.5 mL min^{-1}. Sixty fractions of 10 mL were collected, after the elution of void volume. The degree of polymerization (DP) of fractions was determined by electrospray ionization mass spectrometry (ESI-MS) at positive mode. Fractions with DP ≥ 3 were pooled and freeze dried.

GOS from lactulose (GOSLu) were obtained following the method previously described (**Section 4.1.4**). A solution (450 g/L) of Duphalac® was dissolved in 50 mM sodium phosphate buffer and 1 mM $MgCl_2$, pH 4.5, after addition of 8 U/mL of β-galactosidase from *Aspergillus oryzae* (Sigma-Aldrich), and incubation at 60 ºC for 8 h under continuous agitation at 300 rpm. Following incubation, the mixtures were immediately immersed in boiling water for 5 min to inactivate the enzyme. After enzyme inactivation, the mixture was purified following the method proposed by Morales et al. (Morales, et al., 2006) with some modifications, by dilution (1/50) with water and treatment with 48 g of activated charcoal per 40 g of initial carbohydrates to remove monosaccharides. Oligosaccharides (GOSLu) were recovered using ethanol:water 50:50 (v:v), the ethanol was evaporated using a rotavapor and the remaining solution was freeze dried.. The DP of the sample was determined by ESI-MS at positive mode, containing carbohydrates with DP ≥ 2.

- *Preparation of glycoconjugates*

A previous step of hydrolysis of CMP was necessary in order to increase the number of free primary amino groups available for glycation and, consequently, to yield potentially and highly functional food-grade peptide-carbohydrate conjugates. Thus, CMP was hydrolyzed following the method previously described (**Section 4.2.2**), using a combination of trypsin/chymotrypsin (overnight at 37 ºC and pH 7) at 1 : 0.05 : 0.025, CMP : trypsin : chymotrypsin ratios (w : w : w). Activities of porcine pancreas trypsin (EC 3.4.21.4, type IX-S) and bovine pancreas α-chymotrypsin (EC 3.4.21.1, Type I-S) were 13000 - 20000 U and ≥40 U per mg of protein, respectively. The hydrolyzed CMP was freeze dried. Enzymes were inactivated by heating at 95 ºC for 5 min.

Aliquots of a solution consisting of 60 mg mL^{-1} of hydrolyzed CMP and 60 mg mL^{-1} of the following and individual prebiotic carbohydrates: i) lactulose, ii) GOSLa and iii) GOSLu dissolved in 0.1 M sodium phosphate buffer pH 7.0, were lyophilized. These were kept under vacuum in a desiccator at 40 °C and a water activity of 0.44, achieved with a saturated K_2CO_3 solution (Merck, Darmstadt, Germany), for 9, 16 and 18 days. In addition, control experiments were performed with hydrolyzed CMP stored at 40 °C without carbohydrates during the same periods (heated CMP). Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate. 2-Furoylmethyl-amino acids (2-FM-AA) were determined in order to study the formation of Amadori compounds during Maillard reaction according to Moreno et al. (Moreno, et al., 2002). A previous hydrolysis of the samples using 8 N HCl at 110°C for 23 h under inert conditions (helium) was carried out. Samples were filtered through Whatman no. 40 filter paper and purified through a Sep-Pak C18 cartridge previously activated with 5 mL of methanol and 10 mL of deionized water (Waters, Milford, MA); 2-FM-AA was eluted with 3 mL of 3 N HCl. Analyses were carried out by an ion-pair RP-HPLC method using a C8 (Alltech furosine-dedicated; Alltech, Nicolasville, KY) column (250 x 4.6 mm i.d.) and a variable wavelength detector at 280 nm (LDC Analytical, SM 4000, Salem, NH). Operating conditions were as indicated by Resmini et al. (Resmini, et al., 1990). Quantitative analyses were performed by using known concentrations (from 0.52 to 5.2 mg/L) of a commercial pure standard of furosine (2-furoylmethyl-lysine; Neosystem Laboratories, Strasbourg, France).

After storage, the unconjugated carbohydrates were quantified by gas chromatography (GC-FID) using a previous two-step derivatization procedure: oximation and trimethylsilylation (Sanz, et al., 2004). The samples were mixed with phenyl-β-glucoside as internal standard and evaporated under vacuum. The oximes were formed using 350 µL of hydroxylamine hydrochloride in pyridine (2.5 % w/v) and then silylated using 350 µL of hexamethyldisilazane and 35 µL of trifluoroacetic acid. GC-FID analyses were carried out using an HP-7890A chromatograph (Hewlett-Packard, Palo Alto, CA) and nitrogen as a carrier gas. Carbohydrates were separated using an HT5 column (25 m x 0.22 µm x 0.1 µm) coated with 5% phenyl polysiloxane-carborane (SGE Europe, Milton Keynes, UK). Oven temperature was programmed as indicated by Hernandez et al. (Hernández, et al., 2009).

The unconjugated carbohydrate fraction was removed by ultrafiltration at 4ºC using a diafiltration unit Model 8400 (Millipore) with a molecular cut off of 1 kDa and then, the retentate was analyzed by ESI-MS, in order to confirm the absence of unconjugated carbohydrates.

The quantity of conjugated prebiotic was determined considering the differences between the amounts used at the initial step of the glycation process and the unconjugated carbohydrate fraction determined by GC-FID. Thus, equivalent amounts of the corresponding free prebiotics were used for the *in vitro* fermentation assays, in order to accurately compare with the neoglycoconjugates.

- *Faecal samples and in vitro fermentations.*

Faecal samples were obtained from three healthy donors (one male and two female; 25 to 30 years old) without any known metabolic or gastrointestinal disorders. Samples were collected and kept in an anaerobic cabin for a maximum of 15 min. The samples were diluted (1/10 w/w) with phosphate buffer (0.1 M, pH 7.4) and homogenised in a stomacher for 2 min at normal speed.

Gently stirred pH-controlled small scale fermenters (5 mL working volume) were filled with basal nutrient medium (peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4.7H_2O$ 0.01 g/L, $CaCl_2.6H_2O$ 0.01 g/L,

$NaHCO_3$ 2 g/L, Tween 80 2 mL, hemin 0.02 g/L, vitamin K_1 10µL, cysteine HCl 0.5 g/L, bile salts 0.5 g/L, pH 7.0) and gassed overnight with nitrogen. After addition of faecal solution (final concentration 1% v/v), samples (neoglycoconjugates or free prebiotics), previously sterilized by filtration through 0.22 um filters, were added to a final concentration of 1% w/v. The amount of CMP : prebiotic neoglycoconjugates was determined considering the initial amount of hydrolyzed CMP and the conjugated prebiotic fraction calculated as described above. Additionally, a control vessel without substrate was included (negative control).

The temperature was kept at 37 ºC at the pH between 6.7 and 6.9 and gassed with nitrogen during all the fermentation process. Fermentations were run over a period of 24 h and 1 mL of samples was taken at 0, 10 and 24 h for fluorescent *in situ* hybridization (FISH) and HPLC (SCFA) analyses.

- *Bacterial Enumeration by Fluorescent in Situ Hybridization (FISH).*

The FISH technique was used to quantify some bacterial groups and, in consequence, to monitor changes in bacterial populations. The samples obtained from the fermentations were fixed (1/4) in 4% w/v paraformaldehyde for 6 hours at 4 ºC. Samples were then centrifuged at 1,500 *g* for 10 min and washed twice with phosphate-buffered saline (PBS 0.1M, pH 7.0). The obtained pellet was resuspended in cold PBS solution and ethanol (99 %) (1:1 v:v) and stored for at least 1 hour at -20 ºC.

The samples were diluted with PBS, in order to obtain 10-100 cells per field of view to count after hybridization. The hybridization was carried out following the method proposed by Martin-Pelaez et al. (Martin-Pelaez, et al., 2008) using 16S rRNA-targeted oligonucleotide probes labelled with Cy3. Probes, commercially available (Sigma-Aldrich, Dorset, UK), were as follows: Bif164, specific for *Bifidobacterium* (Langendijk, et al., 1995), Bac303, specific for *Bacteroides* (Manz, et al., 1996), Chis150, for the *Clostridium histolyticum* group clusters I, II (Franks, et al., 1998), Erec482 for the *Clostridium coccoides – Eubacterium rectale* group (Franks, et al., 1998), Lab158 for *Lactobacillus-Enterococcus* group (Harmsen, et al., 1999) and Ato291 for the

Atopobium cluster (Harmsen, et al., 1999). For total counts the nucleic acid stain 4,6-diamino-2-phenylindole (DAPI) was used. Samples were examined under a fluorescent microscope (Nikon Eclipse, E400) and DAPI-stained cells were examined under ultraviolet light. A minimum 15 random fields of view were counted for each sample.

- *SCFA and Lactic Acid analyses.*

Samples from the fermenters were centrifuged at 13,000 *g* for 10 min to remove all particles and the produced lactic, acetic, propionic and butyric acid were quantified using a BioRad HPX-87H HPLC column (Watford, UK) at 50 ºC, with a 0.005 mM H_2SO_4 as mobile phase, in isocratic mode, at a flow rate of 0.6 mL/min (Sanz, et al., 2005).

- *Statistical analyses.*

Statistical analyses was performed using the Statistica for Windows version 6 (2002) by Statsoft Inc. (Tulsa, OK). Differences between bacterial counts and SCFA concentration were tested using one-way ANOVA test, followed by Scheffe test as a post hoc comparison of means ($P<0.05$).

4.3.2.3.3. Results and Discussion

- *Determination of the extent of glycation between prebiotic carbohydrates and CMP.*

Maillard reaction between lactulose, GOSLa and GOSLu and hydrolysed CMP was controlled studying the formation of 2-FM-AA, which is formed upon acid hydrolysis of the peptide-bound Amadori products. The formation of 2-FM-AA was measurement as mg of furosine (2-furoylmethyl-lysine) per 100 mg of neoglycoconjugate. Maximum yields were obtained after 16 days of incubation for CMP:GOSLu (1.57 mg of furosine per 100 mg of neoglycoconjugate) and for CMP:GOSLa (1.90 mg of furosine per 100

mg of neoglycoconjugate) (**Figure 4.30**). It has been described that, in general terms, aldoses react more rapidly than ketoses in order to form the corresponding Schiff base prior to its rearrangement to the more stable Amadori or Heyns compound, because the aldehyde carbonyl groups are relatively more electrophilic than ketone carbonyl groups (Bunn, et al., 1981). In consequence, the higher yield obtained for CMP:GOSLa can be due to the presence of a aldose (mostly glucose) at the reducing end of GOSLa (Otieno, 2010), in comparison with GOSLu that present mainly a ketose (fructose) at the reducing end (Martinez-Villaluenga, et al., 2008). Finally, after 16 days of incubation the levels of furosine decreased in CMP:GOSLa glycoconjugates, which was indicative of the predominant degradation of Amadori compounds to dicarbonyl intermediates that may serve as precursors of the advanced stages of the Maillard reaction, whereas it remained constant in CMP:Lu and CMP:GOSLu (**Figure 4.30**).

Furthermore, the yield after 16 days of incubation for CMP:Lactulose was much higher (3.87 mg of furosine per 100 mg of glycoconjugate) than those obtained for the neoglycoconjugates based on GOSLa or GosLu (**Figure 4.30**). This behaviour is explained by the well-established order of reactivity according to which monosaccharides are more reactive than disaccharides and these more reactive than oligosaccharides due to the smaller the carbonic chain of the sugar is, the more acyclic forms exist and the more reactive is the sugar with the amino groups of proteins (Chevalier, et al., 2001, Corzo-Martínez, et al., 2010).

Likewise, GC-MS analyses of initial GOS concentration and unconjugated carbohydrates after storage revealed that the relation CMP:GOSLu, CMP:GOSLa and CMP:Lactulose was approximately 2:1 (w:w).

Table 4.21 shows changes in bacterial population after fermentation of the neoglyconconjugates, free carbohydrates, hydrolyzed CMP and corresponding controls with fecal samples for 10 and 24 h. The bacterial composition of the inoculum is likely to be affected by several factors such as genetics, age, sex, health status, nutrition and diet of the volunteers (Benson, et al., 2010). Quantitative results obtained for the three donors had a low standard deviation, which is indicative of the data consistency presented in this work. In general, no significant differences were found in total cells,

Bac303, Lab158, Chis150, Ato291 and Erec482 for all samples under study. Similar behavior has been reported for lactulose and GOS from lactose by Rycroft et al. (Rycroft, et al., 2001) after 24 hours of fermentation.

- *Changes in bacterial populations during in vitro batch culture fermentation*

Bif164 values detected for hydrolyzed CMP after both 10 and 24 h of incubation did not show significant differences with the control (**Table 4.21**). The bifidogenic activity of intact CMP had been previously reported by using pure cultures (Azuma, et al., 1984, Idota, et al., 1994, Janer, et al., 2004, Metwally, et al., 2001), although this activity could not be further confirmed following studies with mixed cultures from infant faeces (Brück, 2002, Bruck, et al., 2006). Our results support the non-bifidogenic effect described for the intact CMP, stressing the importance of carrying out mixed culture studies for the evaluation of prebiotic properties. In addition, to the best of our knowledge, this is the first evidence showing the effect on human gut bacteria following *in vitro* fermentation of previously proteolitically digested CMP.

On the contrary, a significant increase in the Bif164 population at both incubation times was detected for the free carbohydrates (lactulose, GOSLu and GOSLa) and their corresponding neoglycoconjugates (CMP:Lactulose, CMP:GOSLa and CMP:GOSLu) (**Table 4.21**). This result confirmed the bifidogenic effect of these carbohydrates as previously reported (Bouhnik, et al., 2004, Cardelle-Cobas, et al., 2009, Rycroft, et al., 2001, Sanz, et al., 2005). Furthermore, it is worth noting that the three neoglyconjugates showed similar bifidogenic effect to those obtained with the corresponding free prebiotics, indicating that the glycation of CMP with prebiotics did not affect to their bifidogenic activity. A possible explanation for this behavior may be that for the formation of the corresponding Amadori or Heyns compounds, the prebiotic moieties do not change drastically since only the carbonyl group react with the free primary amino group from peptides, through a condensation of both groups forming a glycosylamine that undergoes Amadori or Heyns rearrangement. Accordingly, Huebner et al (Huebner, et al., 2008) found that the prebiotic score of fructooligosaccharides did not decrease after their conjugation with glycine.

- *Changes in lactic acid and SCFA profile*

Table 4.22 shows the values obtained for lactic acid and SCFA produced during the incubation with the different carbohydrates and neoglycoconjugates tested. Acetic acid was the most abundant SCFA in all studied samples and, with the exception of CMP:Lactulose conjugate, a notable increase was detected after 10 hours of fermentation. In addition, the formation of this acid was more highly promoted by the prebiotic carbohydrates than by the neoglycoconjugates. Lactic acid was only detected at 10 hours of fermentation of carbohydrates and neoglycoconjugates. A possible cause for the decrease in this acid during fermentation process could be the fact that it can be converted into other SCFA by different bacteria genus (Bourriaud, et al., 2005, Hughes, et al., 2008). The concentrations of lactic acid did not show significant differences for GOSLu with its respective neoglycoconjugate. On the contrary, in the treatment with GOSLa, the concentration of lactic acid was higher than that of CMP:GOSLa, whereas for lactulose the concentration of this acid was significantly lower than that of CMP:Lactulose. Both lactic and acetic acids are considered end products of the bifidobacteria pathway, supporting the increase in bifidobacteria population described in **Table 4.21**. However, it cannot establish a linear relationship between production of SCFA and bacterial genus, considering that several bacteria are involved in the production of these acids.

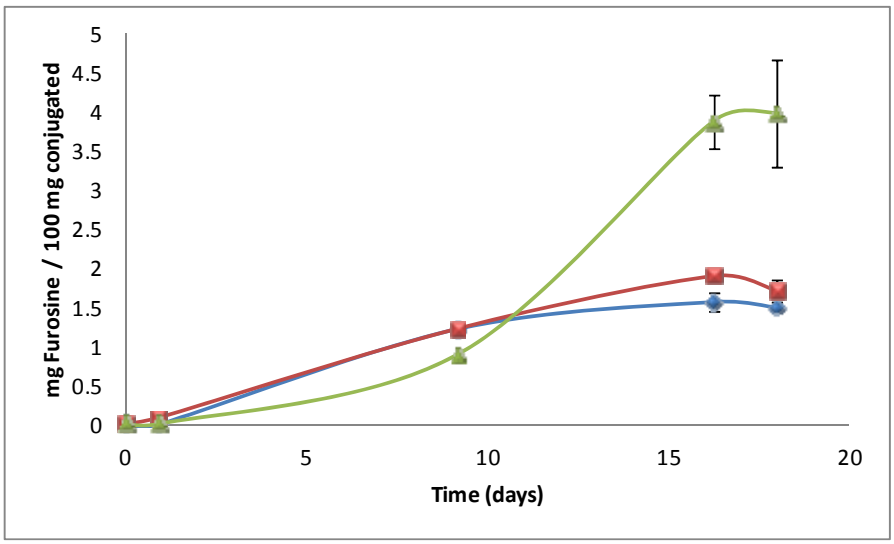

Figure 4.30. Furosine content at different storage time during the glycation of hydrolysed CMP with lactulose (▲) GOSLu (♦) and GOSLa (■).

Table 4.21. Bacterial populations (log 10 cells mL-1 batch culture) in pH controlled cultures at 0, 10 and 24 hours of fermentation using glycated hydrolyzed CMP with lactulose (CMP:Lactulose), galactooligosaccharides from lactulose (CMP:GOSLu) and galactoligosaccharides from lactose (CMP:GOSLa) and their corresponding free carbohydrates and control (no substrate).

Sample	Time (h)	Total cells	Bif164	Bac303	Lab158	Chis150	Ato291	Erec482
Control	**0**	**9.02 (0.02)[§ a*]**	**7.83 (0.01)[a]**	**8.00 (0.07)[a]**	**7.01 (0.11)[a]**	**6.75 (0.05)[a]**	**7.97 (0.48)[a]**	**7.24 (0.06)[a]**
	10	9.50 (0.02)[ab]	8.06 (0.01)[ab]	8.09 (0.02)[a]	6.91 (0.04)[a]	7.32 (0.01)[abc]	7.85 (0.0)1[a]	8.02 (0.20)[a]
	24	9.78 (0.05)[b]	7.92 (0.07)[a]	8.41 (0.07)[a]	6.95 (0.16)[a]	6.97 (0.03)[abc]	7.32 (0.13)[a]	7.77 (0.22)[a]
Lactulose	10	9.33 (0.12)[ab]	9.21 (0.08)[d]	8.48 (0.10)[a]	7.06 (0.14)[a]	7.23 (0.16)[abc]	7.65 (0.06)[a]	7.55 (0.06)[a]
	24	9.73 (0.08)[b]	8.85 (0.12)[cd]	8.52 (0.06)[a]	7.22 (0.03)[a]	7.73 (0.06)[bc]	7.53 (0.03)[a]	7.95 (0.03)[a]
GOSLu	10	9.33 (0.06)[ab]	9.18 (0.05)[d]	8.48 (0.07)[a]	7.09 (0.09)[a]	7.20 (0.07)[abc]	7.67 (0.06)[a]	7.54 (0.13)[a]
	24	9.73 (0.09)[b]	8.95 (0.08)[cd]	8.39 (0.07)[a]	7.38 (0.29)[a]	7.71 (0.04)[abc]	7.52 (0.04)[a]	7.97 (0.12)[a]
GOSLa	10	9.42 (0.02)[ab]	9.29 (0.07)[d]	8.41 (0.08)[a]	7.12 (0.20)[a]	7.30 (0.22)[abc]	7.45 (0.01)[a]	7.51 (0.08)[a]
	24	9.83 (0.01)[b]	9.07 (0.05)[cd]	8.76 (0.05)[a]	7.50 (0.04)[a]	7.69 (0.06)[abc]	7.29 (0.14)[a]	7.92 (0.04)[a]
Hydrolyzed CMP	10	9.42 (0.04)[ab]	8.41 (0.19)[bc]	8.29 (0.08)[a]	6.88 (0.21)[a]	7.37 (0.13)[abc]	7.67 (0.03)[a]	7.99 (0.14)[a]
	24	9.78 (0.09)[b]	8.13 (0.11)[abc]	8.51 (0.08)[a]	6.86 (0.10)[a]	7.26 (0.16)[abc]	7.29 (0.15)[a]	7.69 (0.16)[a]
CMP:Lactulose	10	9.07 (0.53)[ab]	8.71 (0.74)[cd]	8.14 (0.73)[a]	6.89 (0.41)[a]	6.82 (0.61)[ab]	7.51 (0.64)[a]	7.17 (0.51)[a]
	24	9.44 (0.50)[ab]	8.52 (0.69)[cd]	7.99 (0.60)[a]	7.11 (0.48)[a]	7.38 (0.66)[abc]	7.33 (0.62)[a]	7.59 (0.54)[a]
CMP:GOSLu	10	9.28 (0.05)[ab]	9.12 (0.03)[cd]	8.54 (0.01)[a]	7.20 (0.13)[a]	7.11 (0.03)[abc]	7.71 (0.15)[a]	7.59 (0.01)[a]
	24	9.73 (0.06)[b]	8.81 (0.03)[cd]	8.83 (0.03)[a]	7.37 (0.20)[a]	7.64 (0.09)[abc]	7.51 (0.11)[a]	8.02 (0.02)[a]
CMP: GOSLa	10	9.38 (0.05)[ab]	9.21 (0.01)[d]	8.56 (0.13)[a]	7.09 (0.08)[a]	7.23 (0.17)[abc]	7.56 (0.20)[a]	7.56 (0.15)[a]
	24	9.76 (0.08)[b]	8.85 (0.11)[cd]	8.72 (0.04)[a]	7.22 (0.01)[a]	7.97 (0.05)[c]	7.48 (0.11)[a]	7.95 (0.02)[a]

[*]Different letters indicate significant differences ($P \leq 0.05$) for each bacterial group. [§] Standard deviation (n=3).

An increase of propionic acid was found at 24 hours of fermentation for all samples tested compare with 10 h of fermentation. No significant differences between prebiotic carbohydrates and the corresponding neoglyconjugates were found at both fermentation times, with the exception of GOS-La at 24 h. Finally, no significant formation of butyric acid was observed (**Table 4.22**).

In recent years, there has been a demand for production of a "second generation" of novel prebiotic ingredients that have a series of properties that include: i) displaying greater persistence in the large intestine ; ii) having great selectivity with regard to control of the intestinal microbiota (for example, in order to be metabolized by specific bifidobacteria); and iii) having an additional biological activity, exerting beneficial effects on specific physiological functions and/or reducing the risk of disease, for example, through their effect on displacement of pathogens and regulation of the function of the immune system (Reid, et al., 2003). In this context, CMP : prebiotic neoglycoconjugates might represent potential candidates for second generation prebiotics as this study has revealed that glycosylation via Maillard reaction of all assayed prebiotic carbohydrates with hydrolyzed CMP under controlled conditions does not reduce the bifidogenic properties of carbohydrates, and in the case of the neoglyconjugates with GOSLu and lactulose the lactic acid and SCFA production was also comparable to that of free carbohydrates.

Consequently, these findings might represent a new alternative to produce novel multifunctional food ingredient by keeping the prebiotic properties of the carbohydrate portion and adding the biological properties of the peptide fraction. Nevertheless, further gut persistence and *in vivo* studies should be conducted in order to explore the potential health benefits of these neoglycoconjugates on animals and human volunteers, as well as to evaluate the impact of glycation on the protein/peptide structure and, subsequently, on its biological properties.

Table 4.22. Lactic acid and SCFA in pH controlled cultures at 0, 10 and 24 hours of fermentation using glycated hydrolyzed CMP with lactulose (CMP:Lactulose), galactooligosaccharides from lactulose (CMP:GOSLu) and galactoligosaccharides from lactose (CMP:GOSLa) and their corresponding free carbohydrates and control (no substrate).

Sample	Time (h)	mM			
		Lactic acid	Acetic acid	Propionic acid	Butyric acid
Control	**0**	**0.00^{a}**	**0.00^{a}**	**0.00^{a}**	**0.00^{a}**
	10	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
	24	0.00^{a}	0.00^{a}	0.00^{a}	0.00^{a}
Lactulose	10	6.82 (4.58)^{b}	27.07 (0.78)^{bc}	3.11 (0.76)^{ab}	0.00^{a}
	24	0.00^{a}	32.00 (9.22)^{cd}	10.09 (0.37)^{de}	0.00^{a}
GOSLu	10	4.06 (2.63)^{b}	22.48 (1.61)^{bc}	1.68 (0.75)^{a}	0.00^{a}
	24	0.00^{a}	43.35 (3.69)^{d}	9.78 (3.12)^{cde}	0.00^{a}
GOSLa	10	13.69 (1.51)^{d}	31.83 (4.57)^{cd}	0.00^{a}	0.00^{a}
	24	0.00^{a}	62.85 (13.21)^{e}	24.23 (4.21)^{g}	0.00^{a}
Hydrolyzed CMP	10	0.00^{a}	19.5 (5.26)^{bc}	0.00^{a}	0.00^{a}
	24	0.00^{a}	23.47 (6.53)^{bc}	12.75 (1.40)^{e}	0.00^{a}
CMP:Lactulose	10	10.48 (6.01)^{c}	5.72 (0.86)^{a}	3.70 (0.61)^{ab}	0.00^{a}
	24	0.00^{a}	24.45 (14.98)^{bc}	12.54 (1.38)^{e}	0.47 (0.01)^{a}
CMP:GOSLu	10	5.95 (0.19)^{b}	18.89 (1.19)^{b}	0.00^{a}	0.00^{a}
	24	0.00^{a}	34.88 (3.40)^{cd}	6.51 (2.18)^{bc}	0.00^{a}
CMP: GOSLa	10	4.33 (3.92)^{b}	19.99 (1.56)^{bc}	3.10 (0.35)^{ab}	0.00^{a}
	24	0.00^{a}	29.00 (10.13)^{bc}	19.72 (6.48)^{f}	1.32 (0.37)^{a}

*Different letters indicate significant differences ($P \leq 0.05$) for each acid.

§ Standard deviation (n=3).

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4.3.2.4. Neoglycoconjugates of caseinomacropeptide and galactooligosaccharides modify adhesion and inflammatory response(s) of intestinal (Caco-2) cells to pathogens

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Abstract

Dietary glycoconjugates, may constitute new food ingredients with the potential ability to inhibit pathogen infection by blocking or competing for bacterial adhesion sites in intestinal cells. In this study, galactooligosaccharides, obtained from transgalactosylation of lactose (GOS-La) or lactulose (GOS-Lu), were used for the glycation of caseinomacropeptide hydrolysates (hCMP). The effects of these compounds on *Salmonella enterica* CECT 443 and *Listeria monocytogenes* CECT 935 adhesion, induction of inflammatory cytokines production, and "toll-like" (TLR)-2/4 and chemokine CXCR3 receptor expression (mRNA) in intestinal epithelial (Caco-2) cells were evaluated. hCMP:GOS-La or hCMP:GOS-Lu significantly reduced pathogens adhesion. GOS-Lu and hCMP:GOS-Lu inhibited the production of IL-1β by intestinal cells stimulated by the pathogens tested, but this effect was not exerted by GOS-La and hCMP:GOS-La. However, this positive effect was more discrete on the *L. monocytogenes* CECT 935-mediated production of TNFα without affecting the *S. enterica* CECT 443-mediated effect. hCMP:GOS-Lu increased TLR4 and CXCR3 expression levels of cells exposed to *S. enterica* CECT 443 suspensions, but down-regulated the expression of TLR2/4 and CXCR3 suggesting the complete blockage of *L. monocytogenes* CECT 935 interaction with intestinal cells.

4.3.2.4.1. Introduction

Food-borne pathogens are important causes of diverse type of infections in humans (von Sonnenburg, et al., 2000). *Salmonella enteriditis* group are common causes of acute gastroenteritis associated with consumption of raw products in USA

(up to 58.4 cases per million) (Barton Behravesh, et al., 2011), and in Europe (34.6-38.2 cases per 10^5 habitants) (Pires, et al., 2010). In addition, 0.2-0.8 cases of *Listeria monocytogenes* infection per 10^5 habitants have been reported in Europe (Noriega, 2008) associated, firstly, with the consumption of non-pasteurized dairy products and, secondarily, with that of meat, fish and vegetables (Lundén, et al., 2004).

The physiological response of the host to infection leads to a rapid and effective clearance of pathogenic microbes. This response involves innate and acquired cellular and humoral immune reactions, designed to both limit spread of the pathogen and to restore homeostasis avoiding collateral damage to host tissues (O'Mahony, et al., 2008). The intestinal epithelium constitutes the first physiological barrier and an integral and essential component of the innate mucosal immune system (Strober, 1998). The initial interaction of pathogens with the intestinal epithelium may be crucial for colonization and infection and in certain cases for bacterial translocation to extraintestinal sites (Burkholder, et al., 2010, Grzymajlo, et al., 2010). The expression of different virulence factors allows the adherence of pathogens to the intestinal epithelium (Burkholder, et al., 2010).

In addition, intestinal epithelial cells produce a variety of cytokines controlling the extent and length of the immune response(s) (Haller, et al., 2000, Jia, et al., 2009) during the interaction with pathogens (Bahrami, et al., 2010). The recognition of pathogens involves the interaction between host pattern-recognition receptors, like Toll-like receptors (TLRs), and conserved microbial ligands (Akira, et al., 2004). These interactions lead to the activation of the nuclear transcription factor kappa (NFκ)-B with production of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β that promote tissue inflammation and may also affect the integrity of the intestinal barrier (Hoffman, 2000, Ma, et al., 2004). Pathogenic bacteria induce the production of different cytokines by human intestinal epithelial cells with a prevalent production of chemoattractant and pro-inflammatory cytokines (Bahrami, et al., 2010).

The recognition of carbohydrate residues located at the surface of the host tissues by bacterial pathogens can initiate infection (Burkholder, et al., 2010, Roy, et al., 2009). These observations led to consider the use of suitable dietary carbohydrates for preventing bacterial adhesion and infections (Sharon, 2006, Shoaf, et al., 2006). Recent data demonstrated that oligosaccharides might be effective to prevent the infection by

different microorganisms acting as false receptors at the intestinal level (Sarabia-Sainz, et al., 2009, Sharon, 2006). Prebiotic galacto-oligosaccharides have been shown to inhibit adherence of enteropathogenic *Escherichia coli* and vibrio cholerae toxin to tissue culture cells and to the cell-surface toxin receptor, respectively (Shoaf, et al., 2006, Sinclair, et al., 2009). Also animal glycoconjugates are known to inhibit *Salmonella enterica* subsp. *enterica* serotype Typhimurium CECT 443 and *L. monocytogenes* CECT 935 adhesion to intestinal epithelial (Caco-2) cells (Laparra, et al., 2011, Laparra, et al., 2010). In this context, the production of effective and inexpensive neo-glycoproteins under controlled conditions could be an alternative for developing dietary compounds that interfere the interaction between bacterial adhesins and recognition sites on the intestinal cell surface.

Caseinomacropeptide (CMP) is a heterogeneous group of polypeptides derived from α-casein and generated during digestion in the stomach or during cheese-making. CMP heterogeneity is mainly attributed to differences in carbohydrate composition and degree of phosphorylation, as well as to the existence of genetic polymorphisms (Abd-El-Salam, et al., 1996, Thomae-Worringer, et al., 2006). CMP is considered as a multifunctional peptide with many possible biological applications (Manso, et al., 2004, Recio, et al., 2009). For instance, bovine CMP could influence downstream events in the immune inflammatory response through the modulation of cytokine production, suppression of interleukin receptors expression, or induction of receptor antagonists (Otani, et al., 1996, Otani, et al., 1995). Furthermore, CMP displayed anti-inflammatory activity in rats with hapten-induced colitis (Daddaoua, et al., 2005), mediated by mechanisms involving down-regulation of interleukin 17 and regulatory T cells (Requena, et al., 2009). Likewise, it has been described that bovine CMP has the ability to interact with toxins, viruses and bacteria, via its oligosaccharide structures that mimic the carbohydrate receptors recognized by pathogens (Dziuba, et al., 1996). Nevertheless, the interactions of CMP with bacterial pathogens seem to be strain-specific (Recio, et al., 2009). In this context, recent studies have pointed out that production of Maillard-type glycoconjugates, with a high number of attached carbohydrates, may be a simple and cost-effective strategy to block pathogen adhesins involved in mucosal colonization and infections (Laparra, et al., 2011, Ledesma-Osuna, et al., 2009).

The objective of this study was to determine the potential of neoglycoconjugates obtained by glycation of hydrolyzed bovine CMP with either novel or commercial

prebiotic galacto-oligosaccharides derived from transgalactosylation of lactulose and lactose to prevent interactions and excessive inflammatory responses on intestinal epithelial cells by bacterial pathogens.

4.3.2.4.2. Material and Methods

- *Hydrolysis of CMP*

Prior to glycation, bovine CMP (Davisco Foods International, Inc, Le Sueur, Minnesota, USA) was hydrolyzed in order to increase the number of free primary amino groups and, consequently, to increase the glycation rate. Briefly, CMP was subjected to a combined trypsin/chymotrypsin proteolysis (overnight at 37 ºC and pH 7) at 1:0.05:0.025 CMP:trypsin:chymotrypsin weight ratios. Activities of porcine pancreas trypsin (EC 3.4.21.4, type IX-S) and bovine pancreas α-chymotrypsin (EC 3.4.21.1, Type I-S) were 13,000–20,000U and ≥40U per mg of protein, respectively (**Section 4.2.2**).

- *Production of galacto-oligosaccharides*

Two different types of galacto-oligosaccharides, derived from transgalactosylation of lactose (GOS-La) and lactulose (GOS-Lu), respectively, were employed for CMP glycation.

i) GOS-La were obtained from an industrial product, Vivinal-GOS® kindly provided by Friesland Foods Domo (Zwolle, The Netherlands), containing approximately 45% GOS and 30% digestible sugars: lactose (15%), glucose (14%), and galactose (1%) (Anthony et al., 2006). Vivinal-GOS® was subjected to Size-Exclusion Chromatography analyses by using a Bio-Gel P2 (Bio-Rad, Hercules, CA, USA) column (90 x 1.6 cm) equilibrated with water and maintained at 4 °C in order to remove digestible mono- and di-saccharide fractions (Hernandez et al., 2009).

ii) GOS-Lu were synthesized by transgalactosylation of a solution (450 g/L) of lactulose (Duphalac, France) dissolved in 50 mM sodium phosphate buffer and 1 mM $MgCl_2$, pH 6.5, after addition of 8 U/mL of β-galactosidase from *Aspergillus oryzae* (Sigma-Aldrich, St. Louis, USA), and incubation at 50 ºC for 20 h under continuous agitation at 300 rpm (**Section 4.1.4**). Mixtures were immediately immersed in boiling water for 5 min to inactivate the enzymes. Subsequently, GOS-Lu mixture was diluted with water and treated with activated charcoal to remove monosaccharides. Oligosaccharides were

recovered using ethanol:water 50:50 (v:v), following the method proposed by Morales et al. (2006) with some modifications.

iii)

- *Production and analysis of neoglycoconjugates*

Aliquots of a solution consisting of 250 mg/mL of previously hydrolyzed CMP (hCMP) and 250 mg/mL of GOS-Lu or GOS-La in 0.1M sodium phosphate buffer, pH 7 (Merck, Darmstadt, Germany), were lyophilized. These were kept under vacuum in a desiccator at 40 °C and a water activity of 0.44, achieved with a saturated K_2CO_3 solution (Merck), for 9, 16 and 18 days. In addition, control experiments were performed with hCMP stored at 40 °C without carbohydrates during the same periods. Incubations were performed in duplicate, and all analytical determinations were performed at least in duplicate.

After the storage of CMP and carbohydrates, the unconjugated GOS fraction was removed by ultrafiltration using a diafiltration unit Model 8400 (Millipore Corp., Bedford, MA, USA) with a molecular cut off of 1000 Da. The permeates were analysed by gas chromatography-mass spectrometry (GC-MS) using a two-step derivatization (oximation and trimethylsilylation) (**Section 4.1.2**) to determine the percentage of unconjugated GOS. The quantity of conjugated GOS was calculated considering the total GOS used at the initial of the glycation reaction.

The retentates were analyzed by ESI-MS (HP-1100 mass detector; Hewlett-Packard, Palo Alto, CA, USA) to confirm the absence of unconjugated GOS, whereas formation of Amadori compounds during Maillard reaction between carbohydrates and hydrolysed CMP was evaluated through the study of 2-furoylmethyl-amino acids obtained by acid hydrolysis at 110 °C for 23 h under inert conditions (helium) with 8 N HCl (Moreno et al., 2002). Analyses were carried out by an ion-pair RP-HPLC method (Resmini, et al., 1990).

- *Bacterial cultures*

Salmonella enterica subsp. *enterica* serotype Typhimurium CECT 443and *Listeria monocytogenes* CECT 935 were grown in Brain-Heart broth and agar, and incubated at 37 ^{o}C under aerobic conditions. All cultures were grown for 20 h to be used in adhesion experiments.

- *Cells culture conditions*

The Caco-2 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 17 and used in experiments at passage 33-46. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM-AQ media, Sigma) containing 4.5 g/l glucose, and supplemented with 25 mM HEPES buffer, and 10% (v/v) fetal bovine serum (Sigma). The cells were maintained at 37 °C in 5% CO_2, 95% air and the culture media were changed every 2 days.

For adhesion experiments, Caco-2 cells were were seeded at an initial density of 50,000 cells/cm^2 onto 24-well plates (Costar). All cultures were grown in DMEM, and culture media was changed every two days. Experiments were performed 5 days post seeding.

- *Adhesion assay*

Crude mucin (Type II, Sigma-Aldrich) was diluted in a phosphate buffered solution (pH 7.2) (PBS) (0.5 mg/ml). An aliquot (0.5 ml) of this solution was loaded into polycarbonate 96-well plates (Costar, Cambridge, MA, USA), and incubated at 37 ºC for 1 h. To remove unbound mucin, the wells were washed twice with 0.5 ml PBS (Laparra, et al., 2009).

Bacteria from 20-hour-old cultures were collected by centrifugation (4,000 x *g* for 5 minutes at 4 ºC), washed twice and suspended in PBS to reach an optical density of 0.5 ($A_{\lambda 600}$). Suspensions of different bacteria were labelled by incubation with 75 µmol l^{-1} carboxyfluorescein diacetate (CFDA) (Sigma-Aldrich), at 37ºC for 30 minutes. Then, the mixtures were washed twice and suspended in PBS. A volume of 0.2 ml working labelled suspensions was loaded into 96-well plates and incubated at 37ºC for 1 hour. After the incubation period, the media was removed and wells were washed twice with 0.3 ml PBS. Then, 0.3 ml 1% (w/v) sodium dodecyl sulphate in 0.1 mol L^{-1} NaOH were added to the wells and incubated at 37ºC for 1 hour. Afterwards, the mixtures were homogenized by pipetting and the supernatants were transferred to black 96-well plates. The fluorescence was read in a multiscan fluorometer (Fluoroskan Ascent, Labsystem, Oy, Finland) (λ_{ex} 485 nm; λ_{em} 538 nm). Negative controls without bacteria were used throughout the experiment.

Adhesion was expressed as the percentage of fluorescence recovered after binding to mucin, relative to the fluorescence loaded into the wells. Each assay was performed in triplicate, and conducted in two independent experiments.

- *Analysis of inflammatory cytokines*

TNF-α (eBioscience, Hattfield, UK), and IL-1β (eBioscience, Hattfield, UK) were determined by ELISA according to the the manufacturer's instructions. The results of the ELISA assay for TNF-α and IL-1β are expressed as pico-grams per mL (pg/ml) of the cell culture media. The sensitivity for these methods is 4 picogram (pg)/ml.

- *Reverse transcription and PCR analyses*

Total RNA from cell cultures was extracted with an RNeasy mini kit (Qiagen, ciudad, país) according to the manufacturer's instructions. One microgram of total RNA was converted to double-stranded cDNA using AMV Reverse Transcriptase (Promega, USA).

PCR was carried out with primers designed for TLR4 (NM138554.3) (forward: 5'- TGG ACA GTT TCC CAC ATT GA -3'; reverse: 5'- TGC CAT TGA AAG CAA CTC TG -3'), TLR2 (NM003264.3) (forward: 5'- GGC CAG CAA ATT ACC TGT GT -3'; reverse: 5'- CCA CTT GCC AGG AAT GAA GT -3'), chemokine CXCR3 receptor (NM001142797.1) (forward: 5'-AAG AAT GCG AGA GAA GCA GC-3'; reverse: 5'-AAG AGG AGG CTG TAG AGG GC-3'), and β-actin (as housekeeping) gene (NM000251.1) (forward: 5'- GCC ATT TTG GAG AAA GGA CA -3'; reverse: 5'- CTC ACA TGG CAC AAA ACA CC -3'). cDNA levels of the genes of interest were measured by real-time PCR, using SYBR Green I Master mix on the LightCycler® 480 (Roche) system. PCR program consisted of 1 cycle of denaturation at 94 ºC for 2 min, and 35 cycles of amplification at 95 ºC for 10s, at 60 ºC for 20s and at 72 ºC for 35s. mRNA levels of the tested genes relative to β-actin mRNA were determined using the $2^{-\Delta Ct}$ method and expressed as fold induction.

- *Statistical analysis*

One-way analysis of variance (ANOVA) and the *post hoc* Tukey test were applied. Statistical significance was established at $P<0.05$. SPSS v.15 software (SPSS Inc., Chicago, IL, USA) was used for the statistical analysis.

4.3.2.4.3. Results and Discussion

- *Determination of the extent of glycation between hCMP and GOS-Lu or GOS-La*

Maximum yields of 2-furoylmethyl lysine (1.57 and 1.90 mg per 100 mg of hCMP:GOS-Lu and hCMP:GOS-La neoglycoconjugates, respectively), which is an indirect marker of the formation of the corresponding peptide-bound Amadori compounds, were achieved after 16 days of incubation (**Sections 4.3.2 and 4.3.3**). Similar results were reported by Sanz et al. (2007) following the characterization of neoglycoconjugates based on GOS derived from lactose and bovine β-lactoglobulin. After this time of incubation, Amadori compounds started to decrease, indicating that the degradation of Amadori compound to other Maillard reaction products, such as advanced glycation ends products (AGEs) and melanoidins, prevailed over their formation. GC-MS analyses of initial GOS concentration and unconjugated GOS after storage revealed that the relation hCMP:carbohydrates was approximately 2:1 (w:w) in both types of neoglycoconjugates (**Sections 4.3.2 and 4.3.3**), confirming that the glycation process was efficient.

- *Influence of paghogen bacterial interactions with the GOS-Lu and GOS-La, on adhesion*

The ability of glycated derivatives from previously hydrolyzed CMP to interact with intestinal pathogens was evaluated by measuring adhesion to mucin (Type II). The adhesion percentages of intestinal pathogens to mucin, and the influence of GOS-Lu and GOS-La and their glycated derivatives from caseinomacropeptide (CMP) are shown in **Figure 4.31**. *S. enterica* CECT 443 exhibited slightly higher ($P>0.05$) adhesion values than *L. monocytogenes* CECT 935 to mucin (**Figure 4.31**). The adhesion of *S. enterica* CECT 443 in presence of GOS-La was significantly reduced, but not that of *L. monocytogenes* CECT 935. The presence of hCMP:GOS-La significantly ($P<0.05$) decreased the adhesion of *L. monocytogenes* CECT 935, and also compared to the effect caused by GOS-La. In presence of the GOS-Lu the adhesion of both pathogens was not modified ($P>0.05$), but the glycated derivative, hCMP:GOS-Lu, significantly ($P<0.05$) decreased the pathogen adhesion in comparison with those of the pathogens alone. Notably, adhesion of both pathogens in presence of non-glycated hCMP was significantly ($P<0.05$) decreased to similar values to those obtained in presence of hCMP:GOS-Lu.

Adhesion of intestinal pathogens to the intestine may influence their residence time and ability to produce infections since it can constitute a first step of invasion (Burkholder, et al., 2010, Grzymajlo, et al., 2010). It has been reported that interactions with mucin seem to be more relevant for commensals than for pathogenic bacteria in the gut (Laparra, et al., 2009). Several studies support that different oligosaccharides (galactooligosaccharides, lactulose, inulin and inulin-like) inhibit enteric pathogen adhesion because of the blockage of recognition binding sites to the surface of intestinal epithelial cells (Shoaf, et al., 2006). However, it should not be ruled out that in addition to carbohydrate-recognition receptors of epithelial cells, intestinal pathogens express proteins (lectin-like molecules) that can bind to different glycoproteins at the surface of the intestinal cells (Laparra, et al., 2010, Zopf, et al., 1996). The *L. monocytogenes* infection has been related to the expression of several proteins such as ActA (AlvarezDominguez, et al., 1997) and LAP protein (Burkholder, et al., 2010), which mediate the adhesion to intestinal epithelial cells and facilitate its extra-intestinal dissemination *in vivo*. It has been reported that a *LAP*-deficient mutant of *L. monocytogenes* has lower adhesion capacity (3-4% reduction) (Burkholder, et al., 2010, Kim, et al., 2006) to Caco-2 cells that are concordant with the adhesion values presented in this study for *L. monocytogenes* CECT 935. The adhesion of this bacterium to intestinal epithelial Caco-2 cells has been associated with the interaction of the heat shock protein (Hsp)-60 of epithelial cells and the LAP protein of *L. monocytogenes* (Wampler, et al., 2004). The high-adhesive properties of *S. enterica* serovar Enteritidis have been related to the FimH adhesin (Grzymajlo, et al., 2010). Surface Plasmon Resonance analysis of purified fusion FimH proteins revealed the existence of high- and low-adhesive allelic variants not only in *S. enterica* serotype Typhimurium but also in *S. enterica* serotype Enteritidis (Grzymajlo, et al., 2010).

Glycoconjugates (Fleckenstein, et al., 2002, Laparra, et al., 2010), in addition to oligosaccharides, have ability to interfere *in vitro* with the adhesion of intestinal pathogens causing human diseases. In the same way, human lactoferrin reduced the adhesion of *Salmonella enterica* serovar Typhimurium to adhere to HeLa cells in an *in vitro* model (Bessler, et al., 2006). In this study, the glycated derivatives, hCMP:GOS-Lu and hCMP:GOS-La, reduced the adhesion of both pathogens tested to mucin, which

evidences the interaction of *S. enterica* and *L. monocytogenes* molecules by these compounds.

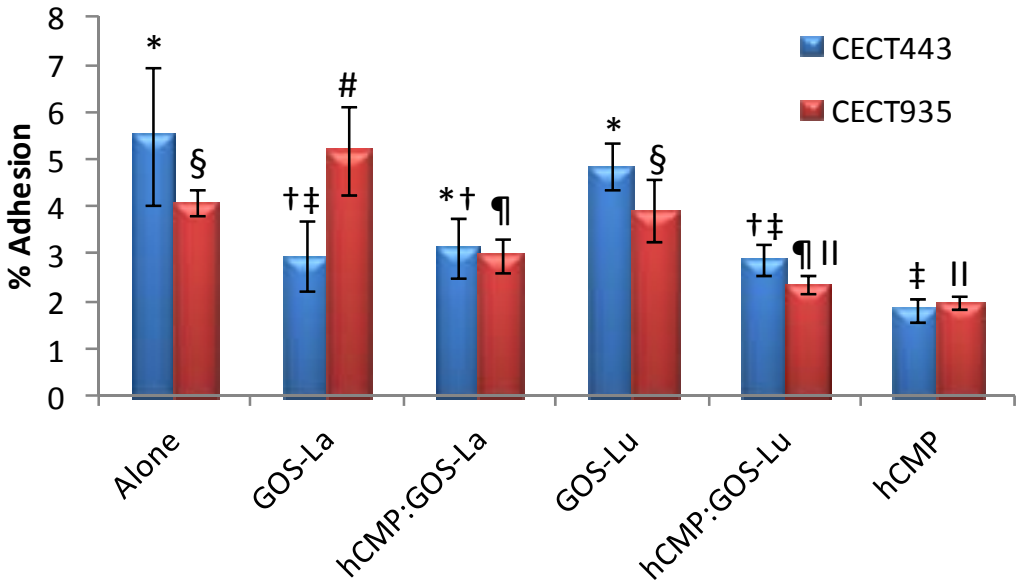

Figure 4.31. Effect of galactooligosaccharides (GOS) from lactose (La) or lactulose (Lu), and their conjugates with caseinmacropeptide (CMP) on the adhesión of *Salmonella enterica* CECT443 and *Listeria monocytogenes* CECT 935 to mucin (Type II). Results are expressed as mean ± SD (n=4). Different symbols indicate statistically significant (P<0.05) differences in adhesion of *S. enterica* CECT443 ($^{*, \dagger, \ddagger}$) or *L. monocytogenes* CECT 935 ($^{\S, \#, \P, ||}$).

