



**UNIVERSIDAD AUTÓNOMA DEL ESTADO
DE MÉXICO**



DOCTORADO EN CIENCIAS AGROPECUARIAS Y RECURSOS NATURALES

TESIS DE INVESTIGACIÓN

**Elaboración de polímeros naturales injertados con compuestos antioxidantes y
evaluación de sus propiedades funcionales**

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Toluca, México; mayo de 2016

EN ESTE DOCTORADO SE DIJO:

El hombre ahora es un animal desconfiado. Sí, desde que conoció las ciencias ha perdido el miedo y ha ganado desconfianza!

Nietzsche; La Gaya Scienza

Todo lo que puede ser conocido tiene un número; ya que es imposible aprender algo con el pensamiento...

Pitágoras de Samos

Todos somos científicos cuando somos niños, pero al crecer, solo algunos conservan un poco de esa curiosidad que es la madre de la ciencia.

Juan Aguilar M. *Biólogo teórico*

No es hacer lo que nos gusta, sino que nos guste lo que hacemos, lo que convierte la vida en una bendición.

Johann W. Goethe (1749-1832) Poeta y escritor alemán

Ver y enseñar no basta. La filosofía debe de ser un poder vivo, y debe tener por esfuerzo y por efecto la mejora del hombre

***Los miserables* de Victor Hugo (1802-1885). Escritor Francés.**

La ciencia no nos ha enseñado aún si la locura es o no lo más sublime de la inteligencia.

Edgar Allan Poe. Escritor Estadunidense (1809-1849)

La ciencia natural, no se limita a descubrir y explicar la naturaleza, sino que es parte de la interacción entre la naturaleza y nosotros mismos

Werner Heisenberg. Físico Alemán (1901-1976)

Un hombre sería afortunado si alguien dibujara la historia de sus hazañas.

Buliwif. Líder y guerrero nórdico

DEDICATORIAS:

Con todo mi amor y alma, a quienes nunca me dejan caer y por quien soy y seré... Verito (siempre estás en mis pensamientos ¡TE AMO!), Niko-Vladi y Kaly (nunca dejan de sorprenderme)... ¡¡¡siempre juntos!!!

Papás; Chintín y Anita, “primero la obligación y luego la vocación, encontré mi equilibrio gracias a ustedes”

Hermanos; Jacinto y Emmanuel, porque siempre han creído en mí, a pesar de ser su polo opuesto. Siempre los haré reír...

Suegros; Adrián y Ceci, por su apoyo, ánimo, comprensión y creer en que alcanzaría esta meta...

Oliver y Adrián Cedillo, siempre es importante cualquier palmada para seguir adelante y su motivación fue esa mano...

Alondra y Alan Arizmendi Vilchis, son grandes y sé que lo serán más...

Hermanos de no sangre; Fernando, Irving, Ricardo, Juan Francisco

AGRADECIMIENTO:

Este trabajo fue posible por el apoyo de muchas personas e instituciones.

- ✓ Dr. Aurelio Domínguez López, columna importante en toda mi formación profesional.
- ✓ Dra. Rosa María Gómez Espinosa, creyó en mí y en este proyecto, espero no haber fallado. Aprendí mucho en cada sesión de revisión.
- ✓ Dr. Octavio Dublán García, por acompañarme nuevamente en mi formación académica y creer en mí para proyectos futuros.
- ✓ Dra. Adriana Villanueva Carvajal por el enorme apoyo en los reportes escritos, asesoría y comentarios.
- ✓ Maestra Virginia Gómez Vidales, sin su albergue en el laboratorio de Resonancia Paramagnética Electrónica y explicaciones, el trabajo no sería el mismo.
- ✓ Al Instituto de Química de la UNAM y al Dr. Fernando Cortez Guzmán por aceptarme en la estancia de investigación.
- ✓ Al Centro Conjunto de Investigación en Química Sustentable (UAEM-UNAM), Maestras Alejandra Núñez Pineda y Lizbeth Triana Cruz por los análisis realizados.
- ✓ UAEMex, PCARN y FCAgrí por darme la oportunidad de estudiar y ser parte de una generación más de doctores.
- ✓ CONACyT y COMECyT por los apoyos económicos para llevar a cabo mis estudios
- ✓ A todos los profesores de la línea de investigación: Dra. Tarín, Dra. Lolita, Dra. Luz Raquel, Dra. Dora Luz, Dr. Nestor

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RESUMEN

Con el fin de generar una nueva fibra dietética antioxidante y prebiótica, el ácido gálico (AG) se injertó en inulina nativa por el método de radicales libres generados mediante el par *Redox* peróxido de hidrógeno/ácido ascórbico (H_2O_2/AA). A través de Resonancia Paramagnética Electrónica (EPR), se evaluaron relaciones molares (RM) a 9, 20, 39 y 49 moles de H_2O_2/AA para determinar el efecto de la oxidación de la inulina y medir la eficiencia en el injerto Inulina- ácido gálico (IGA). El injerto se confirmó mediante UV, espectroscopia de infrarrojo (FT-IR) y difracción de rayos X en polvos. Además, la actividad antioxidante fue evaluada por métodos espectroscópicos y la actividad prebiótica del IGA se determinó por el crecimiento *In vitro* de *Lactobacillus acidophilus*. La concentración más alta de macro-radical en la inulina se obtuvo con relaciones molares de H_2O_2/AA de 20 y 49. El macro-radical de la inulina se forma por la eliminación de un átomo de hidrógeno de los grupos metilo en los monómeros de fructosa. Los espectros UV muestran picos de absorbancia a 214 y 266-268 nm que evidencian la presencia de anillos aromáticos en el injerto IGA y los espectros FT-IR muestran una banda a 1743 cm^{-1} , que confirma el enlace covalente entre la inulina y el AG. Las concentraciones equivalentes de AG en IGA fueron; 30.4 y 16.3 mg AG/ g IGA, con RM = 9 M de H_2O_2/AA . Los nuevos polímeros exhiben capacidad de inhibir radicales libres tales como DPPH, 1O_2 , y para reducir la peroxidación de lípidos. La inulina muestra una significativa capacidad de estimular el crecimiento de *L. acidophilus* que no se ve afectada por la presencia de moléculas de AG injertado. Este trabajo demuestra que es posible proporcionar capacidad de inhibición de radicales libres a los fructo-oligosacáridos, evitando que sus propiedades prebióticas disminuyan, lo que podría extender su potencial como alimentos funcionales.

I. INTRODUCCIÓN

La fibra dietética (FD) contenida en algunos productos vegetales comestibles se puede asociar con compuestos fenólicos y por lo tanto presentar actividad antioxidante significativa (Saura-Calixto, Pérez-Jiménez, y Goñi, 2009). Este tipo de fibra se conoce como fibra dietética antioxidante (FDA) y debido a su estructura polimérica también podría mostrar una capacidad prebiótica considerable. Los polifenoles asociados con esta fibra podrían ser liberados en el tracto gastrointestinal durante la digestión (Mercado-Mercado *et al.*, 2015) actuando contra la formación de radicales libres, mientras que los azúcares del polímero expresan actividad prebiótica.