- *Effects of the GOS-Lu and GOS-La and their glycoconjugates in the pathogen-induced inflammatory response(s)*

Production of pro-inflammatory cytokines, IL-1β and TNFα, by Caco-2 cells exposed to *Salmonella enterica* CECT 443 or *Listeria monocytogenes* CECT 935 in presence of the galactooligosaccharides and their glycoconjugates are shown in **Figure 4.32**. Cell suspensions of *S. enterica* CECT 443 and *L. monocytogenes* CECT 935 induced the production of IL-1β (**Figure 4.32 a**) by intestinal epithelial cells. There was a significant (P<0.05) increase in the production of IL-1β when the pathogens were added in presence of GOS-La. In contrast, the presence of GOS-Lu inhibited the pathogen-induced IL-1β production. An increased production of IL-1β was not detected when both bacteria were mixed with hCMP:GOS-Lu. However, bacterial cell suspensions mixed with hCMP:GOS-La

caused a significant increase in IL-1β production by intestinal cell cultures. When considering TNFα production both pathogens caused the production of this pro-nflammatory cytokine by intestinal Caco-2 cells (**Figure 4.32 b**). None of the compounds tested inhibited its production in intestinal cultures exposed to *S. enterica* CECT 443. However, the oligosaccharides tested and their glycoconjugates significantly (P<0.05) decreased TNFα production mediated by *L. monocytogenes* CECT 935.

S. enterica expresses several virulence factors such as flagella, chemotaxis and motility, and induces the production of IL-1β, IL-6, IL-8 and TNFα in intestinal HT-29 cells (Bahrami, et al., 2010). These data support the results reported in the present study, and are concordant with the increased expression of cytokines, with chemoattractant and proinflammatory functions, by intestinal epithelial cells following infection with enteroinvasive bacteria (Jung, et al., 1995, Kagnoff, et al., 1997). In this context, it has been reported the positive effect of bovine lactoferrin (LF) against *S. typhimurium* infection in mice, reducing the severity, mortality and the degree of inflammation (Mosquito et al., 2010). The authors speculated that this effect was caused by the disruption of the type three secretory system (TTSS), the needle complex mechanism by which the bacteria can attach to or invade host's tissues, binding to the bacterial lipopolysaccharide. The positive effect caused by the hCMP:GOS-Lu inhibiting the production of IL-1β may likely indicate a reduced interaction with molecules triggering inflammation, such as the TTSS system, of *S. enterica* CECT 443.

L. monocytogenes infection is known to impair the barrier properties of intestinal monolayers (Burkholder, et al., 2010). This microorganism invades the intestinal epithelia by a two-step mechanism of synergy between its invasins (InlA and InlB). The InlA protein, expressed on the surface of invading bacteria, interacts with E-cadherin, expressed on the epithelial cells and the InlB protein activates junctional endocytosis and bacterial invasion of the intestine (Pentecost, et al., 2010). In this study, the inhibited production of IL-1β and TNFα caused by GOS-Lu and hCMP:GOS-Lu suggests the blockage of the interaction of *L. monocytogenes*'s invasins with the intestinal cells. These effects could have important consequences on the intestinal barrier function because TNFα increases tight junction dependent permeability (Ma, et al., 2004) that may facilitate bacterial infection.

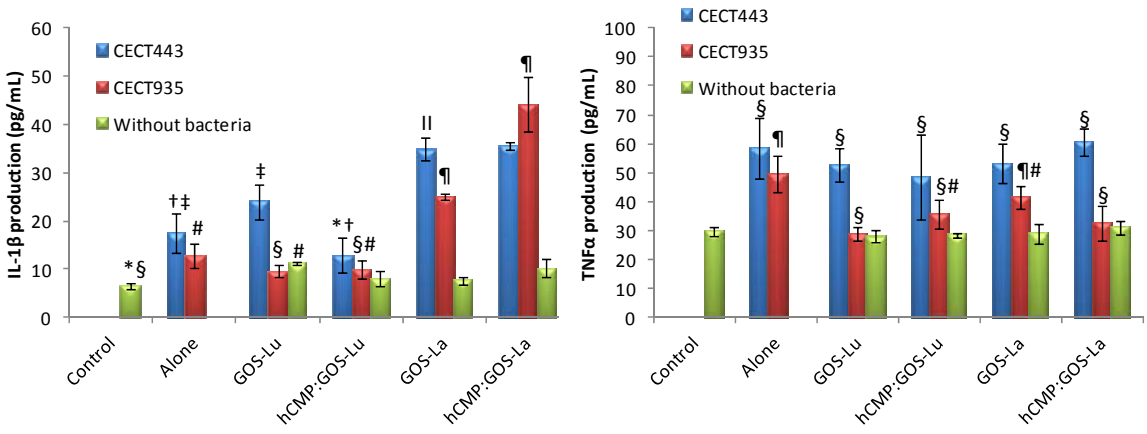

Figura 4.32. Effect of *Salmonella enterica* CECT443 and *Listeria monocytogenes* CECT 935 cell suspensions, in presence of galactooligosaccharides (GOS) from lactose (La) or lactulose (Lu), and their conjugates with caseinmacropeptide (CMP), on IL-1β and TNFα production by Caco-2 cultures. Results are expressed as mean ± SD (n=4). Different symbols indicate statistically significant (P<0.05) differences in adhesion of *S. enterica* CECT443 ($^{*, \dagger, \ddagger, ||}$) or *L. monocytogenes* CECT 935 ($^{\S, \#, \P}$).

- *Expression of the Toll-like (TLRs) and chemokine receptor in intestinal cells*

Mammalian defence against invading bacteria is mediated by the swift and coordinated recruitment of immunocompetent cells to sites of infection. One of the key members of the innate immune system is the family of TLRs acting as sentinels for the detection of microbial products. Chemokine CXCR3 receptor is involved in the macrophages and T cells recruitment. The influence of GOS-Lu and GOS-La, and their glycoconjugates in the TLR2/4 and CXCR3 receptors in intestinal cells is shown in **Figure 4.33**.

Cell suspensions of *S. enterica* CECT 443 alone (**Figure 4.33 a**) only caused a significant (P<0.05) increased expression (mRNA) of TLR2 in intestinal epithelial cell cultures compared to the controls. The presence of GOS-Lu, hCMP:GOS-Lu, and hCMP:GOS-La had a positive effect blocking the interaction of *S. enterica* CECT 443 with the intestinal cell as concluded from reductions in TLR2 expression compared to that of cells stimulated with the bacteria alone. Regarding TLR4 expression, there was detected a significant increased expression of this receptor when cell suspensions of *S. enterica* CECT 443 were exposed

to epithelial Caco-2 cells in presence of hCMP:GOS-Lu. Otherwise, only hCMP:GOS-La reduced TLR4 expression level compared to that of cells stimulated with the bacteria alone. CXCR3 receptor expression was also increased when bacterial suspensions were exposed in presence of hCMP:GOS-Lu; however, its expression levels were significantly (P<0.05) decreased by the addition of either GOS-Lu and hCMP:GOS-La in comparison with the effect of the bacteria alone. When intestinal Caco-2 cells were exposed to cell suspensions of *L. monocytogenes* CECT 935 all of TLR-2, -4, and CXCR3 receptor expression levels were significantly increased (**Figure 4.33 b**). All the compounds tested had a positive effect blocking the interaction of this bacterium with the intestinal cells as supported by the significantly (P<0.05) reduced expression levels of all receptors compared to that of cells stimulated with the bacteria alone.

TLR2, TLR4 and the myeloid differentiation factor (MyD88)-dependent signalling have not been considered necessary for the induction of protective immunity in mice against *S. enterica* infection (Seibert, et al., 2010). The present study also shows that TLR4 expression is not modified by *S. enterica* CECT 443 in intestinal epithelial cells. This result is concordant with the previously reported transient modulation of TLR2, but not TLR4, receptor by *S. enterica* serovar Typhimurium in porcine jejunal epithelial cells (Burkey, et al., 2009). It has been recently demonstrated that TLR2/4 signaling is required for *S. enterica* serovar Typhimurium virulence favoring the enhanced rate of acidification of the *Salmonella*-containing phagosome (Arpaia, et al., 2011). The myeloid differentiation protein 88 (MyD88)-mediated chemokine induction and monocyte recruitment are also required during *L. monocytogenes* infection (Jia, et al., 2009). Accordingly, in this study, it was quantified a significant (P<0.05) increased TLR4 expression levels in intestinal epithelial cell cultures incubated with *L. monocytogenes* CECT 935. In addition, the increased expression levels of TLR2 indicate the concurrent participation of TLR2-dependent signalling in the response of intestinal cells to *L. monocytogenes* CECT 935.

TLRs are central to pathogen recognition by the innate immune system. However, their role in the generation of acquired immunity is less clear. It has been reported that mice deficient in MyD88 and TLR2 can generate a normal CD4+ Th1 and CD8+ T cell responses following infection with *L. monocytogenes* (Way, et al., 2003). Otherwise, TLR4 signalling deficiency in MyD88 might involve the TRAM/TRIF pathway (Seibert, et al., 2010).

The presence of the hCMP:GOS-Lu modifies the expression of all receptors studied in relation to those detected when the intestinal cells were exposed to the pathogens alone. In the case of *S. enterica* CECT 443, this compound seemed to favor the interaction with receptor TLR4 and activation of innate immunity against the pathogen as supported by the increased expression of chemokine CXCR3 receptor (**Figure 4.33 a**). In contrast, cell suspensions of *L. monocytogenes* CECT 935 seemed to be unrecognized by intestinal cells when the hCMP:GOS-Lu was present. The results suggest that these compounds have ability to modify the interaction of virulence factors of this intestinal pathogen with the epithelial cells. This hypothesis is supported by the absence of pro-inflammatory cytokines production found in cell cultures incubated with *L. monocytogenes* CECT 935 (**Figure 4.32**).

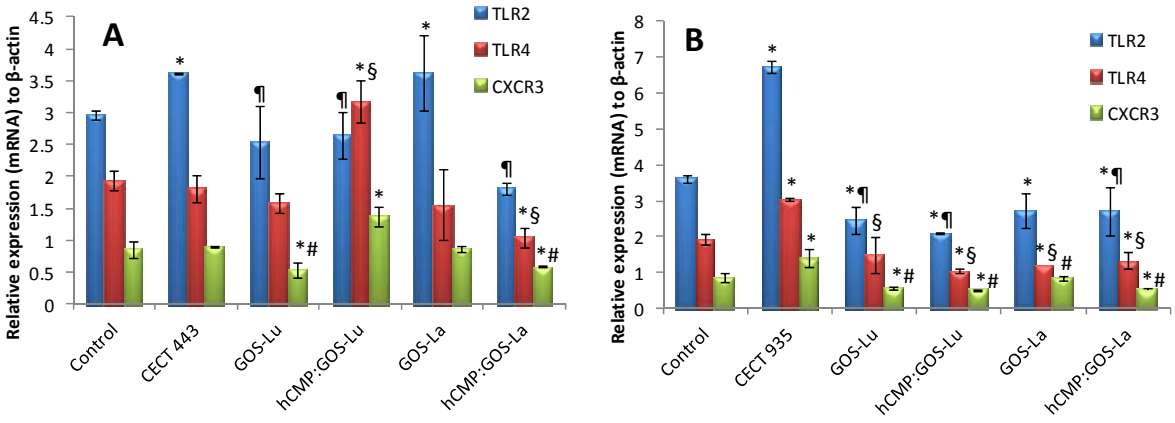

Figure 4.33. Effect of *Salmonella enterica* CECT443 (A) and *Listeria monocytogenes* CECT 935 (B) in presence or not of galactooligosaccharides (GOS) from lactose (La) or lactulose (Lu), and their conjugates with caseinmacropeptide (hCMP) on "Toll"-like (TLR)-2, -4, and chemokine CXCR3 receptor expression (mRNA) in Caco-2 cultures. Results are expressed as mean ± SD (n=4). * Indicate differences (P<0.05) compared to the controls (Caco-2 cultures not exposed to any treatment) for each receptor, and additional symbols ($^{\S, \#, \P}$) indicate significant differences compared to the incubation with the bacteria alone.

4.3.2.4.4. Conclusions

Glycated derivatives from galactooligosaccharides of lactose (GOS-La) and lactulose (GOS-Lu) interfere with bacterial adhesion to mucin, being the most effective the glycated derivatives hCMP:GOS-La and hCMP:GOS-Lu. These compounds exhibited more discrete effects in the case of pro-inflammatory, Il-1β and TNFα, cytokine production since them only reduced the *L. monocytogenes* CECT 935-induced TNFα production by intestinal cells. According to TLRs and CXCR3 receptor expression levels, the results suggest that the use of specific dietary neoglycoproteins can help to interfere with the first interactions between intestinal pathogens and epithelial cells that could lead to infections and excessive inflammation.

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4.3.3. Estudios *In vivo*

4.3.3.1. The structure and composition of galacto-oligosaccharides affects their resistance to ileal digestion and prebiotic properties in rats.

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Abstract

This work addresses a detailed study of the *in vivo* ileal digestibility and modulatory effects of novel galacto-oligosaccharides (GOS) derived from lactulose (GOS-Lu) and commercial GOS derived from lactose (GOS-La) in fecal microbiota of growing rats. Quantitative analysis of carbohydrates from dietary and ileal samples demonstrated that the trisaccharide fraction of GOS-Lu was substantially more resistant to gut digestion than that from GOS-La, according to their ileal digestibility rates (12.5% and 52.9%, respectively); the disaccharide fraction of GOS-Lu was fully resistant to the extreme environment of the upper digestive tract. The minimal ileal digestibility exhibited by the GOS-Lu could be attributed to the great resistance of galactosyl-fructoses to the digestive enzymes, highlighting the key role of the type of monomer and linkage involved in the oligosaccharide chain on the resistance to the action of mammalian digestive enzymes. Likewise, the partial digestion of GOS-La trisaccharides showed that glycosidic linkages (1→6) and (1→2) between galactose and glucose monomers were more resistant to *in vivo* gastrointestinal digestion than linkage type (1→4) between galactoses. The absence of GOS-La and GOS-Lu digestive-resistant oligosaccharides in fecal samples indicated that they were readily fermented within the large intestine, enabling both type of GOS to exert a potential prebiotic function. Indeed, GOS-Lu significantly ($P<0.05$) stimulated the growth of bifidobacteria and *Eubacterium rectale* in fecal samples, resulting in a higher selectivity index compared to commercial GOS-La after 14 d of treatment. These novel

data support a direct relationship between resistance patterns to digestion and prebiotic properties of galacto-oligosaccharides.

4.3.3.1.1. Introduction

The mammalian intestine harbors a complex microbial ecosystem consisting of an extraordinary number of resident commensal bacteria existing in homeostasis with the host (Eckburg, et al., 2005). This endogenous microbiota establishes a symbiotic relationship and impacts numerous physiological functions of the host including nutrition exchange, control of epithelial cell proliferation/differentiation, pathogen exclusion and stimulation of the immune system (Cerf-Bensussan, et al., 2010, Flint, et al., 2007). Currently, there is a growing interest in identifying functional dietary compounds capable of modulating the composition and metabolic activities of the intestinal microbiota. These compounds, named prebiotics, have been recently redefined as non-digestible functional ingredients which are selectively fermented and allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health (Roberfroid, 2007). Among intestinal bacteria stimulated by prebiotics, bifidobacteria and lactobacilli species are the most relevant; both are believed to play an important role in maintaining and promoting a healthy gut environment (Macfarlane, et al., 2008, Tuohy, et al., 2005). The major prebiotic oligosaccharides on the market are fructan inulin, fructo-oligosaccharides (FOS) and galacto-oligosaccharides (GOS) (Rastall, 2010). The latter are non-digestible carbohydrates which are usually comprised by 2 to 10 molecules of galactose and a terminal glucose unit, derived primarily from transgalactosylation reactions of lactose catalyzed by β-galactosidases of fungal, bacterial or yeast origin, to result in oligosaccharides with different glycosidic linkages and degree of polymerization (DP) (Torres, et al., 2010). Recently, the synthesis and detailed chemical characterization of novel lactulose-derived GOS (GOS-Lu) has been accomplished (**Section 4.1.3**) (Cardelle-Cobas, et al., 2008, Martinez-Villaluenga, et al., 2008). GOS-Lu are attracting increasing attention due to their prospective prebiotic applications, being recognized their ability to promote the *in vitro* growth of several probiotic strains of *Lactobacillus* and *Bifidobacterium* (Cardelle-Cobas, et al., 2011), in a

similar way as commercial lactose-derived GOS (GOS-La) (Cardelle-Cobas, et al., 2009, Cardelle-Cobas, et al., 2008).

In order to exert their potential prebiotic properties, oligosaccharides have to resist and survive, at least to some extent, to the acidic environment and enzymatic digestion in the upper digestive tract. Thus, dietary oligosaccharides that escape both digestion by endogenous enzymes and absorption in small intestine become available to microbial fermentation in the large intestine (Roberfroid, et al., 2010). Although a number of *in vitro* and *in vivo* studies have demonstrated the potential health benefits of GOS-La, mainly through modulation of gut microbiota, there have been very few attempts to identify and quantify the intestinal survival of GOS-La and their derivatives (Roberfroid, 2007). Furthermore, the reported results are scarce and controversial mainly due to methodological difficulties (i.e., complexity of analytical techniques, availability of samples and experimental models used). Several authors have claimed that GOS-La could not fully match the criteria of resistance to small intestinal digestion necessary to exert their prebiotic properties within the large intestine (Alles, et al., 1999, Roberfroid, 2007, Smiricky-Tjardes, et al., 2003). Chonan *et al*. (2004) reported a differential digestibility behaviour between di- and trisaccharides of GOS-La, being the former more susceptible to digestion. Ohtsuka *et al*. (1990) showed that only a small amount of 4´-galactosyl-lactose, a major trisaccharide present in GOS-La, was digested by a homogenate of intestinal mucosa of rats. Additional studies to distinguish the differential behaviour on digestibility between major components of these complex mixtures of oligosaccharides, expected to exert health beneficial effects, are clearly necessary.

To the best of our knowledge, no data is available regarding resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption of the recently characterized GOS-Lu (**Section 4.1.3**). The exploitation of these promising oligosaccharides as prebiotic compounds will depend largely upon their ileal survival rates after digestion and their further selective fermentation by microbiota in the large intestine. Therefore, the aims of this work were to carry out a detailed comparative study regarding the ileal digestibility of GOS-La and GOS-Lu, and to evaluate if their major compounds are fermented by the microbiota to promote the selective growth of beneficial bacteria groups in the large intestine. We have demonstrated that

trisaccharides from GOS-Lu are more resistant to gut digestion than those derived from GOS-La, being disaccharides from GOS-Lu fully resistant to the extreme conditions of the gastrointestinal tract (GIT). The absence of di- and trisaccharides in fecal samples suggests that both GOS-La and GOS-Lu were fully fermented by the intestinal microbiota. A strong bifidogenic effect in fecal samples of growing rats fed GOS-Lu was demonstrated whereas a significant increase of lactobacilli in rats fed GOS-La was also observed. These data further our knowledge on the remarkable gastrointestinal survival of GOS and their potential prebiotic function within the large intestine.

4.3.3.1.2. Experimental methods

- *Materials*

Analytical standards of lactulose (Lu) (Gal-β(1→4)-Fru), lactose (Gal-β(1→4)-Glc), sucrose (Glc-α(1→2)-β-Fru), maltose (Glc-α(1→4)-D-Glc), maltotriose (Glc-α(1→4)-Glc-α(1→4)-D-Glc), raffinose (Gal-α(1→6)-Glc-α(1→2)-β-Fru), α,α-threhalose (Glc-α(1→1)-α-Glc), as well as β-galactosidase from *Aspergillus oryzae* (8.0 Units/mg protein), phenyl-β-D-glucoside, hexamethyldisilazane and hydroxylamine hydrochloride were obtained from Sigma (St. Louis, US). 1,6-galactobiose (Gal-β(1→6)-Gal), 1,4-galactobiose (Gal-β(1→4)-Gal), 1,3-galactobiose (Gal-α(1→3)-Gal) were supplied from Dextra Laboratories (Reading, UK). 6´-galactosyl-lactose (Gal-β(1→6)-Gal-β(1→4)-Glc) was a gift from Prof. Nieves Corzo from CIAL-CSIC (Madrid, Spain). Pyridine was obtained from Merck (Darmstadt, Germany). All other chemicals were of analytical grade.

- *Synthesis and preparation of galacto-oligosaccharides*

An industrially available galacto-oligosaccharide mixture derived from lactose (GOS-La) was used in this study. In order to remove their mono- and disaccharides, the mixture was fractionated using size-exclusion chromatography (**Section 4.1.2**). Briefly, 80 mL of GOS-La (25 % w/v) were injected in a Bio-Gel P2 (Bio-Rad Hercules, CA, USA) column (90 x 5 cm) using water as mobile phase at a flow of 1.5 mL/min. Sixty fractions of 10 mL were collected, after the elution of void volume. The DP of collected fractions was determined by electrospray ionization mass spectrometry (ESI-MS) at positive mode. Fractions with

DP ≥ 3 were pooled and freeze-dried to be used in the *in vivo* experiments. The trisaccharides content of GOS-La was determined by hydrophilic interaction chromatography coupled to mass spectrometry (HILIC LC-MS) (**Section 4.1.4**). In brief, 20 µL of sample was injected in a BEH amide column (150 mm x 4.6 mm; 3.5 µm; XBridge, Waters, Hertfordshire, UK). The elution was performed using a linear gradient of acetonitrile : MilliQ water, both having 0.1% NH_4OH, from 80:20 (v:v) to 50:50 (v:v) for 31 min. The separation and detection was carried out by using an Agilent 1200 series HPLC system (Hewlett-Packard, Palo Alto, CA, USA) coupled to a quadrupole HP-1100 mass detector (Hewlett-Packard) provided with an electrospray ionization (ESI) used at positive mode. The trisaccharide fraction was 35.1 % of the total GOS-La purified.

Regarding the galacto-oligosaccharides derived from lactulose (GOS-Lu), its enzymatic synthesis was carried out from the hydrolysis and transgalactosylation of the prebiotic carbohydrate lactulose (Duphalac®, Solvay Pharmaceuticals, Weesp, Holland) by using a β-galactosidase from *Aspergillus oryzae*, and following the procedure previously in the **section 4.1.3**. The GOS mixture was treated with activated charcoal to remove the monosaccharides fraction following the method of Morales *et al*. (2006). A detailed characterization of the main carbohydrates in GOS-Lu has been recently reported, being di- and trisaccharides 78 % and 14 % of total carbohydrates, respectively (**Section 4.1.3**).

- *Animals and diets*

Male weaned Wistar rats (Charles River Laboratories, Barcelona, Spain), matched by weight (40 ± 5 g) were individually housed in metabolism cages throughout the experiment under controlled conditions of temperature (25 °C), moisture (50%) and lighting (12 h cycles). Rats were fed a growth purified diet (AIN-93G, Testdiet, UK) based on corn starch (40%), casein (20%), maltodextrin (13.2%), sucrose (10%) and soybean oil (7%) as main dietary ingredients (Reeves, 1997). A 6-day pre-experimental adaptation period was followed by a 14-day experimental period. At the end of the adaptation period, rats had an average weight of 75 ± 5 g and subsequently entered the experimental period. Thirty-six animals were randomly assigned to three dietary groups of twelve animals each and fed *ad libitum*. Water was freely available at all times. Rats were fed AIN-93G (control group), AIN-93G plus 1% (wt:wt) GOS-Lu (GOS-Lu group) and

AIN-93G plus 1% (wt:wt) GOS-La (GOS-La group). Chromium oxide (Cr_2O_3) was included (2 g kg^{-1}) in all diets as an indigestible marker (Clemente, et al., 2008). Fresh fecal samples from rats subjected to the same dietary treatment were collected weekly (days 0, 7 and 14), pooled by collected day (equal weights from 4 animals per pool) in sterile flasks and frozen prior to storage at –80 °C until further carbohydrate and microbiological analysis (see below).

All the experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the Spanish Council for Scientific Research (CSIC, Spain), and the animals were cared for in accordance with the Spanish Ministry of Agriculture guidelines (RD 1201/2005).

- *Ileal sample collection*

At the end of the dietary intervention period (14 days), rats were deprived of food for 14 h, fed 4 g of corresponding diet and serially euthanized under total anesthesia (40 mg pentobarbital sodium/kg body weight). Ileum (last 20 cm of small intestine) was immediately dissected out and rinsed with sterile distilled water in order to collect the ileal content. Intestinal contents of individual rats were immediately frozen, freeze-dried and stored at –80 °C until further analysis.

- *Quantitative determination of galacto-oligosaccharides (di- and trisaccharides) by GC-MS*

The identification and quantification by GC-MS of di- and trisaccharides from GOS-La and GOS-Lu present in diets, ileum and fecal samples after sequential derivatization, involving oximation and trimethylsilylation steps, was carried out. An amount of 1 mL of a solution prepared by dissolving 50 mg of sample in 2 mL of 70% (v/v, ethanol/water) at 4 ^{o}C and filtered by 0.20 µm was mixed with 0.25 mL of phenyl-β-D-glucoside (1 mg/mL), used as internal standard, and evaporated under vacuum. Derivatized carbohydrates were formed by addition of 0.35 mL of 2.5% (w/v) of hydroxilamine chloride in pyridine after 30 min at 75 ^{o}C; the formed oximes were silylated with 0.35 mL of hexamethyldisilazane and 0.035 mL of trifluoroacetic acid at 45 ^{o}C for 30 min. The samples were centrifuged at 8,000*g* for 10 min and 1 µL of the supernatant was injected.

GC-MS analyses were carried out in an Agilent Technologies 7890A gas chromatograph (Hewlett-Packard) coupled to a 5975C quadrupole mass detector operating in electronic impact mode at 70 eV, using helium as carrier gas (1 mL/min). A 30 m x 0.25 mm i.d x 0.25 µm film thickness fused silica column with cross-linked methyl silicone from Teknokroma (Barcelona, Spain) was used. The temperature program used was previously described by Sanz *et al*. (2004), with some modifications; the oven temperature was held at 200 °C for 15 min, then programmed to 270 °C at a heating rate of 15 °C/min, then programmed to 290 °C at 1 °C/min and finally programmed to 300 °C at 15 °C min^{-1} and held for 20 min. Chromatographic peaks were analyzed using an HPChem Station software (Agilent, PaloAlto, US). All analyses were carried out in triplicate.

Identification of trimethylsilyloximes (TMSO) derivatives of carbohydrates was carried out by comparison of mass spectra and retention indices (I^T) with standard carbohydrates previously derivatized. Previous reported data (Coulier, et al., 2009, Ruiz-Matute, et al., 2010) was also used to identify carbohydrates not commercially available; such identifications were considered as tentative. Carbohydrate quantitative data were obtained from GC-MS peak areas using the internal standard method; standard solutions from 0.003 to 1 mg of lactulose, maltose, sacarose, maltotriose and raffinose were prepared to calculate the corresponding response factors relative to internal standard and used to quantify di- and trisaccharides.

- *Ileal and fecal digestibility*

The ileal apparent digestibility (%) of GOS-Lu and GOS-La was calculated according to the expression: $[(P_f/Cr_2O_{3f}) - (P_i/Cr_2O_{3i})]/(P_f/Cr_2O_{3f}) \times 100$, where P_f and P_i represent the amount of carbohydrates (mg per 100 mg of sample) in feed and ileal samples, respectively, determined by GC-MS analyses, and Cr_2O_{3f} and Cr_2O_{3i} are chromium oxide concentrations (mg per 100 mg) in feed and ileal contents[(24)]. In a similar way, fecal digestibility of the different compounds at the end of the experimental period (14 d) was also evaluated. Chromium oxide content was determined in experimental diets, ileal and fecal samples following the procedure described by Fenton and Fenton (1979).

- *DNA extraction from fecal samples*

Total DNA was isolated from freeze-dried fecal samples (40 mg), using the QIAamp DNA stool kit (Quiagen, West Sussex, UK) and following the manufacturer's instructions. Eluted DNA was treated with RNase (Invitrogen, Paisley, UK) and DNA concentration assessed spectrophotometrically by using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, USA). Purified DNA samples were stored at -80 °C until use.

- *Quantitative PCR (qPCR) analysis*

The different microbial groups including total bacteria, *Bacteroides*, *Lactobacilli*, *Bifidobacteria*, *Eubacterium rectale/Clostridium coccoides* and *Clostridium leptum* were determined in fecal samples using qPCR. The 16S rRNA gene-targeted group-specific primers used in this study are listed in **Table 4.23**. qPCR assays were performed in polypropylene 96-well plates using an iQ5 Cycler Multicolor PCR detection system (BioRad laboratories, Hercules, USA). The reaction mixture (25 µl) comprised of 12.5 µl of iQ SYBR Green Supermix (BioRad), 0.75 µl of each of the specific primers (10 µM; Roche Diagnostics, Barcelona, Spain), 9µl of sterile distilled water and 2 µl of DNA template. For total bacteria, *Bacteroides*, *Bifidobacteria* and La*ctobacilli* group, PCR conditions were an initial denaturation step at 50 ºC for 2 min and 95 ºC for 10 min for initial denaturation, followed by 40 cycles at 95 ºC for 15 s and 60 ºC for 1 min for primer annealing and product elongation. In the case of *Eubacterium rectale/Clostridium coccoides* and *Clostridium leptum* groups, PCR conditions were an initial denaturation step at 94 ºC for 5 min followed by 40 cycles at 94 ºC for 20 s, 50 ºC for 20 s and 72 ºC for 1 min. After amplification, a melting curve analysis to distinguish the targeted PCR product from non-targeted PCR product was carried out (Ririe, et al., 1997). The melting curves were obtained by slow heating at temperatures from 55 ºC to 95 ºC at a rate of 0.5 ºC/10 s, with continuous fluorescence collection. A plasmid standard containing the target region was generated for each specific primer set using DNA extracted from pooled fecal samples of rats fed AIN-93G diet. The amplified products were run on a 2 % agarose gel, purified by MBL-Agarose QuickClean kit (Dominion MBL, Spain), cloned using the TOPO TA cloning kit for Sequencing (Invitrogen) and transformed into *Escherichia coli* One Shot Top 10 cells (Invitrogen). Plasmids were eluted by GenElute™ Plasmid Miniprep Kit (Sigma), sequences were obtained by the sequencing service of the Instituto de Parasitología y Biomedicina

López-Neyra (CSIC, Granada, Spain) and then submitted to the ribosomal RNA database (http://bioinformatics.psb.ugent.be/webtools/rRNA/blastrrna.html) in order to confirm the specificity of the primers. The concentration of the resulted products was determined spectrophotometrically and the copy number was calculated in terms of the product size (Lee, et al., 2006). For quantification of target DNA copy number, standard curves were generated using serial 10-fold dilutions of the extracted products by using at least six non-zero standard concentrations per assay. The bacterial concentration in each sample was measured as $\log_{10}$ copy number by the interpolation of the C_t values obtained by the intestinal samples into the standard calibration curves. Each plate included triplicate reactions per DNA sample and the appropriate set of standards.

- *Selectivity index (SI)*

The equation to estimate the SI values was adapted from Palframan *et al*. (2003) as follows:

$$[(Bif_t/Total_t)/(Bif_0/Total_0)] + [(Lact_t/Total_t)/(Lact_0/Total_0)] + [(Erec_t/Total_t)/(Erec_0/Total_0)] - [(Bact_t/Total_t)/(Bact_0/Total_0)] - [(Clost_t/Total_t)/(Clost_0/Total_0)]$$

where Bif, Lact, Erec, Bact, Clost and Total are the $\log_{10}$ copy number/g of fecal sample of bifidobacteria, lactobacilli, *Eubacterium rectale* group, bacteroides, *Clostridium leptum* subgroup and total bacteria, respectively, at the time of sampling (0, 7 and 14 days).

- *Statistics*

The effect of dietary treatment on fecal microbiota composition was analyzed by two-way repeated measures ANOVA using the GLM procedure (SPSS Statistics version 18.0, Madrid, Spain). Statistical differences of means were determined by MSD test; P values < 0.05 were considered significant.

Table 4.23. Group-specific primers based on 16S rRNA sequences used for quantitative PCR in this study.

Target bacterial group	Primer	Sequence (5'–3')	PCR product size (bp)	Annealing T (°C)	References
All bacteria	F-Eub 338	ACTCCTACGGGAGGCAGCAG	200	60	Guo *et al*. (2008)
	R-Eub 518	ATTACCGCGGCTGCTGG			
Bacteroides	F-AllBac 296	GAGAGGAAGGTCCCCCAC	106	60	Layton et al. (2006)
	R-AllBac 412	CGCTACTTGGCTGGTTCAG			
Bifidobacteria	F-Bifido	CGCGTCYGGTGTGAAAG	244	60	Delroisse *et al*. (2008)
	R-Bifido	CCCCACATCCAGCATCCA			
***Eubacterium rectale/ Clostridium coccoides* group**	F-g-Ccoc	AAATGACGGTACCTGACTAA	440	50	Matsuki *et al*. (2004)
	R-g-Ccoc	CTTTGAGTTTCATTCTTGCGAA			
***Clostridium leptum* group**	F-sg-Clept	GCACAAGCAGTGGAGT	239	50	Matsuki *et al*. (2004)
	R3-sg-Clept	CTTCCTCCGTTTTGTCAA			
Lactobacilli	F-Lacto	GAGGCAGCAGTAGGGAATCTTC	126	60	Delroisse *et al*. (2008)
	R-Lacto	GGCCAGTTACTACCTCTATCCTTCTTC			

4.3.3.1.3. Results

- *Animal performance*

The intake of control, GOS-Lu and GOS-La diets was about 14 g/day per rat during the experimental period (14 d), and no significant differences were observed in body weight gain or feed intake among groups. In addition, dietary treatments did not have any significant effect on relative weight of different organs including stomach, small intestine, cecum and colon, except that rats fed GOS-La had significantly ($P<0.05$) lower relative colon weight compared to those fed control diets (data not shown).

- *Apparent ileal and fecal digestibility of GOS-Lu and GOS-La (di- and trisaccharides)*

Quantitative evaluation of carbohydrates from dietary and ileal samples demonstrated that the disaccharide fraction of GOS-Lu, mostly constituted by β-galactobioses and galactosyl-fructoses (**Section 4.1.3**), were fully resistant to digestion in the small intestine (**Table 4.24**). As can be observed in **Figure 4.34 A**, the GC-MS profiles of GOS-Lu disaccharides from dietary and ileal samples of rats were almost identical, with slight differences corresponding to minor chromatographic peaks which did not match to carbohydrates according to their mass spectra and retention times. The chromatographic profile of fecal samples of rats fed GOS-Lu demonstrated the complete fermentation of the disaccharides fraction in large intestine (**Figure 4.34 A**); none of the detected chromatographic peaks of fecal samples showed the typical mass fragmentation patterns of TMSO derivatives of carbohydrates.

As a result of the high levels of digestible lactose present in the disaccharide fraction, mono- and disaccharides were removed from the starting GOS-La. Therefore, no data regarding the resistance of disaccharides from GOS-La to digestion is reported in this study. As far the GOS-Lu trisaccharide fraction, it exhibited a limited digestion as evaluated in ileal samples of treated animals, showing an average digestibility rate of 12.5% (**Table 4.24**); in contrast, GOS-La trisaccharides were clearly susceptible to small intestinal hydrolysis, having a much higher average digestibility rate (52.9 %) (**Table 4.325**). An exhaustive analysis of their single chromatographic peaks, obtained by GC-MS, was carried out as it is described below. **Figure 4.34 B** shows the chromatographic profile of dietary GOS-Lu trisaccharides and those found in ileal samples of rats fed GOS-Lu. The similarity of both chromatographic profiles confirms the high resistance to digestibility of their major components. As previously reported, the major trisaccharides of GOS-Lu were identified as Gal-(1→6)-Gal-(1→4)-Fru (6´-galactosyl-lactulose) (peaks 15 and 16), and Gal-(1→4)-Gal-(1→1)-Fru (peak 18) (**Figure 4.34 B**) (**Section 4.1.3**) (Cardelle-Cobas, et al., 2008). In contrast, some individual GOS-La trisaccharides were completely digested (**Figure 4.35 B**); thus, the chromatographic peaks 13, 14, 19 and 22 were not found in ileal samples of rats fed GOS-La (**Table 4.25, Figure 4.35 B**). Indeed, such susceptibility to hydrolysis was evidenced by the presence of disaccharides in ileal samples of rats fed GOS-La (**Figure 4.35 A**), demonstrating that some oligosaccharides of DP ≥ 3 were

hydrolyzed. Di- and trisaccharides of GOS-La reaching the large intestine were fully fermented as indicated by the absence of such oligosaccharides in fecal samples (**Figure 4.35**).

- *Tentative identification of GOS-La trisaccharides and disaccharides derived from hydrolysis of oligosaccharides of DP* ≥ 3

In order to determine the chemical structure of GOS-La oligosaccharides digested at the ileum, a tentative identification of the TMSO trisaccharides and disaccharides formed as a result of digestion was carried out by GC-MS analyses. **Table 4.25** shows the retention indices of carbohydrates present in dietary and ileal samples of rats fed GOS-La. The mass spectrum of peak 1 (I^T= 2686) showed a relationship of *m/z* 191:204:217 ions of 1.4:1:1.1, characteristic of non-reducing sugars with 1→1 glycosidic linkages, being consistent with Gal-(1→1)-Gal (1,1-galactobiose) or Gal-(1→1)-Glc (1,1-galactosyl-glucose). Given that 1,1-galactobiose shows an I^T value of 2903 (**Section 4.1.3**), the chromatographic peak was assigned to 1,1-galactosyl-glucose. Peak 2 was composed by a mixture of two disaccharides, Gal-(1→4)-Glc (1,4-galactosyl-glucose) and Gal-(1→3)-Glc (1,3-galactosyl-glucose). The former was assigned by comparison with a GOS previously obtained by using β-galactosidase from *Aspergillus aculeatus* (Cardelle-Cobas, et al., 2008); the latter was assigned by the presence of relatively high abundant *m/z* 205, 244 and 307 ions. Such fragmentation pattern is characteristic of the glycosidic linkage 1→3 and similar to that of Gal-(1→3)-Gal (1,3-galactosyl-galactose) standard but showing different retention indices (I^T= 2699 and 2932, respectively), impairing its tentative identification. Peak 3 showed the typical fragmentation pattern of TMSO carbohydrates; however, it could not be identified by its mass spectrum and retention index. Peak 4 was constituted by a mixture of Gal-(1→4)-Gal (1,4-galactosyl-galactose), identified by comparison with its corresponding commercial standard, and Gal-(1→4)-Glc (1,4-galactosyl-glucose, isomer *Z*), identified in a similar way to its corresponding *E* isomer. Similarly, peak 5 was formed by a mixture of the second peak of Gal-(1→3)-Glc (1,3-galactosyl-glucose, isomer *Z*) and an unknown disaccharide. Peaks 6 and 7 were identified as Gal-(1→2)-Glc (1,2-galactosyl-glucose), isomers *E* and *Z* respectively, by the presence of

the *m/z* 319 ion with a high abundance, which is characteristic of 1→2 glycosidic linkages and that corresponds to the loss of a TMSOH group from the C3-C6 chain of an hexose group. Peaks 8 and 9 were characterized by a high intensity ion of *m/z* 422 corresponding to C1-C4 of the oxime chain, typical of 1→6 glycosidic linkages, being identified as Gal-(1→6)-Glc (1,6-galactosyl-glucose) isomers due to the difference of retention indices between these peaks and the corresponding Gal-(1→6)-Gal (1,6-galactosyl-galactose) standards.

Regarding GOS-La trisaccharides, up to seventeen chromatographic peaks were detected. Peak 10 had a relation of ions 191:204:217, typical of 1→1 glycosidic linkages. Peak 16 was identified as Gal-(1→4)-Gal-(1→4)-Glc (4´-galactosyl-lactose) by comparison with the most abundant trisaccharide of Vivinal-GOS® (Coulier, et al., 2009) and supported by previous data reported by Cardelle-Cobas *et al*. (Cardelle-Cobas, et al., 2008). In peak 17, co-elution of two compounds occurred, being the first one identified as Gal-(1→6)-Gal-(1→4)-Glc (6´-galactosyl-lactose) by comparison with the compound previously isolated and identified by NMR (Martinez-Villaluenga, et al., 2008), being the second one identified as Gal-(1→4)-Gal-(1→2)-Glc 1 by its characteristic *m/z* 319 ion and by comparison with that previously identified in Vivinal-GOS® by NMR (Coulier, et al., 2009). Peak 20 was constituted by two compounds, Gal-(1→4)-Gal-(1→2)-Glc 2 and Gal-(1→6)-Gal-(1→2)-Glc 1, identified in a similar way as the previous peak. Peak 18 corresponded to the isomers of 4´-galactosyl-lactose and co-eluted with other unknown carbohydrate, whereas peak 23 was identified as Gal-(1→6)-Gal-(1→6)-Glc by the presence of the ion at *m/z* 422 and by comparison with 6´-galactosyl-galactose (Cardelle-Cobas, et al., 2008) ; although the mass spectrum was similar, the retention index was different due to the presence of a terminal galactose unit instead of a glucose molecule. Unfortunately, we were unable to determine the chemical structure of some GOS-La trisaccharides having low ileal digestibility (peaks 11, 12, 15, 24, 25 and 26) (**Table 4.25**) due to the absence of diagnostic ions in the mass spectra which, however, were typical of TMSO carbohydrates.

Table 4.24. Apparent ileal digestibility, retention indices (I^T) and tentative structural identification of galacto-oligosaccharides (di- and trisaccharides) of GOS-Lu.

Peak number*	Disaccharides	I^T	Ileal digestibility[a] (%)
1+2	Gal-(1→4)-Fru (**lactulose**)	2878-2887	0.0[a]
3	Gal-(1→1)-Gal + Gal-(1→4)-Gal E	2903	
4	Gal-(1→5)-Fru 1	2915	
5a	Gal-(1→3)-Gal E + Gal-(1→2)-Gal E	2932	
5b	Gal-(1→5)-Fru 2	2937	
6	Gal-(1→4)-Gal Z + unk	2959	
7	Gal-(1→3)-Gal Z – Gal-(1→2)-Gal Z	2979	
8	Gal-(1→6)-Fru 1	3003	
9	Gal-(1→6)-Fru 2	3012	
10	Gal-(1→1)-Fru 1	3029	
11a	Gal-(1→6)-Gal E	3046	
11b	Gal-(1→1)-Fru 2	3049	
12	Gal-(1→6)-Gal Z	3094	
	Trisaccharides		
13	unknown	3785	12.5
14	unknown	3794	
15	Gal-(1→6)-Gal-(1→4)-Fru 1	3809	
16	Gal-(1→6)-Gal-(1→4)-Fru 2	3826	
17	unknown	3835	
18	Gal-(1→4)-Gal-(1→1)-Fru	3841	

Gal: galactose; Fru: fructose.

*Labelled peaks are described in Figure 1.

[a]Calculated as ileal digestibility of the whole fraction of di- and trisaccharides.

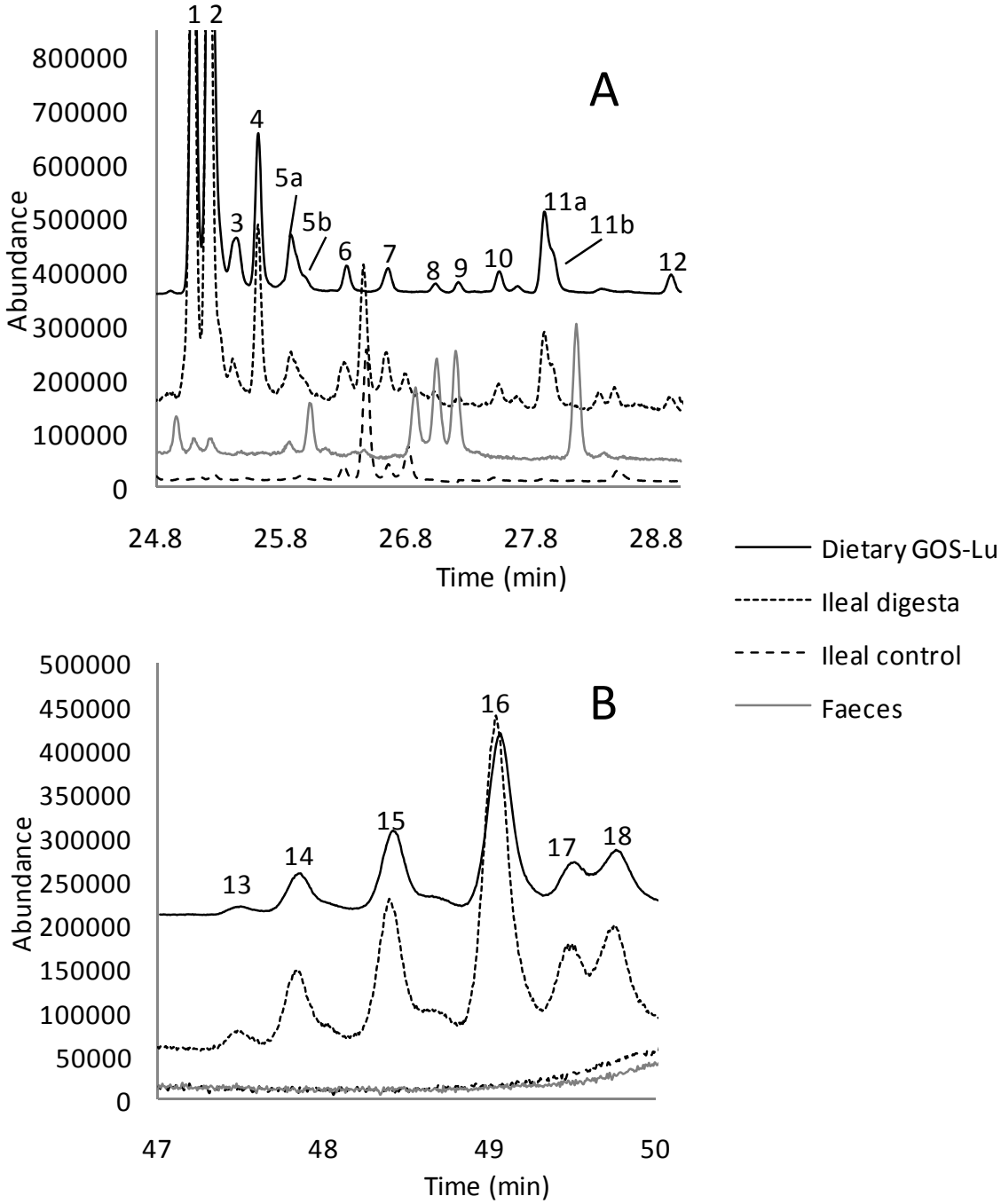

Figure 4.34. Representative GC-MS profiles of TMS oxime of di- (A) and trisaccharides (B) present in dietary GOS-Lu, as well as in ileal and faecal samples of rats fed GOS-Lu. Peak numbers correspond to the carbohydrates indicated in Table 2.

Table 4.25. Apparent ileal digestibility, retention indices (I^T) and tentative structural identification of galacto-oligosaccharides (di- and trisaccharides) of GOS-La.

Peak number*	Disaccharides	*IT*	Individual Ileal digestibility (%)	Ileal digestibility[c] (%)
1	Gal-(1→1)-Gal or Gal-(1→1)-Glc	2685	-	
2	Gal-(1→4)-Glc *E* + Gal-(1→3)-Glc *E*	2699	-	
3	unknown	2701	-	
4	Gal-(1→4)-Glc *Z*+ Gal-(1→4)-Gal *E*	2709	-	
5	Gal-(1→3)-Glc *Z* + unk	2727	-	0.0[a]
6	Gal-(1→2)-Glc *E*	2736	-	
7	Gal-(1→2)-Glc *Z*	2765	-	
8	Gal-(1→6)-Glc *E*	2824	-	
9	Gal-(1→6)-Glc *Z*	2868	-	
	Trisaccharides			
10	(1→1)[b]	3661	6	
11	unknown	3675	47.7	
12	unknown	3711	22.6	
13	unknown	3723	99.5	
14	unknown	3755	100	
15	unknown	3766	30.2	
16	Gal-(1→4)-Gal-(1→4)-Glc *E*	3775	29.5	
17	Gal-(1→6)-Gal-(1→4)-Glc *E* + Gal-(1→4)-Gal-(1→2)-Glc	3794	77	
18	Gal-(1→4)-Gal-(1→4)-Glc *Z* + unk	3801	77.9	52.9
19	unknown	3811	100	
20	Gal-(1→6)-Gal-(1→2)-Glc 1 + Gal-(1→4)-Gal-(1→2)-Glc 2	3826	0	
21	unknown	3862	54.1	
22	unknown	3880	100	
23	Gal-(1→6)-Gal-(1→6)-Glc 1	3889	81.3	
24	unknown	3963	25.3	
25	unknown	3997	30.4	
26	unknown	4012	29.7	

Gal: galactose; Glc: glucose.

*Labelled peaks are described in Figure 2.

[a]Disaccharides were not present in GOS-La diet.

[b]Non-identified monomers.

[c]Calculated as ileal digestibility of the whole fraction of di- and trisaccharides.

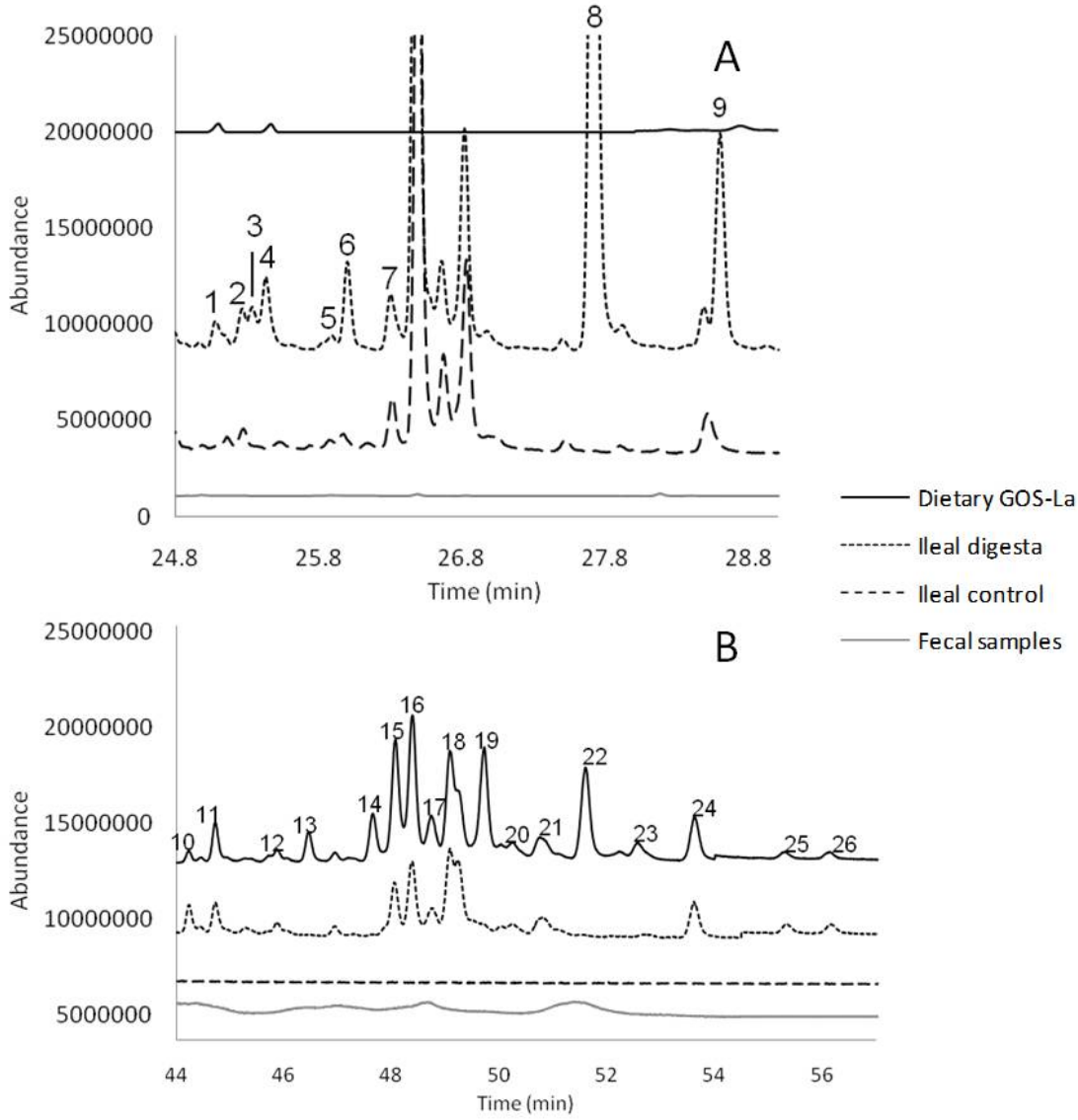

Figure 4.35. Representative GC-MS profiles of TMS oxime of di- (A) and trisaccharides (B) present in GOS-La, as well as well as in ileal and faecal samples of rats fed GOS-La. Peak numbers correspond to the carbohydrates indicated in Table 4.3.10.

- *Effect of GOS on fecal microbiota composition*

Quantitative PCR was used to monitor the modulatory effect of GOS-Lu and GOS-La on fecal microbiota of growing rats during 14 d of treatment. In all treatments, including the control group, bifidobacteria populations from fecal samples of rats increased throughout the experimental period (0-14 d), and a significantly ($P<0.05$) stronger bifidogenic effect was observed in rats fed GOS-Lu compared to those fed GOS-La at the

end of the experimental period (**Table 4.26**). A significant (P<0.05) increase of lactobacilli population in fecal samples of rats fed GOS-La was also shown. In addition, a significant increase in the growth of *Eubacterium rectale/Clostridium coccoides* group was observed by qPCR in response to the dietary treatment with GOS-Lu or GOS-La.

In order to obtain a general quantitative measure of the prebiotic effect, a selectivity index (SI) was calculated for the different treatments. The SI represents a comparative relationship between the growth of `beneficial´ bacteria, including bifidobacteria, lactobacilli and *Eubacterium rectale*, and that of `undesirable´ ones, such as bacteroides and clostridia, in relation to the change in the total number of bacteria (Palframan, et al., 2003). According to the SI, no prebiotic effects were observed after 7d of ingestion of GOS-La or GOS-Lu (**Figure 4.36**). For both GOS, significantly (P<0.05) higher SI values in fecal samples after 14 d of treatment in comparison with the control were observed. GOS-Lu gave place to the highest SI value (1.30 ± 0.02), with that of GOS-La being 1.19 ± 0.04, after 14 d of treatment.

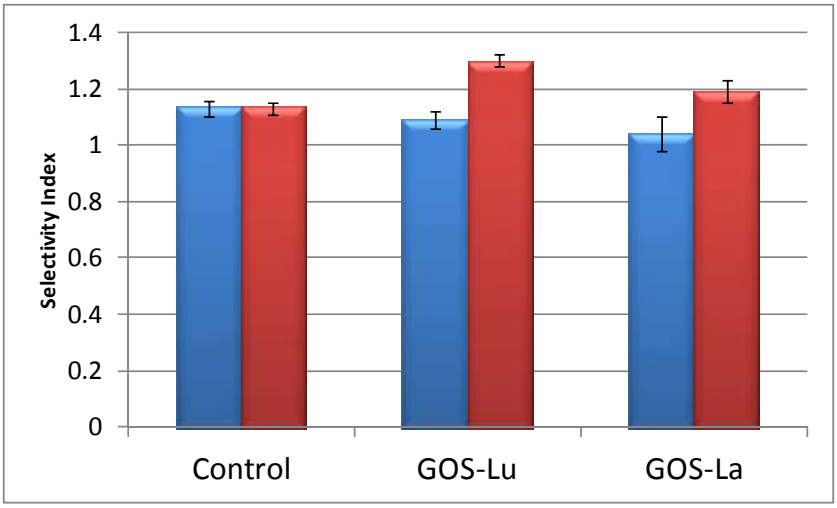

Figure 4.36. Selectivity index (SI) scores from fecal samples of growing rats fed control, GOS-Lu and GOS-La diets for 7 d (white bars) and 14 d (black bars). Data are means of three samples, each having three technical replicates. Means not sharing superscript letters differ significantly (P<0.05; MSD test). Error bars show standard deviations.

Table 4.26. The effect of dietary treatment (control, GOs-Lu and GOS-La) on fecal microbiota composition in growing rats fed for 14 days. Data are means of three samples, each having three technical replicates, expressed as $\log_{10}$ copy number/g of fecal sample.

	Diets			Time (days)			Pooled SEM	Two-way ANOVA*		
Bacterial Groups	**Control**	**GOS- Lu**	**GOS- La**	**0**	**7**	**14**		**Diets**	**Time**	**Interaction**
All bacteria	10,542^{a}	10,742^{b}	10,757^{b}	10,590^{A}	10,568^{A}	10,881^{B}	0.007	<0.001	<0.001	NS
Bacteroides	10,573^{a}	10,681^{b}	10,867^{c}	10,705^{A}	10,612^{B}	10,805^{C}	0.007	<0.001	<0.001	NS
Bifidobacteria	9,819^{a}	10,508^{b}	10,064^{c}	9,682^{A}	10,062^{B}	10,646^{C}	0.024	<0.001	<0.001	NS
Lactobacilli	9,635^{a}	9,777^{b}	10,050^{c}	9,518^{A}	9,765^{B}	10,179^{C}	0.006	<0.001	<0.001	NS
Eubacterium rectale / Clostridium coccoides group	9,472^{a}	10,049^{b}	10,146^{b}	9,591^{A}	9,834^{B}	10,243^{C}	0.019	<0.001	<0.001	NS
Clostrodium leptum subgroup	9,328^{a}	9,476^{b}	9,624^{c}	9,574^{A}	9,382^{B}	9,473^{C}	0.007	<0.001	<0.001	NS

$^{a,b,\ A,B}$Within each set of treatments (diet and treatment time) and within each row, mean values bearing a different superscript letter differ significantly (n=3; MSD test; $P<0.05$).

*Significance main effects (diet and treatment time) were determined by GLM REP (General Linear Model by Two-way Repeated Measures ANOVA).

NS: non-significant.

4.3.3.1.4. Discussion

It is generally accepted that the major beneficial effects of prebiotic carbohydrates occur in the large intestine due to the slow transit of the substrates to be fermented and their incidence on microbiota diversity which plays an important role in host health. In order to exert a positive effect on gut health, prebiotic carbohydrates should be resistant, at least to some extent, to the extreme conditions (acidic environment and action of hydrolytic enzymes) of the upper digestive tract to reach, in physiologically relevant amounts, the large intestine where they should be as much persistent as possible (Gibson, 2004, Roberfroid, et al., 2010). Several studies have demonstrated a modulatory effect of commercially available GOS-La on fecal microbiota of healthy human volunteers (Bouhnik, et al., 2004, Depeint, et al., 2008, Ito, et al., 1993) whereas others have failed to show any significant effect (Satokari, et al., 2001, Smiricky-Tjardes, et al., 2003). Interestingly, the administration to human volunteers of a GOS-La mixture containing mainly β 1→3, as well as β 1→4 and β 1→6 linkages proved to have a better bifidogenic effect than a commercially available GOS-La mixture consisting of GOS having β 1→4 and β 1→6 linkages only (Depeint, et al., 2008). These dissimilarities on the selective growth of bifidobacteria could be attributed to several factors, including resistance to hydrolysis and/or fermentation selectivity of dietary oligosaccharides. More recently, *in vitro* studies performed with human fecal slurries or pure cultures have established that GOS-Lu are also able to exert a positive effect on growth rates of several strains of *Bifidobacterium* and/or *Lactobacillus* (Cardelle-Cobas, et al., 2011, Cardelle-Cobas, et al., 2009), suggesting their potential as novel prebiotic ingredients.

While most of the research interest on prebiotics has focused on their role as modulators of intestinal microbiota, scarce efforts have been made on small intestinal resistance of dietary GOS, one of the three major criteria to determine its prebiotic potential (Bouhnik, et al., 2004, Roberfroid, 2007). Indeed, the elevated complexity of the samples still remain a challenge in analytical chemistry, and this is clearly increased when biological samples are evaluated. Even if there are suggestions that GOS-La reach the large intestinal sections intact, several studies have revealed their susceptibility to partial hydrolysis (Roberfroid, 2007); in addition, no data regarding the *in vivo* digestibility of GOS-Lu had been reported yet. In the present study, we have demonstrated that GOS-La

and GOS-Lu are resistant to *in vivo* digestion at different extent, being the former much more susceptible to hydrolysis (**Tables 4.24 and 4.25**). Di- and trisaccharides resistant to digestion in the upper gastrointestinal tract were completely fermented by intestinal microbiota, as none were found in fecal samples (**Figures 4.34 and 4.35**).

GOS, either produced from lactose or lactulose, are constituted by a very complex mixture of carbohydrates, differing in the linkage type, number and order of monomers in the oligosaccharide chain. Galacto-oligosaccharides derived from lactose and lactulose usually comprise oligomers of different chain lengths with a terminal glucose or fructose, respectively. According to our results, these structural differences seem to have a significant impact on the susceptibility to the *in vivo* gastrointestinal digestion and, as a result, in their potential as prebiotic carbohydrates. Thus, GOS-Lu trisaccharides were more resistant to hydrolysis than those derived from GOS-La (**Tables 4.24 and 4.25**). Several intestinal brush border enzymes are able to catalyze the hydrolysis of glycosidic linkages, such as amylases and sucrases; in addition, other glycolytic enzymes such as isomaltases and β-glycosidases can also contribute to the digestion of dietary carbohydrates (Goodman, 2010). Considering the structures present in GOS-La trisaccharides, it is likely that β-glycosidases present in the brush border of the small intestine hydrolyze glycosidic linkages between galactoses and glucoses. In this study, we have demonstrated the susceptibility to hydrolysis of 4´-galactosyl-lactose (Gal-(1→4)-Gal-(1→4)-Glu) (peaks 16 and 18, **Table 4.25**), as previously reported *in vitro* by Ohtsuka *et al*. (1990). These authors showed partial digestibility of this thisaccharide after treatment with a homogenate of small intestinal mucosa from rats, which could be attributed to the presence of lactase (β-galactosidase). The susceptibility to hydrolysis of GOS-La having DP ≥ 3 was also confirmed by the presence of derivative disaccharides in ileal samples (**Figure 4.35 A**); likewise, the abundance of Gal-(1→6)-Glc and Gal-(1→2)-Glc disaccharides in ileal samples pointed out the high resistance of these glycosidic linkages to extreme conditions of the upper digestive tract (peaks 6, 7, 8 and 9, **Table 4.24 and Figure 4.23 A**). In addition, a partial and selective fermentation of GOS-La by the small intestinal microbiota may also occur and could explain the reported growth stimulation of probiotic bacteria in the ileum of weaned piglets after administration of several prebiotic carbohydrates, including GOS-La (Konstantinov, et al., 2004).

GOS-Lu disaccharides were highly resistant to the acidic environment and enzymatic digestion in the upper digestive tract (**Table 4.24**). Contrary to other reported prebiotics, disaccharides from GOS-Lu demonstrated a higher resistance to hydrolysis when compared to trisaccharides. A plausible explanation for this differential behaviour could be the role that both monomer and linkage type may play on the resistance to the action of the digestive enzymes. Thus, the higher digestibility of GOS-Lu trisaccharides as compared to disaccharides could be attributed to the higher susceptibility to the action of hydrolytic enzymes of some specific linkages between galactose residues, in a similar way to those contained in the 4´-galactosyl-lactose (Gal-(1→4)-Gal-(1→4)-Glc), than those involved in the formation of galactosyl-fructoses. In this context, it is known that lactulose is not hydrolyzed or absorbed in the small intestine, being fermented selectively by the colonic microbiota(Bouhnik, et al., 2004, Olano, et al., 2009). GOS-Lu disaccharides are mostly constituted by β-galactobioses and galactosyl-fructoses (**Section 4.1.3**). Therefore, in a similar way to lactulose, it is very plausible that other galactosyl-fructoses, such as those contained in peaks 4, 5b, 8, 9, 10, 11b, 15, 16 and 18 (**Table 4.24**), are quite resistant to digestion within the mammalian gastrointestinal system, which confers them the ability to reach the large intestine intact as fermentable substrates for the resident intestinal microbiota.

In this study, we have also evaluated the impact of GOS derived from lactose or lactulose, with significant differences on their major components (**Tables 4.24 and 4.25**), on fecal microbiota of growing rats. Interestingly, novel GOS-Lu exerted a stronger bifidogenic effect on fecal microbiota than GOS-La, although the latter still showed a significant increase in lactobacilli population, compared to the control (**Table 4.26**). These results are in good agreement with previous findings reported by Cardelle-Cobas *et al.* (Cardelle-Cobas, et al., 2009) who found a significant *in vitro* bifidogenic effect in human fecal slurry cultures following fermentation with GOS-Lu and, in a lesser extent, GOS-La. It has been suggested that variation in daily dose may contribute to differences in the modulatory effect on intestinal microbiota. A GOS-La administrated at doses of 5 g or higher was bifidogenic as observed in fecal samples of human healthy volunteers, whereas a dose of 2.5 g showed no significant effect (Davis, et al., 2010). These authors suggested that a minimum or `threshold´ dose may exist below which a bifidogenic effect

is not observed. Consequently, it seems likely that GOS having an extended digestion in the upper digestive tract will have a stronger dose-dependent effect on their ability to modulate the intestinal microbiota, being higher doses necessary for such effect. We suggest that the amounts of GOS able to reach the large intestine are dependent of their structural characteristics and, therefore, may influence on their potential prebiotic effects. Our data demonstrate that a detailed chemical characterization and studies of digestibility using *in vivo* model systems, supporting human intervention studies, are relevant for dietary recommendations, helping us to determine criteria to develop novel prebiotics.

In spite of rapid research advances in gut microbial ecology, the systematic understanding of this complex ecosystem and the microbial interactions are still limited. Our microbiological data reflect the effects of GOS-Lu and GOS-La at group level only. Not all bifidobacteria are likely able to utilize or compete for these prebiotics. Regarding this relevant question, further studies are in progress in order to investigate which type/species of bifidobacteria are selectively affected by these prebiotics in the large intestine. Besides, fecal microbiota analysis revealed that not only the stimulation on growth of bifidobacteria or lactobacilli occurred, being the *E. rectale/C. coccoides* group stimulated by both GOS-Lu and GOS-La (**Table 4.26**). An increase in population of *E. rectale/C. coccoides* has been reported *in* human intervention studies after treatment with FOS (Langlands, et al., 2004). These bacteria are known to produce relatively high amounts of butyrate (Barcenilla, et al., 2000) which could exert a protective role in protection against inflammatory bowel disease and colorectal cancer (Hague, et al., 1997). Although its physiological relevance needs to be more deeply investigated, some authors have claimed that the prebiotic concept may be expanded towards other genera, including *Eubacterium* and *Roseburia* (Roberfroid, et al., 2010).

In conclusion, GOS-La and novel GOS-Lu were incorporated in a single dose (1%, wt:wt) to rodents for a period of fourteen days. Under such conditions, they have demonstrated to meet the three main criteria that a food ingredient must satisfy to be considered as prebiotic (Gibson, et al., 2004, Roberfroid, 2007): (*i*) resistance, at least to some extension, to gastric acidity and hydrolysis by mammalian enzymes and gastrointestinal absorption; (*ii*) substrate of fermentation by intestinal microorganisms;

and (*iii*) selective stimulation of the growth of intestinal bacteria associated with health and wellbeing. Our findings revealed that GOS-Lu had a higher resistance to *in vivo* gastrointestinal digestion and absorption in the small intestine, likely due to the presence of a fructose residue in the reducing end of the oligosaccharides. The partial digestion of GOS-La trisaccharides suggested that glycosidic linkages Gal-(1→6)-Glc and Glc-(1→2)-Glc are more resistant to *in vivo* gastrointestinal digestion than linkage type Gal-(1→4)-Gal. The absence of resistant GOS in fecal samples indicated that oligosaccharides served as fermentation substrates in the large intestine. As a result, a stronger bifidogenic effect was observed in fecal samples of rats fed GOS-Lu as compared with those fed GOS-La. Interestingly, a significant increase on population of *E. rectale/C. coccoides* group after treatment with GOS-Lu or GOS-La was also revealed. To the best of our knowledge, this work provides the first evidence of the *in vivo* prebiotic effects of GOS-Lu indicating that these novel oligosaccharides could have the ability to reach the large intestine in physiologically relevant doses due to their low digestibility. Further *in vivo* studies addressing the effect of GOS-Lu on the selective growth of the intestinal microbiota are currently underway.

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4.3.3.3. Galacto-oligosacáridos derivados de lactulosa multifuncionales con actividad inmunomoduladora y prebiótica

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Basado en una parte de la patente española, N. de solicitud: 201130784; Fecha de prioridad: 16 de mayo de 2011

4.3.3.4.1. Introducción

El estado fisiológico del intestino grueso, y en particular del colon, tiene una gran importancia para la salud humana debido en gran parte a las actividades metabólicas de la microbiota que lo coloniza. Un gran número de enfermedades (diarrea, colitis ulcerosa, inflamación, cáncer, etc.) están relacionadas con alteraciones en la composición de la microbiota del colon. Además, es bien conocido que cepas de los géneros *Bifidobacterium* y *Lactobacillus* pueden ejercer efectos beneficiosos sobre estos trastornos mediante la modulación de las funciones fisiológicas, metabólicas e inmunológicas del hospedador. Desde que hace más de una década se introdujera el concepto de "prebiótico", referido a oligosacáridos resistentes a la digestión gastrointestinal que afectan beneficiosamente al individuo al estimular selectivamente el crecimiento y/o actividad de especies bacterianas beneficiosas en el intestino (Gibson y col., 1995), existe un considerable interés en su utilización como ingredientes alimentarios como respuesta a la alta demanda existente en el mercado de alimentos saludables y complementos alimentarios. Por ello, la investigación en este campo se ha centrado en el desarrollo y caracterización de nuevos prebióticos que cumplan los requisitos necesarios para ser considerados como tales. Estas propiedades se basan en ser resistentes a la digestión gastrointestinal, la capacidad de ser fermentables y favorecer selectivamente el crecimiento y/o actividad de especies bacterianas beneficiosas en el colon (fundamentalmente *Bifidobacterium* spp. y *Lactobacillus* spp.) con el objetivo final de mejorar la salud gastrointestinal.

Los principales ingredientes prebióticos disponibles comercialmente en el mercado se basan en los siguientes carbohidratos: i) lactulosa (isómero de la lactosa) que

es probablemente el primer carbohidrato utilizado como factor bifidogénico para el consumo human (Méndez y col., 1979); ii) galactooligosacáridos (GOS) obtenidos tras un proceso de transgalactosilación enzimática a partir de la lactosa; iii) fructooligosacáridos (FOS) producidos mediante la acción enzimática de la fructosiltransferasa sobre la sacarosa; y iv) oligofructosas obtenidas a partir de la hidrólisis parcial de la inulina.

Para evaluar la actividad prebiótica de carbohidratos no digeribles se consideran una serie de aspectos que incluyen: cambios cuantitativos en la población microbiana, formación de productos finales de fermentación, así como la velocidad de fermentación. No obstante, se cree que gran parte de los carbohidratos prebióticos disponibles comercialmente hasta el momento fermentan rápidamente en el intestino grueso de tal modo que su acción tiene lugar principalmente en las zonas proximales (McBain y col., 2001; Rastall y col., 2000; Tzortzis y col., 2005). Estos resultados explican en parte el gran interés actual que existe por disponer de una "segunda generación" de nuevos ingredientes prebióticos que posean una serie de propiedades como:

i) ser activos a dosis bajas;

ii) no presentar efectos secundarios;

iii) tener una velocidad de fermentación más lenta, de modo que puedan presentar una mayor persistencia en el intestino grueso y, especialmente, en el colon ya que es esta región intestinal la que presenta una mayor incidencia de patologías digestivas y oncológicas (Roberfroid, 2007);

iv) presentar una gran selectividad en cuanto al control de la microbiota intestinal (por ejemplo, poder ser metabolizados por bifidobacterias específicas);

v) poseer actividad biológica adicional ejerciendo efectos beneficiosos sobre funciones fisiológicas específicas y/o reduciendo el riesgo de enfermedad, por ejemplo a través de su efecto sobre el desplazamiento de patógenos y la regulación de la función del sistema inmunitario (Gibson y col., 2005).

La mejora de las propiedades de los prebióticos comercializados, de modo que favorezcan de forma más selectiva el crecimiento y actividad de microorganismos beneficiosos (*Bifidobacterium* spp. y *Lactobacilus* spp.) en el colon, sería muy ventajoso

para reforzar su función fisiológica en el epitelio intestinal y sistema inmunológico asociado al intestino y, así, mejorar las defensas del hospedador frente a microorganismos patógenos. En esta línea, se están realizando esfuerzos importantes dirigidos a la identificación de oligosacáridos prebióticos con bioactividades añadidas, aunque por el momento los resultados obtenidos han tenido un éxito limitado (Ouwehand y col., 2005). A modo de ejemplo, una mezcla de GOS comerciales derivados de lactosa mostró tener afinidad por receptores de membrana de microorganismos comensales como *Escherichia coli*; aunque este hecho ocasiona una menor interacción y adhesión con células intestinales (Caco-2) y hepáticas (HepG2) de origen humano (Shoaf y col., 2006), además es necesario emplear elevadas concentraciones del prebiótico, lo que dificulta que su uso en la práctica tenga efectos fisiológicamente relevantes (Macfarlane, et al., 2008).

Teniendo en cuenta estos antecedentes, se han desarrollado nuevos productos basados en mezclas de ingredientes prebióticos con bacterias probióticas (Longoni y col., 2011; Mogna y col., 2009) y/o compuestos con propiedades inmunológicas (Di Pierro, 2009) con el objetivo de reforzar la salud gastrointestinal del consumidor. Sin embargo, estos productos basados en múltiples ingredientes suelen presentar un coste elevado en el mercado y, además, en ciertos casos es difícil garantizar la supervivencia de determinadas bacterias probióticas en matrices alimentarias, así como tras el proceso de digestión. Por tanto, la producción de carbohidratos prebióticos con funciones beneficiosas añadidas presentaría un potencial interés para la industria alimentaria y/o farmacéutica.

Por otra parte, recientemente se han sintetizado una serie de nuevos galactooligosacáridos obtenidos por transgalactosilación a partir de la lactulosa empleando β-galactosidasas procedentes de *Kluyveromyces lactis* (Martínez-Villaluenga y col., 2008), o *Aspergillus aculeatus* (Cardelles-Coba y col., 2008a), así como a partir de la isomerización con aluminato sódico de los galactooligosacáridos obtenidos a partir de lactosa con la β-galactosidasa de *Kluyveromyces lactis* (Cardelles-Coba y col., 2008b). Posteriormente, un estudio *in vitro* realizado con heces humanas ha mostrado el potencial poder bifidogénico de estos nuevos galactooligosacáridos (Cardelles-Coba y col., 2009), aunque en este estudio ni se determinó el patrón de carbohidratos obtenidos tras

transgalactosilación ni se evaluó el efecto de los mismos sobre otros grupos bacterianos presentes en la microbiota intestinal y que pueden tener, igualmente, un efecto en la salud gastrointestinal del individuo. No obstante, para explorar la posible aplicación de estos nuevos GOS como carbohidratos prebióticos es necesario estudiar el perfil de oligosacáridos obtenido, su digestibilidad (íntimamente relacionada con su composición estructural), así como su capacidad para modular la microbiota intestinal con ensayos *in vivo* y su efecto sobre la biodiversidad de grupos bacterianos beneficiosos para la salud. Asimismo, y con el objetivo de satisfacer la demanda actual de prebióticos de segunda generación, sería conveniente evaluar posibles bioactividades añadidas no estudiadas hasta el momento como su capacidad de interacción con bacterias probióticas y/o inmunomoduladora.

La patente citada anteriormente se refiere a la utilización de galacto-oligosacáridos derivados de la lactulosa (GOS-Lu) con propiedades prebióticas mejoradas y adicionales a las de los prebióticos comerciales utilizados habitualmente. En este apartado resumimos únicamente los datos referentes a la evaluación de la actividad inmunomoduladora y la actidad prebiótica *in vivo*, ya que en las anteriores secciones se ha abordado el resto de resultados que forman parte de dicha patente.

4.3.3.4.2. Estudio *in vivo* de la actividad inmunomoduladora

En el estudio se utilizaron ratas Wistar macho en crecimiento divididas aleatoriamente (n=15) en cuatro grupos a las que se suministró respectivamente i) la dieta control, ii) la dieta control + 1 % GOS-Lactulosa + 0.2 % Cr_2O_3 (marcador no digestible), iii) la dieta control + 1 % carbohidratos prebióticos comerciales (GOS-Lactosa)+ 0.2 % Cr_2O_3, iv) la dieta control + 1 % Lactulosa + 0.2 % Cr_2O_3 durante un período de 14 días con una ingesta diaria de 14 gramos. Una vez finalizado el periodo de tratamiento los animales se anestesiaron (40 mg pentobarbital sódico / kg peso corporal) para llevar a cabo la obtención de sangre mediante punción intracardiaca que se congeló rápidamente en nitrógeno líquido, y la disección de tejido intestinal, cuyas secciones se recogieron en un medio crioprotector para el RNA y se congelaron inmediatamente en nitrógeno líquido.

En las muestras homogeneizadas (Tissue lyser, Qiagen) se extrajo el contenido total de RNA utilizando un kit comercial (RNA mini kit, Qiagen) para este fin, de acuerdo a las instrucciones del fabricante. A partir de alícuotas (1 µg) de RNA se obtuvo el cDNA mediante reacción de la transcriptasa reversa (AMV Reverse Transcriptase, Promega). Posteriormente, con técnicas de PCR en tiempo real se monitorizó los cambios en la expresión (mRNA) de biomarcadores del control homeostático de la inflamación y respuesta inmune. Los biomarcadores monitorizados incluyeron interleucinas (IL) pleiotrópicas (IL-6, -10 y -15), el factor de transcripción nuclear kappa B (NFkB) y proinflamatorios como el de necrosis tumoral alfa (TNFα), para los cuales se diseñaron secuencias cebadoras (primer) utilizando las bases de datos del GenBank (NCBI).

Los resultados obtenidos (**Figura 4.37**) ponen de manifiesto que la admininistración de los GOS-Lactulosa induce la expresión de IL-6 e IL-10, lo que puede favorecer la regulación de los procesos inflamatorios a nivel intestinal. La inclusión de los GOS-Lactulosa en la dieta de los animales causó una mayor expresión del NFκB, implicado en diversos procesos de señalización intracelular, crecimiento y supervivencia celular. La cuantificación (ELISA) de TNFα en muestras de sangre periférica confirmó que la inclusión de los GOS-Lactulosa en la dieta de los animales no altera los parámetros basales de este marcador de activación de la inflamación con respecto al grupo control.

Tomando estos resultados en conjunto con los datos *in vivo* sobre la modulación de la flora intestinal (**Sección 4.3.10**), se podría considerar a los GOS-Lactulosa como ingredientes multifuncionales dado que la citada actividad inmunomoduladora se produce preferentemente al aumentar la población de bifidobacterias y/o inducir la expresión de biomarcadores implicados en el control homeostático de la inflamación y/o activación del sistema inmunológico, observándose un efecto positivo de los oligosacáridos en el aumento de ARN (mensajero) de moléculas reguladoras de los procesos de inflamación y activación de la respuesta inmune.

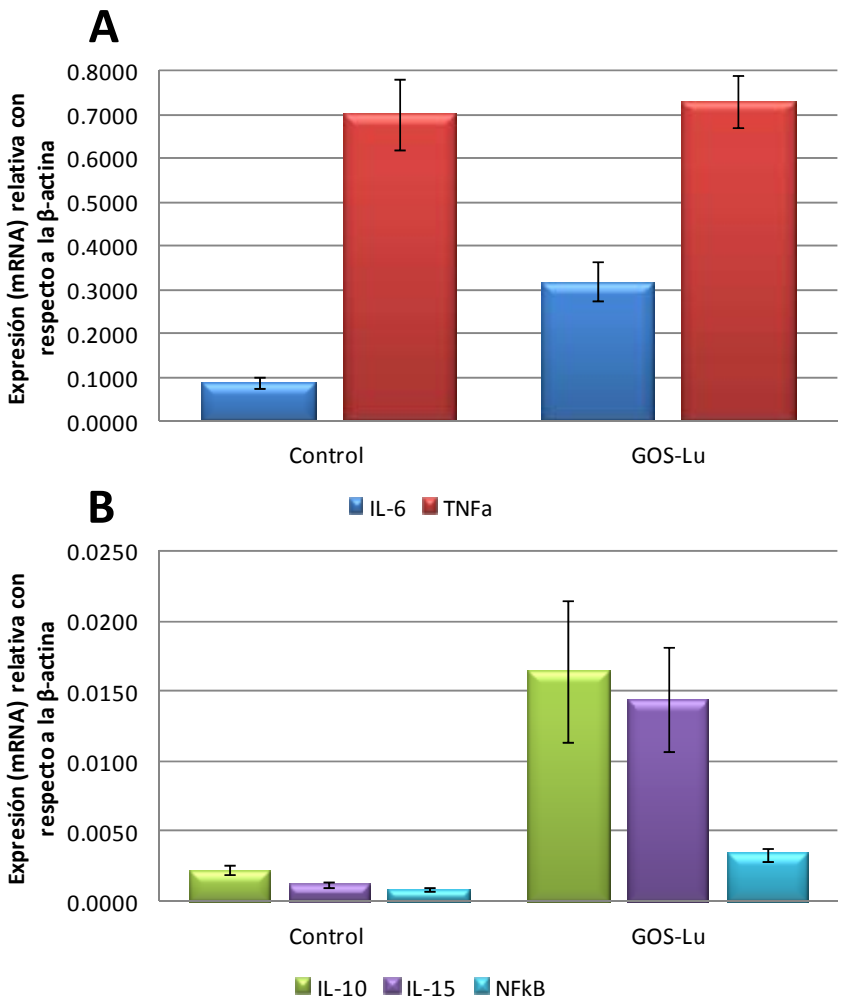


Figura 4.37 Efecto de los GOS-Lactulosa en la expresión (mRNA) de marcadores inmunológicos en secciones de íleon de los animales de experimentación (ratas Wistar). Los resultados se muestran como expresión relativa respecto a β-actina.

4.3.3.4.3. Evaluación *in vivo* de la actividad prebiótica.

En los estudios de actividad prebiótica in vivo se utilizaron ratas Wistar macho en crecimiento divididas en cuatro grupos en las condiciones indicadas en el apartado anterior. El estudio del efecto de GOS-Lu en la modulación de las poblaciones bacterianas intestinales (bacterias totales, *Bacteroides* sp., *Lactobacillus* spp., *Bifidobacterium* spp., grupo de *Clostridium coccoides*/*Eubacterium rectale* y grupo de *Clostridium leptum*) se realizó mediante PCR cuantitativa (qPCR) en diferentes tramos del intestino (íleon, ciego y colon). Como ilustra la **Tabla 4.27** se encontró un efecto bifidogénico significativo en el

ciego del grupo de animales que ingirió GOS-Lu respecto al control, mientras que no se observó incremento alguno en bifidobacterias en el grupo que consumió GOS-La. Por otra parte, también pudo observarse un aumento significativo en el porcentaje de bifidobacterias respecto a bacterias totales en ciego y colon del grupo de animales que consumió GOS-Lu (**Tabla 4.28**). Además, cabe destacar un aumento significativo de bacterias beneficiosas correspondientes al grupo *Clostridium coccoides*/*Eubacterium rectale*, con relevancia en salud gastrointestinal.

Tabla 4.27. Efecto de la dieta control y dietas experimentales (GOS-Lu y GOS-La) sobre la composición microbiana en el ciego de ratas en crecimiento alimentadas durante 14 días.

	Dietas		
Grupos bacterianos (log_{10} número de copias / g de digesta intestinal)	Control	GOS-Lu	GOS-La
Bacterias totales	8.511[a]	8.650[b]	8.609[ab]
Bacteroides	7.37	7.543	7.599
Bifidobacterias	6.207[a]	6.977[b]	6.499[ab]
Lactobacillus	6.520[ab]	6.459[ab]	6.942[b]
Grupo *Clostridium coccoides* / *Eubacterium rectale*	6.711[a]	7.075[b]	7.114[b]
Subgrupo *Clostrodium leptum*	6.377	6.331	6.34
Enterobacterias	6.921[a]	6.971[a]	6.310[b]

[a,b] Dentro de cada fila, los valores medios con diferente superíndice difieren significativamente (n=10; test MSD; P<0.05).

Tabla 4.28. Efecto de la inclusión de prebióticos (Lactulosa, GOS-Lactulosa y GOS-lactosa) sobre el porcentaje de Bifidobacterias respecto a bacterias totales en digesta intestinal (íleon, ciego y colon) en ratas en crecimiento tras tratamiento durante 14 días.

Sección Intestinal	Control	GOS-Lu	GOS-La
Ileon	0.20	3.10	0.89
Ciego	3.32[a]	14.64[b]	3.36[a]
Colon	4.14[a]	11.86[b]	3.69[a]

[a,b] Dentro de cada fila, los valores medios con diferente superíndice difieren significativamente

Es de destacar los estudios realizados relativos al efecto de los GOS-Lu sobre la biodiversidad intestinal del grupo de bifidobacterias comparándolos con la obtenida tras ingesta GOS-La. Tal y como muestra la **Figura 4.38**, un número significativamente superior de especies correspondiente al grupo de bifidobacterias respondieron a la fermentación con GOS-Lu, frente a prebióticos convencionales como los GOS-La. Como resultado, se observó un mayor enriquecimiento (número de bandas electroforéticas) en bifidobacterias presentes en ciego de ratas tratadas con GOS-Lu respecto a las tratadas con GOS-La y dieta control (**Tabla 4.29**). Los índices de diversidad poblacional de Shannon y Evenness mostraron valores superiores en el caso de muestras cecales provenientes de ratas alimentadas con GOS-Lu. Además, los estudios de similitud demostraron que la población de bifidobacterias de ciego en ratas alimentadas con GOS-Lu son muy diferentes respecto a aquellas alimentadas con lactulosa, GOS-La y dieta control (**Figura 4.39**).

Tomados en su conjunto, estos resultados muestran claramente el poder prebiótico superior de los carbohidratos objeto de la patente (GOS-Lu) frente a prebióticos comercialmente disponibles (GOS-La) bajo las mismas condiciones de experimentación. Por tanto, los GOS-Lu podrían ejercer un efecto prebiótico en un número mayor de individuos y a dosis más bajas que las empleadas para prebióticos ya comerciales

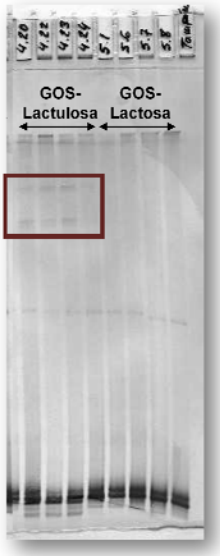


Figura 4.38. Gel de electroforesis en gradiente desnaturalizante (DGGE) de bifidobacterias aisladas de las muestras de ciego de los grupos de ratas que consumieron GOS-Lu y GOS-La**.**

Tabla 4.29. Grado de enriquecimiento (definido como número de bandas electroforéticas correspondientes al grupo de Bifidobacterias tras análisis de DGGE), así como los índices de Shannon y Evenness (relativos a la diversidad poblacional) de muestras procedentes de ciego de ratas tratadas con una dieta control, GOS-Lu o GOS-La tras 14 días de tratamiento.

Muestras	Enriquecimiento (número de bandas electroforéticas)	Índice de biodiversidad Shannon	Índice de biodiversidad Evenness
Control	9.25 (0.96)*	2.22 (0.10)	0.70 (0.03)
GOS-Lu	13 (0.82)	2.56 (0.07)	0.81 (0.02)
GOS-La	9.75 (0.5)	2.28 (0.05)	0.71 (0.02)

*Desviación estándar

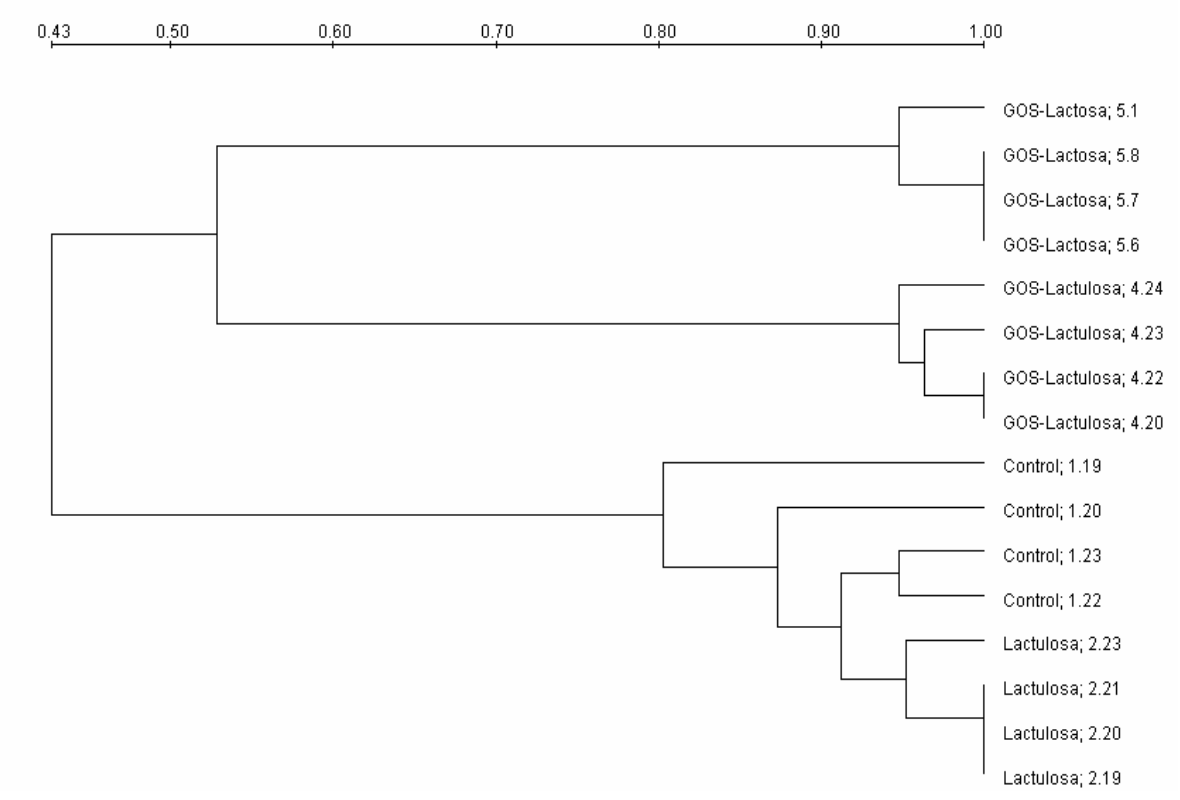


Figura 4.39. Dendograma de similitud de bifidobacterias obtenido tras análisis de DGGE de muestras procedentes de ciego de ratas tratadas con una dieta control, lactulosa, GOS-Lactosa o GOS-Lactulosa tras 14 días de tratamiento. En la parte superior de la figura queda representada la escala de similitud entre muestras.

5. DISCUSIÓN GENERAL

5. Discusión general

Como se ha comentado en secciones anteriores, el desarrollo de nuevos ingredientes alimentarios multifuncionales es un reto que actualmente desafía la Ciencia y Tecnología de Alimentos, fundamentado en la multidisciplinaridad necesaria para la producción de los mismos, que abarca desde la síntesis y/o extracción del ingrediente, pasando por su caracterización estructural y finalmente por pruebas de bioactividad, tanto *in vitro* como *in vivo*, para determinar su eficacia biológica. En esta tesis, se llevó a cabo este trabajo multidisciplinar orientado al estudio de nuevos ingredientes con propiedades prebióticas. Para esta finalidad fue necesaria tanto la obtención de oligosacáridos libres de conocidos carbohidratos digeribles como el desarrollo de nuevos métodos analíticos para la caracterización de los compuestos de interés, que superen las limitaciones de los ya existentes, y el empleo de diferentes técnicas microbiológicas que permitan evaluar su bioactividad.

Aunque los métodos analíticos desarrollados podrían ser aplicados a oligosacáridos y péptidos de diferentes estructuras (u orígenes) en esta tesis se empleó GOS-La, dado el interés industrial y económico existente en dichos productos por su incorporación a diversos alimentos presentes en el mercado actual, los recientemente sintetizados GOS-Lu cuyas propiedades prebióticas parecen prometedoras considerando las mostradas por los GOS-La y la lactulosa, y CMP por sus actividades funcionales ya descritas en la bibliografía.

En el caso de los GOS, el trabajo se inició con la evaluación de los métodos de fraccionamiento más apropiados para la obtención de oligosacáridos de diferente grado de polimerización que fueron empleados en estudios posteriores. Como se muestra en el artículo presentado en la **Sección 4.1.2** la selección de uno u otro tratamiento depende de la finalidad de la muestra presentando todos ellos ventajas e inconvenientes destacados. Así, las muestras de GOS-La y GOS-Lu se sometieron a diferentes tratamientos en función de la recuperación deseada de sus oligosacáridos. En el caso de los GOS-Lu, y dado el interés existente en la fracción de disacáridos que al igual que la lactulosa podían resultar no digeribles en el tracto digestivo y con potenciales propiedades bioactivas, el método de fraccionamiento elegido fue el

tratamiento con carbón activo empleando etanol 1% (v:v) para eliminar los monosacáridos seguido por etanol 50% (v:v) para recuperar los di- y oligosacáridos. Este tratamiento fue elegido por su rapidez y facilidad de manejo de muestra. Otro tratamiento útil hubiera sido el empleo de S. cerevisiae, ya que la lactulosa y sus isómeros no son fermentados por dichas levaduras (Ruiz-Matute, 2007), sin embargo la formación de trehalosa durante su metabolismo podría afectar a los posteriores estudios tanto analíticos como de bioactividad. Por su parte, la SEC, como se puede observar en los resultados mostrados en la **Sección 4.1.2** resulta una técnica muy útil para la separación de oligosacáridos en sus distintos DPs, aunque su empleo resulta algo tedioso y la muestra se recupera empleando grandes volúmenes de disolvente. En el caso de los GOS-La, para los que se requería la eliminación total de disacáridos tales como lactosa, digeribles en el tracto digestivo, ésta fue la técnica de elección, ya que los tratamientos anteriormente citados no permitían alcanzar dicho objetivo sin perder oligosacáridos de DP superior (incluido el tratamiento con carbón activo empleando etanol 10% (v:v) sugerido en dicha sección como apto para el estudio de GOS, en el que se perdían cantidades elevadas de trisacáridos (≈45%)).

Una vez obtenidas las muestras de interés resulta fundamental el desarrollo de métodos que permitan su caracterización. En esta tesis este objetivo se ha abordado por dos vías diferentes y complementarias, el empleo de GC y de HPLC ambas acopladas a MS. Dado que la GC-MS es una herramienta muy apropiada para el análisis de di- y trisacáridos debido a su sensibilidad y capacidad de resolución, el estudio detallado de los espectros de masas de dichos compuestos de GOS-Lu, fracciones mayoritarias, ha permitido su caracterización detallada, no descrita hasta el momento en la bibliografía, determinándose de forma tentativa sus estructuras químicas (**Sección 4.1.3**). Dicho método se aplicó también a los GOS-La comerciales cuyas estructuras habían sido ya descritas por otros autores empleando una combinación de diferentes técnicas (RMN, HPAEC-MS, SEC, etc.; Coulier y col. 2009). Los resultados obtenidos del análisis por GC-MS de estos compuestos se recogen en la **Sección 4.3.3.1** y resultaron de gran utilidad para el estudio de su digestibilidad empleando sistemas *in vivo* tal y como se comentará más adelante.

Ambas mezclas, GOS-La y GOS-Lu, se encuentran compuestas por carbohidratos de diferente peso molecular y distintos enlaces glicosídicos de abundancia variable y difieren ampliamente en la composición monomérica de dichos compuestos. En el caso de los GOS-La la mayor parte de los carbohidratos posee unidades terminales de glucosa mientras que en el caso de GOS-Lu son unidades terminales de fructosa. Esta diferencia estructural podría afectar en gran medida sus propiedades bioactivas, tal y como se mostrará en los estudios posteriores.

Por otra parte, la cromatografía de líquidos permite el análisis de oligosacáridos de mayor DP. La cromatografía de carbón grafitizado es uno de los modos de HPLC más útiles para el análisis de carbohidratos empleados en la actualidad (Koizumi, 2002), por lo que fue empleado para el análisis de GOS-Lu (**Sección 4.1.3**). Sin embargo, estudios llevados a cabo en el laboratorio (Brokl y col., 2011) demostraron que aunque la cromatografía de carbón grafitizado fue útil para la separación de isómeros de oligosacáridos, la separación entre distintos DPs era limitada, siendo la HILIC una técnicas más apropiada para tal fin. Por tanto, considerando estos estudios preliminares y dadas las ventajas que proporciona la HILIC para el análisis de compuestos polares tales como los carbohidratos y su posibilidad de acoplamiento a MS, se seleccionó esta técnica para la optimización y validación de un método apropiado que permitiera la caracterización de mezclas tan complejas como los GOS-La, de las que se dispone de una muy limitada información estructural (**Sección 4.1.4**). El método desarrollado empleando la columna BEH acoplada a MS^n sería también de gran utilidad en un futuro para el análisis de los oligosacáridos desconocidos de los GOS-Lu.