La inulina es un grupo heterogéneo de polisacáridos de plantas compuestas básicamente de monómeros fructosa unidos por enlaces β (2 \rightarrow 1). Se encuentran en algunas especies pertenecientes a la familia *Liliaceae*, *Asteraceae* y recientemente se ha reportado en hojas de *Agave tequilana* (Madrigal & Sangronis, 2007; Montañez-Soto, Venegas-González, Vivar-Vera, & Ramos-Ramirez, 2011). La importancia de la inulina se encuentra en su contribución como parte de la FD y porque es considerada como un compuesto prebiótico (Glenn R Gibson, Probert, Loo, Rastall, y Roberfroid, 2004b; Kolida y Gibson, 2007). El consumo de inulina está asociado a diversos beneficios para la salud. Entre estos, se han observado la prevención de diabetes y cáncer, mejora del metabolismo de lípidos, reducción de colesterol en suero, y el aumento de la absorción de calcio y magnesio, entre otros (Lou *et al.*, 2009; Pejin *et al.*, 2013). Por otro lado, los fructanos tipo inulina no presentan actividad antioxidante. Sin embargo, fructanos y compuestos fenólicos podrían combinarse mediante injertos moleculares con la finalidad de sintetizar sistemas redox capaces de disminuir las especies reactivas de oxígeno (ROS) (Bolouri-Moghaddam, Le Roy, Xiang, Rolland, y Van den Ende, 2010; Hernández-Marín y Martínez, 2012).

Los injertos moleculares son métodos para la modificación de materiales poliméricos que permiten imponer características naturales a los polímeros sintéticos, otorgándoles nuevas propiedades al polímero modificado (Spizzirri, *et al.*, 2011; Spizzirri, *et al.*, 2009. Cirillo, *et al.*, 2010; Curcio, *et al.*, 2009). La reciente investigación se ha dirigido a la síntesis de FDA a través del injerto molecular de ácido gálico en inulina mediante la donación de un radical libre inducido. El proceso de injerto implica la generación de un sistema par *Redox*, a través

de la reacción peróxido de hidrógeno/ácido ascórbico (H_2O_2/AA) (Cirillo *et al.*, 2010; Curcio *et al.*, 2009; Spizzirri *et al.*, 2009, 2011). El radical hidroxilo ($\bullet OH$) que inicia la reacción se forma cuando el H_2O_2 oxida al AA. Después de esto, el proceso de injerto de la molécula tiene lugar en dos pasos. El primero consiste en la generación de un macro-radical en la cadena de biopolímero a través de la reacción con el radical libre inducido entre AA y H_2O_2 . En el segundo paso, la molécula antioxidante se une al macro-radical a través de un enlace covalente (Curcio *et al.*, 2009; Spizzirri *et al.*; 2009, 2010; Toti y Aminabhavi, 2004).

Dado que algunos de estos biopolímeros, antes de ser injertados, tienen una actividad prebiótica significativa, los nuevos conjugados podrían mostrar un uso potencial como compuestos prebióticos y antioxidantes (Arizmendi-Cotero, Gómez-Espinosa, Dublán-García, Gómez-Vidales, y Dominguez- López, 2016). Según Saura-Calixto, (2011) alrededor de 50% de los antioxidantes de la dieta total, principalmente fenoles, pasan a través del intestino delgado unidos a la FD. Estos se liberan de la matriz de fibra en el colon por la acción de la microflora bacteriana, los metabolitos que producen y por el entorno antioxidante. En consecuencia, el transporte de antioxidantes en la dieta a través del tracto gastrointestinal puede ser una función esencial de la FD. Los conjugados de inulina-ácido gálico (IAG) sintetizados podrían ser un modelo adecuado y relativamente simple para estudiar el efecto antioxidante y prebiótico de fibra dietética natural asociada con polifenoles. Por lo tanto, los objetivos de este trabajo doctoral fueron; (i) sintetizar conjugados Inulina-AG, (ii) evaluar la relación (H_2O_2/AA) sobre el proceso de radicalización de la inulina, (iii) caracterizar los conjugados Inulina-AG, (iv) medir su actividad antioxidante y (v) evaluar la capacidad prebiótica *In vitro* de los conjugados a través de cultivos de *Lactobacillus acidophilus*.

II. ANTECEDENTES

2.1.Fibra dietética e Inulina

2.1.1. Definición y componentes de la fibra dietética

Se considera fibra dietética (FD) a los polisacáridos vegetales y la lignina, que son resistentes a la hidrólisis enzimática del tracto digestivo. En 2011 la Asociación Americana de Química de Cereales (AACC) la definió como “parte comestible de las plantas o hidratos de carbono, resistentes a la digestión y absorción en el intestino delgado, con fermentación parcial o completa en el intestino grueso” (Escudero Álvarez & González Sánchez, 2006). Las fibras dietéticas contienen una mezcla de componentes bioactivos, que incluyen almidones resistentes, vitaminas, minerales, fitoquímicos y antioxidantes (Lattimer & Haub, 2010). Sin embargo, el principal componente de la FD son polisacáridos y oligosacáridos resistentes.

Los carbohidratos resistentes a la digestión en el intestino delgado, que requieren de la fermentación bacteriana en el intestino grueso con un grado de polimerización mayor a tres, son considerados FD (Lattimer & Haub, 2010), una descripción de los componentes se describe en la Tabla 1

Tabla 1. Componentes de la fibra dietética.

Polisacáridos no almidón y Oligosacáridos	Carbohidratos análogos	Lignina y sustancias asociadas a carbohidratos no estructurados
<ul style="list-style-type: none">• Celulosa• Hemicelulosa• Arobinoxilanos• Arabinogalactanos• Polifruktosas• Inulina• Oligofruktanos• Galacto-Oligosacáridos• Gomas• Mucílagos• Pectinas	<ul style="list-style-type: none">• Dextrinas indigestibles• Maltodextrinas resistentes• Dextrinas resistentes de papa• Compuestos carbohidratos sintetizados• Polidextrosas• Metil celulosa• Hidroxipropilmetil celulosa• Almidones resistentes	<ul style="list-style-type: none">• Ceras• Saponinas• Suberinas• Taninos

Fuente: (DeVries *et al.*, 2001).

2.1.2. Fructanos e Inulina

La inulina es un polímero de monómeros de fructuosa, unidos por enlaces (2→1)-β-D-fructosil-fructosa, de varias longitudes, generalmente terminadas por una unidad simple de glucosa, unida por un enlace α-D-glucopiranosil (Figura 1).

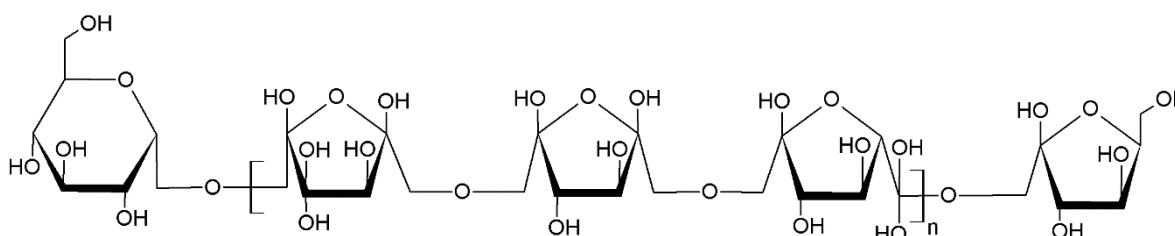


Figura 1. Estructura química de la inulina

Después del almidón, los fructanos tipo inulina son los polisacáridos más abundantes en la naturaleza. Entre las especies de plantas que producen fructanos se encuentran algunas pertenecientes a la familia *Liliaceae* (ajo, cebolla, espárrago, ajoporro), *Asteraceae* (achicoria, patata o tupinambo, yacón) y recientemente se reportan fructanos tipo inulina en hojas de *Agave tequilana* Weber azul (Madrigal & Sangronis, 2007; Montañez-Soto, Venegas-González, Vivar-Vera, & Ramos-Ramirez, 2011). La Tabla 2 muestra el contenido de inulina en algunas especies vegetales.

Tabla 2. Contenido promedio de inulina en especies vegetales.