La información obtenida sirvió para llevar a cabo una correlación entre la estructura de los GOS y sus posibles efectos biológicos, tal y como se discutirá más adelante; esta relación estructura-función ya ha sido previamente descrita (Rastall y col., 2005), lo cual afianza la necesidad de un análisis estructural de posibles nuevos carbohidratos prebióticos, previo a sus análisis de bioactividad.

Los estudios de bioactividad abarcaron desde el empleo de cultivos puros y mezclas fecales en sistemas *in vitro* hasta el estudio del efecto *in vivo*. En la bibliografía

ya existían diversos estudios que demostraban los efectos positivos de los GOS-La para el crecimiento selectivo de bacterias beneficiosas, sin embargo los estudios llevados a cabo en este trabajo han puesto de manifiesto la mayor resistencia a las condiciones gastrointestinales de Lactobacillus crecidos empleando estos carbohidratos y lo que es más relevante, su cierta digestibilidad ileal en modelos de ratas Wistar.

En el caso de los GOS-Lu, los estudios existentes hasta el momento eran más limitados y referidos únicamente a su poder bifidogénico *in vitro*. Los efectos de los GOS-Lu en el crecimiento de Lactobacillus y en la resistencia de los mismos a las condiciones gastrointestinales fueron positivos, sin embargo, su diferencia estructural con respecto a los GOS-La no afectó a dichas propiedades. Estos resultados están de acuerdo con los obtenidos con bacterias fecales humanas, donde no se observaron diferencias entre los GOS-Lu y los GOS-La en cuanto a crecimiento bacteriano, pero si un marcado efecto bifidogénico de ambas mezclas. Sin embargo, las diferencias *in vivo* fueron notables, observándose una menor digestibilidad para los GOS-Lu que además presentaron un mayor índice de selectividad. Este efecto positivo de los GOS-Lu, sobre los GOS-La, se ve aumentado por la capacidad inhibitoria de estos compuestos en la producción de citoquininas pro-inflamatorias y la capacidad de modular la antiadherencia de patógenos en células intestinales. Con todos estos resultados, se evidencia que los GOS-Lu, poseen un efecto beneficioso adicional en comparación a los conocidos GOS-La, representando un nuevo posible ingrediente prebiótico multifuncional.

Si bien son conocidos los efectos positivos de los prebióticos, uno de los mayores retos que se presentan en la actualidad es el desarrollo de nuevos compuestos que sean capaces de llegar a las zonas más distales del colón donde tienen lugar diversas enfermedades. En este sentido, el compuesto de Amadori podría ser una alternativa ya que se ha comprobado que el mismo es capaz de alcanzar estas zonas distales del colon (Faist y col. 2001; Erbersdobler y col., 2001). Además, hoy en día existe un gran interés en la búsqueda de una nueva generación de ingredientes alimentarios multifuncionales. En este sentido, en el presente trabajo se sintetizó una serie de compuestos de Amadori, a partir de GOS-Lu y GOS-La, y de los hidrolizados de CMP, conocida secuencia peptídica con diversas propiedades funcionales como de

antiadherencia de patógenos en el intestino. Sin embargo, y debido a que dicha secuencia se somete a una hidrólisis enzimática, es de gran importancia conocer su estructura.

Para la caracterización de O-glicopéptidos de hidrolizados de CMP se ha empleado también la HILIC acoplada a MS^n, sin embargo, en este caso se obtuvieron buenos resultados de separación empleando la columna zwiteriónica. En cambio, para el análisis de fosfopéptidos se observó una mejor separación mediante RP, debido a las repulsiones entre el grupo sulfonato de la columna zwiteriónica y el grupo fosfato de los fosfopéptidos.

Con los experimentos de bioactividad *in vitro* llevados a cabo, se logró constatar que la conjugación péptido : carbohidrato no afecta a las propiedades prebióticas de este último y que además su habilidad de evitar procesos anti-inflamatorios y de adhesión de patógenos en células intestinales es incrementado. Estos resultados abren una nueva posibilidad de un nuevo ingrediente multi-funcional y de fácil producción. Sin embargo, es necesario determinar la supervivencia de estos compuestos hasta las zonas más distales del colon o la fermentación de los mismos en sistemas *in vivo*, con el fin de constatar de manera fehaciente todas las posibles propiedades beneficiosas que puedan ejercer estos compuestos. En este sentido, nuestro grupo de investigación está realizando este tipo de pruebas *in vivo* y optimizando metodologías analíticas apropiadas para la detección del compuesto de Amadori en diferentes tramos del intestino grueso de los sistemas biológicos empleados.

6. CONCLUSIONES-CONCLUSIONS

6. Conclusiones

- La cromatografía de exclusión molecular resultó ser la técnica más efectiva para la obtención de GOS-La sin la presencia de mono- y disacáridos, mientras que el tratamiento con carbón activo con 1% de etanol permitió eliminar selectivamente la fracción de monosacáridos de GOS-Lu.

- El análisis por GC-MS de distintas mezclas de GOS-Lu obtenidos empleando β-galactosidasas de diferentes orígenes permitió caracterizar de forma tentativa los di- y trisacáridos formados por unidades de galactosil-galactosas y galactosil-fructosas.

- El método desarrollado de HILIC-MSn empleando una columna de BEH amida fue eficaz para la separación y posterior caracterización estructural tentativa de mezclas complejas de GOS-La comerciales.

- El método desarrollado de ZIC-HILIC-MSn permitió una óptima separación y caracterización de 41 O-glicopéptidos procedentes de la hidrólisis enzimática del CMP bovino sin la necesidad de usar ningún paso previo de derivatización y/o enriquecimiento. Asimismo, el mecanismo de separación por ZIC-HILIC no sólo se debió a fenómenos de partición sino también a importantes interacciones electrostáticas tanto de atracción como de repulsión con el grupo sulfonato terminal de la fase estacionaria.

- Con el método desarrollado de RPLC-MSn empleando un analizador de masas tipo Q-TOF se logró la identificación de un nuevo sitio de fosforilación minoritario (S^{166}) en la α-caseína bovina, indicando la utilidad de dicho método para la identificación de modificaciones post-traduccionales de poca abundancia.

- El empleo de GOS-Lu y GOS-La como fuente de carbono por distintas especies de lactobacilos mejoró la resistencia de estos microorganismos a las condiciones gastrointestinales, así como su capacidad de adhesión a células intestinales. Igualmente, los conjugados de hCMP:GOS-Lu y hCMP:GOS-La sirvieron de fuente de carbono a distintas especies de lactobacilos aumentando

la resistencia de estos probióticos a las sales biliares. Este comportamiento pone de manifiesto el potencial uso de estos compuestos en productos simbióticos.

- La glicación de GOS-La, GOS-Lu y lactulosa con hidrolizados de CMP mantuvo las propiedades bifidogénicas presentadas por estos carbohidratos libres en sistemas *in vitro* empleando heces humanas.

- Los conjugados de hCMP:GOS-Lu y hCMP:GOS-La mostraron capacidades antiadherentes de bacterias patógenas a mucinas. Estos compuestos así como los GOS-Lu libres inhibieron la producción *in vitro* de citoquinas pro-inflamatorias.

- Los trisacáridos de GOS-Lu presentaron una mayor resistencia a la digestión gastrointestinal (digestibilidad ileal del 12,5%) frente a los GOS-La (digestibilidad ileal del 52,9%) empleando ratas como modelo animal, mientras que la fracción de disacáridos de GOS-Lu fue completamente indigerible. Además, estos oligosacáridos promovieron el crecimiento selectivo de las bacterias beneficiosas del intestino grueso, siendo el índice de selectividad mayor para los GOS-Lu.

- Los GOS-Lu promovieron una mayor biodiversidad de bifidobacterias en el intestino grueso en comparación con los GOS-La y la lactulosa.

- Los GOS-Lu indujeron una mayor expresión de NFkB, implicado en procesos de señalización, crecimiento y supervivencia celular, y de IL-6 e IL-10 favoreciendo la regulación de procesos inflamatorios en el intestino empleando ratas como modelo animal.

- Los GOS-Lu pueden constituir una nueva fuente de carbohidratos prebióticos con propiedades biológicas añadidas y mejoradas que los GOS-La, siendo la conjugación con péptidos bioactivos una posible ruta para la obtención de ingredientes multifuncionales de grado alimentario.

6. Conclusions

- The GC-MS analysis of different GOS-Lu mixtures obtained by using β-galactosidases from different sources allowed the tentative characterization of di- and trisaccharides constituted by galactosyl-galactose and galactosyl-fructose units.

- Size exclusion chromatography resulted to be the most effective technique to obtain GOS-La without presence of mono- and disaccharides, while activated charcoal treatment with 1% of ethanol allowed the selective removal of the monosaccharide fraction from GOS-Lu.

- The developed HILIC-MSn method using a BEH amide column was effective for the separation and further tentative structural characterization of commercial GOS-La complex mixtures.

- The developed ZIC-HILIC-MSn method allowed the optimal separation and structural characterization of 41 O-glycopeptides from enzymatically hydrolyzed bovine CMP without requiring previous derivatization and/or enrichment procedures. Likewise, the separation mechanism in the ZIC-HILIC column occurred due not only to partition phenomena but also to important electrostatic interactions of both attraction and repulsion nature with the sulphonate terminal group of the stationary phase.

- The developed RPLC-MSn method using a Q-TOF mass analyzer allowed the identification of a new minor phosphorylation site (S^{166}) in the bovine α-casein, indicating the utility of this method for the identification of low-abundance post-translational modifications.

- The use of GOS-Lu and GOS-La as carbon source by different Lactobacillus strains improved the resistance of these microorganisms to gastrointestinal conditions, as well as their capacity of adhesion to intestinal cells. Similarly, hCMP:GOS-Lu and hCMP:GOS-La conjugates were used from different Lactobacillus strains as carbon source increasing the resistance of

these probiotics to bile salts. This behaviour points out the potential application of these compounds to symbiotic products.

- The glycation of GOS-La and GOS-Lu with hydrolyzed CMP kept the bifidogenic properties exhibited by these carbohydrates in *in vitro* systems using human fecal samples.

- The hCMP:GOS-Lu and hCMP:GOS-La conjugates showed to possess anti-adhesive properties against pathogenic bacteria to mucins. These compounds, as well as free GOS-Lu, inhibited the *in vitro* production of pro-inflammatory cytokines.

- The GOS-Lu trisaccharides had a higher resistance to gastrointestinal digestion (ileal digestibility 12.5 %) than GOS-La (ileal digestibility 52.9 %) using rats as animal models, while the GOS-Lu disaccharide fraction was completely undigestible. Moreover, these oligosaccharides promoted the selective growth of beneficial bacteria in the large intestine, being the selectivity index higher for GOS-Lu than for GOS-La.

- The GOS-Lu promoted a higher bifidobacteria biodiversity in the large intestine than GOS-La and lactulose.

- GOS-Lu induced a higher NFkB expression, involved in signaling, growing and survival cell processes, and also in IL-6 and IL-10 favouring the regulation of inflammatory processes in the intestine using rats as animal model.

- GOS-Lu can represent a new source of prebiotic carbohydrates with additional and improved biological properties in comparison to GOS-La. Likewise, the conjugation with bioactive peptides explores a possible alternative to obtain food multifunctional ingredients.

7. REFERENCIAS

7. Referencias

Abd-El-Salam, M.H., El-Shibiny, S., y Buchheim, W. 1996. Characteristics and potential uses of the casein macropeptide. International Dairy Journal. **6**: 327-341.

Abdel-Salam, A.M. y Effat, L.K. 2010. Preparation and evaluation of a novel therapeutic dairy-based drink for phenylketonuria. North American Journal of medical Sciences. **2**: 66-70.

Aguiar, M., Haas, W., Beausoleil, S.A., Rush, J., y Gygi, S.P. 2010. Gas-phase rearrangements do not affect site localization reliability in phosphoproteomics data sets. Journal of Proteome Research. **9**: 3103-3107.

Akira, S. y Takeda, K. 2004. Toll-like receptor signalling. Nature Reviews Immunology. **4**: 499-511.

Akpinar, O. y Penner, M.H. 2008. Preparation of cellooligosaccharides: Comparative study. Journal of Food Agriculture & Environment. **6**: 55-61.

Alaiz, M., Hidalgo, F.J., y Zamora, R. 1997. Comparative antioxidant activity of Maillard- and oxidized lipid-damaged bovine serum albumin. Journal of Agricultural and Food Chemistry. **45**: 3250-3254.

Albrecht, S., Schols, H.A., Klarenbeek, B., Voragen, A.G.J., y Gruppen, H. 2010. Introducing Capillary Electrophoresis with Laser-Induced Fluorescence (CE−LIF) as a Potential Analysis and Quantification Tool for Galactooligosaccharides Extracted from Complex Food Matrices. Journal of Agricultural and Food Chemistry. **58**: 2787-2794.

Alexander, L.J., Stewart, A.F., Mackinlay, A.G., Kapelinskaya, T.V., Tkach, T.M., y Gorodetsky, S.I. 1988. Isolation and characterization of the bovine κ-casein gene. European Journal of Biochemistry. **178**: 395-401.

Allen, S.G., Schulman, D., Lichwa, J., Antal, M.J., Laser, M., y Lynd, L.R. 2001. A comparison between hot liquid water and steam fractionation of corn fiber. Industrial & Engineering Chemistry Research. **40**: 2934-2941.

Alles, M.S., Hartemink, R., Meyboom, S., Harryvan, J.L., Van Laere, K.M.J., Nagengast, F.M., y Hautvast, J. 1999. Effect of transgalactooligosaccharides on the composition of the human intestinal microflora and on putative risk markers for colon cancer. American Journal of Clinical Nutrition. **69**: 980-991.

Alpert, A.J. 1990. Hydrophilic-Interaction chromatography for the separation of peptides, nucleics-acids and other polar compounds. Journal of Chromatography. **499**: 177-196.

Alpert, A.J., Shukla, M., Shukla, A.K., Zieske, L.R., Yuen, S.W., Ferguson, M.A.J., Mehlert, A., Pauly, M., y Orlando, R. 1994. Hydrophilic-interaction chromatography of complex carbohydrates. Journal of Chromatography A. **676**: 191-202.

Alvarez-Manilla, G., Atwood III, J., Guo, Y., Warren, N.L., Orlando, O., y Pierce, M. 2006. Tools for Glycoproteomic Analysis: Size Exclusion Chromatography Facilitates Identification of Tryptic Glycopeptides with N-linked Glycosylation Sites. Journal of Proteome Research **5**: 701-708.

AlvarezDominguez, C., VazquezBoland, J.A., CarrascoMarin, E., LopezMato, P., y LeyvaCobian, F. 1997. Hose cell heparan sulfate proteoglycans mediate attachment and entry of Listeria monocytogenes, and the listerial surface protein ActA is involved in heparan sulfate receptor recognition. Infection and Immunity. **65**: 78-88.

Amadori, M. 1931. Products of condensation between glucose and p-phenetidine. Accademia Nazionale Dei Lincei **2**: 337-342.

Ames, J.M., Wynne, A., Hofmann, A., Plos, S., y Gibson, G.R. 1999. The effect of a model melanoidin mixture on faecal bacterial populations in vitro. British Journal of Nutrition. **82**: 489-495.

Amon, S., Plematl, A., y Rizzi, A. 2006. Capillary zone electrophoresis of glycopeptides under controlled electroosmotic flow conditions coupled to electrospray and matrix-assisted laser desorption/ionization mass spectrometry. Electrophoresis. **27**: 1209-1219.

AOAC, Official Methods of Analysis (1990). 15th ed.: association of Official Analytical Chemists, no. 920.184. Vo. II, 1028-1029, Arlington, VA.

Antonio, C., Larson, T., Gilday, A., Graham, I., Bergström, E., y Thomas-Oates, J. 2008. Hydrophilic interaction chromatography/electrospray mass spectrometry analysis of carbohydrate-related metabolites from Arabidopsis thaliana leaf tissue. Rapid Communications in Mass Spectrometry. **22**: 1399-1407.

Arpaia, N., Godec, J., Lau, L., Sivick, K.E., McLaughlin, L.M., Jones, M.B., Dracheva, T., Peterson, S.N., Monack, D.M., y Barton, G.M. 2011. TLR Signaling Is Required for Salmonella typhimurium Virulence. Cell. **144**: 675-688.

Ashizuka, Y., Nakagawa, R., Hori, T., Yasutake, D., Tobiishi, K., y Sasaki, K. 2008. Determination of brominated flame retardants and brominated dioxins in fish collected from three regions of Japan. Molecular Nutrition & Food Research. **52**: 273-283.

Avila-Fernandez, A., Galicia-Lagunas, N., Rodriguez-Alegria, M.E., Olvera, C., y Lopez-Munguia, A. 2011. Production of functional oligosaccharides through limited acid hydrolysis of agave fructans. Food Chemistry. **129**: 380-386.

Azuma, N., Yamauchi, K., y Mitsuoka, T. 1984. Bifidus growth-promoting activity of a glycomacropeptide derived from human k-casein. Agricultural and Biological Chemistry. **48**: 2159-2162.

Bahrami, B., Macfarlane, S., y Macfarlane, G. 2010. Induction of cytokine formation by human intestinal bacteria in gut epithelial cell lines. Journal of Applied Microbiology. **110**: 353-363.

Barboza, M., Sela, D.A., Pirim, C., LoCascio, R.G., Freeman, S.L., German, J.B., Mills, D.A., y Lebrilla, C.B. 2009. Glycoprofiling Bifidobacterial Consumption of Galacto-Oligosaccharides by Mass Spectrometry Reveals Strain-Specific, Preferential Consumption of Glycans. Applied and Environmental Microbiology. **75**: 7319-7325.

Barcenilla, A., Pryde, S.E., Martin, J.C., Duncan, S.H., Stewart, C.S., Henderson, C., y Flint, H.J. 2000. Phylogenetic relationships of butyrate-producing bacteria from the human gut. Applied and Environmental Microbiology. **66**: 1654-1661.

Barton Behravesh, C., Mody, R.K., Jungk, J., Gaul, L., Redd, J.T., Chen, S., Cosgrove, S., Hedican, E., Sweat, D., Chavez-Hauser, L., Snow, S.L., Hanson, H., Thai-An, N., Sodha, S.V., Boore, A.L., Russo, E., Mikoleit, M., Theobald, L., Gerner-Smidt, P., Hoekstra, R.M., Angulo, F.J., Swerdlow, D.L., Tauxe, R.V., Griffin, P.M., y Williams, I.T. 2011. 2008 outbreak of Salmonella Saintpaul infections associated with raw produce. New England Journal of Medicine. **364**: 918-927.

Baumgartner, S., Gennerritzmann, R., Haas, J., Amado, R., y Neukom, H. 1986. Isolation and identification of cyclitols in carob pods (Ceratonia siliqua l). Journal of Agricultural and Food Chemistry. **34**: 827-829.

Baxter, J.H. 1995. Free amino-acid stability in reducing sugar systems. Journal of Food Science. **60**: 405-408.

Begley, M., Gahan, C.G.M., y Hill, C. 2005. The interaction between bacteria and bile. Fems Microbiology Reviews. **29**: 625-651.

Begley, M., Hill, C., y Gahan, C.G.M. 2006. Bile salt hydrolase activity in probiotics. Applied and Environmental Microbiology. **72**: 1729-1738.

Bekedam, E.K., Loots, M.J., Schols, H.A., Van Boekel, M., y Smit, G. 2008. Roasting effects on formation mechanisms of coffee brew melanoidins. Journal of Agricultural and Food Chemistry. **56**: 7138-7145.

Belenguer, A., Duncan, S.H., Calder, A.G., Holtrop, G., Louis, P., Lobley, G.E., y Flint, H.J. 2006. Two routes of metabolic cross-feeding between Bifidobacterium adolescentis and butyrate-producing anaerobes from the human gut. Applied and Environmental Microbiology. **72**: 3593-3599.

Bell, L.N. 1997. Maillard reaction as influenced by buffer type and concentration. Food Chemistry. **59**: 143-147.

Benson, A.K., Kelly, S.A., Legge, R., Ma, F., Low, S.J., Kim, J., Zhang, M., Oh, P.L., Nehrenberg, D., Hua, K., Kachman, S.D., Moriyama, E.N., Walter, J., Peterson, D.A., y Pomp, D. 2010. Individuality in gut microbiota composition is a complex polygenic trait shaped by multiple environmental and host genetic factors. Proceedings of the National Academy of Sciences of the United States of America. **107**: 18933-18938.

Berthod, A., Chang, S.S.C., Kullman, J.P.S., y Armstrong, D.W. 1998. Practice and mechanism of HPLC oligosaccharide separation with a cyclodextrin bonded phase. Talanta. **47**: 1001-1012.

Bessler, H.C., de Oliveira, I.R., y Giugliano, L.G. 2006. Human milk glycoproteins inhibit the adherence of Salmonella typhimurium to HeLa cells. Microbiology and Immunology. **50**: 877-882.

Blanch, M., Sanchez-Ballesta, M.T., Escribano, M.I., y Merodio, C. 2011. Fructo-oligosaccharides in table grapes and response to storage. Food Chemistry. **129**: 724-730.

Boersema, P.J., Mohammed, S., y Heck, A.J.R. 2009. Phosphopeptide fragmentation and analysis by mass spectrometry. Journal of Mass Spectrometry. **44**: 861-878.

Boersema, P.J., Divecha, N., Heck, A.J.R., y Mohammed, S. 2007. Evaluation and optimization of ZIC-HILIC-RP as an alternative MudPIT strategy. Journal of Proteome Research. **6**: 937-946.

Bolondi, L., Bortolotti, M., Santi, V., Calletti, T., Gaiani, S., y Labo, G. 1985. Measurement of gastric-emptying time by real-time ultrasonography. Gastroenterology. **89**: 752-759.

Borrelli, R.C. y Fogliano, V. 2005. Bread crust melanoidins as potential prebiotic ingredients. Molecular Nutrition & Food Research. **49**: 673-678.

Borromei, C., Cavazza, A., Corradini, C., Vatteroni, C., Bazzini, A., Ferrari, R., y Merusi, P. 2010. Validated HPAEC-PAD method for prebiotics determination in synbiotic fermented milks during shelf life. Analytical and Bioanalytical Chemistry. **397**: 127-135.

Bouhnik, Y., Attar, A., Joly, F.A., Riottot, M., Dyard, F., y Flourié, B. 2004. Lactulose ingestion increases faecal bifidobacterial counts: A randomised double-blind study in healthy humans. European Journal of Clinical Nutrition. **58**: 462-466.

Bouhnik, Y., Neut, C., Raskine, L., Michel, C., Riottot, M., Andrieux, C., Guillemot, F., Dyard, F., y Flourie, B. 2004. Prospective, randomized, parallel-group trial to evaluate the effects of lactulose and polyethylene glycol-4000 on colonic flora in chronic idiopathic constipation. Alimentary Pharmacology & Therapeutics. **19**: 889-899.

Bourriaud, C., Robins, R.J., Martin, L., Kozlowski, F., Tenailleau, E., Cherbut, C., y Michel, C. 2005. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. Journal of Applied Microbiology. **99**: 201-212.

Boutrou, R., Jardin, J., Blais, A., Tome, D., y Leonil, J. 2008. Gluycosylations of κ-casein-derived caseinomacropeptide reduce its accessibility to endo- but not

exointestinal brush border membrane peptidases. Journal of Agricultural and Food Chemistry. **56**: 8166-8173.

Brody, E.P. 2000. Biological activities of bovine glycomacropeptide. The British Journal of Nutrition. **84**: S39-S46.

Brokl, M., Hernández-Hernández, O., Soria, A.C., y Sanz, M.L. 2011. Evaluation of different operation modes of high performance liquid chromatography for the analysis of complex mixtures of neutral oligosaccharides. Journal of Chromatography A. **1218**: 7697-7703.

Brokl, M., Soria, A.C., Martínez-Castro, I., Sanz, M.L., y Ruiz-Matute, A.I. 2009. Characterization of O-trimethylsilyl oximes of trisaccharides by gas chromatography–mass spectrometry. Journal of Chromatography A. **1216**: 4689-4692.

Bruck, W.M., Kelleher, S.L., Gibson, G.R., Graverholt, G., y Lannerdal, B.L. 2006. The effects of α-lactalbumin and glycomacropeptide on the association of CaCo-2 cells by enteropathogenic Escherichia coli, Salmonella typhimurium and Shigella flexneri. FEMS Microbiology Letters. **259**: 158-162.

Brück, W.M., Graverholt, G., Gibson, G. R. (2002). . , 41, 231-237. 2002. Use of batch culture and a two-stage continuous culture system to study the effect of supplemental α-lactalbumin and glycomacropeptide on mixed populations of human gut bacteria. FEMS Microbiology Ecology. **41**: 231-237.

Brummer, Y. y Cui, S.W., Understanding carbohydrates analysis, en Food Carbohydrates. Chemistry, physical properties and applications, Editor S.W. Cui, 2005, Taylor & Francis Group: Boca Raton.

Brunati, A.M., Marin, O., Bisinella, A., Salviati, A., y Pinna, L.A. 2000. Novel consensus sequence for the Golgi apparatus casein kinase, revealed using proline-rich protein-1 (PRP1)-derived peptide substrates. Biochemical Journal. **351**: 765-768.

Bunn, H.F. y Higgins, P.J. 1981. Reaction of monosaccharides with proteins - possible evolutionary significance. Science. **213**: 222-224.

Burdock, G.A., Carabin, I.G., y Griffiths, J.C. 2006. The importance of GRAS to the functional food and nutraceutical industries. Toxicology. **221**: 17-27.

Burkey, T.E., Skjolaas, K.A., Dritz, S.S., y Minton, J.E. 2009. Expression of porcine Toll-like receptor 2, 4 and 9 gene transcripts in the presence of lipopolysaccharide and Salmonella enterica serovars Typhimurium and Choleraesuis. Veterinary Immunology and Immunopathology. **130**: 96-101.

Burkholder, K.M. y Bhunia, A.K. 2010. Listeria monocytogenes Uses Listeria Adhesion Protein (LAP) To Promote Bacterial Transepithelial Translocation and Induces Expression of LAP Receptor Hsp60. Infection and Immunity. **78**: 5062-5073.

Burvall, A., Asp, N.G., Bosson, A., Sanjose, C., y Dahlqvist, A. 1978. Storage of lactose-hydrolyzed dried milk - effect of water activity on protein nutritional-value. Journal of Dairy Research. **45**: 381-389.

Candela, M., Maccaferri, S., Turroni, S., Carnevali, P., y Brigidi, P. 2010. Functional intestinal microbiome, new frontiers in prebiotic design. International Journal of Food Microbiology. **140**: 93-101.

Cardelle-Cobas, A., Synthesis, characterization and prebiotic properties of oligosaccharides derived from lactulose, Tesis Doctoral. 2009, Universidad Autónoma de Madrid: Madrid.

Cardelle-Cobas, A., Villamiel, M., Olano, A., y Corzo, N. 2008. Study of galacto-oligosaccharide formation from lactose using Pectinex Ultra SP-L. Journal of the Science of Food and Agriculture. **88**: 954-961.

Cardelle-Cobas, A., Corzo, N., Villamiel, M., y Olano, A. 2008. Isomerization of Lactose-Derived Oligosaccharides: A Case Study Using Sodium Aluminate. Journal of Agricultural and Food Chemistry. **56**: 10954-10959.

Cardelle-Cobas, A., Martínez-Villaluenga, C., Sanz, M.L., y Montilla, A. 2009. Gas chromatographic–mass spectrometric analysis of galactosyl derivatives obtained by the action of two different β-galactosidases. Food Chemistry. **114**: 1099-1105.

Cardelle-Cobas, A., Martínez-Villaluenga, C., Villamiel, M., Olano, A., y Corzo, N. 2008. Synthesis of Oligosaccharides Derived from Lactulose and Pectinex Ultra SP-L. Journal of Agricultural and Food Chemistry. **56**: 3328-3333.

Cardelle-Cobas, A., Corzo, N., Olano, A., Peláez, C., Requena, T., y Ávila, M. 2011. Galactooligosaccharides derived from lactose and lactulose: Influence of

structure on Lactobacillus, Streptococcus and Bifidobacterium growth. International Journal of Food Microbiology. **149**: 81-87.

Cardelle-Cobas, A., Fernández, M., Salazar, N., Martínez-Villaluenga, C., Villamiel, M., Ruas-Madiedo, P., y de los Reyes-Gavilán, C.G. 2009. Bifidogenic effect and stimulation of short chain fatty acid production in human faecal slurry cultures by oligosaccharides derived from lactose and lactulose. Journal of Dairy Research. **76**: 317-325.

Careri, M. y Mangia, A. 2003. Analysis of food proteins and peptides by chromatography and mass spectrometry. Journal od Chromatography A. **1000**: 609-635.

Cerf-Bensussan, N. y Gaboriau-Routhiau, V. 2010. The immune system and the gut microbiota: friends or foes? Nature Reviews Immunology. **10**: 735-744.

Chapman, C.M.C., Gibson, G.R., y Rowland, I. 2011. Health benefits of probiotics: are mixtures more effective than single strains? European Journal of Nutrition. **50**: 1-17.

Charles-Bernard, M., Kraehenbuehl, K., Rytz, A., y Roberts, D.D. 2005. Interactions between volatile and nonvolatile coffee components. 1. Screening of nonvolatile components. Journal of Agricultural and Food Chemistry. **53**: 4417-4425.

Charles, A.L., Huang, T.C., y Chang, Y.H. 2008. Structural analysis and characterization of a mucopolysaccharide isolated from roots of cassava (Manihot esculenta Crantz L.). Food Hydrocolloids. **22**: 184-191.

Charteris, W.P., Kelly, P.M., Morelli, L., y Collins, J.K. 1997. Selective detection, enumeration and identification of potentially probiotic Lactobacillus and Bifidobacterium species in mixed bacterial populations. International Journal of Food Microbiology. **35**: 1-27.

Charteris, W.P., Kelly, P.M., Morelli, L., y Collins, J.K. 1998. Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. Journal of Applied Microbiology. **84**: 759-768.

Chávarri, M., Marañón, I., Ares, R., Ibáñez, F.C., Marzo, F., y Villarán, M.d.C. 2010. Microencapsulation of a probiotic and prebiotic in alginate-chitosan capsules

improves survival in simulated gastro-intestinal conditions. International Journal of Food Microbiology. **142**: 185-189.

Chen, C. 2003. Synthesis of galactooligosaccharides and transgalactosylation modeling in reverse micelles. Enzyme and Microbial Technology. **33**: 497-507.

Chen, Y.S., Srionnual, S., Onda, T., y Yanagida, F. 2007. Effects of prebiotic oligosaccharides and trehalose on growth and production of bacteriocins by lactic acid bacteria. Letters in Applied Microbiology. **45**: 190-193.

Chevalier, F., Chobert, J.M., Genot, C., y Haertle, T. 2001. Scavenging of free radicals, antimicrobial, and cytotoxic activities of the Maillard reaction products of beta-lactoglobulin glycated with several sugars. Journal of Agricultural and Food Chemistry. **49**: 5031-5038.

Chonan, O., Shibahara-Sone, H., Takahashi, R., Ikeda, M., Kikuchi-Hayakawa, H., Ishikawa, F., Kimura, K., y Matsumoto, K. 2004. Undigestibility of galactooligosaccharides. Journal of the Japanese Society for Food Science and Technology-Nippon Shokuhin Kagaku Kogaku Kaishi. **51**: 28-33.

Christensen, B., Nielsen, M.S., Haselmann, K.F., Petersen, T.E., y Sorensen, E.S. 2005. Post-translationally modified residues of native human osteopontin are located in clusters: identification of 36 phosphorylation and five O-glycosylation sites and their biological implications. Biochemical Journal. **390**: 285-292.

Claverol, S., Burlet-Schiltz, O., Gairin, J.E., y Monsarrat, B. 2003. Characterization of protein variants and post-translational modifications: ESI-MS^n analyses of intact proteins eluted from polyacrylamide gels. Molecular y Cellular Proteomics. **2**: 483-493.

Clemente, A., Jimenez, E., Marin-Manzano, M.C., y Rubio, L.A. 2008. Active Bowman-Birk inhibitors survive gastrointestinal digestion at the terminal ileum of pigs fed chickpea-based diets. Journal of the Science of Food and Agriculture. **88**: 513-521.

Collins, M.E. y Rastall, R., Oligosaccharides in Food and Agriculture, en Glycoscience, Editores B. Fraser-Reid, K. Tatsuta, and J. Thiem,. 2008, Springer-Verlag: Berlin. 1186-1200.

Coolbear, K.P., Elgar, D.F., y Ayers, J.S. 1996. Profiling of genetic variants of bovine κ-casein macropeptide by electrophoretic and chromatographic techniques. International Dairy Journal. **6**: 1055-1068.

Corzo-Martínez, M., Moreno, F.J., Villamiel, M., y Harte, F.M. 2010. Characterization and improvement of rheological properties of sodium caseinate glycated with galactose, lactose and dextran. Food Hydrocolloids. **24**: 88-97.

Côté, G.L. 2009. Acceptor products of alternansucrase with gentiobiose. Production of novel oligosaccharides for food and feed and elimination of bitterness. Carbohydrate Research. **344**: 187-190.

Côté, G.L. y Sheng, S. 2006. Penta-, hexa-, and heptasaccharide acceptor products of alternansucrase. Carbohydrate Research. **341**: 2066-2072.

Côté, G.L., Dunlap, C.A., y Vermillion, K.E. 2009. Glucosylation of raffinose via alternansucrase acceptor reactions. Carbohydrate Research. **344**: 1951-1959.

Coulier, L., Timmermans, J., Bas, R., Van Den Dool, R., Haaksman, I., Klarenbeek, B., Slaghek, T., y Van Dongen, W. 2009. In-Depth Characterization of Prebiotic Galacto-oligosaccharides by a Combination of Analytical Techniques. Journal of Agricultural and Food Chemistry. **57**: 8488-8495.

Crittenden, R.G. y Playne, M.J. 1996. Production, properties and applications of food-grade oligosaccharides. Trends in Food Science & Technology. **7**: 353-361.

Crittenden, R.G. y Playne, M.J. 2002. Purification of food-grade oligosaccharides using immobilised cells of Zymomonas mobilis. Applied Microbiology and Biotechnology. **58**: 297-302.

Cubbon, S., Bradbury, T., Wilson, J., y Thomas-Oates, J. 2007. Hydrophilic interaction chromatography for mass spectrometric metabonomic studies of urine. Analytical Chemistry. **79**: 8911-8918.

Cummings, J.H. y Macfarlane, G.T. 1991. The control and consequences of bacterial fermentation in the human colon. Journal of Applied Bacteriology. **70**: 443-459.

Cummings, J.H., Macfarlane, G.T., y Englyst, H.N. 2001. Prebiotic digestion and fermentation. American Journal of Clinical Nutrition. **73**: 415S-420S.

Daddaoua, A., Puerta, V., Zarzuelo, A., Suárez, M.D., Sánchez de Medina, F., y Martínez-Augustin, O. 2005. Bovine glycomacropeptide is anti-inflammatory in rats with hapten-induced colitis. The Journal of Nutrition. **135**: 1164-1170.

Dalpathado, D.S. y Desaire, H. 2008. Glycopeptide analysis by mass spectrometry. Analyst **133**: 731-738.

Davies, D.T. y Law, A.J.R. 1977. Improved method for quantitative fractionation of casein mixtures using ion-exchange chromatography. Journal of Dairy Research. **44**: 213-221.

Davis, L.M.G., Martinez, I., Walter, J., y Hutkins, R. 2010. A dose dependent impact of prebiotic galactooligosaccharides on the intestinal microbiota of healthy adults. International Journal of Food Microbiology. **144**: 285-292.

De Lagemaat, J.v., Silvan, J.M., Moreno, F.J., Olano, A., y del Castillo, M.D. 2007. In vitro glycation and antigenicity of soy proteins. Food Research International. **40**: 153-160.

De Preter, V., Vanhoutte, T., Huys, G., Swings, J., Rutgeerts, P., y Verbeke, K. 2006. Effect of lactulose and Saccharomyces boulardii administration on the colonic urea-nitrogen metabolism and the bifidobacteria concentration in healthy human subjects. Alimentary Pharmacology and Therapeutics. **23**: 963-974.

De Sousa, V.M.C., Dos Santos, E.F., y Sgarbieri, V.C. 2011. The importance of prebiotics in functional foods and clinical practice. Food and Nutrition, 133-144.

Deguchi, K., Ito, H., Takegawa, Y., Shinji, N., Nakagawa, H., y Nishimura, S.I. 2006. Complementary structural information of positive- and negative-ion MS^n spectra of glycopeptides with neutral and sialylated N-glycans. Rapid Communications in Mass Spectrometry. **20**: 741-746.

Deguchi, K., Keira, T., Yamada, K., Ito, H., Takegawa, Y., Nakagawa, H., y Nishimura, S. 2008. Two-dimensional hydrophilic interaction chromatography coupling anion-exchange and hydrophilic interaction columns for separation of 2-pyridylamino derivatives of neutral and sialylated N-glycans. Journal of Chromatography A. **1189**: 169-174.

Delfour, A., Jolles, J., Alais, C., y Jolles, P. 1965. Caseino-glycopeptides: Characterization of a methionin residue and of the N-terminal sequence. Biochemical and Biophysical Research Communications. **19**: 452-455.

Delgado-Andrade, C., Seiquer, I., Garcia, M.M., Galdo, G., y Navarro, M.P. 2011. Increased Maillard reaction products intake reduces phosphorus digestibility in male adolescent mice. Nutrition. **27**: 86-91.

Depeint, F., Tzortzis, G., Vulevic, J., I'Anson, K., y Gibson, G.R. 2008. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of Bifidobacterium bifidum NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. American Journal of Clinical Nutrition. **87**: 785-791.

Desrosiers, T., Savoie, L., Bergeron, G., y Parent, G. 1989. Estimation of lysine damage in heated whey proteins by furosine determinations in conjunction with the digestion cell technique. Journal of Agricultural and Food Chemistry. **37**: 1385-1391.

Dl Pierro, F., - Patente EP 2014181. Compositions based on prebiotic and immunogenic ingredients for the prevention and treatment of gastroenteric disorders caused by dysbiosis and/or alterations of the normal intestinal flora.

Dohrn, R., Bunz, A.P., Devlieghere, F., y Thelen, D. 1993. experimental measurements of phase-equilibria for ternary and quaternary systems of glucose, water, CO_2 and ethanol with a novel apparatus. Fluid Phase Equilibria. **83**: 149-158.

Dsouza, R. y Teja, A.S. 1988. High-pressure phase-equilibria in the system glucose + fructose + water + ethanol + carbon-dioxide. Fluid Phase Equilibria. **39**: 211-224.

Dumas, B.R., Mercier, J.C., Brignon, G., y Grosclau.F. 1972. Primary structure of bovine beta-caseine - complete sequence. European Journal of Biochemistry. **25**: 505-&.

Dunn, J.D., Reid, G.E., y Bruening, M.L. 2010. Techniques for phosphopeptide enrichment prior to analysis by mass spectrometry. Mass Spectrometry Reviews. **29**: 29-54.

Dziuba, J. y Minkiewicz, P. 1996. Influence of glycosylation on micelle-stabilizing ability and biological properties of C-terminal fragments of cow's kappa-casein. International Dairy Journal. **6**: 1017-1044.

Eckburg, P.B., Bik, E.M., Bernstein, C.N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S.R., Nelson, K.E., y Relman, D.A. 2005. Diversity of the human intestinal microbial flora. Science. **308**: 1635-1638.

Edeas, M., Attaf, D., Mailfert, A.S., Nasu, M., y Joubet, R. 2010. Maillard Reaction, mitochondria and oxidative stress: Potential role of antioxidants. Pathologie Biologie. **58**: 220-225.

Edwards, C.A. y Parrett, A.M. 2002. Intestinal flora during the first months of life: new perspectives. British Journal of Nutrition. **88**: S11-S18.

El Rassi, Z., Reversed-phase and hydrophobic interaction chromatography of carbohydrates and glycoconjugates, en Journal of Chromatography Library, Editor R. Ziad El, 2002, Elsevier. 41-102.

Enomoto, H., Hayashi, Y., Li, C.P., Ohki, S., Ohtomo, H., Shiokawa, M., y Aoki, T. 2009. Glycation and phosphorylation of alpha-lactalbumin by dry heating: Effect on protein structure and physiological functions. Journal of Dairy Science. **92**: 3057-3068.

Erbersdobler, H.F. y Faist, V. 2001. Metabolic transit of Amadori products. Nahrung-Food. **45**: 177-181.

Eremeeva, T. 2003. Size-exclusion chromatography of enzymatically treated cellulose and related polysaccharides: a review. Journal of Biochemical and Biophysical Methods. **56**: 253-264.

Espinosa, S., Bosch, E., y Roses, M. 2000. Retention of ionizable compounds on HPLC. 5. pH scales and the retention of acids and bases with acetonitrile-water mobile phases. Analytical Chemistry. **72**: 5193-5200.

Faist, V. y Erbersdobler, H.F. 2001. Metabolic transit and *in vivo* effects of melanoidins and precursor compounds deriving from the Maillard reaction. Annals of Nutrition and Metabolism. **45**: 1-12.

Falony, G. y De Vuyst, L., Ecological interections of bacteria in the human gut, en Prebiotics and probiotics science and technology, Editores D. Charalampopoulos and R. Rastall. 2009, Springer: New York. 641-682.

Falony, G., Vlachou, A., Verbrugghe, K., y De Vuyst, L. 2006. Cross-feeding between Bifidobacterium longum BB536 and acetate-converting, butyrate-producing

colon bacteria during growth on oligofructose. Applied and Environmental Microbiology. **72**: 7835-7841.

FAO/WHO (2003) Probiotics in Food. Health and nutritional properties and guidelines for evaluation. **85**.

Fayle, S.E. y Gerrard, J.A., The Maillard Reaction2002, Cambridge: The Royal Society of Chemistry.

Fenton, T.W. y Fenton, M. 1979. Improved procedure for the determination of chromic oxide in feed and feces. Canadian Journal of Animal Science. **59**: 631-634.

Fernandez, M.F., Boris, S., y Barbes, C. 2003. Probiotic properties of human lactobacilli strains to be used in the gastrointestinal tract. Journal of Applied Microbiology. **94**: 449-455.

Fiat, A.M. y Jolles, P. 1989. Caseins of various origins and biologically active casein peptides and oligosaccharides - structural and physiological aspects. Molecular and Cellular Biochemistry. **87**: 5-30.

Finot, P.A. 2005. The absorption and metabolism of modified amino acids in processed foods. Journal of AOAC International. **88**: 894-903.

Fitzgerald, G.F. y Meisel, H., Milk protein hydrolysates and bioactive peptides, en Advanced dairy chemistry: Proteins, Editores P.F. Fox y P.L.H. McSweeney,. 2003, Kluwer Academic/Plenum Publisher: New York. 675-698.

FitzGerald, R.J. 1998. Potential uses of caseinophosphopeptides. International Dairy Journal. **8**: 451-457.

Fitzgerald, R.J. y Murray, B.A. 2006. Bioactive peptides and lactic fermentations. International Journal of Dairy Technology. **59**: 118-125.

Fleckenstein, J.M., Holland, J.T., y Hasty, D.L. 2002. Interaction of an outer membrane protein of enterotoxigenic Escherichia coli with cell surface heparan sulfate proteoglycans. Infection and Immunity. **70**: 1530-1537.

Flint, H.J., Duncan, S.H., Scott, K.P., y Louis, P. 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. Environmental Microbiology. **9**: 1101-1111.

Foley, J.P. y Dorsey, J.G. 1984. Clarification of the limit of detection in chromatography. Chromatographia. **18**: 503-511.

Fortina, M.G., Ricci, G., Mora, D., Guglielmetti, S., y Manachini, P.L. 2003. Unusual organization for lactose and galactose gene clusters in Lactobacillus helveticus. Applied and Environmental Microbiology. **69**: 3238-3243.

Franks, A.H., Harmsen, H.J.M., Raangs, G.C., Jansen, G.J., Schut, F., y Welling, G.W. 1998. Variations of bacterial populations in human feces measured by fluorescent in situ hybridization with group-specific 16S rRNA-Targeted oligonucleotide probes. Applied and Environmental Microbiology. **64**: 3336-3345.

Fransen, C.T.M., Van Laere, K.M.J., van Wijk, A.A.C., Brull, L.P., Dignum, M., Thomas-Oates, J.E., Haverkamp, J., Schols, H.A., Voragen, A.G.J., Kamerling, J.P., y Vliegenthart, J.F.G. 1998. alpha-D-Glcp-(1-1)-beta-D-Galp-containing oligosaccharides, novel products from lactose by the action of beta-galactosidase. Carbohydrate Research. **314**: 101-114.

Fu, Q., Liang, T., Zhang, X., Du, Y., Guo, Z., y Liang, X. 2010. Carbohydrate separation by hydrophilic interaction liquid chromatography on a 'click' maltose column. Carbohydrate Research. **345**: 2690-2697.

Funcke, W. y Vonsonntag, C. 1979. Syn and anti forms of some monosaccharide O-methyl oximes:a 13C-n.m.r. and g.l.c. study Carbohydrate Research. **69**: 247-251.

Garcia-Ruiz, C., Garcia, C., Cifuentes, A., y Marina, L. 2007. Characterization and differentiation of diverse transgenic and nontransgenic soybean varieties from CE protein profiles. Electrophoresis. **28**: 2314-2323.

Garcia, M.M., Seiquer, I., Delgado-Andrade, C., Galdo, G., y Navarro, M.P. 2009. Intake of Maillard reaction products reduces iron bioavailability in male adolescents. Molecular Nutrition & Food Research. **53**: 1551-1560.

Geisser, A., Hendrich, T., Boehm, G., y Stahl, B. 2005. Separation of lactose from human milk oligosaccharides with simulated moving bed chromatography. Journal of Chromatography A. **1092**: 17-23.

Gelders, G.G., Bijnens, L., Loosveld, A.M., Vidts, A., y Delcour, J.A. 2003. Fractionation of starch hydrolysates into dextrins with narrow molecular mass distribution and their detection by high-performance anion-exchange chromatography with pulsed amperometric detection. Journal of Chromatography A. **992**: 75-83.

Geng, M., Ji, J., y Regnier, F.E. 2000. Signature-peptide approach to detecting proteins in complex mixtures. Journal of Chromatography A. **870**: 295-313.

Ghishan, F.K. y Kiela, P.R. 2011. From probiotics to therapeutics: another step forward? Journal of Clinical Investigation. **121**: 2149-2152.

Gibson, G. 2004. Fibre and effects on probiotics (the prebiotic concept). Clinical Nutrition Supplements. **1**: 25-31.

Gibson, G.R. 2004. From probiotics to prebiotics and a healthy digestive system. Journal of Food Science. **69**: M141-M143.

Gibson, G.R. y Roberfroid, M.B. 1995. Dietary modulation of the human colonic microbiota - introducing the concept of prebiotics. Journal of Nutrition. **125**: 1401-1412.

Gibson, G.R., McCartney, A.L., y Rastall, R.A. 2005. Prebiotics and resistance to gastrointestinal infections. British Journal of Nutrition. **93**: S31-S34.

Gibson, G.R., Probert, H.M., Loo, J.V., Rastall, R.A., y Roberfroid, M.B. 2004. Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. Nutrition Research Reviews. **17**: 259.

Gilar, M., Bouvier, E.S.P., y Compton, B.J. 2001. Advances in sample preparation in electromigration, chromatographic and mass spectrometric separation methods. Journal of Chromatography A. **909**: 111-135.

Gilar, M., Yu, Y.Q., Ahn, J., Fournier, J., y Gebler, J.C. 2008. Mixed-mode chromatography for fractionation of peptides, phosphopeptides and sialylated glycopeptides. Journal of Chromatography A. **1191**: 162-170.

Gniechwitz, D., Reichardt, N., Meiss, E., Ralph, J., Steinhart, H., Blaut, M., y Bunzel, M. 2008. Characterization and fermentability of an ethanol soluble high molecular weight coffee fraction. Journal of Agricultural and Food Chemistry. **56**: 5960-5969.

Godejohann, M. 2007. Hydrophilic interaction chromatography coupled to nuclear magnetic resonance spectroscopy and mass spectroscopy - A new approach for the separation and identification of extremely polar analytes in bodyfluids. Journal of Chromatography A. **1156**: 87-93.

Godshall, M.A., Roberts, E.J., y Miranda, X.M. 2001. Composition of the soluble, nondialyzable components in raw cane sugar. Journal of Food Processing and Preservation. **25**: 323-335.

Gonzalez de Paredo, A., Klein, D., Macek, B., Hess, D., Katalinic, J.P., y Hofsteenge, J. C-Mannosylation and O-Fucosylation of Thrombospondin Type 1 Repeats. Molecular Cell Proteomics. **1**: 11-18.

Goodman, B.E. 2010. Insights into digestion and absorption of major nutrients in humans. Advances in Physiology Education. **34**: 44-53.

Gosling, A., Stevens, G.W., Barber, A.R., Kentish, S.E., y Gras, S.L. 2010. Recent advances refining galactooligosaccharide production from lactose. Food Chemistry. **121**: 307-318.

Goulas, A., Tzortzis, G., y Gibson, G. 2007. Development of a process for the production and purification of α- and β-galactooligosaccharides from Bifidobacterium bifidum NCIMB 41171. International Dairy Journal. **17**: 648-656.

Goulas, A.K., Grandison, A.S., y Rastall, R.A. 2003. Fractionation of oligosaccharides by nanofiltration. Journal of the Science of Food and Agriculture. **83**: 675-680.

Grzymajlo, K., Kuzminska-Bajor, M., Jaworski, J., Dobryszycki, P., y Ugorski, M. 2010. The high-adhesive properties of the FimH adhesin of Salmonella enterica serovar Enteritidis are determined by a single F118S substitution. Microbiology-Sgm. **156**: 1738-1748.

Guerra, A., Gaspar, A.R., Contreras, S., Lucia, L.A., Crestini, C., y ArgyropouloS, D.S. 2007. On the propensity of lignin to associate: A size exclusion chromatography study with lignin derivatives isolated from different plant species. Phytochemistry. **68**: 2570-2583.

Guggenheim, B., Schmid, R., Aeschlimann, J.M., Berrocal, R., y Neeser, J.R. 1999. Powdered milk micellar casein prevents oral colonization by Streptococcus sobrinus and dental caries in rats: a basis for the caries-protective effect of dairy products. Caries Research. **33**: 446-454.

Guo, Y. y Huang, A.H. 2003. A HILIC method for the analysis of tromethamine as the counter ion in an investigational pharmaceutical salt. Journal of Pharmaceutical and Biomedical Analysis. **31**: 1191-1201.

Guo, Y. y Gaiki, S. 2005. Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography. Journal of Chromatography A. **1074**: 71-80.

Hagg, M.B. 1998. Membranes in chemical processing - A review of applications and novel developments. Separation and Purification Methods. **27**: 51-168.

Hague, A., Singh, B., y Paraskeva, C. 1997. Butyrate acts as a survival factor for colonic epithelial cells: Further fuel for the *in vivo* versus in vitro debate. Gastroenterology. **112**: 1036-1040.

Haller, D., Blum, S., Bode, C., Hammes, W.P., y Shiffrin, L.J. 2000. Activation of peripheral blood mononuclear cells by non-pathogenic bacteria in vitro: evidence of NK cells as primary targets. Infection and Immunity. **68**: 752-759.

Hao, Z., Xiao, B., y Weng, N. 2008. Impact of column temperature and mobile phase components on selectivity of hydrophilic interaction chromatography (HILIC). Journal of Separation Science. **31**: 1449-1464.

Harmsen, H.J.M., Elfferich, P., Schut, F., y Welling, G.W. 1999. A 16S rRNA-targeted Probe for Detection of Lactobacilli and Enterococci in Faecal Samples by Fluorescent In Situ Hybridization. Microbial Ecology in Health and Disease. **11**: 3-12.

Hartmann, R. y Meisel, H. 2007. Food-derived peptides with biological activity: from research to food applications. Current Opinion in Biotechnology. **18**: 163-169.

Hayashi, F. 1937. The adsorption of sugars by charcoal. Journal of Biochemistry. **16**: 1-16.

Hemström, P. y Irgum, K. 2006. Hydrophilic interaction chromatography. Journal of Separation Science. **29**: 1784-1821.

Hernandez-Hernandez, O., Moreno, F.J., Kolida, S., Rastall, R.A., y Sanz, M.L. 2011. Effect of glycation of bovine β-lactoglobulin with galactooligosaccharides on the growth of human faecal bacteria. International Dairy Journal. **21**: 949-952.

Hernandez, O., Emaldi, U., y Tovar, J. 2008. In vitro digestibility of edible films from various starch sources. Carbohydrate Polymers. **71**: 648-655.

Heyns, K., Paulsen, H., Eichstedt, R., y Rolle, M. 1957. The formation of 2-aminoaldoses by rearrangement of ketosylamines. Chemische Berichte. **90**: 2039-2049.

Hijova, E. y Chmelarova, A. 2007. Short chain fatty acids and colonic health. Bratislava Medical Journal-Bratislavske Lekarske Listy. **108**: 354-358.

Hodge, J.E. 1953. Dehydrated Foods, Chemistry of Browning Reactions in Model Systems. Journal of Agricultural and Food Chemistry. **1**: 928-943.

Hoffman, R.A. 2000. Intraepithelial lymphocytes coinduce nitric oxide synthase in intestinal epithelial cells. American Journal of Physiology-Gastrointestinal and Liver Physiology. **278**: G886-G894.

Holland, J.W., Deeth, H.C., y Alewood, P.F. 2004. Proteomic analysis of K-casein micro-heterogeneity. Proteomics. **4**: 743-752.

Holland, J.W., Deeth, H.C., y Alewood, P.F. 2005. Analysis of O-glycosylation site occupancy in bovine kappa-casein glycoforms separated by two-dimensional gel electrophoresis. Proteomics. **5**: 990-1002.

Holland, J.W., Deeth, H.C., y Alewood, P.F. 2006. Resolution and characterisation of multiple isoforms of bovine kappa-casein by 2-DE following a reversible cysteine-tagging enrichment strategy. Proteomics. **6**: 3087-3095.

Holzman, D.C. 2011. Major host health effects ascribed to gut microbiome. Microbe. **6**: 259-260.

Horvath, K. y Molnar-Perl, I. 1997. Simultaneous quantitation of mono-, di- and trisaccharides by GC-MS of their TMS ether oxime derivatives in honey. Chromatographia. **45**: 328-335.

Huck, C.W., Huber, C.G., y Bonn, G.K., HPLC of carbohydrates with cation- and anion exchanges silica and resin based stationary phases., en Carbohydrate analysis by modern chromatography and electrophoresis, Editor Z. El Rassi, 2002, Elsevier Science B.V. 165-205.

Huddleston, M.J., Bean, M.F., y Carr, S.A. 1993. Collisional fragmentation of glycopeptides by electrospray ionization LC/MS and LC/MS/MS: methods for selective detection of glycopeptides in protein digests. Analytical Chemistry. **65**: 877-884.

Huebner, J., Wehling, R., y Hutkins, R. 2007. Functional activity of commercial prebiotics. International Dairy Journal. **17**: 770-775.

Huebner, J., Wehling, R., Parkhurst, A., y Hutkins, R. 2008. Effect of processing conditions on the prebiotic activity of commercial prebiotics. International Dairy Journal.

Hughes, S.A., Shewry, P.R., Gibson, G.R., McCleary, B.V., y Rastall, R.A. 2008. *In vitro* fermentation of oat and barley derived β-glucans by human faecal microbiota. FEMS Microbiology Ecology. **64**: 482-493.

Hughes, S.A., Shewry, P.R., Li, L., Gibson, G.R., Sanz, M.L., y Rastall, R.A. 2007. *In vitro* fermentation by human fecal microflora of wheat arabinoxylans. Journal of Agricultural and Food Chemistry. **55**: 4589-4595.

Hunt, D.F., Yates, J.R., Shabanowitz, J., Winston, S., y Hauer, C.R. 1986. Protein sequencing by tandem mass-spectrometry. Proceedings of the National Academy of Sciences USA. **83**: 6233-6237.

Hunter, T. 1987. A thounsand and one protein kinases. Cell. **50**: 823-829.

Iannitti, T. y Palmieri, B. 2010. Therapeutical use of probiotic formulations in clinical practice. Clinical Nutrition. **29**: 701-725.

Idota, T., Kawakami, H., y Nakajima, I. 1994. Bifidus growth-promoting activity effect of N-acetylneuraminic acid-containing substances. Bioscience, Biotechnology and Biochemistry. **58**: 1720-1722.

Ikegami, T., Tomomatsu, K., Takubo, H., Horie, K., y Tanaka, N. 2008. Separation efficiencies in hydrophilic interaction chromatography. Journal of Chromatography A.

Ikegami, T., Horie, K., Saad, N., Hosoya, K., Fiehn, O., y Tanaka, N. 2008. Highly efficient analysis of underivatized carbohydrates using monolithic-silica-based capillary hydrophilic interaction (HILIC) HPLC. Analytical and Bioanalytical Chemistry. **391**: 2533-2542.

Ito, M., Kimura, M., Deguchi, Y., Miyamoriwatabe, A., Yajima, T., y Kan, T. 1993. Effects of transgalactosylated disaccharides on the human intestinal microflora and their metabolism. Journal of Nutritional Science and Vitaminology. **39**: 279-288.

Ito, M., Deguchi, Y., Miyamori, A., Matsumoto, K., Kikuchi, H., Matsumoto, K., Kobayashi, Y., Yajima, T., y Kan, T. 1990 Effects of administration of

galactooligosaccharides on the human faecal microflora, stool weight and abdominal sensation. Microbial Ecology in Health and Disease. **3**: 285-292.

Iwase, Y., Honma, S., Matsuzaki, M., Miyakawa, Y., Kanno, T., Ishii, K., Furuichi, N., Furukawa, K., y Horigome, T. 2010. A fully automated phosphopeptide purification system for large-scale phosphoproteome analysis. Journal of Biochemistry. **147**: 689-696.

Jain, P.K., McNaught, C.E., Anderson, A.D.G., MacFie, J., y Mitchell, C.J. 2004. Influence of synbiotic containing Lactobacillus acidophilus La5, Bifidobacterium lactis Bb 12, Streptococcus thermophilus, Lactobacillus bulgaricus and oligofructose on gut barrier function and sepsis in critically ill patients: A randomised controlled trial. Clinical Nutrition. **23**: 467-475.

Jandera, P. 2008. Stationary phases for hydrophilic interaction chromatography, their characterization and implementation into multidimensional chromatography concepts. Journal of Separation Science **31**: 1421-1437.

Jandera, P. 2011. Stationary and mobile phases in hydrophilic interaction chromatography: a review. Analytica Chimica Acta. **692**: 1-25.

Janer, C., Pelaez, C., y Requena, T. 2004. Caseinomacropeptide and whey protein concentrate enhance Bifidobacterium lactis growth in milk. Food Chemistry. **86**: 263-267.

Jenness, R. y Sloan, R.E. 1970. The composition of milk of various species: A review. Dairy Sciences Abstract. **32**: 599-612.

Jia, T., Leiner, I., Dorothee, G., Brandl, K., y Pamer, E.G. 2009. MyD88 and Type I Interferon Receptor-Mediated Chemokine Induction and Monocyte Recruitment during Listeria monocytogenes Infection. Journal of Immunology. **183**: 1271-1278.

Jin, F.M., Zhou, Z.Y., Enomoto, H., Moriya, T., y Higashijima, H. 2004. Conversion mechanism of cellulosic biomass to lactic acid in subcritical water and acid-base catalytic effect of subcritical water. Chemistry Letters. **33**: 126-127.

Jollès, P., Lévy-Toledano, S., Fiat, A.M., Soria, C., Gillessen, D., Thomaidis, A., Dunn, F.W., y Caen, J.P. 1986. Analogy between fibrinogen and casein. Effect of an undecapeptide isolated from κ-casein on platelet function. European Journal of Biochemistry. **158**: 379-382.

Joung, I., Kim, T.U., Stolz, L.A., Payne, G., Winkler, D.G., Walsh, C.T., Strominger, J.L., y Shin, J. 1995. Modification of ser(59) in the unique n-terminal region of tyrosine kinase p56(lck) regulates specificity of its src homology 2 domain. Proceedings of the National Academy of Sciences of the United States of America. **92**: 5778-5782.

Jules, M., Guillou, V., Francois, J., y Parrou, J.L. 2004. Two distinct pathways for trehalose assimilation in the yeast Saccharomyces cerevisiae. Applied and Environmental Microbiology. **70**: 2771-2778.

Jung, H.C., Eckmann, L., Yang, S.K., Panja, A., Fierer, J., Morzyckawroblewska, E., y Kagnoff, M.F. 1995. A distinct array of proinflammatory cytokines is expressed in human colon epithelial-cells in response to bacterial invasion. Journal of Clinical Investigation. **95**: 55-65.

Kagnoff, M.F. y Eckmann, L. 1997. Epithelial cells as sensors for microbial infection. Journal of Clinical Investigation. **100**: S51-S55.

Karlsson, G., Winge, S., y Sandberg, H. 2005. Separation of monosaccharides by hydrophilic interaction chromatography with evaporative light scattering detection. Journal of Chromatography A. **1092**: 246-249.

Kemp, B.E. y Pearson, R.B. 1990. Protein-kinase recognition sequence motifs. Trends in Biochemical Sciences. **15**: 342-346.

Kim, J.S. y Lee, Y.S. 2009. Enolization and racemization reactions of glucose and fructose on heating with amino-acid enantiomers and the formation of melanoidins as a result of the Maillard reaction. Amino Acids. **36**: 465-474.

Kim, K.P., Jagadeesan, B., Burkholder, K.M., Jaradat, Z.W., Wampler, J.L., Lathrop, A.A., Morgan, M.T., y Bhunia, A.K. 2006. Adhesion characteristics of Listeria adhesion protein (LAP)-expressing Escherichia coli to Caco-2 cells and of recombinant LAP to eukaryotic receptor Hsp60 as examined in a surface plasmon resonance sensor. FEMS Microbiology Letters. **256**: 324-332.

Kimoto-Nira, H., Kobayashi, M., Nomura, M., Sasaki, K., y Suzuki, C. 2009. Bile resistance in Lactococcus lactis strains varies with cellular fatty acid composition: Analysis by using different growth media. International Journal of Food Microbiology. **131**: 183-188.

Kimoto-Nira, H., Suzuki, C., Sasaki, K., Kobayashi, M., y Mizumachi, K. 2010. Survival of a Lactococcus lactis strain varies with its carbohydrate preference under *in vitro* conditions simulated gastrointestinal tract. International Journal of Food Microbiology. **143**: 226-229.

Kimura, K., Matsumoto, K., Ishihara, C., Harada, K., y Miyagi, A. 1995. Structure determination of galacto-oligosaccharides by pyridylamination and nmr-spectroscopy. Carbohydrate Research. **270**: 33-42.

Kjeldsen, F., Savitski, M.M., Nielsen, M.L., Shi, L., y Zubarev, R.A. 2007. On studying protein phosphorylation patterns using bottom-up LC-MS/MS: the case of human alpha-casein. Analyst. **132**: 768-776.

Knutsen, S.H., Sletmoen, M., Kristensen, T., Barbeyron, T., Kloareg, B., y Potin, P. 2001. A rapid method for the separation and analysis of carrageenan oligosaccharides released by iota- and kappa-carrageenase. Carbohydrate Research. **331**: 101-106.

Koizumi, K. 1996. High-performance liquid chromatographic separation of carbohydrates on graphitized carbon columns. Journal of Chromatography A. **720**: 119-126.

Koizumi, K., HPLC of carbohydrates on graphitizaed carbon columns, en Carbohydrate analysis by modern chromatography and elcetrophoresis, Editor Z. El Rassi, 2002, Elsevier: Amsterdam. 103-118.

Koizumi, K., Kubota, Y., Ozaki, H., Shigenobu, K., Fukuda, M., y Tanimoto, T. 1992. Analyses of isomeric mono-o-methyl-d-glucoses, d-glucobioses and d-glucose monophosphates by high-performance anion-exchange chromatography with pulsed amperometric detection. Journal of Chromatography. **595**: 340-345.

Kolida, S. y Gibson, G.R. 2007. Prebiotic capacity of inulin-type fructans. Journal of Nutrition. **137**: 2503S-2506S.

Koll, P., Mandar, R., Smidt, I., Hutt, P., Truusalu, K., Mikelsaar, R.H., Shchepetova, J., Krogh-Andersen, K., Marcotte, H., Hammarstrom, L., y Mikelsaar, M. 2010. Screening and Evaluation of Human Intestinal Lactobacilli for the Development of Novel Gastrointestinal Probiotics. Current Microbiology. **61**: 560-566.

Konstantinov, S.R., Awati, A., Smidt, H., Williams, B.A., Akkermans, A.D.L., y de Vos, W.A. 2004. Specific response of a novel and abundant Lactobacillus

amylovorus-like phylotype to dietary prebiotics in the guts of weaning piglets. Applied and Environmental Microbiology. **70**: 3821-3830.

Kuhn, R. y Weygand, F. 1937. The Amadori rearrangement. Chemische Berichte. **70B**: 769-772.

Kunz, C., Rudloff, S., Hintelmann, A., Pohlentz, G., y Egge, H. 1996. High-pH anion-exchange chromatography with pulsed amperometric detection and molar response factors of human milk oligosaccharides. Journal of Chromatography B: Biomedical Sciences and Applications. **685**: 211-221.

Labuza, T.P. y Saltmarch, M. 1982. Kinetics of browning and protein-quality loss in whey powders during steady-state and non-steady state storage-conditions. Journal of Food Science. **47**: 92-&.

LaClair, C.E., Ney, D.M., MacLeod, E.L., y Etzel, M.R. 2009. Purification and Use of Glycomacropeptide for Nutritional Management of Phenylketonuria. Journal of Food Science. **74**: E199-E206.

Langendijk, P.S., Schut, F., Jansen, G.J., Raangs, G.C., Kamphuis, G.R., Wilkinson, M.H.F., y Welling, G.W. 1995. Quantitative fluorescence in-situ hybridization of Bifidobacterium spp with genus-specific 16S ribosomal-RNA-targeted probes and its application in fecal samples. Applied and Environmental Microbiology. **61**: 3069-3075.

Langlands, S.J., Hopkins, M.J., Coleman, N., y Cummings, J.H. 2004. Prebiotic carbohydrates modify the mucosa associated microflora of the human large bowel. Gut. **53**: 1610-1616.

Laparra, J.M. y Sanz, Y. 2009. Comparison of *in vitro* models to study bacterial adhesion to the intestinal epithelium. Letters in Applied Microbiology. **49**: 695-701.

Laparra, J.M., Lopez-Rubio, A., Lagaron, J.M., y Sanz, Y. 2010. Dietary glycosaminoglycans interfere in bacterial adhesion and gliadin-induced pro-inflammatory response in intestinal epithelial (Caco-2) cells. International Journal of Biological Macromolecules. **47**: 458-464.

Laparra, J.M., Corzo-Martinez, M., Villamiel, M., Moreno, F.J., y Sanz, Y. 2011. Maillard-type glycoconjugates from dairy proteins inhibit adhesion of Escherichia coli to mucin. Food Chemistry. **129**: 1435-1443.

Laroque, D., Inisan, C., Berger, C., Vouland, É., Dufossé, L., y Guérard, F. 2008. Kinetic study on the Maillard reaction. Consideration of sugar reactivity. Food Chemistry. **111**: 1032-1042.

Larsen, M.R., Hojrup, P., y Roepstorff, P. 2005. Characterization of Gel-separated Glycoproteins Using Two-step Proteolytic Digestion Combined with Sequential Microcolumns and Mass Spectrometry. Molecular Cell Proteomics. **4**: 107-119.

Ledesma-Osuna, A.I., Ramos-Clamont, G., y Vazquez-Moreno, L. 2009. Biorecognition of Chemically Modified Bovine Serum Albumin with Lactose Prepared under Different Conditions. Journal of Agricultural and Food Chemistry. **57**: 9734-9739.

Lee, C., Kim, J., Shin, S.G., y Hwang, S. 2006. Absolute and relative QPCR quantification of plasmid copy number in Escherichia coli. Journal of Biotechnology. **123**: 273-280.

Lee, Y.K. y Salminen, S. 1995. The coming of age of probiotics. Trends in Food Science & Technology. **6**: 241-245.

Lehmann, W.D., Krueger, R., Salek, M., Hung, C.-W., Wolschin, F., y Weckwerth, W. 2007. Neutral loss-based phosphopeptide recognition: A collection of caveats. Journal of Proteome Research. **6**: 2866-2873.

Léonil, J. y Mollé, D. 1990. Liberation of tryptic fragments from caseinomacropeptide of bovine κ-casein involved in platelet function. Biochemical Journal. **271**: 247-252.

Lespinet, O. y Labedan, B. 2006. Puzzling over orphan enzymes. Cellular and Molecular Life Sciences. **63**: 517-523.

Li, W.Y., Li, J.D., Chen, T.Q., y Chen, C.X. 2004. Study on nanotiltration for purifying fructo-oligosaccharides I. Operation modes. Journal of Membrane Science. **245**: 123-129.

Liem, A.K.D. 1999. Important developments in methods and techniques for the determination of dioxins and PCBs in foodstuffs and human tissues. Trac-Trends in Analytical Chemistry. **18**: 499-507.

Lindgren, S.E. y Dobrogosz, W.J. 1990. Antagonistic activities of lactic acid bacteria in food and feed fermentations. FEMS Microbiology Letters. **87**: 149-163.

Longoni, V. y Penci, M., -Patente WO 2010081126. Compositions comprising probiotic and prebiotic components and mineral salts, with lactoferrin.

Loo, J.v., Cummings, J., Delzenne, N., Englyst, H., Franck, A., Hopkins, M., Kok, N., Macfarlane, G., Newton, D., Quigley, M., Roberfroid, M., Vliet, T.v., y Heuvel, E.v.d. 1999. Functional food properties of non-digestible oligosaccharides: a consensus report from the ENDO project (DGXII AIRII-CT94-1095). British Journal of Nutrition. **81**: 121-132.

Lu, L., Gu, G., Xiao, M., y Wang, F. 2010. Separation and structure analysis of trisaccharide isomers produced from lactose by Lactobacillus bulgaricus L3 β-galactosidase. Food Chemistry. **121**: 1283-1288.

Lundén, J., Tolvanen, R., y Korkeala, H. 2004. Human Listeriosis Outbreaks Linked to Dairy Products in Europe. Journal of Diary Science. **87**: 6-12.

Luthje, K., Hyotylainen, T., Rautiainen-Rama, M., y Riekkola, M.L. 2005. Pressurised hot water extraction-microporous membrane liquid-liquid extraction coupled on-line with gas chromatography-mass spectrometry in the analysis of pesticides in grapes. Analyst. **130**: 52-58.

Lyte, M. 2011. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. Bioessays. **33**: 574-581.

Ma, T.Y., Iwamoto, G.K., Hoa, N.T., Akotia, V., Pedram, A., Boivin, M.A., y Said, H.M. 2004. TNF-alpha-induced increase in intestinal epithelial tight junction permeability requires NF-kappa B activation. American Journal of Physiology-Gastrointestinal and Liver Physiology. **286**: G367-G376.

Macfarlane, G.T., Steed, H., y Macfarlane, S. 2008. Bacterial metabolism and health-related effects of galacto-oligosaccharides and other prebiotics. Journal of Applied Microbiology. **104**: 305-344.

Macfarlane, S. y Macfarlane, G.T. 2003. Regulation of short-chain fatty acid production. Proceedings of the Nutrition Society. **62**: 67-72.

Macfarlane, S., Macfarlane, G.T., y Cummings, J.H. 2006. Review article: prebiotics in the gastrointestinal tract. Alimentary Pharmacology & Therapeutics. **24**: 701-714.

Madureira, A.R., Amorim, M., Gomes, A.M., Pintado, M.E., y Malcata, F.X. 2011. Protective effect of whey cheese matrix on probiotic strains exposed to simulated gastrointestinal conditions. Food Research International. **44**: 465-470.

Man, J.C. 1960. A medium for teh cultivation of lactobacilli. Journal of Applied Bacteriology. **23**: 130-135.

Manso, M.A. y López-Fandiño, R. 2003. Angiotensin I converting enzyme-inhibitory activity of bovine, ovine and caprine κ-casein macropeptides and their tryptic hydrolyzates. Journal of Food Protection. **66**: 1686-1692.

Manso, M.A. y Lopez-Fandino, R. 2004. kappa-casein macropeptides from cheese whey: Physicochemical, biological, nutritional, and technological features for possible uses. Food Reviews International. **20**: 329-355.

Manso, M.A. y Lopez-Fandino, R. 2004. Angiotensin I converting enzyme-inhibitory activity of bovine, ovine, and caprine kappa-casein macropeptides and their tryptic hydrolysates. Journal of Food Protection. **66**: 1686-1692.

Manz, W., Amann, R., Ludwig, W., Vancanneyt, M., y Schleifer, K.H. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum cytophaga-flavobacter-bacteroides in the natural environment. Microbiology-Uk. **142**: 1097-1106.

Martín-Diana, A.B., Frías, J., y Fontecha, J. 2005. Emulsifying properties of whey protein concentrate and caseinomacropeptide of cow, ewe and goat. Milchwissenschaft **60**: 363-367.

Martin-Pelaez, S., Gibson, G.R., Martin-Orue, S.M., Klinder, A., Rastall, R.A., La Ragione, R.M., Woodward, M.J., y Costabile, A. 2008. *In vitro* fermentation of carbohydrates by porcine faecal inocula and their influence on Salmonella typhimurium growth in batch culture systems. FEMS Microbiology Ecology. **66**: 608-619.

Martinez-Ferez, A., Rudloff, S., Guadix, A., Henkel, C.A., Pohlentz, G., Boza, J.J., Guadix, E.M., y Kunz, C. 2006. Goats' milk as a natural source of lactose-derived oligosaccharides: Isolation by membrane technology. International Dairy Journal. **16**: 173-181.

Martinez-Villaluenga, C., Cardelle-Cobas, A., Olano, A., Corzo, N., Villamiel, M., y Jimeno, M.L. 2008. Enzymatic synthesis and identification of two trisaccharides produced from lactulose by transgalactosylation. Journal of Agricultural and Food Chemistry. **56**: 557-563.

Martínez-Villaluenga, C., Cardelle-Cobas, A., Corzo, N., Olano, A., y Villamiel, M. 2008. Optimization of conditions for galactooligosaccharide synthesis during lactose hydrolysis by β-galactosidase from Kluyveromyces lactis (Lactozym 3000 L HP G). Food Chemistry. **107**: 258-264.

Martinez, R.C.R., Aynaou, A.-E., Albrecht, S., Schols, H.A., De Martinis, E.C.P., Zoetendal, E.G., Venema, K., Saad, S.M.I., y Smidt, H. 2011. *In vitro* evaluation of gastrointestinal survival of Lactobacillus amylovorus DSM 16698 alone and combined with galactooligosaccharides, milk and/or Bifidobacterium animalis subsp. lactis Bb-12. International Journal of Food Microbiology. **149**: 152-158.

Marx, S.P., Winkler, S., y Hartmeier, W. 2000. Metabolization of beta-(2,6)-linked fructose-oligosaccharides by different bifidobacteria. FEMS Microbiology Letters. **182**: 163-169.

Maugard, T., Gaunt, D., Legoy, M.D., y Besson, T. 2003. Microwave-assisted synthesis of galacto-oligosaccharides from lactose with immobilized beta-galactosidase from Kluyveromyces lactis. Biotechnology Letters. **25**: 623-629.

Mazzotti, M., Storti, G., y Morbidelli, M. 1997. Optimal operation of simulated moving bed units for nonlinear chromatographic separations. Journal of Chromatography A. **769**: 3-24.

McBain, A.J. y MacFarlane, G.T. 2001. Modulation of genotoxic enzyme activities by nondigestible oligosaccharide metabolism in in-vitro human gut bacterial ecosystems. Journal of Medical Microbiology. **50**: 833-842.

McCalley, D.V. 2010. Study of the selectivity, retention mechanisms and performance of alternative silica-based stationary phases for separation of ionised solutes in hydrophilic interaction chromatography. Journal of Chromatography A. **1217**: 3408-3417.

McGookin, B.J. y Augustin, M.A. 1991. Antioxidant activity of casein and maillard reaction-products from casein-sugar mixtures. Journal of Dairy Research. **58**: 313-320.

McNulty, D.E. y Annan, R.S. 2008. Hydrophilic interaction chromatography reduces the complexity of the phosphoproteome and improves global phosphopeptide isolation and detection. Molecular & Cellular Proteomics. **7**: 971-980.

Medzihradszky, K.F., GilleceCastro, B.L., Townsend, R.R., Burlingame, A.L., y Hardy, M.R. 1996. Structural elucidation of O-linked glycopeptides by high energy collision-induced dissociation. Journal of the American Society for Mass Spectrometry. **7**: 319-328.

Meisel, H. 1997. Biochemical properties of bioactive peptides derived from milk proteins: Potential nutraceuticals for food and pharmaceutical applications. Livestock Production Science. **50**: 125-138.

Meisel, H. 1998. Overview on milk protein-derived peptides. International Dairy Journal. **8**: 363-373.

Meisel, H. y Schlimme, E. 1990. Milk proteins: precursors of bioactive peptides. Trends in Food Science & Technology. **1**: 41-43.

Melmer, M., Stangler, T., Premstaller, A., y Lindner, W. 2011. Comparison of hydrophilic-interaction, reversed-phase and porous graphitic carbon chromatography for glycan analysis. Journal of Chromatography A. **1218**: 118-123.

Méndez, A. y Olano, A. 1979. Lactulose: A review on some chemical properties and applications in infant nutrition and medicine. Dairy Science Abstract. **41**: 531-535.

Mercier, J.-C., Chobert, J.-M., y Addeo, F. 1976. Comparative study of the amino acid sequences of the caseinomacropeptides from seven species. FEBS Letters. **72**: 208-214.

Mercier, J.C. 1981. Phosphorylation of caseins, present evidence for an amino-acid triplet code post-translationally recognized by specific kinases. Biochimie. **63**: 1-17.

Mercier, J.C., Addeo, F., y Pélissier, J.P. 1977. Structure primaire du caséinomacropeptide de la caseine K caprine. Biochimie. **58**: 1303-1310.

Metwally, M.M., El-Shibiny, S., Dieb, S.M.E., El-Etriby, H.M.M., y Assem, F.A. 2001. Large scale preparation and growth promoting effects on Bifidobacterium of

glycomacropeptide from sweet whey. Egyptian Journal of Dairy Science. **29**: 29-36.

Miguel, M., Manso, M.A., López-Fandiño, R., Alonso, M.J., y Salaices, M. 2007. Vascular effects and antihypertensive properties of κ-casein macropeptide. International Dairy Journal **17**: 1473-1477.

Mikkelsen, T.L., Rasmussen, E., Olsen, A., Barkholt, V., y Frokiaer, H. 2006. Immunogenicity of kappa-casein and glycomacropeptide. Journal of Dairy Science. **89**: 824-830.

Mills, D.J.S., Tuohy, K.M., Booth, J., Buck, M., Crabbe, M.J.C., Gibson, G.R., y Ames, J.M. 2008. Dietary glycated protein modulates the colonic microbiota towards a more detrimental composition in ulcerative colitis patients and non-ulcerative colitis subjects. Journal of Applied Microbiology. **105**: 706-714.

Minkiewicz, P., Slangen, C.J., Lagerwerf, F.M., Haverkamp, J., Rollema, H.S., y Visser, S. 1996. Reversed-phase high-performance liquid chromatographic separation of bovine k-casein macropeptide and characterization of isolated fractions. Journal of Chromatography A. **743**: 123-135.

Mischerikow, N., Altelaar, A.F.M., Navarro, J.D., Mohammed, S., y Heck, A.J.R. 2010. Comparative Assessment of Site Assignments in CID and Electron Transfer Dissociation Spectra of Phosphopeptides Discloses Limited Relocation of Phosphate Groups. Molecular & Cellular Proteomics. **9**: 2140-2148.

Mogna, G., Strozzi, G.P., y Mogna, L., -Patente WO 2009071578. Composition based on probiotic bacteria in association with a prebiotic and use thereof in the prevention and/or treatment of respiratory pathologies and/or infections and in the improvement of intestinal functionality.

Molle, D. y Leonil, J. 1995. Heterogeneity of the bovine kappa-casein caseinomacropeptide, resolved by liquid-chromatography online with electrospray-ionization mass-spectrometry. Journal of Chromatography A. **708**: 223-230.

Molnar-Perl, I. y Horvath, K. 1997. Simultaneous quantitation of mono-, di- and trisaccharides as their TMS ether oxime derivatives by GC-MS .1. In model solutions. Chromatographia. **45**: 321-327.

Montanes, F., Fornari, T., Martin-Alvarez, P.J., Corzo, N., Olano, A., y Ibanez, E. 2006. Selective recovery of tagatose from mixtures with galactose by direct extraction with supercritical CO2 and different cosolvents. Journal of Agricultural and Food Chemistry. **54**: 8340-8345.

Montanes, F., Corzo, N., Olano, A., Reglero, G., Ibanez, E., y Fornari, T. 2008. Selective fractionation of carbohydrate complex mixtures by supercritical extraction with CO2 and different co-solvents. Journal of Supercritical Fluids. **45**: 189-194.

Montilla, A., Lagemaat, J., Olano, A., y del Castillo, M.D. 2006. Determination of Oligosaccharides by Conventional High-Resolution Gas Chromatography. Chromatographia. **63**: 453-458.

Morales, V., Sanz, M.L., Olano, A., y Corzo, N. 2006. Rapid Separation on Activated Charcoal of High Oligosaccharides in Honey. Chromatographia. **64**: 1-6.

Moreno, F., Quintanilla-Lopez, J., Lebron-Aguilar, R., Olano, A., y Sanz, M. 2008. Mass Spectrometric Characterization of Glycated β-Lactoglobulin Peptides Derived from Galacto-oligosaccharides Surviving the *In Vitro* Gastrointestinal Digestion. Journal of the American Society for Mass Spectrometry. **19**: 927-937.

Moreno, F.J. y López-Fandiño, R., Estructura y funcionalidad del glicomacropéptido bovino, en Funcionalidad de Componentes Lácteos, Editores J. Fontecha and I. Recio,. 2009, Universidad Miguel Hernández: Elche. 223-249.

Moreno, F.J., Lopez-Fandino, R., y Olano, A. 2002. Characterization and functional properties of lactosyl caseinomacropeptide conjugates. Journal of Agricultural and Food Chemistry. **50**: 5179-5184.

Moreno, F.J., Recio, I., Olano, A., y Lopez-Fandino, R. 2000. Chromatographic characterization of ovine kappa-casein macropeptide. Journal of Dairy Research. **67**: 349-359.

Moreno, F.J., Recio, I., Olano, A., y Lopez-Fandino, R. 2001. Heterogeneity of caprine kappa-casein macropeptide. Journal of Dairy Research. **68**: 197-208.

Morishita, Y., Oowada, T., Ozaki, A., y Mizutani, T. 2002. Galactooligosaccharide in combination with Bifidobacterium and Bacteroides affects the population of Clostridium perfringens in the intestine of gnotobiotic mice. Nutrition Research. **22**: 1333-1341.

Moro, G.E., Stahl, B., Fanaro, S., Jelinek, J., Boehm, G., y Coppa, G.V. 2005. Dietary prebiotic oligosaccharides are detectable in the faeces of formula-fed infants. Acta Paediatrica. **94**: 27-30.

Mossine, V.V. y Mawhinney, T.P. 2007. N-alpha-(1-DeOXY-D-fructos-1-yl)-L-histidine ("D-fructose-L-histidine"): a potent copper chelator from tomato powder. Journal of Agricultural and Food Chemistry. **55**: 10373-10381.

Mussatto, S. y Mancilha, I. 2007. Non-digestible oligosaccharides: A review. Carbohydrate Polymers. **68**: 587-597.

Nakamura, S., Kato, A., y Kobayashi, K. 1992. Enhanced antioxidative effect of ovalbumin due to covalent binding of polysaccharides. Journal of Agricultural and Food Chemistry. **40**: 2033-2037.

Naranjo, G.B., Malec, L.S., y Vigo, M. 1998. Reducing sugars effect on available lysine loss of casein by moderate heat treatment. Food Chemistry. **62**: 309-313.

Neeser, J.R., Chambaz, A., Del Vedovo, S., Prigent, M.J., y Guggenheim, B. 1988. Specific and nonspecific inhibition of adhesion of oral actinomyces and streptococcii to erythrocytes and polystyrene by caseinoglycopeptide derivatives. Infection and Immunity. **56**: 3201-3208.

Neeser, J.R., Golliard, M., Woltz, A., Rouvet, M., Gillmann, M.L., y Guggenheim, B. 1994. *In vitro* modulation of oral bacterial adhesion to saliva-coated hydroxyapatite beads by milk casein derivatives. Oral Microbiology and Immunology **9**: 193-201.

Neish, A.S. 2009. Microbes in Gastrointestinal Health and Disease. Gastroenterology. **136**: 65-80.

Nelson, D. y Cox, M., Lehninger principles of biochemistry 2008, New York: W. H. Freeman and Company, co.

Neri, D.F.M., Balcão, V.M., Cardoso, S.M., Silva, A.M.S., Domingues, M.d.R.M., Torres, D.P.M., Rodrigues, L.R.M., Carvalho Jr, L.B., y Teixeira, J.A.C. 2011. Characterization of galactooligosaccharides produced by β-galactosidase immobilized onto magnetized Dacron. International Dairy Journal. **21**: 172-178.

Newcombe, G., Drikas, M., Assemi, S., y Beckett, R. 1997. Influence of characterised natural organic material on activated carbon adsorption .1. Characterisation of concentrated reservoir water. Water Research. **31**: 965-972.

Ney, D.M., Hull, A.K., van Calcar, S.C., Liu, X., y Etzel, M.R. 2008. Dietary glycomacropeptide supports growth and reduces the concentrations of phenylalanine in plasma and brain in a murine model of Phenylketonuria(1,2). Journal of Nutrition. **138**: 316-322.

Nguyen, H.P., Yang, S.H., Wigginton, J.G., Simpkins, J.W., y Schug, K.A. 2010. Retention behavior of estrogen metabolites on hydrophilic interaction chromatography stationary phases. Journal of Separation Science. **33**: 793-802.

Nielsen, P.M. y Tromholt, N. 1994. Method for production of a kappa-casein glycomacropeptide and use of a κ-casein glycomacropeptide. Brevet PCT WO/94. **15952**.

Noriega, L. 2008. Listeria monocytogenes: Informe de un aumento de casos en mujeres embarazadas y revisión de la literatura. revista Chilena de Infectología. **25**: 342-349.

O'Mahony, C., Scully, P., O'Mahony, D., Murphy, S., O'Brien, F., Lyons, A., Sherlock, G., MacSharry, J., Kiely, B., Shanahan, F., y O'Mahony, L. 2008. Commensal-induced regulatory T cells mediate protection against pathogen-stimulated NF-kappa B activation. Plos Pathogens. **4**.

Obana, H., Kikuchi, K., Okihashi, M., y Hori, S. 1997. Determination of organophosphorus pesticides in foods using an accelerated solvent extraction system. Analyst. **122**: 217-220.

Obrien, J. y Morrissey, P.A. 1989. Nutritional and toxicological aspects of the maillard browning reaction in foods. Critical Reviews in Food Science and Nutrition. **28**: 211-248.

Ohtsuka, K., Tsuji, K., Nakagawa, Y., Ueda, H., Ozawa, O., Uchida, T., y Ichikawa, T. 1990. Availability of 4'-galactosyllactose (o-beta-d-galactopyranosyl-(1- 4)-o-beta-d-galactopyranosyl-(1- 4)-d-glu copyranose) in rat. Journal of Nutritional Science and Vitaminology. **36**: 265-276.

Okatch, H., Torto, N., y Armateifio, J. 2003. Characterisation of legumes by enzymatic hydrolysis, microdialysis sampling, and micro-high-performance anion-exchange chromatography with electrospray ionisation mass spectrometry. Journal of Chromatography A. **992**: 67-74.

Okuda, T., Baes, A.U., Nishijima, W., y Okada, M. 2001. Isolation and characterization of coagulant extracted from Moringa oleifera seed by salt solution. Water Research. **35**: 405-410.

Olano-Martin, E., Gibson, G.R., y Rastall, R.A. 2002. Comparison of the *in vitro* bifidogenic properties of pectins and pectic-oligosaccharides. Journal of Applied Microbiology. **93**: 505-511.

Olano, A. y Corzo, N. 2009. Lactulose as a food ingredient. Journal of the Science of Food and Agriculture. **89**: 1987-1990.

Oliver, C., Melton, L., y Stanley, R. 2006. Creating Proteins with Novel Functionality via the Maillard Reaction: A Review. Critical Reviews in Food Science and Nutrition. **46**: 337-350.

Oliver, C.M., Melton, L.D., y Stanley, R.A. 2006. Creating proteins with novel functionality via the Maillard reaction: A review. Critical Reviews in Food Science and Nutrition. **46**: 337-350.

Ong, E.S. y Len, S.M. 2003. Pressurized hot water extraction of berberine, baicalein and glycyrrhizin in medicinal plants. Analytica Chimica Acta. **482**: 81-89.

Otani, H., Horimoto, Y., y Monnai, M. 1996. Suppression of interleukin-2 receptor expression on mouse CD4+T cells by bovine κ-caseinoglycopeptide. Bioscience, Biotechnology, and Biochemistry **60:** 1017-1019.

Otani, H., Monnai, M., Kawasaki, Y., Kawakami, H., y Tanimoto, M. 1995. Inhibition of mitogen-induced proliferative responses of lymphocytes by bovine κ-caseinoglycopeptides having different carbohydrate chains. Journal of Dairy Research. **62**: 349-357.

Otani, H., Horimoto, Y., Matin, M.A., Ohnuki, H., y Kawahara, T. 2005. Effect of bovine κ-caseinoglycopeptide on mitogen-induced proliferative responses of spleen cells from different strains of mice. Milchwissenschaft. **60**: 245-248.

Otieno, D.O. 2010. Synthesis of β-Galactooligosaccharides from Lactose Using Microbial β-Galactosidases. Comprehensive Reviews in Food Science and Food Safety. **9**: 471-482.

Ouwehand, A.C., Derrien, M., de Vos, W., Tiihonen, K., y Rautonen, N. 2005. Prebiotics and other microbial substrates for gut functionality. Current Opinion in Biotechnology. **16**: 212-217.

Palframan, R., Gibson, G.R., y Rastall, R.A. 2003. Development of a quantitative tool for the comparison of the prebiotic effect of dietary oligosaccharides. Letters in Applied Microbiology. **37**: 281-284.

Palumbo, A.M. y Reid, G.E. 2008. Evaluation of Gas-Phase Rearrangement and Competing Fragmentation Reactions on Protein Phosphorylation Site Assignment Using Collision Induced Dissociation-MS/MS and MS^3. Analytical Chemistry. **80**: 9735-9747.

Panagiotopoulos, C., Repeta, D.J., y Johnson, C.G. 2007. Characterization of methyl sugars, 3-deoxysugars and methyl deoxysugars in marine high molecular weight dissolved organic matter. Organic Geochemistry. **38**: 884-896.

Park, C.W., Youn, M., Jung, Y.-M., Kim, H., Jeong, Y., Lee, H.-K., Kim, H.O., Lee, I., Lee, S.W., Kang, K.H., y Park, Y.-H. 2008. New functional probiotic Lactobacillus sakei probio 65 alleviates atopic symptoms in the mouse. Journal of Medicinal Food. **11**: 405-412.

Park, S., Cho, H., Kim, Y., Ahn, S., y Chang, T. 2007. Fast size-exclusion chromatography at high temperature. Journal of Chromatography A. **1157**: 96-100.

Parracho, H., McCartney, A.L., y Gibson, G.R. 2007. Probiotics and prebiotics in infant nutrition. Proceedings of the Nutrition Society. **66**: 405-411.

Pastell, H., Westermann, P., Meyer, A.S., Tuomainen, P.i., y Tenkanen, M. 2009. *In Vitro* Fermentation of Arabinoxylan-Derived Carbohydrates by Bifidobacteria and Mixed Fecal Microbiota. Journal of Agricultural and Food Chemistry. **57**: 8598-8606.

Patel, S. y Goyal, A. 2010. Functional oligosaccharides: production, properties and applications. World Journal of Microbiology and Biotechnology. **27**: 1119-1128.

Pentecost, M., Kumaran, J., Ghosh, P., y Amieva, M.R. 2010. Listeria monocytogenes Internalin B Activates Junctional Endocytosis to Accelerate Intestinal Invasion. Plos Pathogens. **6**.

Pereyra Gonzales, A.S., Naranjo, G.B., Leiva, G.E., y Malec, L.S. 2010. Maillard reaction kinetics in milk powder: Effect of water activity at mild temperatures. International Dairy Journal. **20**: 40-45.

Perrin, S., Grill, J.P., y Schneider, F. 2000. Effects of fructooligosaccharides and their monomeric components on bile salt resistance in three species of bifidobacteria. Journal of Applied Microbiology. **88**: 968-974.

Phelan, M., Aherne, A., FitzGerald, R.J., y O'Brien, N.M. 2009. Casein-derived bioactive peptides: Biological effects, industrial uses, safety aspects and regulatory status. International Dairy Journal. **19**: 643-654.

Picariello, G., Ferranti, P., Mamone, G., Roepstorff, P., y Addeo, F. 2008. Identification of N-linked glycoproteins in human milk by hydrophilic interaction liquid chromatography and mass spectrometry. Proteomics. **8**: 3833-3847.

Pires, S.M., Vigre, H., Makela, P., y Hald, T. 2010. Using Outbreak Data for Source Attribution of Human Salmonellosis and Campylobacteriosis in Europe. Foodborne Pathogens and Disease. **7**: 1351-1361.

Pisano, A., Packer, N.H., Redmond, J.W., Williams, K.L., y Gooley, A.A. 1994. Characterization of O-linked glycosylation motifs in the glycopeptides domain of bovine kappa-casein. Glycobiology. **4**: 837-844.

Pitino, I., Randazzo, C.L., Mandalari, G., Lo Curto, A., Faulks, R.M., Le Marc, Y., Bisignano, C., Caggia, C., y Wickham, M.S.J. 2010. Survival of Lactobacillus rhamnosus strains in the upper gastrointestinal tract. Food Microbiology. **27**: 1121-1127.

Pool-Zobel, B.L. 2006. Inulin-type fructans and reduction in colon cancer risk: review of experimental and human data. British Journal of Nutrition. **93**: S73-S90.

Poolman, B. 1993. Energy transduction in lactic-acid bacteria. Fems Microbiology Reviews. **12**: 125-148.

Popovici, S.T. y Schoenmakers, P.J. 2005. Fast size-exclusion chromatography - Theoretical and practical considerations. Journal of Chromatography A. **1099**: 92-102.

Prinzenberg, E.M., Krause, I., y Erhardt, G. 1999. SSCP analysis at the bovine CSN3 locus discriminates six alleles corresponding to known protein variants (A, B, C, E, F,

G) and three new DNA polymorphisms (H, I, A(1)). Animal Biotechnology **10**: 49-62.

Quigley, E.M.M. 2010. Prebiotics and probiotics; modifying and mining the microbiota. Pharmacological Research. **61**: 213-218.

Ramos, J.J., Diez, C., Camara, C., y Ramos, L., Dispositivo miniaturizado de extracción con líquidos a presión, U.C.d. Madrid-CSIC, Editor. 2006: España.

Rasmussen, L.K., Sorensen, E.S., Petersen, T.E., Nielsen, N.C., y Thomsen, J.K. 1997. Characterization of phosphate sites in native ovine, caprine, and bovine casein micelles and their caseinomacropeptides: A solid-state phosphorus-31 nuclear magnetic resonance and sequence and mass spectrometric study. Journal of Dairy Science. **80**: 607-614.

Rastall, R., Gibson, G., Fuller, R., y Gaskins, H.R., Colonic Functional Foods, in Functional Foods - Concept to product, G. Gibson and C.M. Williams, Editors. 2000, Wodhead Publishing: Cambridge. 71-93.

Rastall, R., Gibson, G.R., Gill, H.S., Guarner, F., Klaenhammer, T.R., Pot, B., Reid, G., Rowland, I.R., y Sanders, M.E. 2005. Modulation of the microbial ecology of the human colon by probiotics, prebiotics and synbiotics to enhance human health: An overview of enabling science and potential applications. FEMS Microbiology Ecology. **52**: 145-152.

Rastall, R.A. 2010. Functional Oligosaccharides: Application and Manufacture. Annual Review of Food Science and Technology **1**: 305-339.

Rastall, R.A. y Maitin, V. 2002. Prebiotics and synbiotics: towards the next generation. Current Opinion in Biotechnology. **13**: 490-496.

Raynie, D.E. 2010. Modern Extraction Techniques. Analytical Chemistry. **82**: 4911-4916.

Read, N.W., Aljanabi, M.N., Holgate, A.M., Barber, D.C., y Edwards, C.A. 1986. Simultaneous measurement of gastric-emptying, small-bowel residence and colonic filling of a solid meal by the use of the gamma-camera. Gut. **27**: 300-308.

Recio, I., Moreno, F.J., y López-Fandiño, R., Glycosylated dairy components: their roles in nature and ways to make use of their biofunctionality in dairy products, en In

Dairy-derived ingredients: food and nutraceutical uses, Editor M. Corredig, 2009, Woodhead Publishing Ltd: Cambridge. 170-211.

Reeves, P.G. 1997. Components of the AIN-93 Diets as Improvements in the AIN-76A Diet. Journal of Nutrition. **127**: 838S-841S

Reichardt, N., Gniechwitz, D., Steinhart, H., Bunzel, M., y Blaut, M. 2009. Characterization of high molecular weight coffee fractions and their fermentation by human intestinal microbiota. Molecular Nutrition & Food Research. **53**: 287-299.