Nombre común	Nombre científico	Inulina (g/100 g materia seca)
Patata	<i>Helianthus tuberosus</i>	8.9
Achicoria	<i>Cichorium intybus</i>	7.9
Agave tequilero azul	<i>Agave tequilana</i>	6.8
Raíz de Dalia	<i>Dhalia spp</i>	5.9
Cebolla	<i>Allium cepa</i> L.	4.8
Ajoporro	<i>Allium porro</i> L.	3.7
Ajo	<i>Allium sativum</i>	2.9
Yacón	<i>Smallanthus sonchifolius</i>	2.7
Espárrago	<i>Asparragus officinalis</i> L.	0.4
Cambur	<i>Musa cavendishii</i>	0.2
Centeno	<i>Secale cereale</i>	0.1

Dependiendo del origen y método de extracción, los fructanos tipo inulina suelen tener diferentes grados de polimerización (GP) que puede ir desde 12 a 46 (López-Molina *et al.*, 2005), estas diferencias condicionan sus propiedades físicas y químicas. La inulina nativa

(GP=12), contiene azúcares libres, mientras que la inulina de alta pureza (GP=25) presenta menor solubilidad que la inulina nativa, debido a la casi total ausencia de azúcares libres (0.5 % de materia seca) (Franck, 2002). Propiedades como la digestibilidad, la actividad prebiótica y aportación calórica, poder edulcorante, entre otras, difieren sustancialmente por el GP (De Gennaro, Birch, Parke, & Stancher, 2000). Otras propiedades que se ven influenciadas por el GP de los fructanos son la capacidad antioxidante, cuando el GP es mayor, su capacidad para reducir radicales libres (DPPH, •OH, ¹O₂) disminuye considerablemente (Je, Park, & Kim, 2004; Park, Je, & Kim, 2003). Sin embargo, algunos compuestos fenólicos pueden estar asociados naturalmente a la inulina y otras FD aumentando significativamente su actividad antioxidante (Palafox-Carlos, Ayala-Zavala, & González-Aguilar, 2011; Saura-Calixto, Pérez-Jiménez, & Goñi, 2009)

2.1.3. Fibra dietética antioxidante (FDA)

Los antioxidantes de las frutas comúnmente están mezclados con diferentes macromoléculas, como los carbohidratos, conformando la matriz del alimento (Manach, *et al.*, 2005). Lecumberry, *et al.*, (2006), encontraron que el cacao es fuente de fibra dietética (83%) con presencia de compuesto fenólicos (1.15%). Sayago-Ayerdi, Arranz, Serrano & Goñi (2007), reportan que la infusión de flor de jamaica (*Hibiscus sabdariffa* L.), preparada en México de forma tradicional, contiene 0.66 g de FD soluble por litro. Pero este valor es menor comparado con el contenido de FD en la planta, lo que sugiere que una gran cantidad de compuestos fenólicos (CF) están asociados a la fibra de la flor de jamaica. Existe evidencia de que estos compuestos pueden tener importancia nutricional muy significativa, además de poseer una alta capacidad antioxidante (Saura-Calixto, Serrano, & Goñi, 2007; Goñi & Serrano, 2005).

Teniendo en cuenta que en los alimentos vegetales hay otros componentes diferentes a polisacáridos y lignina, componentes bioactivos tales como polifenoles, carotenoides y fitoesteroles, el concepto de FD puede ampliarse al de fibra dietética antioxidante (FDA). El conjunto de estos componentes del alimento o de la materia prima, constituye la denominada fracción indigestible de los alimentos (Saura-Calixto, Garcia-Alonso, Goni, & Bravo, 2000). En el caso de los polifenoles, una parte considerable de ellos puede estar asociada a la fracción de fibra insoluble, principalmente los compuestos de mayor grado de polimerización

como taninos condensados (proantocianidinas) y taninos hidrolizables. Mientras que a la fracción de fibra soluble, se suelen asociar polifenoles de menor peso molecular tales como algunos flavonoides, ácidos fenólicos, dímeros y trímeros de proantocianidinas (Saura-Calixto, Serrano, & Goñi, 2007; Goñi & Serrano, 2005). Estos compuestos son los más abundantes en la dieta, sin embargo sólo los polifenoles asociados a la fibra dietética son capaces de pasar la mucosa del intestino delgado y llegar al colon, donde se convierten en sustrato fermentable por la microflora bacteriana junto con los hidratos de carbono no digeribles y la proteína (Manach, Williamson, Morand, Scalbert, & Rémésy, 2005).

Los fructuoligosacáridos (FOS) son los componentes más abundantes de la FD y son considerados como una fuente importante de ácidos fenólicos, los cuales pueden ser liberados por enzimas bacterianas en el intestino grueso (Guo & Beta, 2013). Los FOS, presentes en muchos alimentos exhiben ligados a su estructura compuestos fenólicos diversos. Campos *et al.*, (2012) reportan de 6.4 a 65 g de FOS/100 g de MS, en muestras de partes vegetales de Yacon (*Smallanthus sonchifolius* Poepp. & Endl), en presencia de ácido hidroxicinámico (17.7 mg/100 g PF) y ácido clorogénico (5.58 mg/100 g PF). Fuentes-Alventosa *et al.*, (2009) cuantificaron, en fibra de espárrago (*Asparagus officinalis* L.), ácido hidroxicinámico (2.31 a 4,91 mg), saponinas (2.14 a 3.64 mg), flavonoides (0.6-1.8 mg) y fructanos (0.2 a 1.4 mg), por cada 100 gramos de fibra. Rufino *et al.*, (2011), reportan en frutos de acaí (*Euterpe oleraceae*) 27.5 mg de FOS/g MS y 11.2 mg/g MS de polifenoles. Existe evidencia de que estos compuestos pueden tener importancia nutricional muy significativa, además de poseer una alta capacidad antioxidante (Saura-Calixto, Serrano, & Goñi, 2007; Goñi & Serrano, 2005). Esta clase especial de FDA posee además la capacidad de ser resistente a la hidrólisis enzimática del intestino delgado y ser fermentada por la flora microbiana del intestino grueso (Van den Ende, *et al.*, 2011), exhibiendo capacidad prebiótica y antioxidante a la vez.

2.2.Capacidad prebiótica de fructanos tipo inulina

2.2.1. Tipos de prebióticos

Los prebióticos están definidos como ingredientes de alimentos que promueven el crecimiento o actividad de bacterias benéficas para la salud (Gibson & Roberfroid, 1995). En términos simples, los prebióticos son el alimento de bacterias de algunas especies de los géneros *Bifidobacterium* y *Lactobacillus* (Douglas & Sanders, 2008).

Los polisacáridos que contienen de 2 a 20 unidades de azúcares, que incluyen; fructanos tipo inulina, *trans*-galactooligosacáridos, isomalto-oligosacáridos, xylo-oligosacáridos, soy-oligosacáridos, gluco-oligosacáridos y lacto-sucrosa han despertado interés en los últimos años como prebióticos (Kolida & Gibson, 2007), siendo los fructanos tipo inulina los más estudiados recientemente como prebióticos. Gibson, Probert, Loo, Rastall, & Roberfroid (2004a), establecieron los siguientes criterios que deben cumplir los polisacáridos para ser asignados como prebióticos:

- Resistencia a la acidez gástrica, la hidrólisis por las enzimas de mamíferos y la absorción gastrointestinal.
- Ser fermentados por la microflora intestinal.
- Estimular y/o actividad de bacterias intestinales asociadas a la salud y el bienestar.