Reid, G. 2008. Probiotics and prebiotics – Progress and challenges. International Dairy Journal. **18**: 969-975.

Reid, G., Younes, J.A., Van der Mei, H.C., Gloor, G.B., Knight, R., y Busscher, H.J. 2011. Microbiota restoration: natural and supplemented recovery of human microbial communities. Nature Reviews Microbiology. **9**: 27-38.

Reid, G., Sanders, M.E., Gaskins, H.R., Gibson, G.R., Mercenier, A., Rastall, R., Roberfroid, M., Rowland, I., Cherbut, C., y Klaenhammer, T.R. 2003. New scientific paradigms for probiotics and prebiotics. Journal of Clinical Gastroenterology. **37**: 105-118.

Reinders, J. y Sickmann, A. 2005. State of the art in phosphoproteomics. Proteomics. **5**: 4052-4061.

Requena, P., Daddaoua, A., Guadix, E., Zarzuelo, A., Dolores Suarez, M., Sanchez de Medina, F., y Martinez-Augustin, O. 2009. Bovine glycomacropeptide induces cytokine production in human monocytes through the stimulation of the MAPK and the NF-kappa B signal transduction pathways. British Journal of Pharmacology. **157**: 1232-1240.

Resmini, P., Pellegrino, L., y Battelli, G. 1990. Accurate quantification of furosine in milk and dairy products by a direct HPLC method. Italian Journal of Food Science. **2**: 173-183.

Ririe, K.M., Rasmussen, R.P., y Wittwer, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. Analytical Biochemistry. **245**: 154-160.

Roberfroid, M. 2007. Prebiotics: The concept revisited. Journal of Nutrition. **137**: 830S-837S.

Roberfroid, M., Gibson, G.R., Hoyles, L., McCartney, A.L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M.J., Leotoing, L., Wittrant, Y., Delzenne, N.M., Cani, P.D., Neyrinck, A.M., y Meheust, A. 2010. Prebiotic effects: metabolic and health benefits. British Journal of Nutrition. **104**: S1-S63.

Roberfroid, M.B. 2000. Prebiotics and probiotics: are they functional foods? American Journal of Clinical Nutrition. **71**: 1682S-1687S.

Robert, L., Labat-Robert, J., y Robert, A.M. 2010. The Maillard reaction. From nutritional problems to preventive medicine. Pathologie Biologie. **58**: 200-206.

Rocklin, R.D. y Pohl, C.A. 1983. Determination of carbohydrates by anion-exchange chromatography with pulsed amperometric detection. Journal of Liquid Chromatography. **6**: 1577-1590.

Rodrigues, R.C.R., Canhoto, T.J.S.B., Araujo, J.M.M., y Mota, J.P.B. 2008. Two-column simulated moving-bed process for binary separation. Journal of Chromatography A. **1180**: 42-52.

Roe, J.H. 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. Journal of Biological Chemistry. **212**: 335-343.

Roepstorff, P. y Fohlman, J. 1984. Proposal for a common nomenclature for sequence ions in mass-spectra of peptides. Biomedical Mass Spectrometry. **11**: 601-601.

Roy, C.C., Kien, C.L., Bouthillier, L., y Levy, E. 2006. Short-Chain Fatty Acids: Ready for Prime Time? Nutrition in Clinical Practice. **21**: 351-366.

Roy, K., Hilliard, G.M., Hamilton, D.J., Luo, J., Ostmann, M.M., y Fleckenstein, J.M. 2009. Enterotoxigenic Escherichia coli EtpA mediates adhesion between flagella and host cells. Nature. **457**: 594-U103.

Rufian-Henares, J.A. y de la Cueva, S.P. 2009. Antimicrobial Activity of Coffee Melanoidins. A Study of Their Metal-Chelating Properties. Journal of Agricultural and Food Chemistry. **57**: 432-438.

Ruiz-Matute, A., Desarrollo de nuevas metodologías para la caracterización de los carbohidratos de la miel y su utilidad en la detección de adulteraciones, Tesis Doctoral. 2007, Universidad Autónoma de Madrid: Madrid.

Ruiz-Matute, A., Soria, A.C., Martinez-Castro, I., y Sanz, M.L. 2007. A new methodology based on GC-MS to detect honey adulteration with commercial syrups. Journal of Agricultural and Food Chemistry. **55**: 7264-7269.

Ruiz-Matute, A.I., Ramos, L., Martinez-Castro, I., y Sanz, M.L. 2008. Fractionation of honey carbohydrates using pressurized liquid extraction with activated charcoal. Journal of Agricultural and Food Chemistry. **56**: 8309-8313.

Ruiz-Matute, A.I., Brokl, M., Soria, A.C., Sanz, M.L., y Martínez-Castro, I. 2010. Gas chromatographic–mass spectrometric characterisation of tri- and tetrasaccharides in honey. Food Chemistry. **120**: 637-642.

Ruiz-Matute, A.I., Hernández-Hernández, O., Rodríguez-Sánchez, S., Sanz, M.L., y Martínez-Castro, I. 2011. Derivatization of carbohydrates for GC and GC-MS analyses. Journal of Chromatography B. **879**: 1226-1240.

Ruiz-Matute, A.I., Sanz, M.L., Corzo, N., Martin-Alvarez, P.J., Ibanez, E., Martinez-Castro, I., y Olano, A. 2007. Purification of lactulose from mixtures with lactose using pressurized liquid extraction with ethanol-water at different temperatures. Journal of Agricultural and Food Chemistry. **55**: 3346-3350.

Rumessen, J.J., Bode, S., Hamberg, O., y Gudmandhoyer, E. 1990. Fructans of Jerusalem artichokes: intestinal transport, absorption, fermentation, and influence on blood glucose, insulin, and C-peptide responses in healthy subjects. American Journal of Clinical Nutrition. **52**: 675-681.

Rycroft, C.E., Jones, M.R., Gibson, G.R., y Rastall, R.A. 2001. A comparative *in vitro* evaluation of the fermentation properties of prebiotic oligosaccharides. Journal of Applied Microbiology. **91**: 878-887.

Saito, T. y Itoh, T. 1992. Variations and distributions of o-glycosidically linked sugar chains in bovine kappa-casein. Journal of Dairy Science. **75**: 1768-1774.

Sako, T., Matsumoto, K., y Tanaka, R. 1999. Recent progress on research and applications of non-digestible galacto-oligosaccharides. International Dairy Journal. **9**: 69-80.

Salminen, S., Ouwehand, A., Benno, Y., y Lee, Y.K. 1999. Probiotics: how should they be defined? Trends in Food Science & Technology. **10**: 107-110.

Salvi, M., Cesaro, L., Tibaldi, E., y Pinna, L.A. 2010. Motif Analysis of Phosphosites Discloses a Potential Prominent Role of the Golgi Casein Kinase (GCK) in the

Generation of Human Plasma Phospho-Proteome. Journal of Proteome Research. **9**: 3335-3338.

Sanders, M.E. y Marco, M.L. 2010. Food Formats for Effective Delivery of Probiotics. Annual Review of Food Science and Technology. **1:** 65-85.

Sandhu, K.S. y Lim, S.-T. 2008. Structural characteristics and *in vitro* digestibility of mango kernel starches (Mangifera indica L.). Food Chemistry. **107**: 92-97.

Sanz, M. y Martinezcastro, I. 2007. Recent developments in sample preparation for chromatographic analysis of carbohydrates. Journal of Chromatography A. **1153**: 74-89.

Sanz, M., Sanz, J., y Martinezcastro, I. 2004. Gas chromatographic–mass spectrometric method for the qualitative and quantitative determination of disaccharides and trisaccharides in honey. Journal of Chromatography A. **1059**: 143-148.

Sanz, M., Gibson, G.R., y Rastall, R.A. 2005. Influence of disaccharide structure on prebiotic selectivity *in vitro*. Journal of Agricultural and Food Chemistry. **53**: 5192-5199.

Sanz, M., Cote, G.L., Gibson, G.R., y Rastall, R.A. 2006. Influence of glycosidic linkages and molecular weight on the fermentation of maltose-based oligosaccharides by human gut bacteria. Journal of Agricultural and Food Chemistry. **54**: 9779-9784.

Sanz, M., Corzo-Martinez, M., Rastall, R.A., Olano, A., y Moreno, F.J. 2007. Characterization and *in vitro* digestibility of bovine beta-lactoglobulin glycated with galactooligosaccharides. Journal of Agricultural and Food Chemistry. **55**: 7916-7925.

Sanz, M., Polemis, N., Morales, V., Corzo, N., Drakoularakou, A., Gibson, G.R., y Rastall, R.A. 2005. *In vitro* investigation into the potential prebiotic activity of honey oligosaccharides. Journal of Agricultural and Food Chemistry. **53**: 2914-2921.

Sanz, M.a.L., Côté, G.L., Gibson, G.R., y Rastall, R.A. 2006. Selective fermentation of gentiobiose-derived oligosaccharides by human gut bacteria and influence of molecular weight. FEMS Microbiology Ecology. **56**: 383-388.

Sanz, M.L., Sanz, J., y Martinez-Castro, I. 2002. Characterization of O-trimethylsilyl oximes of disaccharides by gas chromatography-mass spectrometry. Chromatographia. **56**: 617-622.

Sanz, M.L., Côté, G.L., Gibson, G.R., y Rastall, R.A. 2006. Influence of glycosidic linkages and molecular weight on the fermentation of maltose-based oligosaccharides by human gut bacteria. Journal of Agricultural and Food Chemistry. **54**: 9779-9784.

Sanz, M.L., Ruiz-Matute, A., Corzo, N., y Martínez-Castro, I., Analysis of Prebiotic Oligosaccharides, en Prebiotics and Porbiotics Science and technology, Editores D. Charalampopoulos y R.A. Rastall,. 2009, Springer: New York. 465-534.

Sanz, M.L., Corzo-Martinez, M., Rastall, R.A., Olano, A., y Moreno, F.J. 2007. Characterization and *in vitro* digestibility of bovine beta-lactoglobulin glycated with galactooligosaccharides. Journal of Agricultural and Food Chemistry. **55**: 7916-7925.

Sarabia-Sainz, A.-i., Ramos-Clamont, G., del Carmen Candia-Plata, M.M., y Vazquez-Moreno, L. 2009. Biorecognition of Escherichia coli K88 adhesin for glycated porcine albumin. International Journal of Biological Macromolecules. **44**: 175-181.

Satokari, R.M., Vaughan, E.E., Akkermans, A.D.L., Saarela, M., y de Vos, W.M. 2001. Bifidobacterial diversity in human feces detected by genus-specific PCR and denaturing gradient gel electrophoresis. Applied and Environmental Microbiology. **67**: 504-513.

Satomi, Y., Shimonishi, Y., Hase, T., y Takao, T. 2004. Site-specific carbohydrate profiling of human transferrin by nano-flow liquid chromatography/electrospray ionization mass spectrometry. Rapid Communications in Mass Spectrometry. **18**: 2983-2988.

Saz, J.M. y Marina, M.L. 2007. High performance liquid chromatography and capillary electrophoresis in the analysis of soybean proteins and peptides in foodstuffs. Journal of Separation Science. **30**: 431-451.

Schaaff, T.G., Cargile, B.J., Stephenson, J.L., y McLuckey, S.A. 2000. Ion trap collisional activation of the (M+2H)(2+)-(M+17H)(17+) ions of human hemoglobin beta-chain. Analytical Chemistry. **72**: 899-907.

Scheinth.Bm y Bettelhe.Fa. 1968. Multiple forms of sialic acids. Carbohydrate Research. **6**: 257-&.

Schulte, M. y Strube, J. 2001. Preparative enantioseparation by simulated moving bed chromatography. Journal of Chromatography A. **906**: 399-416.

Schumacher, D. y Kroh, L.W. 1995. A rapid method for separation of anomeric saccharides using a cyclodextrin bonded phase and for investigation of mutarotation. Food Chemistry. **54**: 353-356.

Schumann, C. 2002. Medical, nutritional and technological properties of lactulose. An update. European Journal of Nutrition. **41**: 1-1.

Schupbach, P., Neeser, J.R., Golliard, M., Rouvet, M., y Guggenheim, B. 1996. Incorporation of caseinoglycomacropeptide and caseinophosphopeptide into the salivary pellicle inhibits adherence of mutans streptococci. Journal of Dental Research **75**: 1779-1788.

Schuster-Wolff-Bühring, R., Fischer, L., y Hinrichs, J. 2010. Production and physiological action of the disaccharide lactulose. International Dairy Journal. **20**: 731-741.

Seibert, S.A., Mex, P., Koehler, A., Kaufmann, S.H.E., y Mittruecker, H.-W. 2010. TLR2-, TLR4-and Myd88-independent acquired humoral and cellular immunity against Salmonella enterica serovar Typhimurium. Immunology Letters. **127**: 126-134.

Seipert, R.R., Dodds, E.D., y Lebrilla, C.B. 2009. Exploiting Differential Dissociation Chemistries of O-Linked Glycopeptide Ions for the Localization of Mucin-Type Protein Glycosylation. Journal of Proteome Research. **8**: 493-501.

Seipert, R.R., Barboza, M., Ninonuevo, M.R., LoCascio, R.G., Mills, D.A., Freeman, S.L., German, J.B., y Lebrilla, C.B. 2008. Analysis and quantitation of fructooligosaccharides using matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry. Analytical Chemistry. **80**: 159-165.

Seiquer, I., Delgado-Andrade, C., y Navarro, P. 2000. Effects of the heat treatment of casein on Zn transport and uptake by Caco-2 cells. Proceedings of the Nutrition Society. **59**: 134A-134A.

Seiquer, I., Delgado-Andrade, C., Haro, A., y Navarro, M.P. 2010. Assessing the effects of severe heat treatment of milk on calcium bioavailability: *In vitro* and *in vivo* studies. Journal of Dairy Science. **93**: 5635-5643.

Shammet, K.M., Brown, R.J., y McMahon, D.J. 1992. Proteolytic activity of proteinases on macropeptide isolated from kappa-casein. Journal of Dairy Science. **75**: 1380-1388.

Sharon, N. 2006. Carbohydrates as future anti-adhesion drugs for infectious diseases. Biochimica Et Biophysica Acta-General Subjects. **1760**: 527-537.

Shoaf, K., Mulvey, G.L., Armstrong, G.D., y Hutkins, R.W. 2006. Prebiotic Galactooligosaccharides Reduce Adherence of Enteropathogenic Escherichia coli to Tissue Culture Cells. Infection and Immunity. **74**: 6920-6928.

Silvan, J., Vandelagemaat, J., Olano, A., y Delcastillo, M. 2006. Analysis and biological properties of amino acid derivates formed by Maillard reaction in foods. Journal of Pharmaceutical and Biomedical Analysis. **41**: 1543-1551.

Sinclair, H.R., de Slegte, J., Gibson, G.R., y Rastall, R.A. 2009. Galactooligosaccharides (GOS) Inhibit Vibrio cholerae Toxin Binding to Its GM1 Receptor. Journal of Agricultural and Food Chemistry. **57**: 3113-3119.

Skorepova, J. y Moresoli, C. 2007. Carbohydrate and mineral removal during the production of low-phytate soy protein isolate by combined electroacidification and high shear tangential flow ultrafiltration. Journal of Agricultural and Food Chemistry. **55**: 5645-5652.

Sleator, R.D. 2010. Probiotic therapy - recruiting old friends to fight new foes. Gut pathogens. **2**: 5-5.

Sleator, R.D. y Hill, C. 2008. New frontiers in probiotic research. Letters in Applied Microbiology. **46**: 143-147.

Smiricky-Tjardes, M.R., Grieshop, C.M., Flickinger, E.A., Bauer, L.L., y Fahey Jr, G.C. 2003. Dietary galactooligosaccharides affect ileal and total-tract nutrient digestibility, ileal and fecal bacterial concentrations, and ileal fermentative characteristics of growing pigs. Journal of Animal Science. **81**: 2535-2545.

Smith, R. 2003. Before the injection—modern methods of sample preparation for separation techniques. Journal of Chromatography A. **1000**: 3-27.

Smithers, G.W., Pearce, R.J., Regester, G.O., y Bradford, R.S. 1991. New casein protein productsfor the food industry: physical, chemical and enzymatic manipulation of milk. Food Australia. **43**: 252–254.

Soccol, C.R., de Souza Vandenberghe, L.P., Spier, M.R., Pedroni Medeiros, A.B., Yamaguishi, C.T., Lindner, J.D.D., Pandey, A., y Thomaz-Soccol, V. 2010. The Potential of Probiotics: A Review. Food Technology and Biotechnology. **48**: 413-434.

Somboonpanyakul, P., Wang, Q., Cui, W., Barbut, S., y Jantawat, P. 2006. Malva nut gum. (Part I): Extraction and physicochemical characterization. Carbohydrate Polymers. **64**: 247-253.

Somoza, V. 2005. Five years of research on health risks and benefits of Maillard reaction products: An update. Molecular Nutrition & Food Research. **49**: 663-672.

Song, C.X., Ye, M.L., Han, G.H., Jiang, X.N., Wang, F.J., Yu, Z.Y., Chen, R., y Zou, H.F. 2010. Reversed-Phase-Reversed-Phase Liquid Chromatography Approach with High Orthogonality for Multidimensional Separation of Phosphopeptides. Analytical Chemistry. **82**: 53-56.

Stahl, M., von Brocke, A., y E., B., Mass Spectrometry of Oligosaccharides, en Carbohydrate Analysis by modern Chromatography and Electrophoresis, Editor Z. El Rassi, 2002, Elsevier Science. 961-1042.

Steed, H. y Macfarlane, S., Mechanisms of Prebiotic Impact on Health, en Prebiotics and Probiotics Sciences and Technology, Editores D. Charalampopoulos y R. Rastall,. 2009, Springer: New York. 135-162.

Stimson, E., Hope, J., Chong, A., y Burlingame, A.L. 1999. Site-Specific Characterization of the N-Linked Glycans of Murine Prion Protein by High-Performance Liquid Chromatography/Electrospray Mass Spectrometry and Exoglycosidase Digestions. Biochemistry. **38**: 4885–4895.

Strege, M.A. 1998. Hydrophilic interaction chromatography electrospray mass spectrometry analysis of polar compounds for natural product drug discovery. Analytical Chemistry. **70**: 2439-2445.

Strober, W., Interactions between epithelial cells and immune cells in the intestine, en Intestinal Plasticity in Health and Disease, Editor B.R.S.Z.M.R.E.O. Wiedenmann, 1998. 37-45 New York Academy of Sciences.

Stromqvist, M., Falk, P., Bergstrom, S., Hansson, L., Lonnerdal, B., Normark, S., y Hernell, O. 1995. Human-milk k-casein and inhibition oh Helicobacter pylori

adhesion to human gastric-mucosa. Journal of Pediatric Gastroenterology and Nutrition. **21**: 288-296.

Sugita-Konishi, Y., Kobayashi, K., Sakanaka, S., Juneja, L.R., y Amano, F. 2004. Preventive effect of sialylglycopeptide - Nondigestive polysaccharide conjugates on Salmonella infection. Journal of Agricultural and Food Chemistry. **52**: 5443-5448.

Summa, C., McCourt, J., Cämmerer, B., Fiala, A., Probst, M., Kun, S., Anklam, E., y Wagner, K.-H. 2008. Radical scavenging activity, anti-bacterial and mutagenic effects of Cocoa bean Maillard Reaction products with degree of roasting. Molecular Nutrition & Food Research. **52**: 342-351.

Takegawa, Y., Hato, M., Deguchi, K., Nakagawa, H., y Nishimura, S.-I. 2008. Chromatographic deuterium isotope effects of derivatized N-glycans and N-glycopeptides in a zwitterionic type of hydrophilic interaction chromatography. Journal of Separation Science. **31**: 1594-1597.

Takegawa, Y., Deguchi, K., Keira, T., Ito, H., Nakagawa, H., y Nishimura, S. 2006. Separation of isomeric 2-aminopyridine derivatized N-glycans and N-glycopeptides of human serum immunoglobulin G by using a zwitterionic type of hydrophilic-interaction chromatography. Journal of Chromatography A. **1113**: 177-181.

Takegawa, Y., Deguchi, K., Ito, H., Keira, T., Nakagawa, H., y Nishimura, S.-I. 2006. Simple separation of isomeric sialylated N-glycopeptides by a zwitterionic type of hydrophilic interaction chromatography. Journal of Separation Science. **29**: 2533-2540.

Takeuchi, M., Tsuda, E., Yoshikawa, M., Sasaki, R., y Chiba, H. 1985. Fractionation and characterisation of 9 subcomponents of bovine k-casein A. Agricultural and Biological Chemistry. **49**: 2269-2276.

Talbo, G.H., Suckau, D., Malkoski, M., y Reynolds, E.C. 2001. MALDI-PSD-MS analysis of the phosphorylation sites of caseinomacropeptide. Peptides. **22**: 1093-1098.

Tanaka, R., Takayama, H., Morotomi, M., Kuroshima, T., Ueyama, S., Matsumoto, K., Kuroda, A., y Mutai, M. 1983. Effects of administration of transgalacto-oligosaccharides and Bifidobacterium breve 4006 on the human fecal flora. Bifidobacteria Microflora. **2**: 17-24.

Thammarutwasik, P., Hongpattarakere, T., Chantachum, S., kijroongrojana, K., Itharat, A., reanmongkol, W., Tewtrakul, S., y Ooraikul, B. 2009. Prebiotics- A review. Songklanakarin Journal of Science and Technology. **31**: 401-408.

Thingholm, T.E., Jensen, O.N., y Larsen, M.R. 2009. Analytical strategies for phosphoproteomics. Proteomics. **9**: 1451-1468.

Thomae-Worringer, C., Sorensen, J., y Lopez-Findino, R. 2006. Health effects and technological features of caseinomacropeptide. International Dairy Journal. **16**: 1324-1333.

Thomas, D.W., Greer, F.R., y Comm, N. 2010. Clinical Report-Probiotics and Prebiotics in Pediatrics. Pediatrics. **126**: 1217-1231.

Tiihonen, J., Peuha, E.L., Latva-Kokko, M., Silander, S., y Paatero, E. 2005. Subcritical water as eluent for chromatographic separation of carbohydrates using cation-exchange resins. Separation and Purification Technology. **44**: 166-174.

Tolstikov, V.V. y Fiehn, O. 2002. Analysis of Highly Polar Compounds of Plant Origin: Combination of Hydrophilic Interaction Chromatography and Electrospray Ion Trap Mass Spectrometry. Analytical Biochemistry. **301**: 298-307.

Torres, D.P.M., Gonçalves, M.d.P.F., Teixeira, J.A., y Rodrigues, L.R. 2010. Galacto-Oligosaccharides: Production, Properties, Applications, and Significance as Prebiotics. Comprehensive Reviews in Food Science and Food Safety. **9**: 438-454.

Toumi, A., Engell, S., Diehl, A., Bock, H.G., y Schloeder, J. 2007. Efficient optimization of simulated moving bed processes. Chemical Engineering and Processing. **46**: 1067-1084.

Troyer, J.K., Stephenson, K.K., y Fahey, J.W. 2001. Analysis of glucosinolates from broccoli and other cruciferous vegetables by hydrophilic interaction liquid chromatography. Journal of Chromatography A. **919**: 299-304.

Tuohy, K.M., Rouzaud, G.C.M., Bruck, W.M., y Gibson, G.R. 2005. Modulation of the human gut microflora towards improved health using prebiotics - Assessment of efficacy. Current Pharmaceutical Design. **11**: 75-90.

Tuohy, K.M., J., Z.C., Klinder, A., Knobel, Y., Pool-Zobel, B.L., y Gibson, G.R. 2002. A human volunteer study to determine the prebiotic effects of lactulose powder

on human colonic microbiota. Microbial Ecology in Health and Disease. **14**: 165-173.

Tuohy, K.M., Hinton, D.J.S., Davies, S.J., Crabbe, M.J.C., Gibson, G.R., y Ames, J.M. 2006. Metabolism of Maillard reaction products by the human gut microbiota – implications for health. Molecular Nutrition & Food Research. **50**: 847-857.

Turpin, W., Humblot, C., Thomas, M., y Guyot, J.-P. 2010. Lactobacilli as multifaceted probiotics with poorly disclosed molecular mechanisms. International Journal of Food Microbiology. **143**: 87-102.

Tzortzis, G. y Vulevic, J., Galacto-oligosaccharide Prebiotics, en Prebiotics and Probiotics Science and Technology, Editores D. Charalampopoulos y R. Rastall,. 2009, Springer: New York. 207-244.

Tzortzis, G., Goulas, A.K., Gee, J.M., y Gibson, G.R. 2005. A novel galactooligosaccharide mixture increases the bifidobacterial population numbers in a continuous *in vitro* fermentation system and in the proximal colonic contents of pigs *in vivo*. Journal of Nutrition. **135**: 1726-1731.

Tzortzis, G., Jay, A.J., Baillon, M.L.A., Gibson, G.R., y Rastall, R.A. 2003. Synthesis of alpha-galactooligosaccharides with alpha-galactosidase from Lactobacillus reuteri of canine origin. Applied Microbiology and Biotechnology. **63**: 286-292.

Tzortzis, G., Goulas, A.K., Baillon, M.L.A., Gibson, G.R., y Rastall, R.A. 2004. *In vitro* evaluation of the fermentation properties of galactooligosaccharides synthesised by α-galactosidase from Lactobacillus reuteri. Applied Microbiology and Biotechnology. **64**: 106-111.

Vacca Smith, A.M. y Bowen, W.H. 2000. The effects of milk and κ-casein on salivary pellicle formed on hydroxyapatite discs in situ. Caries Research. **34**: 88-93.

Vaisar, T. y Urban, J. 1996. Probing the proline effect in CID of protonated peptides. Journal of Mass Spectrometry. **31**: 1185-1187.

Valerio, F., De Bellis, P., Lonigro, S.L., Morelli, L., Visconti, A., y Lavermicocca, P. 2006. and *in vivo* survival and transit tolerance of potentially probiotic strains carried by artichokes in the gastrointestinal tract. Applied and Environmental Microbiology. **72**: 3042-3045.

van Boekel, M. 2001. Kinetic aspects of the Maillard reaction: a critical review. Nahrung. **45**: 45 (3) 150-159.

van Boekel, M., Fogliano, V., Pellegrini, N., Stanton, C., Scholz, G., Lalljie, S., Somoza, V., Knorr, D., Rao Jasti, P., y Eisenbrand, G. 2010. A review on the beneficial aspects of food processing. Molecular Nutrition and Food Research. **54**: 54 (9) 1215-1247.

Van Laere, K.M.J., Hartemink, R., Bosveld, M., Schols, H.A., y Voragen, A.G.J. 2000. Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. Journal of Agricultural and Food Chemistry. **48**: 1644-1652.

Van Loo, J.A.E. 2004. Prebiotics promote good health: The basis, the potential, and the emerging evidence. Journal of Clinical Gastroenterology. **38**: S70-S75.

Vance, B.A., Wu, W., Ribaudo, R., Segal, D.M., y Kearse, K.P. 1997. CD69 heterogeneity results from usage of both typical (N-X-T) and atypical (N-X-C) N-linked glycosylation motifs. Journal of Allergy and Clinical Immunology. **99**: 1869-1869.

Vegas, R., Moure, A., Dominguez, H., Parajo, J.C., Alvarez, J.R., y Luque, S. 2008. Evaluation of ultra- and nanofiltration for refining soluble products from rice husk xylan. Bioresource Technology. **99**: 5341-5351.

Vente, J.A., Bosch, H., de Haan, A.B., y Bussmann, P.J.T. 2005. Comparison of sorption isotherms of mono- and disaccharides relevant to oligosaccharide separations for Na, K, and Ca loaded cation exchange resins. Chemical Engineering Communications. **192**: 23-33.

Vernazza, C.L., Gibson, G.R., y Rastall, R.A. 2006. Carbohydrate preference, acid tolerance and bile tolerance in five strains of Bifidobacterium. Journal of Applied Microbiology. **100**: 846-853.

Via, A., Diella, F., Gibson, T.J., y Helmer-Citterich, M. 2011. From sequence to structural analysis in protein phosphorylation motifs. Frontiers in Bioscience-Landmark. **16**: 1261-1275.

von Sonnenburg, F., Tornieporth, N., Waiyaki, P., Lowe, B., Peruski, L.F., DuPont, H.L., Mathewson, J.J., y Steffen, R. 2000. Risk and aetiology of diarrhoea at various tourist destinations. Lancet. **356**: 133-134.

Vreeman, H.J., Visser, S., Slangen, C.J., y Vanriel, J.A.M. 1986. Characterization of bovine kappa-casein fractions and the kinetics of chymosin-induced

macropeptide release from carbohydrate-free and carbohydrate-containing fractions determined by high-performance gel-permeation chromatography. Biochemical Journal. **240**: 87-97.

Wadstrom, T., Andersson, K., Sydow, M., Axelsson, L., Lindgren, S., y Gullmar, B. 1987. Surface-properties of lactobacilli isolated from the small-intestine of pigs. Journal of Applied Bacteriology. **62**: 513-520.

Wampler, J.L., Kim, K.P., Jaradat, Z., y Bhunia, A.K. 2004. Heat shock protein 60 acts as a receptor for the Listeria adhesion protein in Caco-2 cells. Infection and Immunity. **72**: 931-936.

Wang, B., Yu, B., Karim, M., Hu, H., Sun, Y., McGreevy, P., Petocz, P., Held, S., y Brand-Miller, J. 2007. Dietary sialic acid supplementation improves learning and memory in piglets. American Journal of Clinical Nutrition. **85**: 561-569.

Wang, H.-Y., Qian, H., y Yao, W.-R. 2011. Melanoidins produced by the Maillard reaction: Structure and biological activity. Food Chemistry. **128**: 573-584.

Wang, Y., Wu, S.L., y Hancock, W.S. 2006. Approaches to the Study of N-linked Glycoproteins in Human Plasma Using Lectin Affinity Chromatography and Nano-HPLC Coupled to Electrospray Linear Ion Trap-Fourier Transform Mass Spectrometry (LTQ/FTMS). Glycobiology. **16**: 514-523.

Waniska, R.D. y Kinsella, J.E. 1980. Comparison of methods for separating oligosaccharides - ultrafiltration, gel-permeation and adsorption chromatography. Journal of Food Science. **45**: 1259-1261.

Warmbier, H.C., Schnickels, R.A., y Labuza, T.P. 1976. Nonenzymatic browning kinetics in an intermediate moisture model system - effect of glucose to lysine ratio. Journal of Food Science. **41**: 981-983.

Way, S.S., Kollmann, T.R., Hajjar, A.M., y Wilson, C.B. 2003. Cutting edge: Protective cell-mediated immunity to Listeria monocytogenes in the absence of myeloid differentiation factor 88. Journal of Immunology. **171**: 533-537.

Whistler, R.L. y Durso, D.F. 1950. Chromatographic separation of sugars on charcoal. Journal of the American Chemical Society. **72**: 677-679.

Whitehead, K., Versalovic, J., Roos, S., y Britton, R.A. 2008. Genomic and genetic characterization of the bile stress response of probiotic Lactobacillus reuteri ATCC 55730. Applied and Environmental Microbiology. **74**: 1812-1819.

Wiame, E. 2002. Identification of a Pathway for the Utilization of the Amadori Product Fructoselysine in Escherichia coli. Journal of Biological Chemistry. **277**: 42523-42529.

Wiame, E., Lamosa, P., Santos, H., y Van Schaftingen, E. 2005. Identification of glucoselysine-6-phosphate deglycase, an enzyme involved in the metabolism of the fructation product glucoselysine. Biochemical Journal. **392**: 263.

Wichienchot, S., Prasertsan, P., Hongpattarakere, T., Gibson, G.R., y Rastall, R.A. 2006. *In vitro* fermentation of mixed linkage glucooligosaccharides produced by Gluconobacter oxydans NCIMB 4943 by the human colonic microflora. Current Issues in Intestinal Microbiology. **7**: 7-12.

Wohlgemuth, S., Loh, G., y Blaut, M. 2010. Recent developments and perspectives in the investigation of probiotic effects. International Journal of Medical Microbiology. **300**: 3-10.

Wuhrer, M., Hokke, C.H., y Deelder, A.M. 2004. Glycopeptide analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry reveals novel features of horseradish peroxidase glycosylation. Rapid Communications in Mass Spectrometry. **18**: 1741-1748.

Wuhrer, M., Koeleman, C.A.M., Hokke, C.H., y Deelder, A.M. 2005. Protein glycosylation analyzed by normal-phase nano-liquid chromatography-mass spectrometry of glycopeptides. Analytical Chemistry. **77**: 886-894.

Wuhrer, M., Koeleman, C.A.M., Hokke, C.H., y Deelder, A.M. 2005. Protein glycosylation analyzed by normal-phase nano-liquid chromatography-mass spectrometry of glycopeptides. Analytical Chemistry. **77**: 886-894.

Wuhrer, M., Catalina, M.I., Deelder, A.M., y Hokke, C.H. 2007. Glycoproteomics base don tándem mass spectrometry of glycopeptides. Journal of Chromatography B. **849**: 115-128.

Yang, Y., Boysen, R.I., y Hearn, M.T.W. 2009. Hydrophilic interaction chromatography coupled to electrospray mass spectrometry for the separation of peptides and protein digests. Journal of Chromatography A. **1216**: 5518-5524.

Yau, J.S. y Tsai, F.N. 1994. Solubilities of d(-)-fructose and d(+)-glucose in subcritical and supercritical carbon-dioxide. Journal of Supercritical Fluids. **7**: 129-133.

Yokota, A., Veenstra, M., Kurdi, P., van Veen, H.W., y Konings, W.N. 2000. Cholate resistance in Lactococcus lactis is mediated by an ATP-dependent multispecific organic anion transporter. Journal of Bacteriology. **182**: 5196-5201.

Yoon, S. 2003. Specificity of yeast (Saccharomyces cerevisiae) in removing carbohydrates by fermentation. Carbohydrate Research. **338**: 1127-1132.

Yoshida, T. 2004. Peptide separation by Hydrophilic-Interaction Chromatography: a review. Journal of Biochemical and Biophysical Methods. **60**: 265-280.

Zaia, J. 2004. Mass spectrometry of oligosaccharides. Mass Spectrometry Reviews. **23**: 161-227.

Zamfir, A. y Katalinic, J.P. 2001. Glycoscreening by on-line sheathless capillary electrophoresis/electrospray ionization-quadrupole time of flight-tandem mass spectrometry. Electrophoresis. **22**: 2448-2457.

Zhang, H., Brokman, S.M., Fang, N., Pohl, N.L., y Yeung, E.S. 2008. Linkage position and residue identification of disaccharides by tandem mass spectrometry and linear discriminant analysis. Rapid Communications in Mass Spectrometry. **22**: 1579-1586.

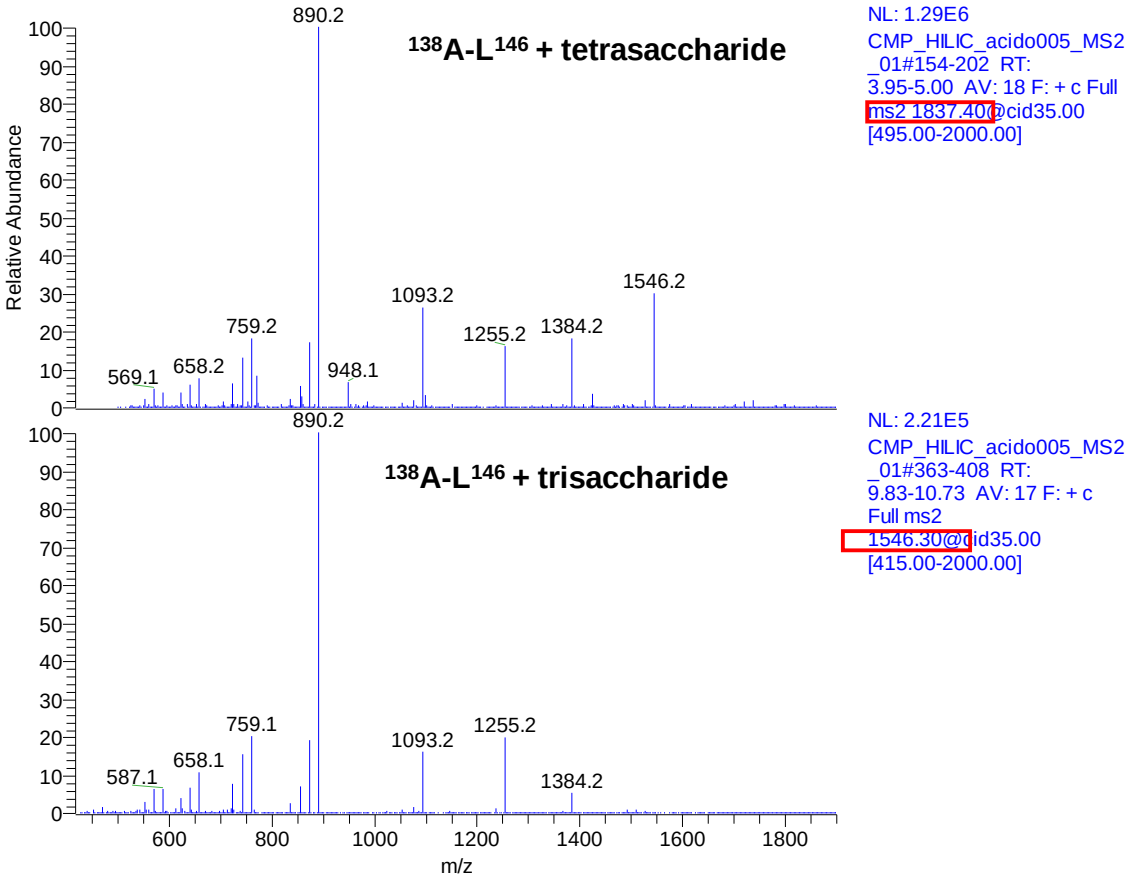
Zill, H., Bek, S., Hofmann, T., Huber, J., Frank, O., Lindenmeier, M., Weigle, B., Erbersdobler, H.F., Scheidler, S., Busch, A.E., y Faist, V. 2003. RAGE-mediated MAPK activation by food-derived AGE and non-AGE products. Biochemical and Biophysical Research Communications. **300**: 311-315.

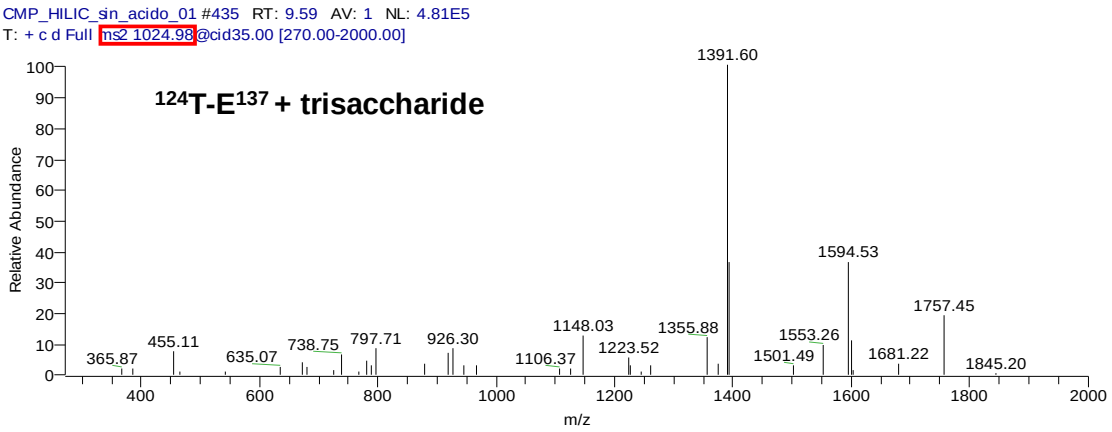
Zopf, D. y Roth, S. 1996. Oligosaccharide anti-infective agents. Lancet. **347**: 1017-1021.

8. ANEXO – SUPPORTING INFORMATION

Mass spectra´s corresponding to the section 4.2.2

1. HILIC-ESI-MS^2 spectra

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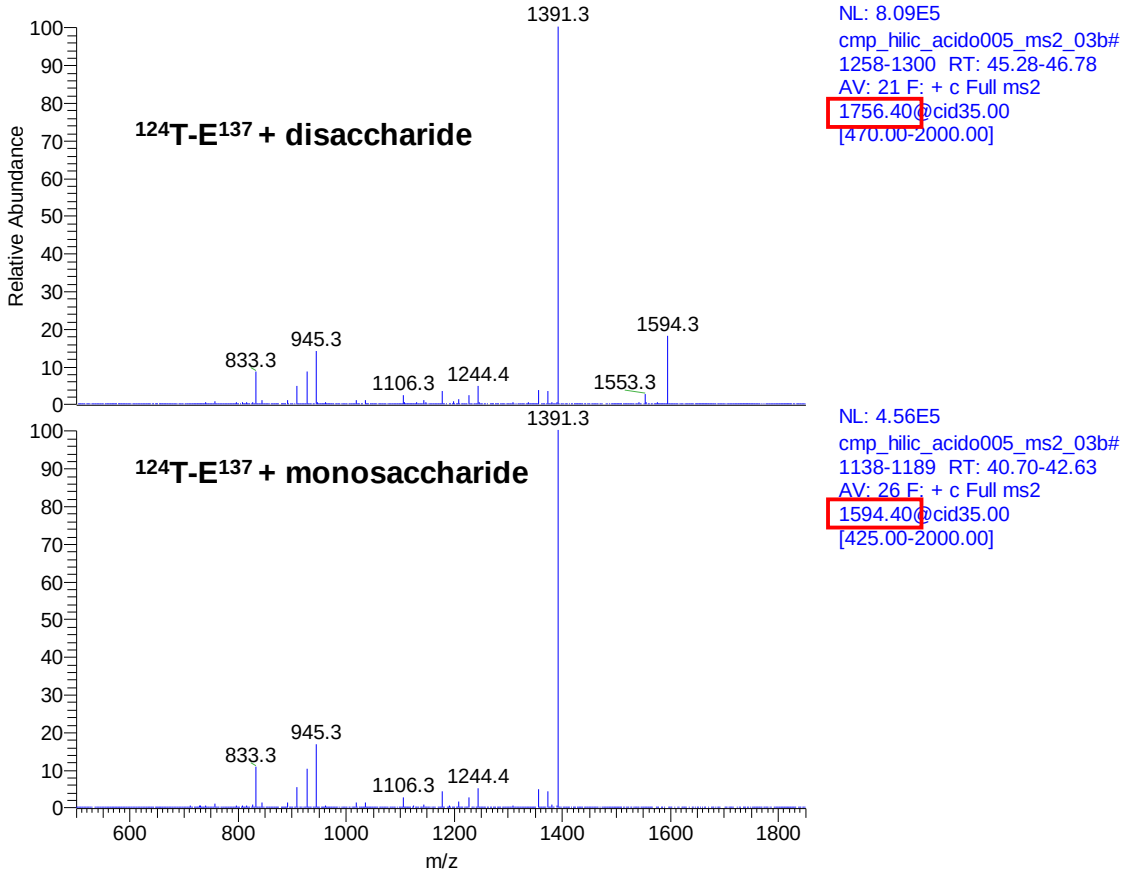
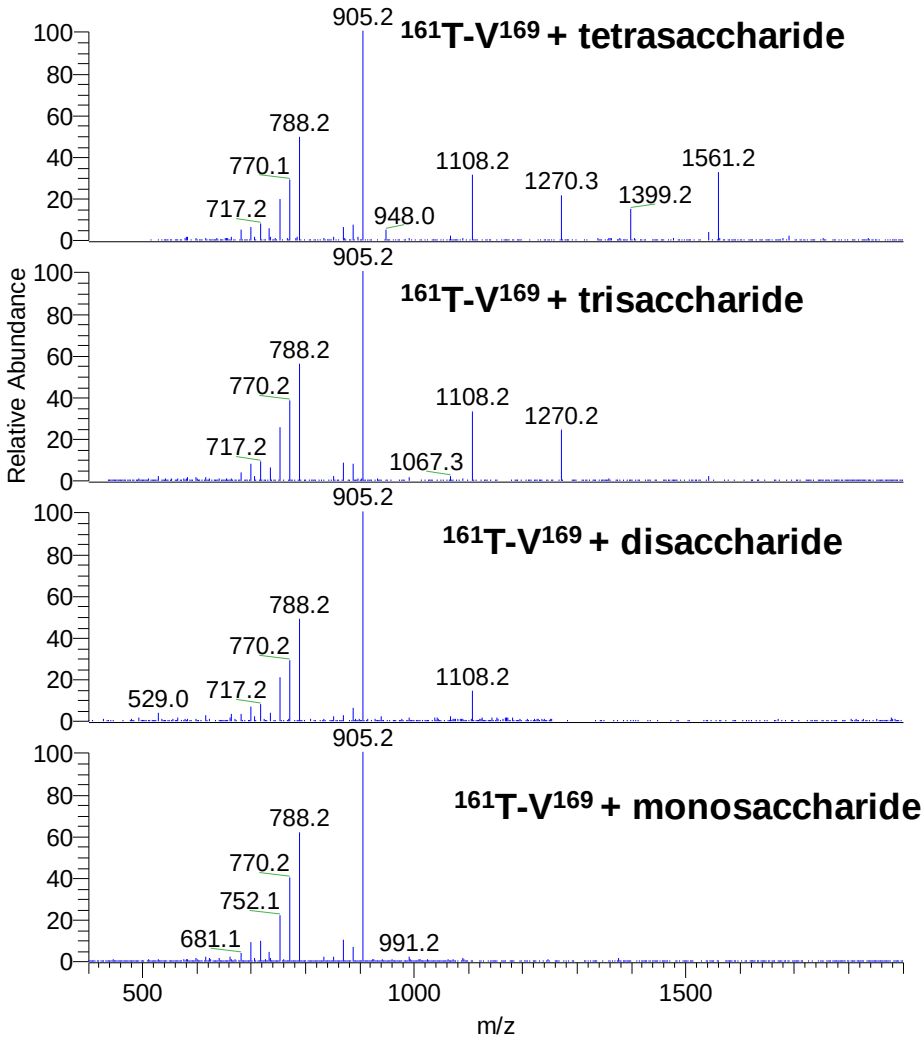
T: + c d Full ms2 1024.98@cid35.00 [270.00-2000.00]
124T-E137 + trisaccharide
Relative Abundance
m/z
1391.60
1594.53
1757.45
1148.03
1355.88
1553.26
455.11
738.75
797.71
926.30
1223.52
1681.22
365.87
635.07
1106.37
1501.49
1845.20


124T-E137 + disaccharide
Relative Abundance
1391.3
945.3
833.3
1594.3
1106.3
1244.4
1553.3
NL: 8.09E5
cmp_hilic_acido005_ms2_03b#
1258-1300 RT: 45.28-46.78
AV: 21 F: + c Full ms2
1756.40@cid35.00
[470.00-2000.00]
124T-E137 + monosaccharide
1391.3
945.3
833.3
1106.3
1244.4
NL: 4.56E5
cmp_hilic_acido005_ms2_03b#
1138-1189 RT: 40.70-42.63
AV: 26 F: + c Full ms2
1594.40@cid35.00
[425.00-2000.00]
m/z

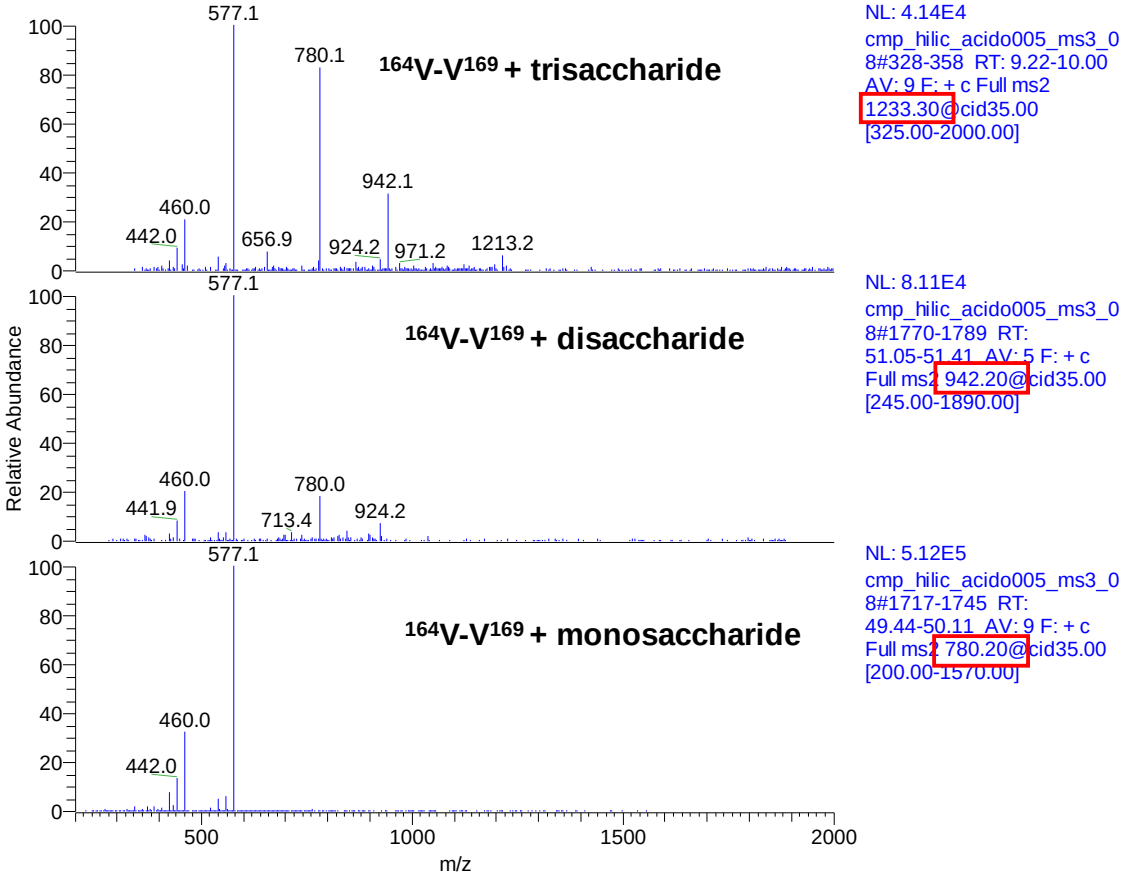


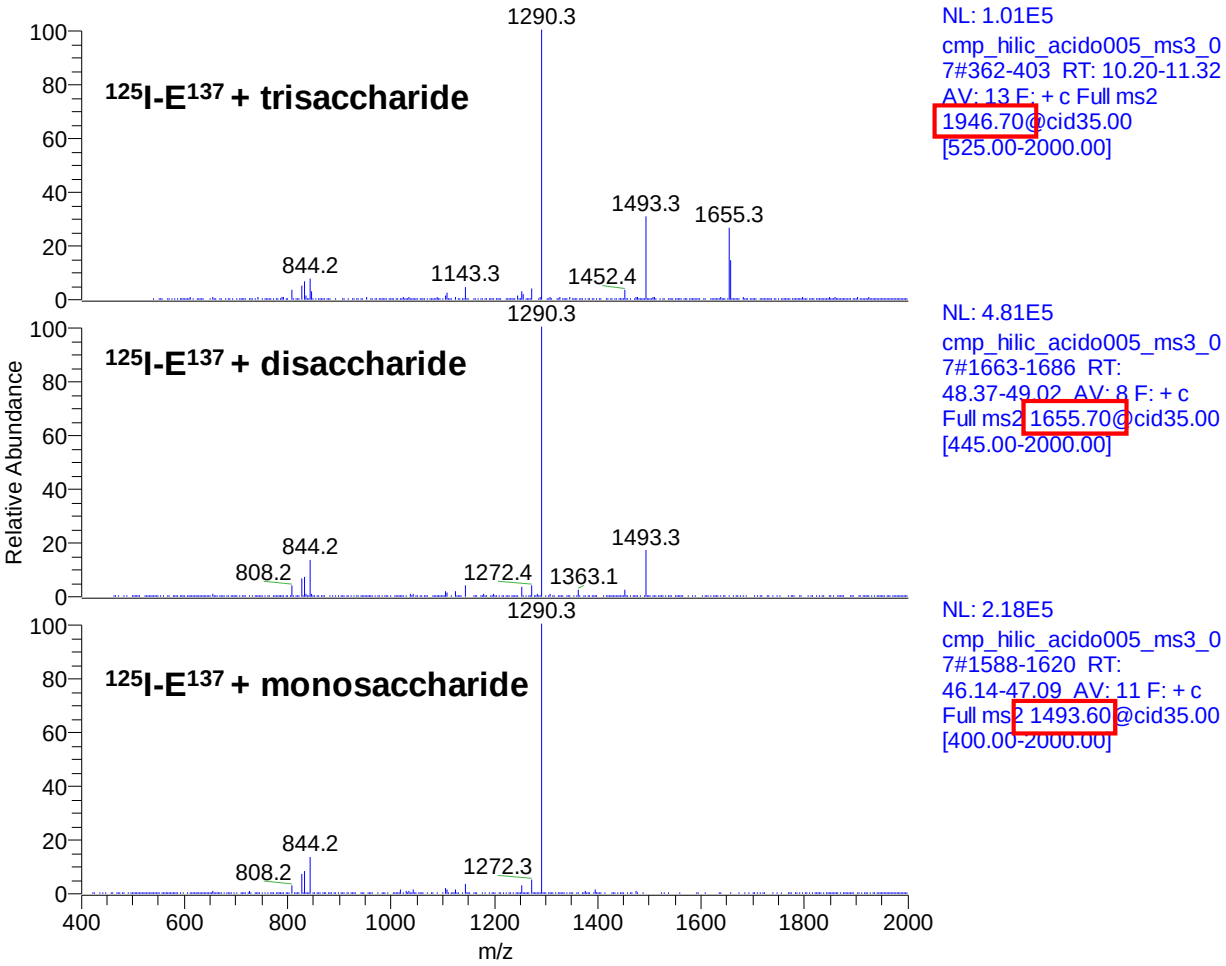
NL: 5.54E5
CMP_HILIC_acido005_MS2_02b#153-158 RT: 4.63-4.76 AV: 3 F: + c Full ms2 1852.50@cid35.00 [500.00-2000.00]

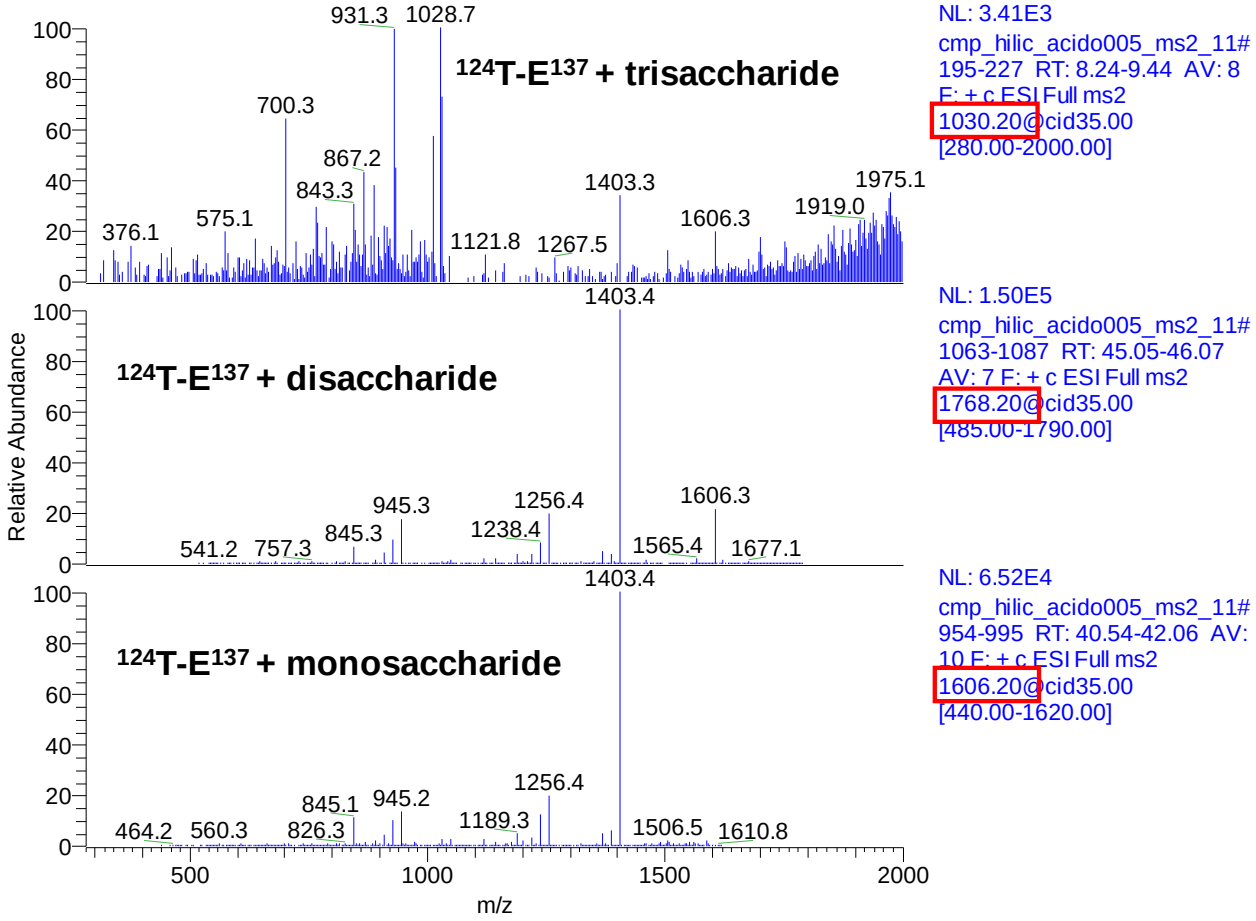
NL: 2.20E5
CMP_HILIC_acido005_MS2_02b#277-294 RT: 9.30-9.77 AV: 7 F: + c Full ms2 1561.40@cid35.00 [415.00-2000.00]

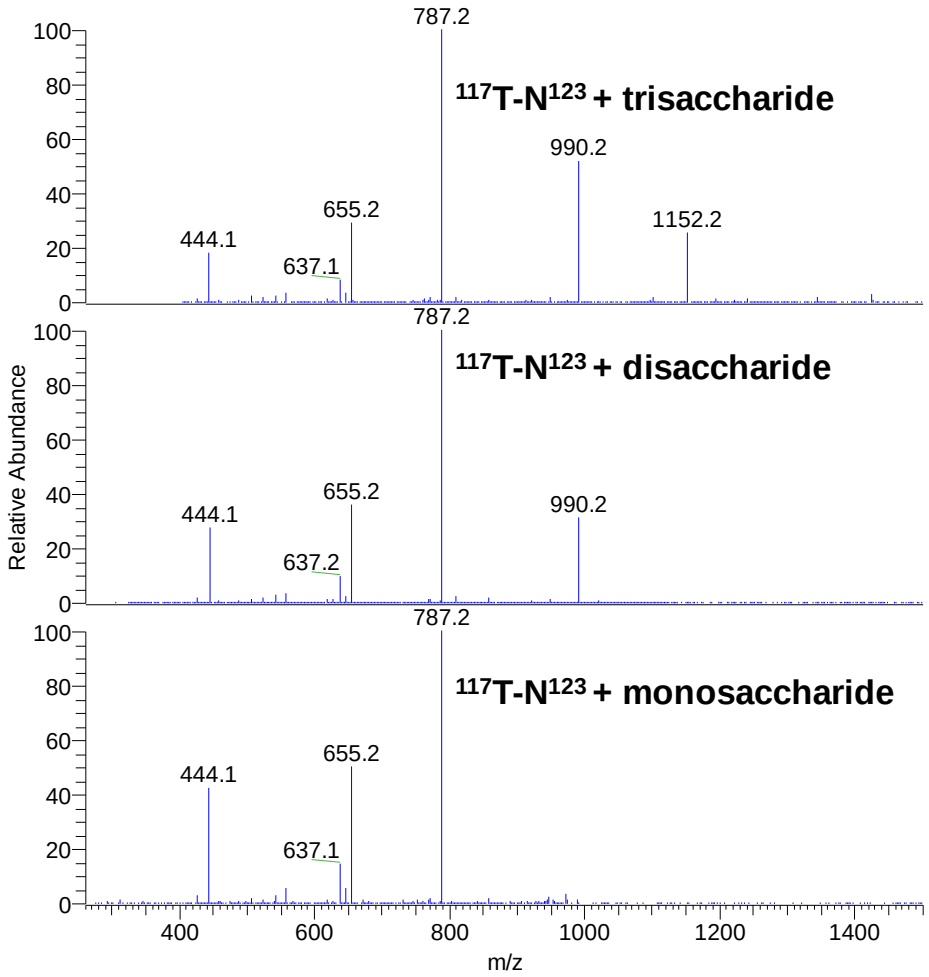
NL: 7.81E4
CMP_HILIC_acido005_MS2_02b#1400-1411 RT: 49.08-49.40 AV: 5 F: + c Full ms2 1270.30@cid35.00 [335.00-2000.00]

NL: 1.13E5
CMP_HILIC_acido005_MS2_02b#1331-1354 RT: 46.28-47.16 AV: 12 F: + c Full ms2 1108.30@cid35.00 [295.00-2000.00]


577.1
780.1
164V-V169 + trisaccharide
942.1
460.0
442.0
656.9
924.2
971.2
1213.2
NL: 4.14E4
cmp_hilic_acido005_ms3_08#328-358 RT: 9.22-10.00 AV: 9 F: + c Full ms2 1233.30@cid35.00 [325.00-2000.00]
577.1
164V-V169 + disaccharide
460.0
441.9
780.0
713.4
924.2
NL: 8.11E4
cmp_hilic_acido005_ms3_08#1770-1789 RT: 51.05-51.41 AV: 5 F: + c Full ms2 942.20@cid35.00 [245.00-1890.00]
577.1
164V-V169 + monosaccharide
460.0
442.0
NL: 5.12E5
cmp_hilic_acido005_ms3_08#1717-1745 RT: 49.44-50.11 AV: 9 F: + c Full ms2 780.20@cid35.00 [200.00-1570.00]
Relative Abundance
m/z
0
20
40
60
80
100
500
1000
1500
2000


125I-E137 + trisaccharide
1290.3
1493.3
1655.3
844.2
1143.3
1452.4
NL: 1.01E5
cmp_hilic_acido005_ms3_07#362-403 RT: 10.20-11.32 AV: 13 F: + c Full ms2 1946.70@cid35.00 [525.00-2000.00]
125I-E137 + disaccharide
1290.3
844.2
808.2
1272.4
1363.1
1493.3
NL: 4.81E5
cmp_hilic_acido005_ms3_07#1663-1686 RT: 48.37-49.02 AV: 8 F: + c Full ms2 1655.70@cid35.00 [445.00-2000.00]
125I-E137 + monosaccharide
1290.3
844.2
808.2
1272.3
NL: 2.18E5
cmp_hilic_acido005_ms3_07#1588-1620 RT: 46.14-47.09 AV: 11 F: + c Full ms2 1493.60@cid35.00 [400.00-2000.00]
Relative Abundance
m/z
0
20
40
60
80
100
400
600
800
1000
1200
1400
1600
1800
2000


124T-E137 + trisaccharide
931.3
1028.7
700.3
867.2
843.3
575.1
376.1
1121.8
1267.5
1403.3
1606.3
1919.0
1975.1
NL: 3.41E3
cmp_hilic_acido005_ms2_11#
195-227 RT: 8.24-9.44 AV: 8
F: + c ESI Full ms2
1030.20@cid35.00
[280.00-2000.00]
124T-E137 + disaccharide
1403.4
945.3
845.3
541.2
757.3
1238.4
1256.4
1565.4
1606.3
1677.1
NL: 1.50E5
cmp_hilic_acido005_ms2_11#
1063-1087 RT: 45.05-46.07
AV: 7 F: + c ESI Full ms2
1768.20@cid35.00
[485.00-1790.00]
124T-E137 + monosaccharide
1403.4
845.1
945.2
464.2
560.3
826.3
1189.3
1256.4
1506.5
1610.8
NL: 6.52E4
cmp_hilic_acido005_ms2_11#
954-995 RT: 40.54-42.06 AV:
10 F: + c ESI Full ms2
1606.20@cid35.00
[440.00-1620.00]
Relative Abundance
m/z
0
20
40
60
80
100
500
1000
1500
2000



NL: 2.39E5
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_01#306-359 RT: 8.20-9.48
AV: 24 F: + c Full ms2
1443.30@cid35.00
[385.00-2000.00]

NL: 1.30E6
CMP_HILIC_acido005_MS2
_01#1634-1724 RT:
44.68-47.05 AV: 46 F: + c
Full ms2 1152.20@cid35.00
[305.00-2000.00]

NL: 5.67E4
CMP_HILIC_acido005_MS2
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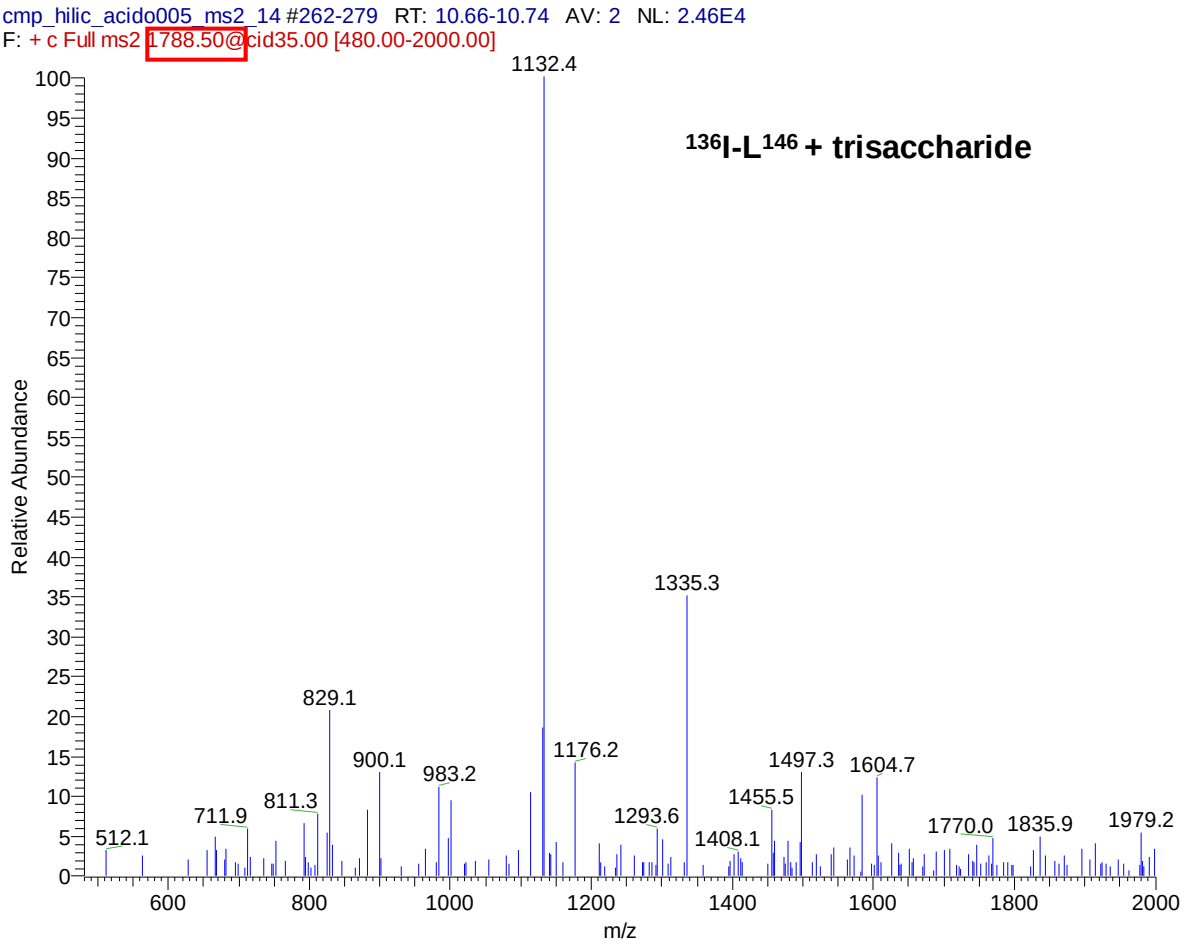
40.46-41.93 AV: 27 F: + c
Full ms2 990.20@cid35.00
[260.00-1990.00]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F: + c Full ms2 1788.50@cid35.00 [480.00-2000.00]
136I-L146 + trisaccharide
Relative Abundance
m/z
1132.4
1335.3
829.1
1176.2
900.1
983.2
1497.3
1604.7
811.3
711.9
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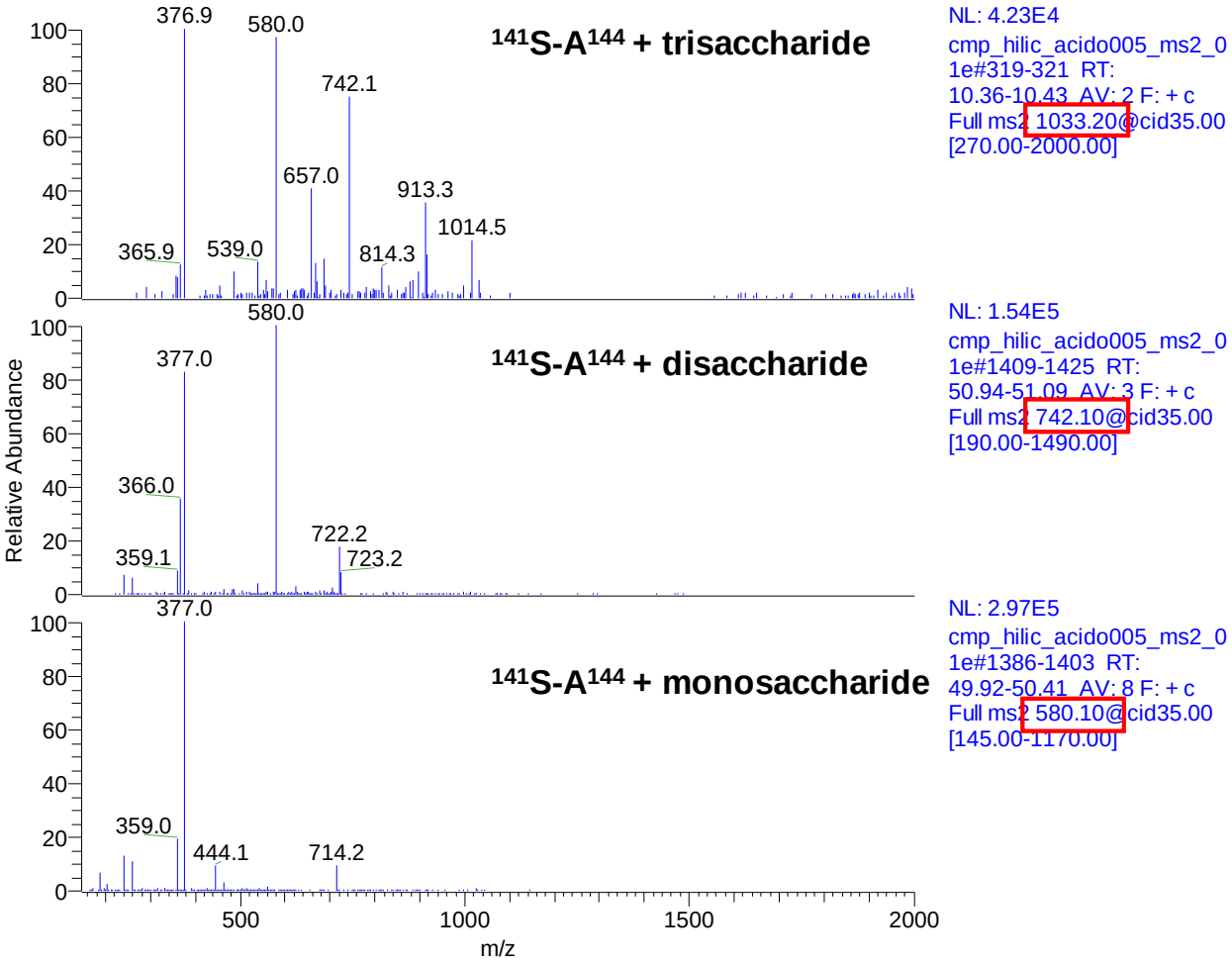
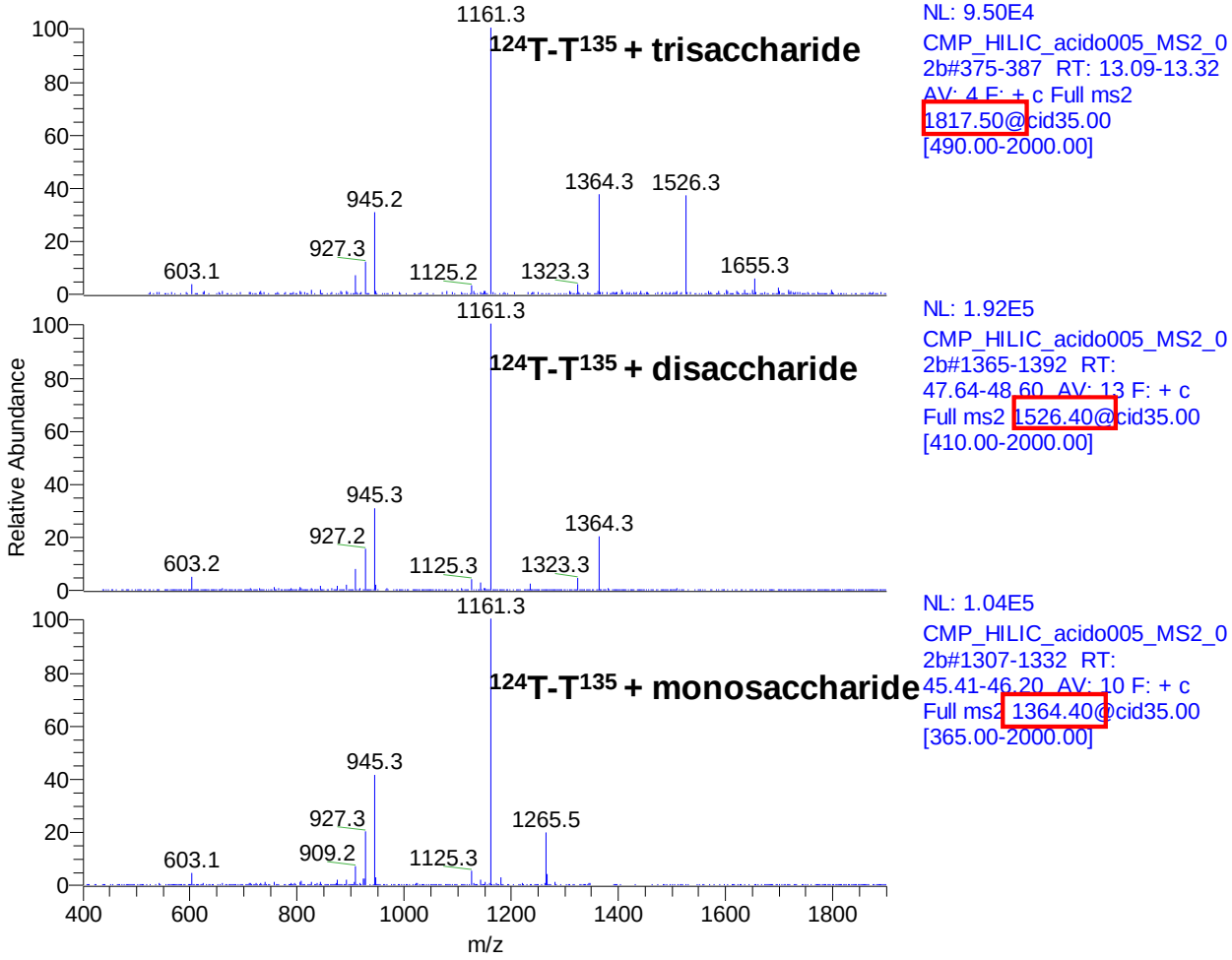
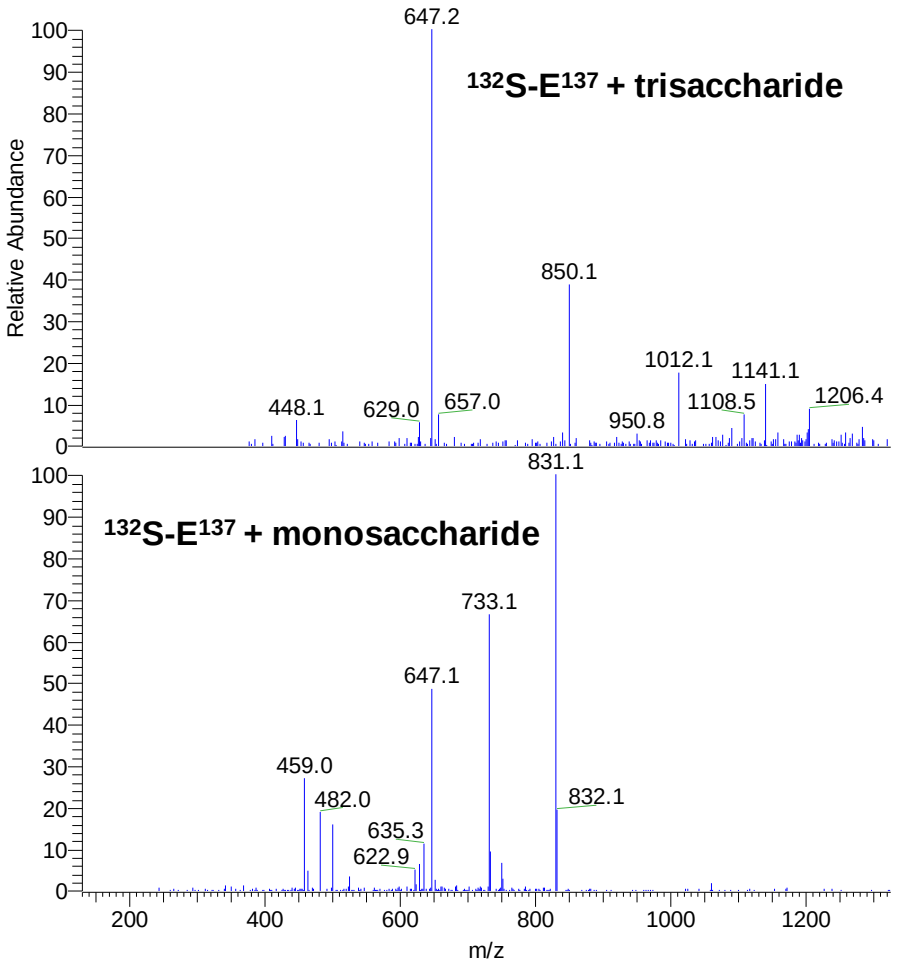
1770.0
1835.9
1979.2
512.1
1408.1

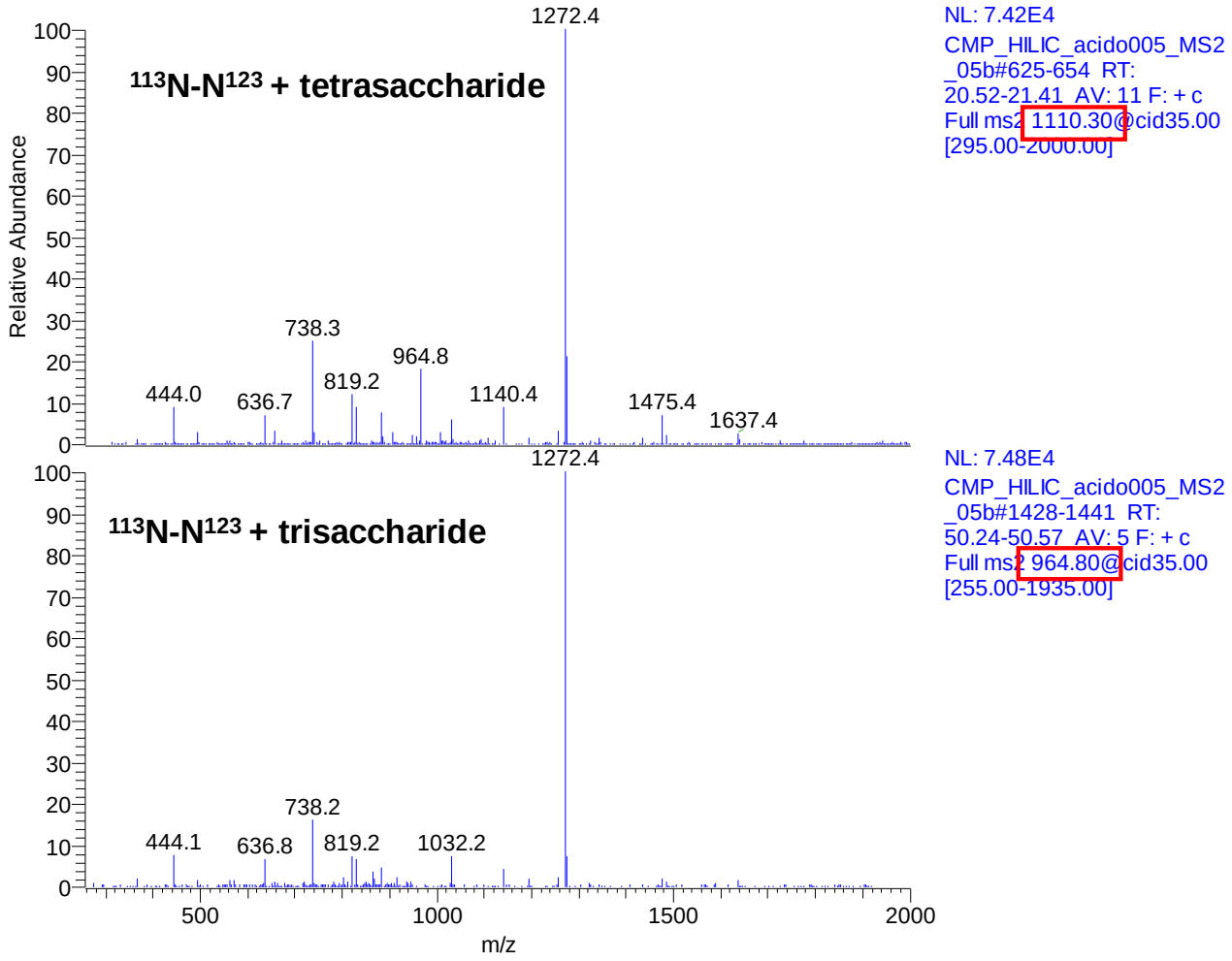

141S-A144 + trisaccharide
376.9
580.0
742.1
657.0
913.3
1014.5
365.9
539.0
814.3
NL: 4.23E4
cmp_hilic_acido005_ms2_01e#319-321 RT: 10.36-10.43 AV: 2 F: + c Full ms2 1033.20@cid35.00 [270.00-2000.00]
141S-A144 + disaccharide
580.0
377.0
366.0
722.2
359.1
723.2
NL: 1.54E5
cmp_hilic_acido005_ms2_01e#1409-1425 RT: 50.94-51.09 AV: 3 F: + c Full ms2 742.10@cid35.00 [190.00-1490.00]
141S-A144 + monosaccharide
377.0
359.0
444.1
714.2
NL: 2.97E5
cmp_hilic_acido005_ms2_01e#1386-1403 RT: 49.92-50.41 AV: 8 F: + c Full ms2 580.10@cid35.00 [145.00-1170.00]
Relative Abundance
m/z
0
20
40
60
80
100
500
1000
1500
2000

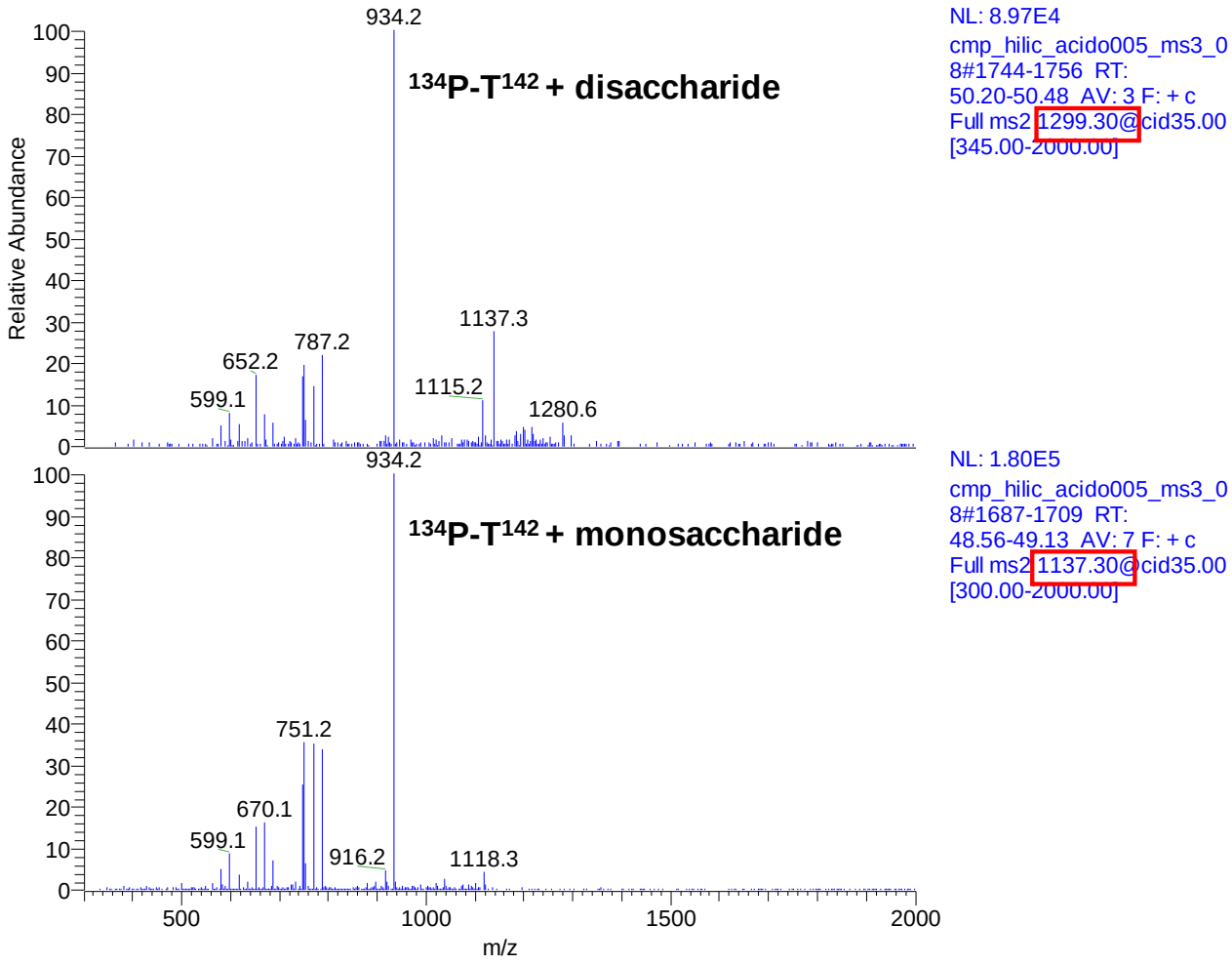

1161.3
124T-T135 + trisaccharide
945.2
927.3
603.1
1125.2
1323.3
1364.3
1526.3
1655.3
NL: 9.50E4
CMP_HILIC_acido005_MS2_02b#375-387 RT: 13.09-13.32 AV: 4 F: + c Full ms2 1817.50@cid35.00 [490.00-2000.00]
1161.3
124T-T135 + disaccharide
945.3
927.2
603.2
1125.3
1323.3
1364.3
NL: 1.92E5
CMP_HILIC_acido005_MS2_02b#1365-1392 RT: 47.64-48.60 AV: 13 F: + c Full ms2 1526.40@cid35.00 [410.00-2000.00]
1161.3
124T-T135 + monosaccharide
945.3
927.3
909.2
603.1
1125.3
1265.5
NL: 1.04E5
CMP_HILIC_acido005_MS2_02b#1307-1332 RT: 45.41-46.20 AV: 10 F: + c Full ms2 1364.40@cid35.00 [365.00-2000.00]
Relative Abundance
m/z
0
20
40
60
80
100
400
600
800
1000
1200
1400
1600
1800



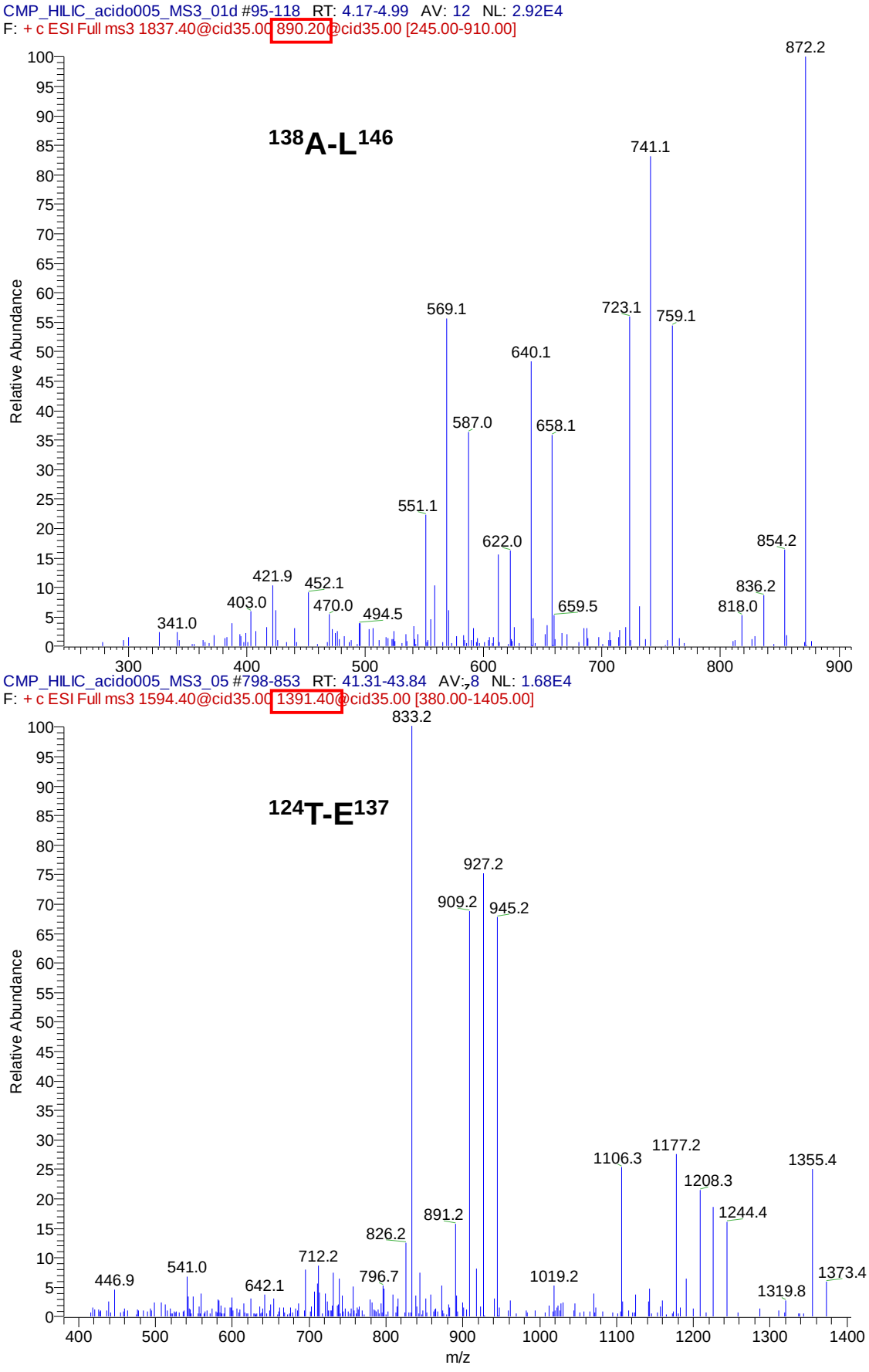
NL: 5.44E4
CMP_HILIC_acido005_MS3_12#527-542 RT: 15.37-15.65 AV: 4 F: + c Full ms2 1303.40@cid35.00 [345.00-2000.00]

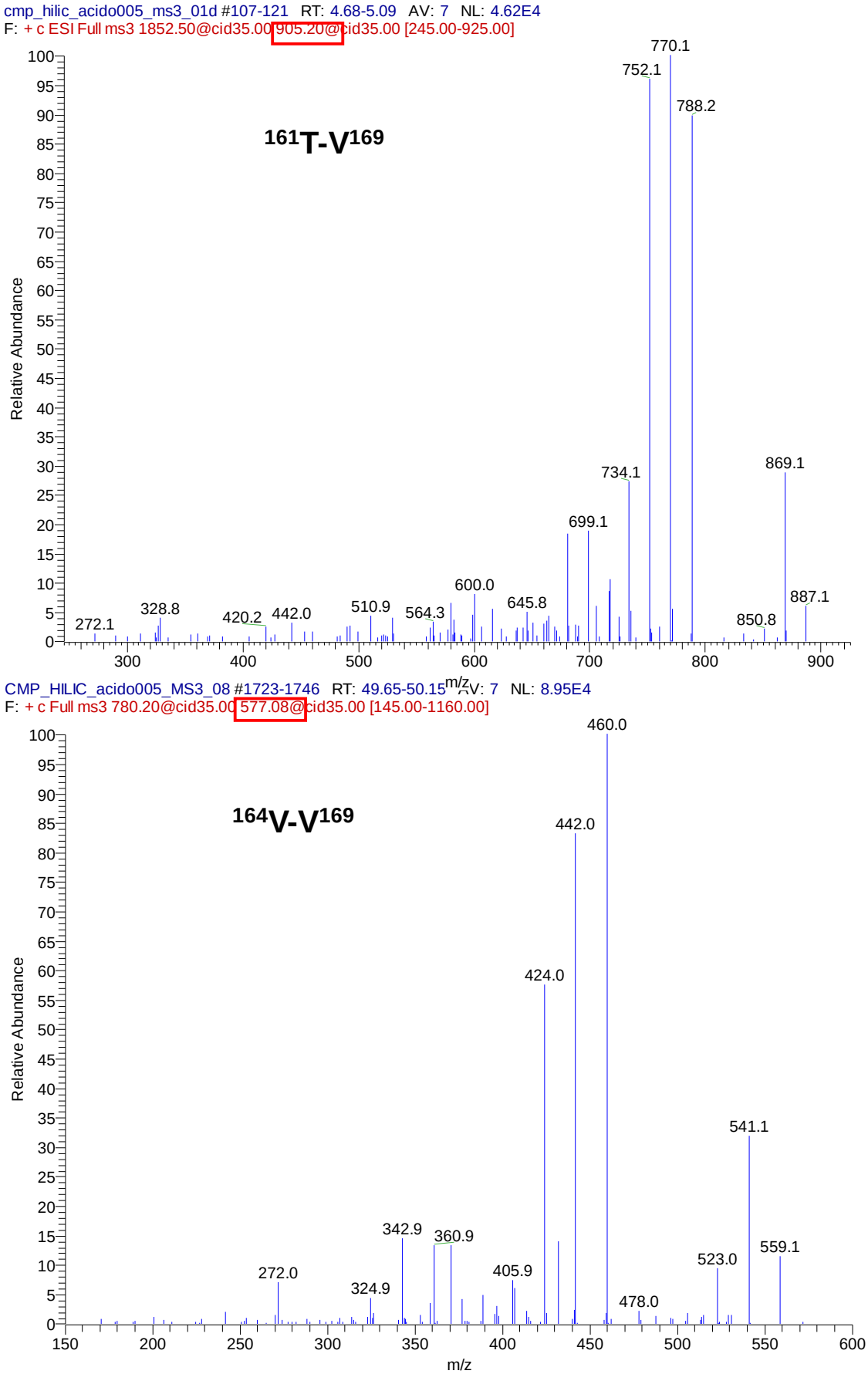
NL: 7.61E4
CMP_HILIC_acido005_MS3_12#1559-1590 RT: 45.46-46.30 AV: 10 F: + c Full ms2 850.10@cid35.00 [220.00-1710.00]