En la Tabla 3 se mencionan algunos polisacáridos que cumplen con los criterios mencionados y pueden clasificarse como prebióticos

Tabla 3. Polisacáridos clasificados como prebióticos

Nombre	Composición	Fuente	GP²
Inulina	$\beta(2-1)$ Fructanos	Raíz de achicoria	11 – 65
Fructo-Oligosacáridos	$\beta(2-1)$ Fructanos	Hidrólisis de inulina de achicoria	3 – 5
Galacto-oligosacáridos	Oligo-galactosa (85%) con fracciones de glucosa y lactosa	Producida de lactosa por β -galactosidasa	2 – 5
Soya-oligosacáridos	Mezcla de Rafinosa (F-Gal-G) ¹ y estaquirosa (F-Gal-Gal-G)	Extracción de suero de leche de soya	3 – 4
Xylo-oligosacáridos	$\beta(1-4)$ -xilosa	Hidrólisis enzimática de xilanos	2 – 4
Pyrodextrinas	Mezcla de oligosacáridos que contiene G	Pirólisis de papa o almidón de maíz	Variable
Isomalto-oligosacáridos	$\alpha(1-4)$ G con ramificaciones $\alpha(1-6)$ G	Transgalactosilación de maltosa	2 – 8

¹ F: fructosa, Gal: galactosa, G: glucosa

² Grado de Polimerización.

Fuente: (Macfarlane, Macfarlane, & Cummings, 2006)

La importancia de los prebióticos se debe a: (i) el equilibrio entre flora intestinal benéfica y perjudicial, (ii) los prebióticos pueden alterar la composición de la microbiota hacia un perfil más saludable, (iii) como una alternativa a los probióticos, que pueden ser difíciles de

manejar en algunos alimentos, y (iv) porque los prebióticos empleados actualmente, especialmente la inulina y sus derivados, son relativamente baratos de fabricar o extraer, aportan beneficios al consumidor y suelen ser ingredientes funcionales capaces de mejorar las propiedades organolépticas de alimentos (Macfarlane *et al.*, 2006; Madrigal & Sangronis, 2007).

2.2.2. Beneficios en el consumo de fructanos tipo inulina

La mayoría de los beneficios determinados al consumo de prebióticos tipo fructanos, se asocian a la optimización del metabolismo y a la función del colon. Tales como; mejora en el metabolismo de lípidos, absorción de calcio y magnesio, prevención de la diabetes y cáncer, reducción del colesterol, entre otros (Lou, Wang, Wang, & Zhang, 2009; Macfarlane *et al.*, 2006; Pejin, Savic, Petkovic, Radotic, & Mojovic, 2013). Diversos estudios han demostrado la capacidad prebiótica de la inulina en la salud humana (Tabla 4), en general una dosis de inulina de 5-8 g/día debe ser suficiente para provocar un efecto positivo en la microflora intestinal (Kolida & Gibson, 2007).

Tabla 4. Beneficios del consumo de fructanos tipo inulina en la salud humana

Tipo	Dosis (g/día)	Beneficio	Sujetos de estudio	Fuente
Inulina u Oligofructosa	15	Aumento de bifidobacterias fecales.	8 adultos sanos	Gibson & Roberfroid, (1995)
Oligofructosa	4	Incremento de bifidobacterias y lactobacilos.	6 y 12 adultos sano	Williams, Witherly, & Buddington, (1994), Buddington, Williams, Chen, & Witherly (1996)
Inulina	20 – 40	Aumento bifidobacterias, disminución de enterococos y enterobacterias.	35 ancianos estreñidos	Kleessen, Sykura, Zunft, & Blaut (1997)
Inulina	15	Aumento en frecuencia y masa de deposiciones fecales.	6 adultos sanos	Den Hond, Geypers, & Ghoois (2000)
Inulina	34	Incremento de bifidobacterias fecales.	6 adultos sanos	Kruse, Kleessen, & Blaut (1999)
Oligofructosa	10	Aumento recuento de bifidobacterias fecales.	40 adultos sanos	Bouhnik, <i>et al.</i> (1999)
Oligofructosa	6.6	Efecto bifidogénico.	30 adultos sanos	Tuohy, Kolida, Lustenberger, & Gibson (2001)

Los microorganismos habitualmente estudiados son las bifidobacterias y lactobacilos, con aumentos significativos de la población. Se han observado aumentos numéricos de 0.5-1.0 log₁₀ que constituye un cambio importante en la microflora intestinal.

2.2.3. Evaluación prebiótica *In vitro* de fructanos tipo inulina

La mayoría de las cepas de *Bifidobacterium* y *Lactobacillus* cuando han sido estudiadas *In vitro* han demostrado ser capaces de fermentar los fructanos de cadena larga. Sin embargo, esta capacidad de utilizar $\beta(2-1)$ fructanos como fuente de carbono y energía no parece ser una característica general en estas dos familias bacterianas. Por otra parte, los fructanos con diferentes grados de polimerización difieren en la expresión de la capacidad para estimular el crecimiento bacteriano (Corral, Valdivieso-Ugarte, Ferna, Adrio, & Velasco, 2008; Pompei *et al.*, 2008).

Pompei *et al.*, (2008), investigaron el efecto prebiótico de oligofruktosa e inulina de alta solubilidad. Sus resultados revelan que las bifidobacterias logran el máximo crecimiento a las 6 h, usando oligofruktosa como fuente de energía, y a las 12 h, con inulina de alta solubilidad. Por su parte, los lactobacilos alcanzaron su mayor expresión a las 24 y 48 h, con oligofruktosa e inulina, respectivamente. Sin embargo, la concentración final fue mayor para ambas cepas cuando crecieron en presencia de inulina de alta solubilidad. Li, Kim, Jin, & Zhou, (2008) evaluaron el potencial prebiótico de inulina de bardana (*Arctium minus* Hill.) a concentraciones de 0.5 a 4% p/v. Sus resultados indican que una suplementación del 1% de esta inulina es suficiente para estimular el crecimiento de *Bifidobacterium adolescentis*. Corral *et al.*, (2008) reportan cepas de *Lactobacillus gasseri* y *Lactobacillus fermentum*, alcanzando niveles de crecimiento más altas en medios de cultivo que contienen mezclas de fructuoligosacáridos producidos por síntesis enzimática, en comparación con los obtenidos por hidrólisis de inulina.

En general, el crecimiento de cepas de *Bifidobacterium* y *Lactobacillus* depende del grado de polimerización de la fuente de carbono. Los fructanos de cadena corta son metabolizados más fácilmente que los de cadena larga, tal es el caso de la inulina. Sin embargo, el empleo de fructanos de alto grado de polimerización podría resultar en un mayor crecimiento, después de un periodo más largo de fermentación.

2.3. Injerto molecular por donación de radical libre

Los injertos moleculares representan un método para la modificación de materiales poliméricos (Contreras-García, Burillo, Aliev, & Bucio, 2008) que permiten imponer características naturales a los polímeros sintéticos, otorgándole nuevas características al polímero modificado (Spizzirri, *et al.*, 2010). El injerto de una molécula simple a un polímero natural, tal como almidón, celulosa, quitosano, gelatina, inulina, entre otros es de gran importancia para el desarrollo de nuevos materiales, combinando las propiedades de la molécula injertada y del polímero natural (Spizzirri, *et al.*, 2011; Spizzirri, *et al.*, 2009; Cirillo, *et al.*, 2010; Curcio, *et al.*, 2009).

Para desarrollar polímeros biodegradables a partir de ésteres de azúcar se han experimentado síntesis enzimáticas y polimerización con reactivo de *Fenton* en agua (Kitagawa & Tokiwa, 2006). La polimerización por oxidación usando ion ferroso y compuestos peroxidativos ha demostrado ser útil para las reacciones de injerto de fibras naturales como el quitosano (Lagos & Reyes, 1988). También se ha sustituido el ion ferroso por ácido ascórbico (Larpen & Tadros, 1991). Miura, Ikeda, & Kobayashi, (2003) emplearon ácido ascórbico para producir oxígeno activado en la síntesis de polímeros lineales de azúcares derivados de alcohol que contenían grupos vinil. Esta catálisis, promueve reacciones bajo temperatura ambiente, presión atmosférica, pH neutral y usando agua como solvente (Kitagawa & Tokiwa, 2006).

Las reacciones de injerto se llevan a cabo por medios químicos y los iniciadores de la reacción son agentes reductores (*redox*), tales como el persulfato de potasio, nitrato cérico de amonio y persulfato de amonio que producen una especie de radical libre después de un calentamiento a 40° C (Spizzirri *et al.*, 2009). Sin embargo, se han desarrollado nuevas técnicas que permiten injertar moléculas simples a biopolímeros usando un sólo paso, reacciones a temperatura ambiente y sin la generación de productos tóxicos de reacción.

Kitagawa & Tokiwa, (2006) evaluaron la polimerización de ésteres de azúcar usando ácido ascórbico (10 mM) y peróxido de hidrógeno (7mM) como agentes de oxidación. Sus resultados muestran que un incremento hasta 10 mM de la concentración de ácido ascórbico aumenta el rendimiento de los polímeros. Por el contrario, una alta concentración de peróxido

Este método ha sido reportado con éxito por diferentes autores para generar nuevos polímeros antioxidantes. En la Tabla 5, se muestra un resumen de resultados de algunos reportes de investigación. Curcio, *et al.* (2009) y Spizzirri, *et al.* (2009, 2010) reportan la misma concentración de antioxidante como valor óptimo para generar un polímero con alta eficiencia (0.7 mmol de antioxidante por gramo de polímero). Sin embargo, Cho, Kim, Ahn, & Je, (2011) reportan que la expresión de la actividad antioxidante aumenta cuando se incrementa la alimentación de antioxidante en la reacción. Por otro lado, este mismo autor reporta como valor óptimo de alimentación una relación molar 1:0.1 entre quitosano y ácido gálico, debido a que esta mostró el porcentaje de injerto más alto (53.88%).

Tabla 5. Resultados de polímeros injertados con compuestos antioxidantes (AO) y capacidad de inhibición de diversos oxidantes.

Polímero	AO ¹	AO injetado (mg/g MS)	% inhibición ²				Fuente	
			DPPH	•OH	PAL	H ₂ O ₂		O ₂ ⁻
Quitosano	AG	45.8	75	73	80	76	74	Liu <i>et al.</i> , (2013)
	AC	38.5	65	62	71	62	62	
	AF	36.7	59	47	66	59	55	
Quitosano	AC	73.4		64	58	56	68	Liu, <i>et al.</i> (2014)
	AF	66.7		57	50	36	57	
Quitosano	C	4.0	98	95	98			Curcio, <i>et al.</i> (2009)
	AG	7.0	92	60	85			
Gelatina	AG	0.7	66	64	98			Spizzirri, <i>et al.</i> (2009)
	C	0.9	98	76	99			
Alginato	C	0.53	61		50			Spizzirri, <i>et al.</i> (2010)
Inulina		1.27	74		60			
Quitosano	AG	119	92			93		Cho, <i>et al.</i> (2011)

¹ Molécula Antioxidante: AG: ácido gálico, AC: ácido cafeico, AF: ácido ferúlico, C: catequina

² DPPH: 1,1-difeneil-2-picril-hidrazilo, •OH: radical hidroxilo, PAL: Peroxidación de ácido linoleico, H₂O₂: peróxido de hidrógeno, O₂⁻: radical superóxido.

Spizzirri, *et al.* (2010), sintetizaron polisacáridos-antioxidantes de alginato e inulina injertados con catequina como molécula antioxidante. Un posible mecanismo de reacción entre la molécula antioxidante y el polisacárido, se explica en dos pasos: El radical hidroxil generado por sistema iniciador (ácido ascórbico/H₂O₂), extrae el átomo de Hidrógeno del grupo hidroxilo del polisacárido (paso 1), consecuentemente se forma un radical libre en el que se inserta la molécula de catequina mediante un enlace covalente (paso 2) (Toti & Aminabhavi, 2004).

La reactividad de la catequina hacia el radical libre se puede explicar con base en la caracterización de su estructura química por la presencia de grupos fenólicos. En la literatura muchos estudios reportan que los grupos fenólicos son compatibles con este tipo de polimerización. Los monómeros con grupos funcionales activos (grupos fenólicos) como sustituyentes laterales, de hecho se usan para la preparación del sistema de injerto polimérico (Nanjundan, Selvamalar, & Jayakumar, 2004) usando iniciadores de radicales libres. Por otro lado, grupos fenólicos podrían involucrarse directamente en el proceso de polimerización. Se ha reportado, que el radical fenólico experimenta un proceso de dimerización por reacción entre el radical hidroxilo y el anillo aromático en las posiciones *orto* o *para* relativas al grupo hidroxilo (Uyama, Maruichi, Tonami, & Kobayashi, 2002). Con base en estos datos, se plantea la hipótesis de que la inserción de antioxidantes en la cadena de polisacáridos y polímeros ocurre en las posiciones 2 y 5 del anillo aromático del ácido gálico y en las posiciones 2', 5' (anillo B) y 6, 8 (anillo A) de la catequina, respectivamente (Cho *et al.*, 2011; Curcio *et al.*, 2009; Spizzirri *et al.*, 2010).

2.4. Resonancia paramagnética electrónica en detección de radicales libres

La Resonancia Paramagnética Electrónica (EPR) es una técnica espectroscópica similar a la resonancia magnética nuclear (RMN), ambas basadas en la interacción de radiación electromagnética con momentos magnéticos de la materia. En EPR el momento magnético surge de electrones desapareados, por lo que esta técnica se utiliza principalmente en la detección de especies químicas con electrones desapareados tales como radicales libres y metales de transición (Solano & Sosa, 2000; Yu & Cheng, 2008). Cuando los electrones desapareados se someten a un campo magnético, estos se alinean paralelamente o antiparalelamente al campo magnético, que corresponde a un nivel bajo y alto de energía, respectivamente (Figura 3). La diferencia de energía entre estos dos niveles es positivamente proporcional a la intensidad del campo de magnético (Lurie & Mäder, 2005). Estas energías están en la región de microondas del espectro electromagnético, los espectros de EPR se obtiene mediante la medición de la absorción de microondas de una energía fija mientras con un campo magnético variado (Goodman, 2013).

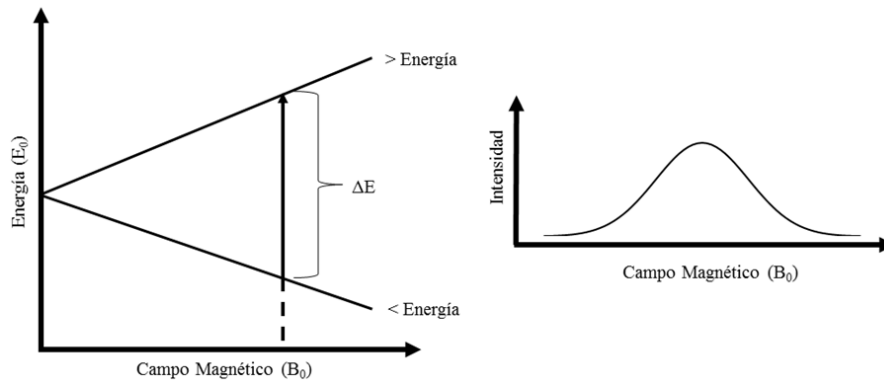


Figura 3. División de los niveles energéticos de un electrón desapareado

2.4.1. Detección de especies radicales estables

Algunos radicales pueden medirse de manera directa por medio de la técnica de EPR, tales como 1,1-difeneil-2-picril-hidrazilo (DPPH), el cual se usa para medir la interacción radicales libres-compuestos antioxidantes y estimar la capacidad de secuestrar radicales de muestras con potencial antioxidante (Biyik & Tapramaz, 2009; Pasanphan, Buettner, & Chirachanchai, 2010; Pejín *et al.*, 2013; Polak, Bartoszek, & Stanimirova, 2013). En general, una solución de DPPH se mezcla con una solución antioxidante. Se registra la intensidad de la señal EPR en condiciones experimentales seleccionadas, (concentración o tiempo) y se mide la intensidad de la señal generada (Figura 4). En ausencia de compuestos antioxidantes la intensidad de la señal de EPR, en todos los radicales, es la más alta y esta disminuye a medida que el compuesto antioxidante se hace presente en la mezcla de reacción (Kopáni, Celec, Danisovic, Michalka, & Biró, 2006). El porcentaje de inhibición puede calcularse con base a la ecuación:

$$\text{Capacidad antioxidante} = (H_0 - H_c) / H_0 * 100 \quad (1)$$

donde, H_0 es la altura de la señal del espectro en ausencia del compuesto antioxidantes y H_c , la altura de la señal del espectro en presencia del compuesto antioxidante (AO).