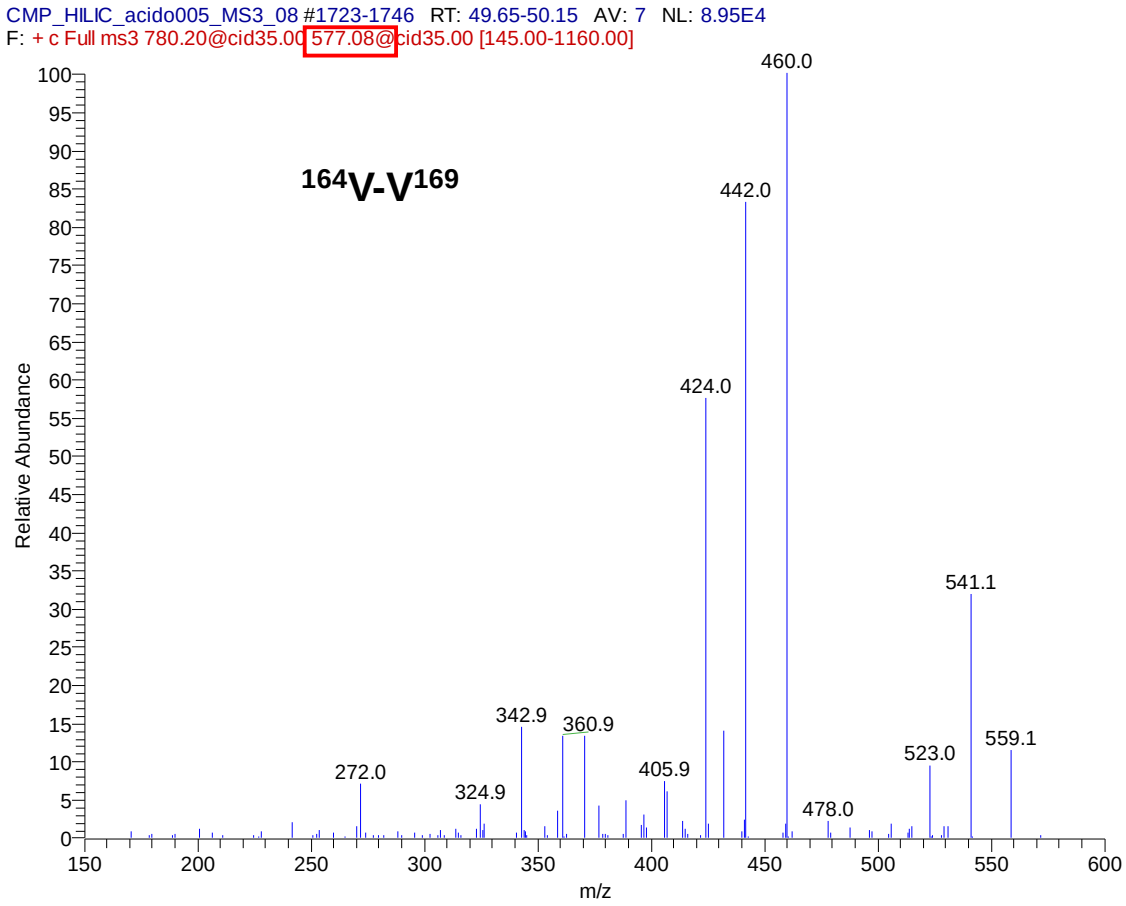

113N-N123 + tetrasaccharide
1272.4
738.3
964.8
819.2
444.0
636.7
1140.4
1475.4
1637.4
NL: 7.42E4
CMP_HILIC_acido005_MS2_05b#625-654 RT: 20.52-21.41 AV: 11 F: + c Full ms2 1110.30@cid35.00 [295.00-2000.00]
113N-N123 + trisaccharide
1272.4
738.2
444.1
636.8
819.2
1032.2
NL: 7.48E4
CMP_HILIC_acido005_MS2_05b#1428-1441 RT: 50.24-50.57 AV: 5 F: + c Full ms2 964.80@cid35.00 [255.00-1935.00]
Relative Abundance
m/z
0
10
20
30
40
50
60
70
80
90
100
500
1000
1500
2000

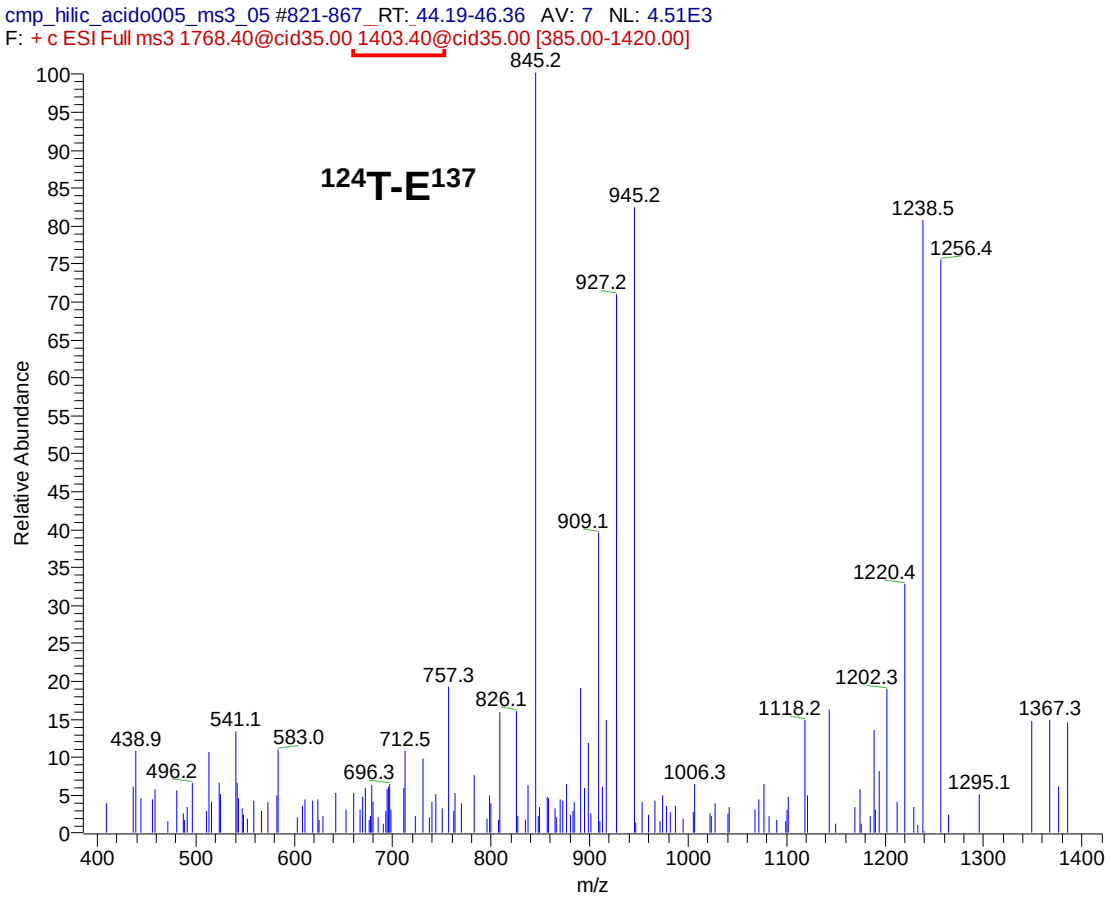

934.2
134P-T142 + disaccharide
1137.3
787.2
652.2
599.1
1115.2
1280.6
Relative Abundance
NL: 8.97E4
cmp_hilic_acido005_ms3_08#1744-1756 RT: 50.20-50.48 AV: 3 F: + c Full ms2 1299.30@cid35.00 [345.00-2000.00]
934.2
134P-T142 + monosaccharide
751.2
670.1
599.1
916.2
1118.3
NL: 1.80E5
cmp_hilic_acido005_ms3_08#1687-1709 RT: 48.56-49.13 AV: 7 F: + c Full ms2 1137.30@cid35.00 [300.00-2000.00]
500
1000
1500
2000
m/z

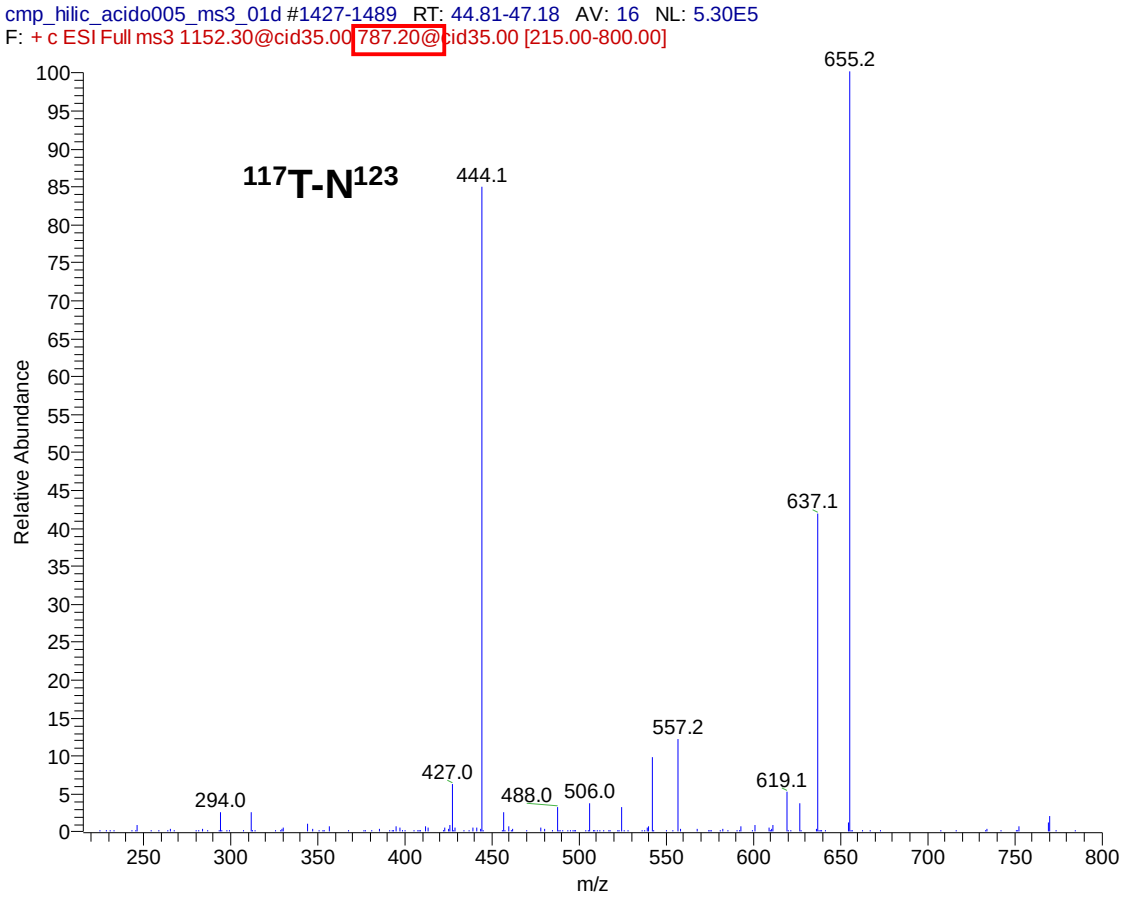
2. HILIC-ESI-MS^3 spectra

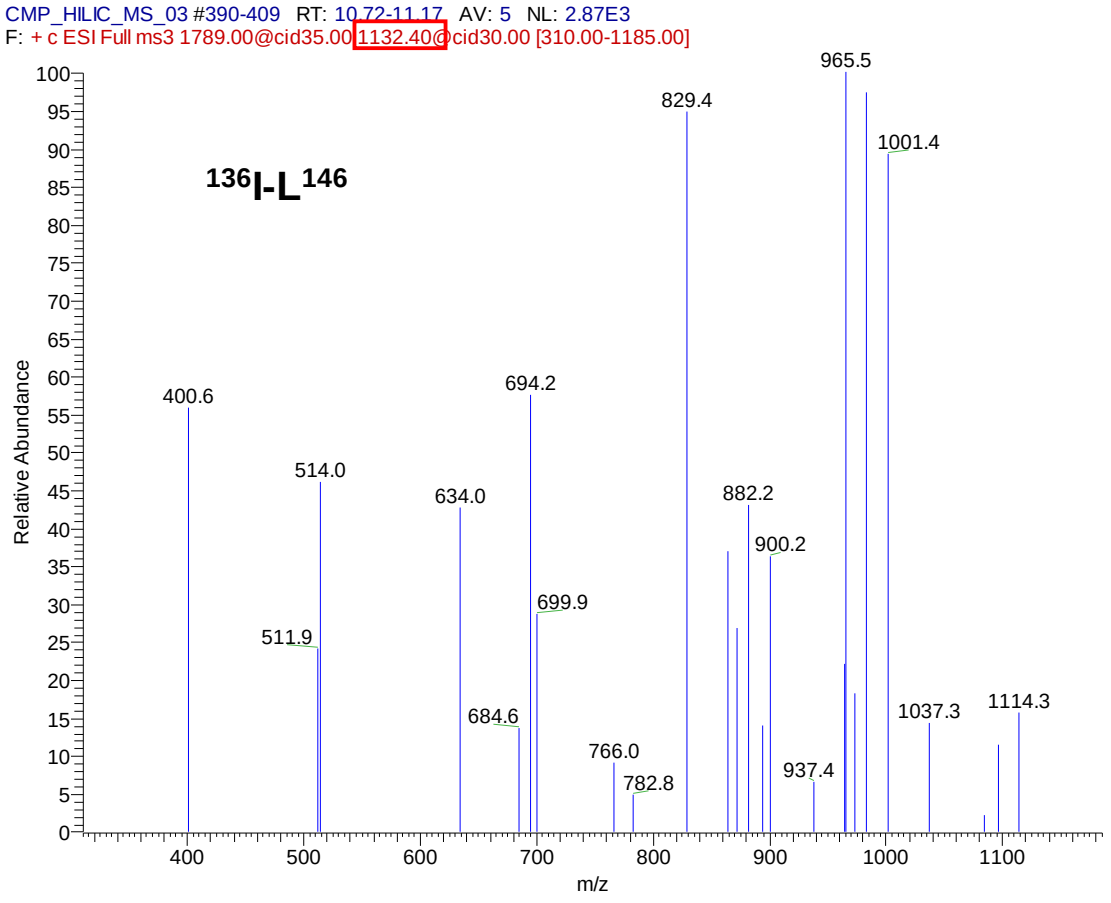



cmp_hilic_acido005_ms3_01d #107-121 RT: 4.68-5.09 AV: 7 NL: 4.62E4
F: + c ESI Full ms3 1852.50@cid35.00 905.20@cid35.00 [245.00-925.00]
161T-V169
Relative Abundance
m/z
CMP_HILIC_acido005_MS3_08 #1723-1746 RT: 49.65-50.15 AV: 7 NL: 8.95E4
F: + c Full ms3 780.20@cid35.00 577.08@cid35.00 [145.00-1160.00]
164V-V169
Relative Abundance
m/z

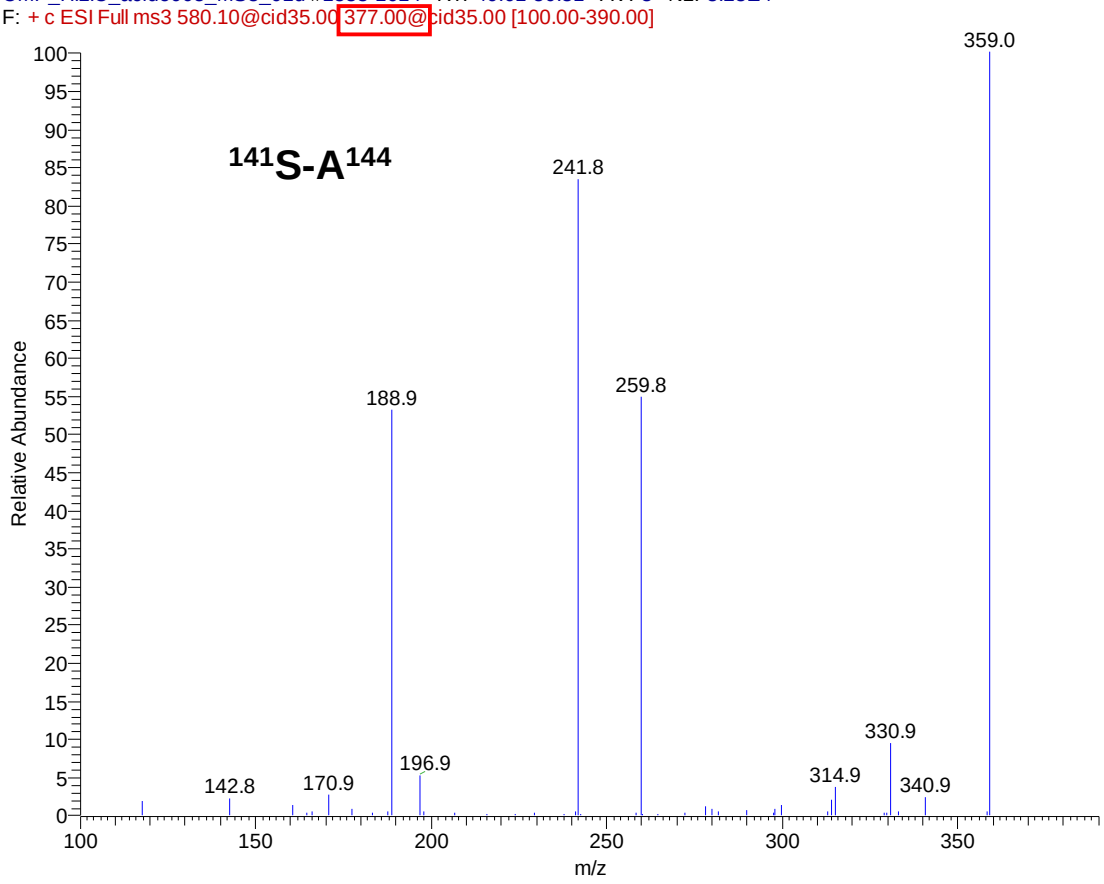

CMP_HILIC_acido005_MS3_08 #1723-1746 RT: 49.65-50.15 AV: 7 NL: 8.95E4
F: + c Full ms3 780.20@cid35.00 577.08@cid35.00 [145.00-1160.00]
164V-V169
Relative Abundance
m/z
460.0
442.0
424.0
541.1
342.9
360.9
559.1
523.0
272.0
405.9
324.9
478.0


cmp_hilic_acido005_ms3_05 #821-867 RT: 44.19-46.36 AV: 7 NL: 4.51E3
F: + c ESI Full ms3 1768.40@cid35.00 1403.40@cid35.00 [385.00-1420.00]
124T-E137
Relative Abundance
m/z
845.2
945.2
1238.5
1256.4
927.2
909.1
1220.4
757.3
826.1
1202.3
1118.2
1367.3
541.1
438.9
583.0
712.5
496.2
696.3
1006.3
1295.1

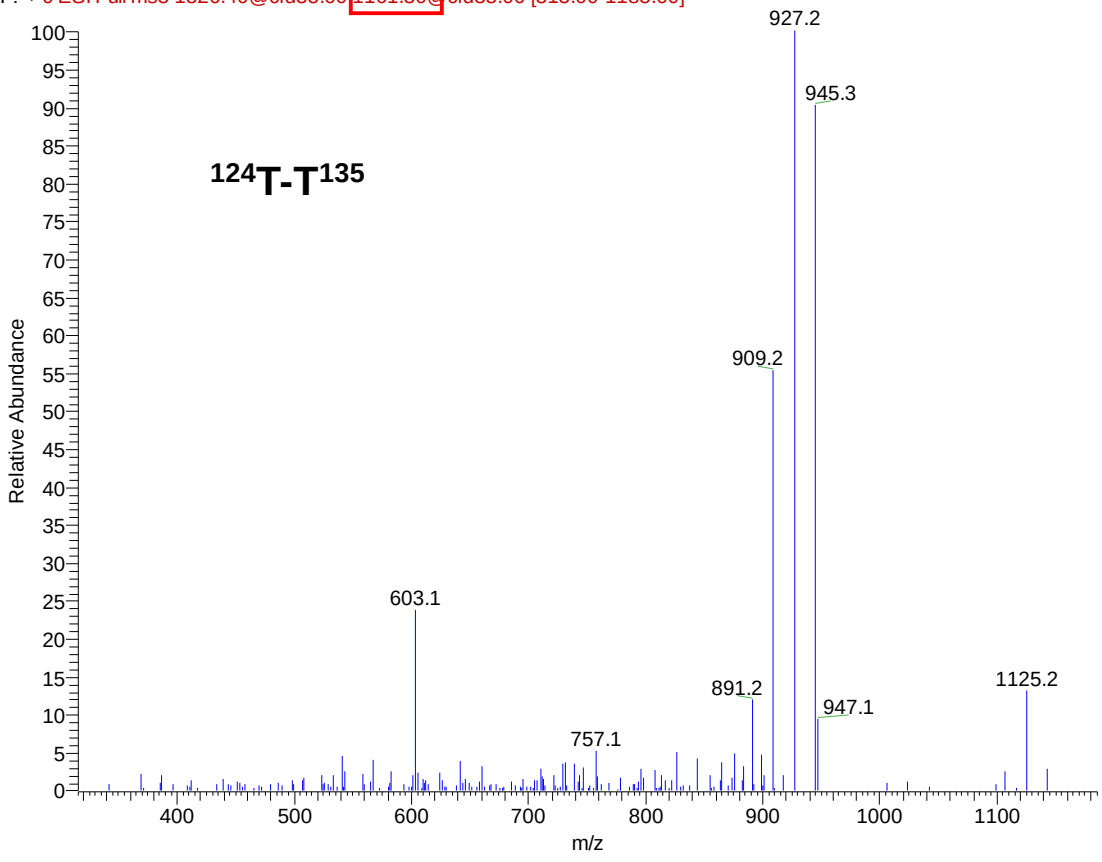

cmp_hilic_acido005_ms3_01d #1427-1489 RT: 44.81-47.18 AV: 16 NL: 5.30E5
F: + c ESI Full ms3 1152.30@cid35.00 787.20@cid35.00 [215.00-800.00]
117T-N123
655.2
444.1
637.1
557.2
427.0
619.1
294.0
488.0
506.0
Relative Abundance
m/z

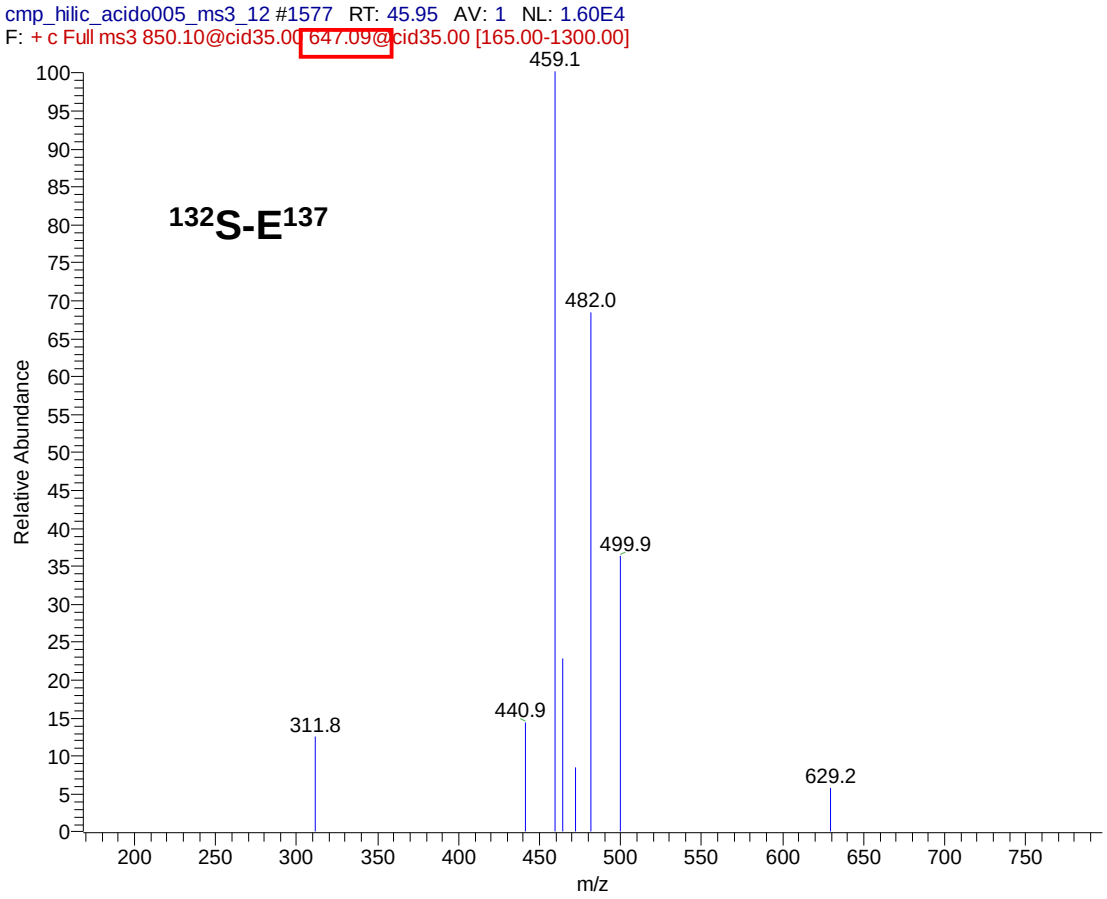

CMP_HILIC_MS_03 #390-409 RT: 10.72-11.17 AV: 5 NL: 2.87E3
F: + c ESI Full ms3 1789.00@cid35.00 1132.40@cid30.00 [310.00-1185.00]
136I-L146
965.5
829.4
1001.4
400.6
694.2
514.0
634.0
882.2
900.2
699.9
511.9
684.6
1037.3
1114.3
766.0
937.4
782.8
Relative Abundance
m/z

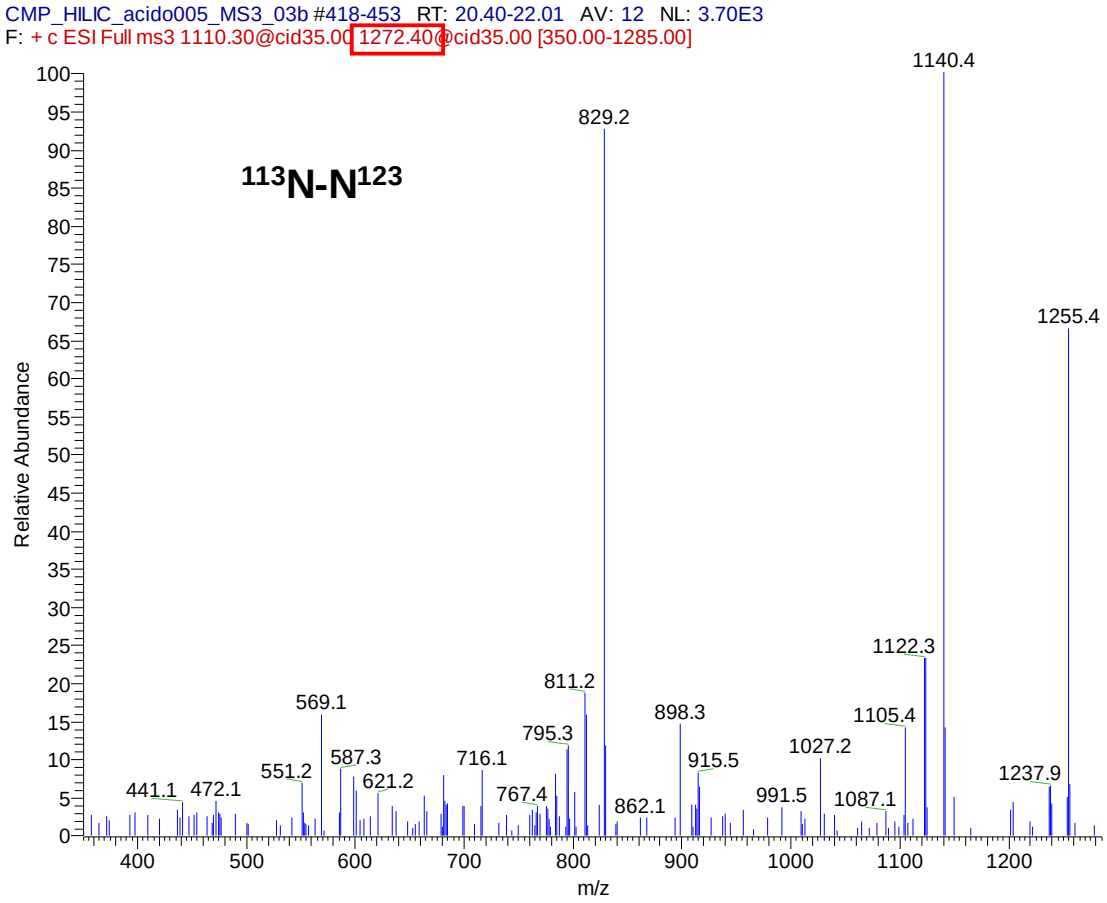
CMP_HILIC_acido005_MS3_01d #1583-1614 RT: 49.61-50.81 AV: 8 NL: 3.28E4
F: + c ESI Full ms3 580.10@cid35.00 377.00@cid35.00 [100.00-390.00]

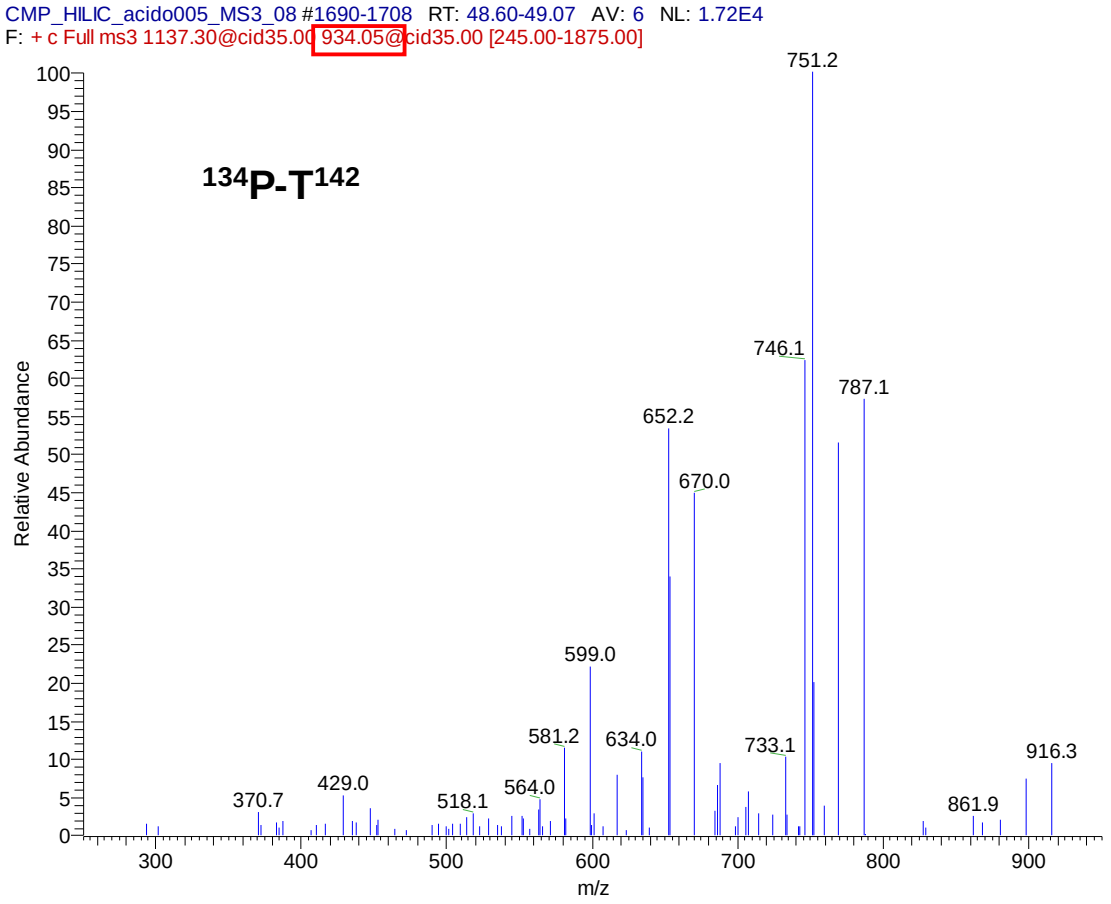


cmp_hilic_acido005_ms3_01d #1553-1573 RT: 48.29-48.97 AV: 5 NL: 3.67E4
F: + c ESI Full ms3 1526.40@cid35.00 1161.30@cid35.00 [315.00-1185.00]

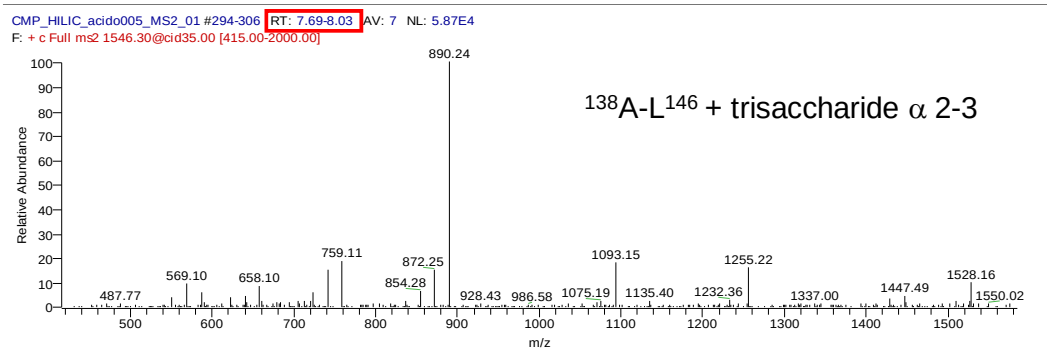


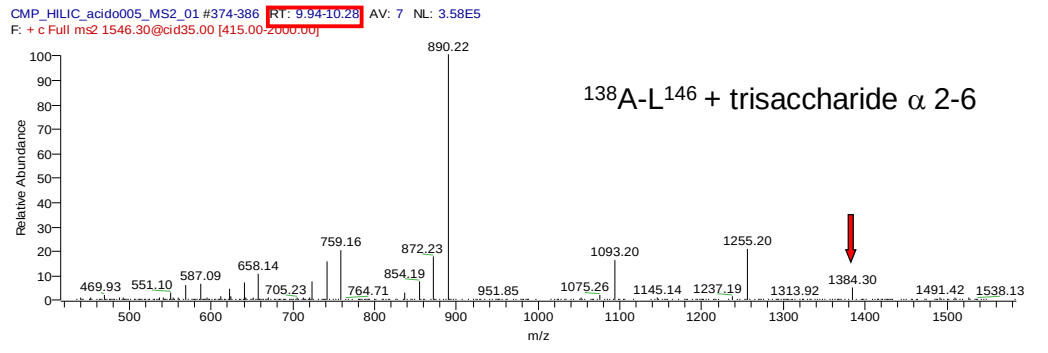

cmp_hilic_acido005_ms3_12 #1577 RT: 45.95 AV: 1 NL: 1.60E4
F: + c Full ms3 850.10@cid35.00 647.09@cid35.00 [165.00-1300.00]
132S-E137
459.1
482.0
499.9
440.9
311.8
629.2
Relative Abundance
m/z

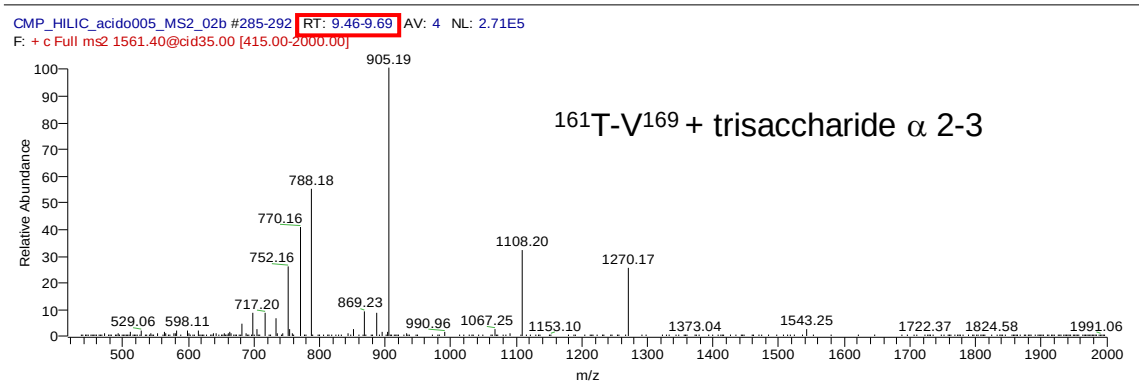

CMP_HILIC_acido005_MS3_03b #418-453 RT: 20.40-22.01 AV: 12 NL: 3.70E3
F: + c ESI Full ms3 1110.30@cid35.00 1272.40@cid35.00 [350.00-1285.00]
113N-N123
1140.4
829.2
1255.4
1122.3
811.2
569.1
898.3
1105.4
795.3
1027.2
915.5
587.3
716.1
551.2
621.2
1237.9
441.1
472.1
767.4
862.1
991.5
1087.1
Relative Abundance
m/z

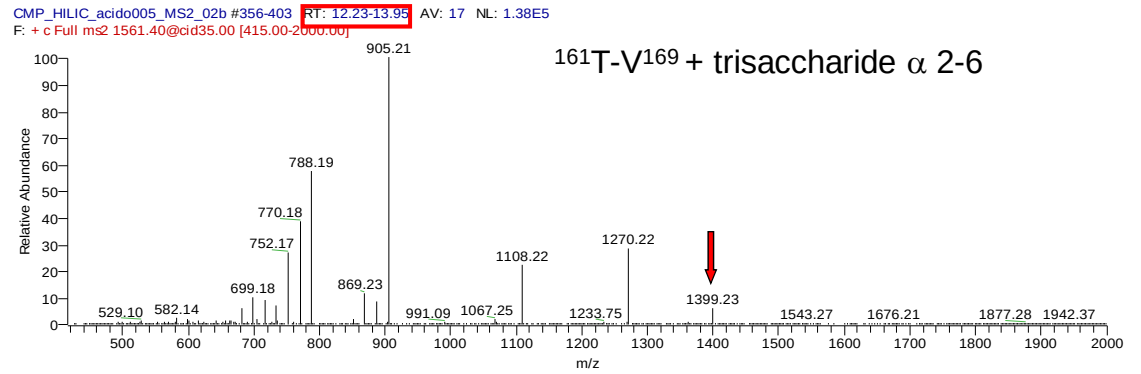


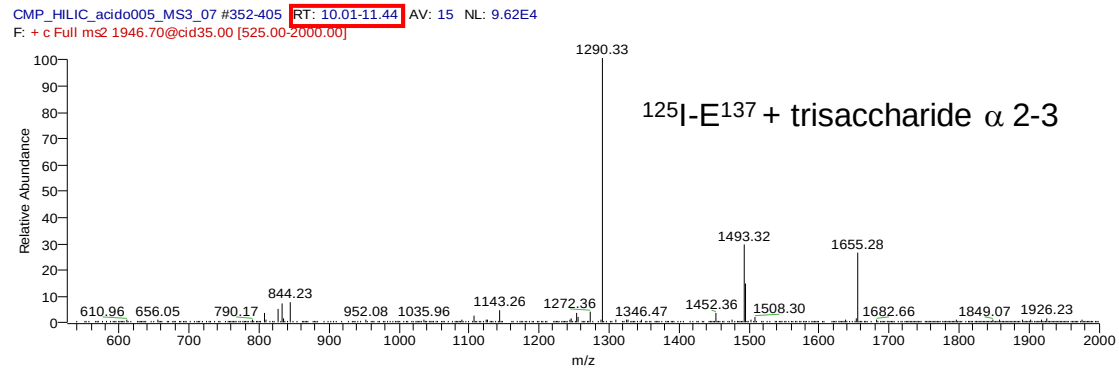
3. Differentiation of isomeric sialylated *O*-glycopeptides (HILIC-ESI-MS2 spectra)

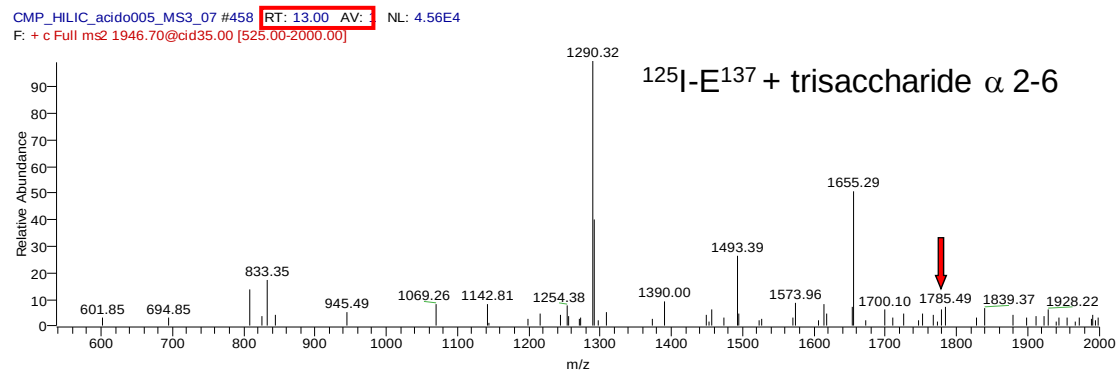


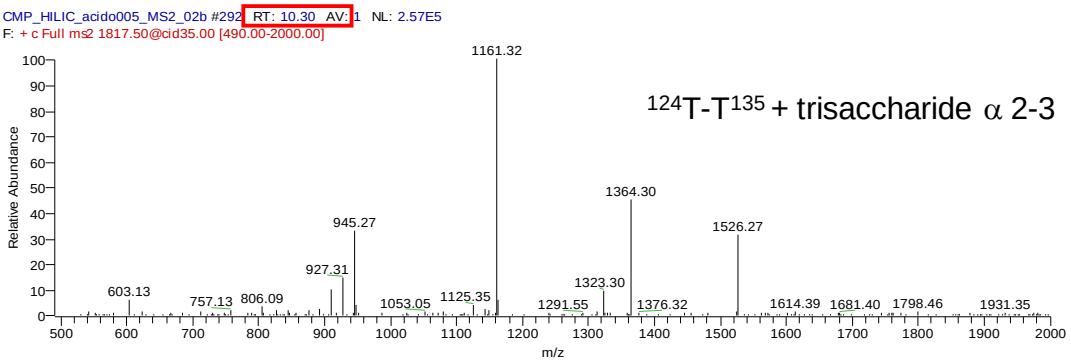


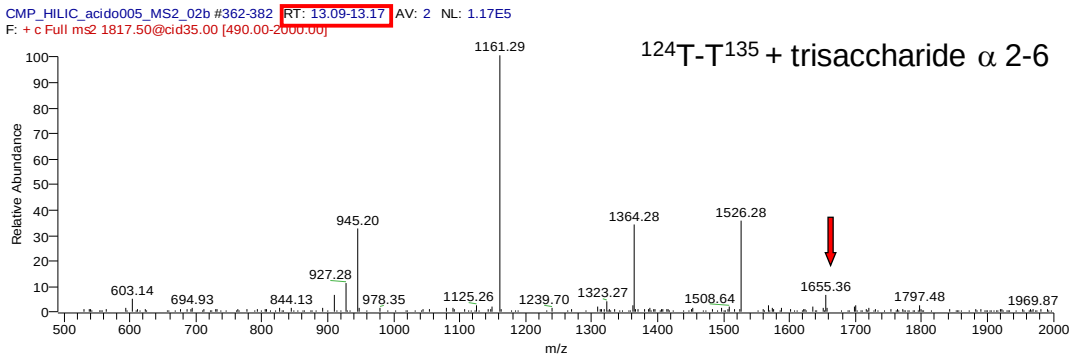

CMP_HILIC_acido005_MS2_02b #285-292 RT: 9.46-9.69 AV: 4 NL: 2.71E5
F: + c Full ms2 1561.40@cid35.00 [415.00-2000.00]
161T-V169 + trisaccharide α 2-3
Relative Abundance
m/z
905.19
788.18
770.16
752.16
1108.20
1270.17
717.20
869.23
529.06
598.11
990.96
1067.25
1153.10
1373.04
1543.25
1722.37
1824.58
1991.06

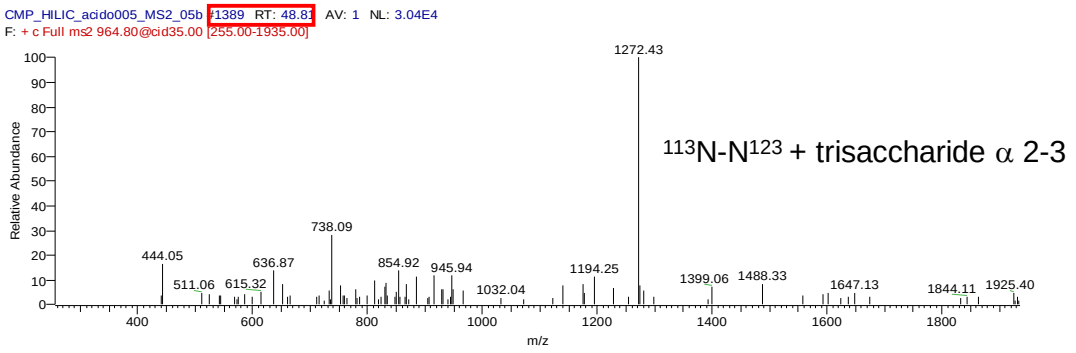

CMP_HILIC_acido005_MS2_02b #356-403 RT: 12.23-13.95 AV: 17 NL: 1.38E5
F: + c Full ms2 1561.40@cid35.00 [415.00-2000.00]
161T-V169 + trisaccharide α 2-6
Relative Abundance
m/z
905.21
788.19
770.18
752.17
1108.22
1270.22
1399.23
699.18
869.23
529.10
582.14
991.09
1067.25
1233.75
1543.27
1676.21
1877.28
1942.37

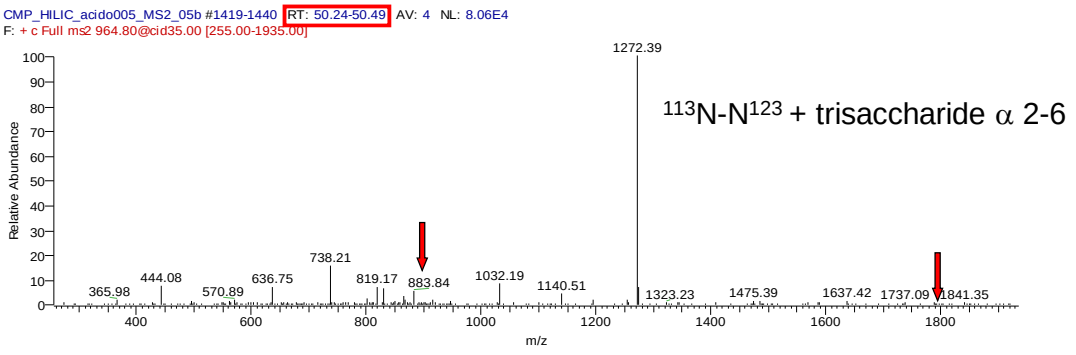

CMP_HILIC_acido005_MS3_07 #352-405 RT: 10.01-11.44 AV: 15 NL: 9.62E4
F: + c Full ms2 1946.70@cid35.00 [525.00-2000.00]
125I-E137 + trisaccharide α 2-3
Relative Abundance
m/z
1290.33
1493.32
1655.28
844.23
610.96
656.05
790.17
952.08
1035.96
1143.26
1272.36
1346.47
1452.36
1508.30
1682.66
1849.07
1926.23


CMP_HILIC_acido005_MS3_07 #458 RT: 13.00 AV: 1 NL: 4.56E4
F: + c Full ms2 1946.70@cid35.00 [525.00-2000.00]
125I-E137 + trisaccharide α 2-6
Relative Abundance
m/z
1290.32
1655.29
1493.39
833.35
601.85
694.85
945.49
1069.26
1142.81
1254.38
1390.00
1573.96
1700.10
1785.49
1839.37
1928.22


CMP_HILIC_acido005_MS2_02b #292 RT: 10.30 AV: 1 NL: 2.57E5
F: + c Full ms2 1817.50@cid35.00 [490.00-2000.00]
^{124}T-T^{135} + trisaccharide α 2-3
Relative Abundance
m/z
1161.32
1364.30
945.27
1526.27
927.31
1323.30
603.13
757.13
806.09
1053.05
1125.35
1291.55
1376.32
1614.39
1681.40
1798.46
1931.35


CMP_HILIC_acido005_MS2_02b #362-382 RT: 13.09-13.17 AV: 2 NL: 1.17E5
F: + c Full ms2 1817.50@cid35.00 [490.00-2000.00]
^{124}T-T^{135} + trisaccharide α 2-6
Relative Abundance
m/z
1161.29
945.20
1364.28
1526.28
927.28
1655.36
603.14
694.93
844.13
978.35
1125.26
1239.70
1323.27
1508.64
1797.48
1969.87


CMP_HILIC_acido005_MS2_05b #1389 RT: 48.81 AV: 1 NL: 3.04E4
F: + c Full ms2 964.80@cid35.00 [255.00-1935.00]
^{113}N-N^{123} + trisaccharide α 2-3
Relative Abundance
m/z
1272.43
738.09
444.05
636.87
854.92
945.94
511.06
615.32
1032.04
1194.25
1399.06
1488.33
1647.13
1844.11
1925.40


CMP_HILIC_acido005_MS2_05b #1419-1440 RT: 50.24-50.49 AV: 4 NL: 8.06E4
F: + c Full ms2 964.80@cid35.00 [255.00-1935.00]
^{113}N-N^{123} + trisaccharide α 2-6
Relative Abundance
m/z
1272.39
738.21
444.08
636.75
819.17
883.84
1032.19
365.98
570.89
1140.51
1323.23
1475.39
1637.42
1737.09
1841.35