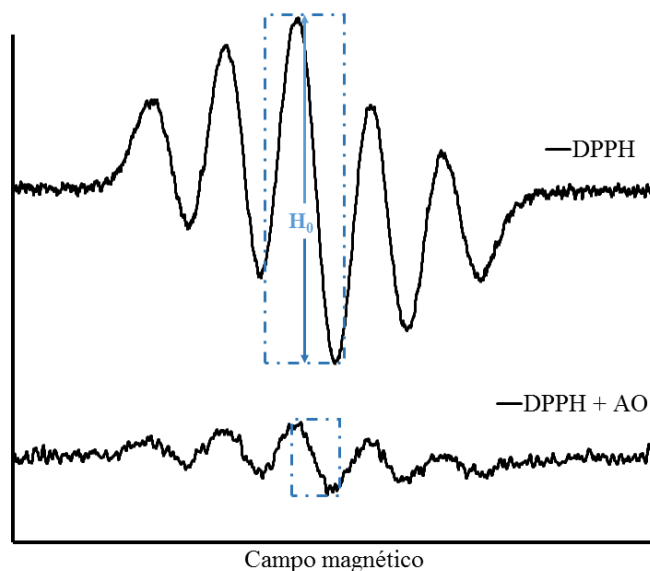


Figura 4. Espectro EPR típico de radical DPPH en ausencia y presencia de compuestos antioxidante (AO).

El radical ascorbato ($\text{Asc}^{\bullet-}$), producto de la oxidación de la vitamina C, también puede medirse directamente a través de EPR. Su espectro típico se observa en la Figura 5a.

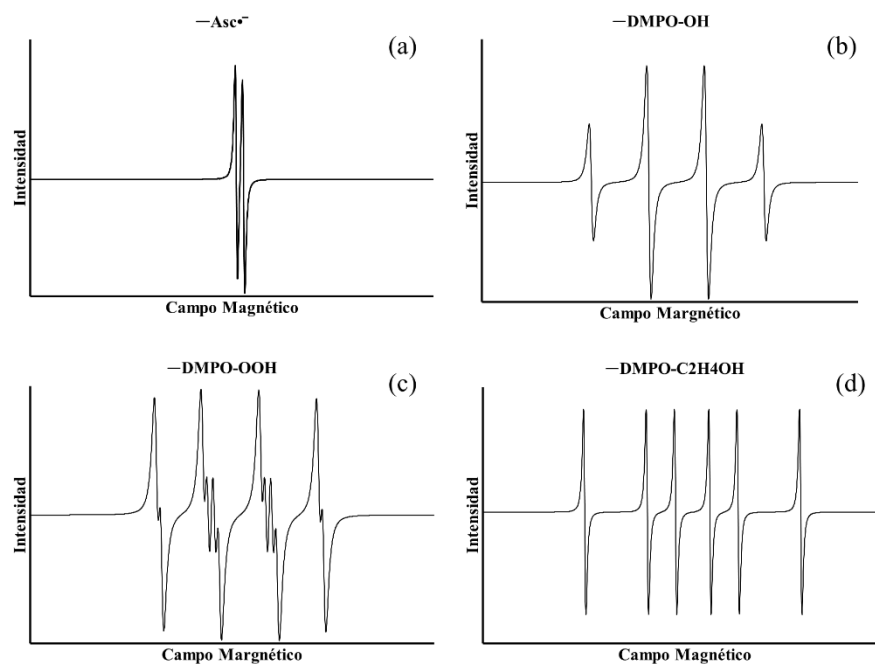


Figura 5. Espectros ERP típicos de radicales (a) Radical ascorbato ($\text{Asc}^{\bullet-}$), (b) Radical Aducto DMPO-OH [Hidroxilo], (c) Radical Aducto DMPO-OOH [Superóxido], (d) Radical Aducto DMPO- C2H4OH [Metilo].

2.4.2. Detección de especies radicales de vida media corta

Los radicales libres de vida corta, tales como, radicales hidroxilo ($\cdot\text{OH}$), radicales superóxido (O_2^-) o radicales centrados en carbono sólo pueden ser detectados por trampas de espín, debido a su alta reactividad (Faure, Andersen, & Nyström, 2012). Las trampas de spin son compuestos diamagnéticos que en presencia de radicales libres reaccionan rápidamente con ellos formando aductos cuya estabilidad permite su detección. El 5,5-dimetil-1-pirrolina N-óxido (DMPO) es el compuesto trampa de spin más utilizado y es empleado en la detección de los radicales libres en polisacáridos (Pejin *et al.*, 2013; Yamaguchi, Yoshimura, Nakazawa, & Ariga, 1999). Los espectros típicos de estos aductos se observan en la Figura 5b-d.

Además del espectro típico, los radicales pueden ser identificados por dos parámetros importantes, el valor de la constante giromagnética (g), que corresponde al desplazamiento químico y las constantes de acoplamiento hiperfino (A_h). Para todos los radicales libres orgánicos, el valor g es de 2.0057 con variaciones insignificantes (Goodman, 2013; Yu & Cheng, 2008). Los valores para las constantes de acoplamiento dependen del electrón al cual se asocia la energía. Buettner & Schafer, (2006), reportan que el radical ascorbato se detecta a una doble señal con A_H a 1.6 Gauss. El radical aducto DMPO-C₂H₄OH está definido por un espectro de seis líneas (Figura 5d) con constantes de acoplamiento $A_N = 15.72$ y $A_H = 22.59$ Gauss, según los reportes de Granados-Oliveros *et al.*, (2013). El aducto DMPO-OH muestra valores típicos A_H y $A_N = 16.4$ Gauss (Granados-Oliveros *et al.*, 2013; Yamaguchi *et al.*, 1999). Mientras que el aducto DMPO-OOH se detecta con valores $A_N = 14.3$, $A_{H\beta} = 11.7$ y $A_{H\gamma} = 1.3$ Gauss. La especificación de especies de radicales utilizando espectros de EPR se facilita mediante la simulación por computadora y el cálculo teórico de las constantes de acoplamiento.

III. PLANTEAMIENTO DEL PROBLEMA

3.1. Justificación

El estado físico de la matriz de los alimentos juega un papel clave en la liberación, transferencia de masa, accesibilidad y estabilidad bioquímica de muchos compuestos del alimento. Los antioxidantes a menudo se localizan en compartimentos celulares o dentro de conjuntos producidos durante su procesamiento. En cualquiera de los casos, necesitan ser liberados para que puedan ser absorbidos en el intestino. Además se conoce poco sobre la interacción de los antioxidantes con otros componentes del alimento, como la fibra dietética.

El tipo de interacciones químicas entre los compuestos fenólicos y la fibra dietética incluye la formación de uniones ordenadas y estables por matrices de enlaces covalentes y no covalentes entre los grupos hidroxilo de los compuestos fenólicos y el grupo polar de las moléculas de polisacáridos (enlaces de hidrógeno, interacciones electrostáticas y dipolares, fuerza de Van der Waals). Los enlaces de los polifenoles necesitan ser hidrolizados por las enzimas en el área superior del intestino; de otro modo estos compuestos no estarían bioaccesibles para absorberse en el intestino humano y serían susceptibles a la degradación por la microflora del colon en el intestino grueso.

La capacidad antioxidante de la fibra dietética podría incrementarse por el injerto de moléculas antioxidantes en sus estructuras. La inserción de un agente antioxidante biocompatible dentro de la estructura de un prebiótico podría además mejorar la estabilidad de este tipo de ingredientes alimenticios, ya que este tipo de sistemas se caracterizan por una alta estabilidad y baja velocidad de degradación de los compuestos. Además, podrían ser aplicados en aquellos campos donde está prohibido el uso de moléculas simples con actividad antioxidante. De este modo, podrían desarrollarse alimentos o vehículos capaces de controlar la liberación de compuestos fenólicos antioxidantes y ser depositados en sitios específicos del tracto digestivo. Además, los conjugados de polímero-antioxidante sintetizados podrían ser un modelo adecuado y relativamente simple para estudiar el efecto antioxidante y prebiótico de fibras dietéticas naturales asociada con polifenoles.

3.2. Objetivos

3.2.1. Generales

- ✓ Evaluar el efecto de la relación molar entre peróxido de hidrógeno/ácido ascórbico y tiempo de reacción, en el injerto molecular de ácido gálico en la cadena de inulina.
- ✓ Determinar las propiedades antioxidantes del conjugado inulina-ácido gálico y evaluar su capacidad prebiótica, mediante cultivos de *Lactobacillus acidophilus*.

3.2.2. Específicos

- (i) Sintetizar conjugados Inulina-Ácido Gálico, mediante el método de donación de un radical libre inducido, generado por el sistema par redox H_2O_2/AA
- (ii) Evaluar la relación molar H_2O_2/AA sobre el proceso de radicalización de la inulina, a través de espectroscopía de resonancia paramagnética electrónica.
- (iii) Caracterizar los conjugados Inulina-Ácido Gálico, por medio de espectroscopía infrarrojo de transformada de Fourier (FTIR), difracción de rayos X en polvos y espectroscopia de luz ultravioleta (UV-vis).
- (iv) Medir la actividad antioxidante de conjugados Inulina-Ácido Gálico, contra radical DPPH, oxígeno singulete y peroxidación lipídica.
- (v) Evaluar la capacidad prebiótica *In vitro* de los conjugados a través de cultivos de *Lactobacillus acidophilus*.

3.3. Preguntas de investigación

1. ¿Es posible incorporar moléculas antioxidantes en la cadena principal de polímeros naturales mediante el proceso de injerto por donación de un radical libre inducido?
2. ¿La incorporación de moléculas de compuestos antioxidantes en las cadenas de polímeros naturales permitirá una mayor expresión de su acción antioxidante?
3. ¿La eficiencia del injerto molecular depende de la concentración de ácido ascórbico y peróxido de hidrógeno en el sistema iniciador redox, además del tiempo de reacción?
4. ¿Las propiedades prebióticas de polímeros naturales se verán afectadas por la incorporación de moléculas antioxidantes en su estructura química?

3.4.Hipótesis

El presente trabajo doctoral plantea las siguientes hipótesis de trabajo:

- El injerto molecular de ácido gálico en la cadena de inulina, depende de la relación molar óptima entre peróxido de hidrógeno/ácido ascórbico del sistema iniciador de reacción, además del tiempo de reacción.
- La presencia de moléculas de ácido gálico en la cadena principal de la inulina, determina las propiedades antioxidantes del conjugado inulina-ácido gálico, sin interferir en la capacidad prebiótica del polímero.

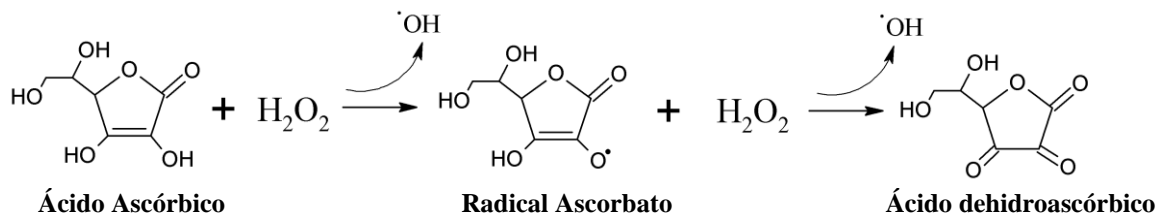
IV. MATERIALES Y MÉTODOS

De acuerdo con los objetivos del estudio, el presente trabajo de investigación se dividió en dos etapas: (1) Optimización del sistema de oxidación H_2O_2 /ácido ascórbico en la reacción de injerto de moléculas de ácido gálico (AG) en la cadena de inulina, mediante ensayos de resonancia paramagnética electrónica (EPR) y (2) Evaluación de la capacidad antioxidante y efecto prebiótico de inulina injertada con moléculas de ácido gálico.

4.1. Optimización del sistema de oxidación (H_2O_2 /AA) en la reacción de injerto de moléculas de ácido gálico (AG) en la cadena de inulina

4.1.1. Método de injerto Inulina-Acido Gálico

El proceso de injerto de AG en inulina, se llevó a cabo de acuerdo con la metodología propuesta por Spizzirri *et al.*, (2011) la cual se esquematiza brevemente en la Figura 6. La primera etapa de este proceso es la formación del sistema par de oxidación a través de la reacción entre el peróxido de hidrógeno (H_2O_2) y el ácido ascórbico (ver Figura 2):



Con la finalidad de conocer la formación de radicales libres en esta reacción, se llevaron a cabo estudios de EPR. Los espectros del radical ascorbato ($Asc^{\cdot-}$) en el sistema iniciador (H_2O_2 /AA) se obtuvieron de manera directa por EPR y la concentración de este radical fue medida como la altura del primer pico en el doblete del espectro resultante. Mediante un diseño factorial de superficie de respuesta Box-Behnken, se evaluó el efecto del H_2O_2 (5.88, 7.84 y 9.80 M), del Ácido Ascórbico (0.119, .213 y .307 M) y del tiempo (15, 30 y 45 min) sobre el incremento de la concentración del radical.

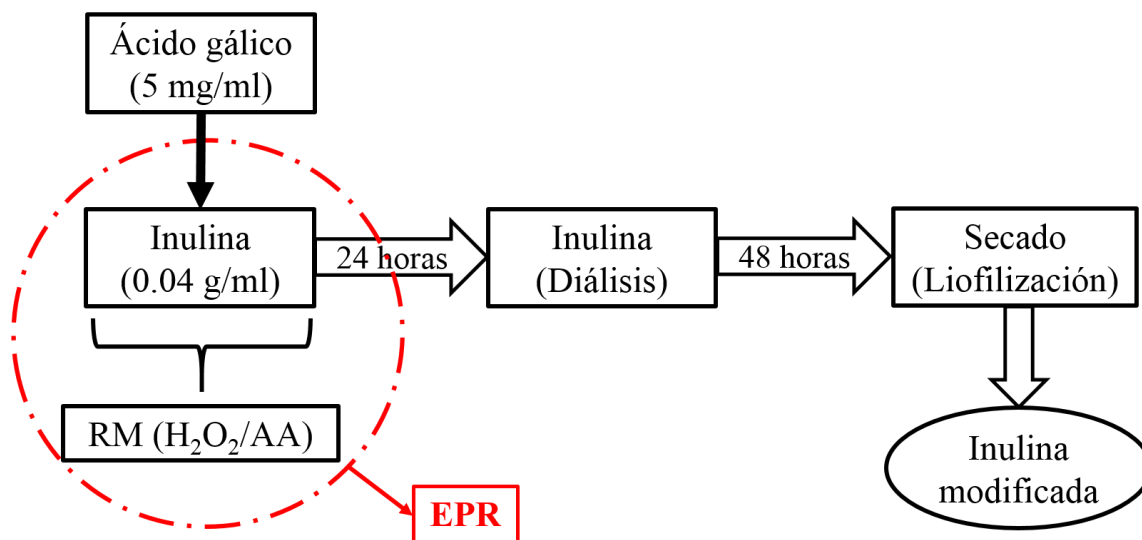


Figura 6. Método de injerto Inulina-Ácido Gálico

Los radicales formados en el primer paso sustraen un electrón de la molécula de inulina, dando lugar a la producción de un macro-radical al cual se unirá la molécula de ácido gálico. En este paso del proceso de injerto, se llevaron a cabo ensayos de EPR para evaluar el efecto de la relación molar (RM) H_2O_2/AA sobre la formación y tipo de radical libre formado por la reacción $H_2O_2/AA + Inulina$.

La obtención y manipulación de los espectros de EPR se realizaron con el programa ES-IPRIT/TE. Los espectros experimentales fueron comparados con espectros estándar para identificar el tipo de radical formado y determinar los valores de constante giromagnética (g) y constantes de acoplamiento hiperfino (A_h).

4.1.2. Caracterización de los conjugados inulina-ácido gálico

Para verificar el injerto de moléculas de ácido gálico en la cadena principal de inulina se efectuaron ensayos de espectroscopía infrarrojo de transformada de Fourier, difracción de rayos X en polvos y espectroscopía de luz ultravioleta (UV-vis). Todos los ensayos incluyeron a las muestras modificadas (injertos IAG-1 a 4) y las materias primas (inulina y ácido gálico).

4.1.3. FT-IR

La espectroscopía infrarrojo de transformada de Fourier (FTIR) se hizo en un equipo de infrarrojo marca Bruker, modelo tensor 27 equipado con un accesorio Platinum ATR. Los análisis se efectuaron en el Centro Conjunto de Investigación en Química Sustentable UAEM-UNAM.

4.1.4. Espectroscopía Ultravioleta-Visible

Los espectros de Ultravioleta-Visible (UV-Vis) se determinaron empleando un espectrofotómetro (Thermo Spectronic 10UV Scanning Modelo Genesys., NY, USA). Se disolvieron 5 mg de cada muestra de IAG-1 a 4 en 1 mL de agua destilada. El ácido gálico fue preparado a una concentración de 0.1 mg/ml en agua destilada y la línea de base correspondió a la inulina sin modificar y disuelta en agua. Las muestras se transfirieron a celdas de cuarzo y se efectuó un barrido de UV-vis desde 190 a 300 nm a una velocidad de paso de 2 nm/s. Los espectros de IAG-1 a 4 y del ácido gálico se graficaron para comparar su perfil e identificar los picos de mayor absorbancia.

4.1.5. Difracción de Rayos X en polvos

El estudio de difracción de rayos X se efectuó con un difractor de polvos Bruker D8 Advance con geometría Bragg-Bretano, descarga con ánodo de cobre ($K\alpha_1 = 0.154 \text{ \AA}$), y detector Linxeye. La dispersión de la radicación fue detectada con voltaje de 30 kV y corriente de 25 mA. Tiempo de paso de 15.9 segundos, en un rango 2θ de 5 a 60° . Las muestras secas y pulverizadas se colocaron en una celda plástica, suficiente cantidad para cubrir por completo el centro de la celda. Los espectros de los patrones de difracción de las muestras IAG-1 a 4, inulina y ácido gálico se recobraron y se compararon para evaluar el cambio en la cristalinidad.

4.2. Evaluación de la capacidad antioxidante y efecto prebiótico de inulina injertada con moléculas de ácido gálico

En esta etapa de la investigación se empleó inulina nativa que fue modificada de acuerdo a la metodología propuesta por Spizzirri *et al.*, (2011) y esquematizada en la Figura 6. Se

empleó una relación $\text{H}_2\text{O}_2/\text{AA}$ de 9 moles y una concentración de 80 mg/ml de inulina. El producto resultante fue caracterizado por UV-Vis y FT-IR.

Los métodos de análisis correspondientes a capacidad antioxidante y efecto prebiótico de las muestras estudiadas (inulina, inulina-ácido gálico, ácido gálico y glucosa) se describen a detalle en los artículos resultantes del presente estudio.

V. RESULTADOS

5.1. Electron paramagnetic resonance study of hydrogen peroxide/ascorbic acid ratio as initiator redox pair in the inulin-gallic acid molecular grafting reaction

Ms. Ref. No.: CARBPOL-D-15-01546R1

Title: Electron Paramagnetic Resonance Study of Hydrogen Peroxide/Ascorbic Acid Ratio as Initiator Redox Pair in the Inulin-Gallic Acid Molecular Grafting Reaction

Carbohydrate Polymers

Dear Dr. Aurelio Dominguez-Lopez,

I am pleased to confirm that your paper "Electron Paramagnetic Resonance Study of Hydrogen Peroxide/Ascorbic Acid Ratio as Initiator Redox Pair in the Inulin-Gallic Acid Molecular Grafting Reaction" has been accepted for publication in Carbohydrate Polymers.

Thank you for submitting your work to this journal.

With kind regards,

Manuel A Coimbra, PhD

Receiving Editor

Carbohydrate Polymers

Cita bibliográfica de este artículo:

Arizmendi-Cotero, D., Gómez-Espinosa, R.M., Dublán García, O., Gómez-Vidales, V. Dominguez-Lopez, A. (2016). Electron paramagnetic resonance study of hydrogen peroxide/ascorbic acid ratio as initiator redox pair in the inulin-gallic acid molecular grafting reaction. *Carbohydrate Polymers*, 136 (20):350-357.

<http://dx.doi.org/10.1016/j.carbpol.2015.09.037>

Electron Paramagnetic Resonance Study of Hydrogen Peroxide/Ascorbic Acid Ratio as Initiator Redox Pair in the Inulin-Gallic Acid Molecular Grafting Reaction

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Abstract

Gallic acid (GA) was grafted onto inulin using the free radicals method, generated by the hydrogen peroxide/ascorbic acid (H₂O₂/AA) redox pair. Molar ratios of H₂O₂/AA at 9, 20, 39 and 49 were evaluated by Electron Paramagnetic Resonance in order to find the effect of the oxidation of the inulin and the efficiency in the Inulin-gallic acid grafting (IGA). The highest concentration of the inulin macro-radical was obtained with H₂O₂/AA molar ratios of 20 and 49 with the removal of a hydrogen atom from a methyl group of the inulin fructose monomers. The highest grafting ratio (30.4 mg GA eq / g IGA) was obtained at 9 M of H₂O₂/AA. UV-Vis, FT-IR-ATR and XDR results confirmed a successful IGA grafting. The efficiency of the grafting reaction depends on the concentration of the macro-radical, it depends on the molar ratio of H₂O₂/AA, being affected by simultaneous reactions between components of the mixture (H₂O₂, AA, Inulin, GA and eventually atmospheric oxygen) as well.

Keywords

Inulin; Molecular Grafting; Phenolic compounds; Antioxidants; Electron Paramagnetic Resonance; Prebiotics.

1. Introduction

The inulin is a heterogeneous group of plant polysaccharides composed basically of fructosyl-fructose monomers linked by $\beta(2\rightarrow1)$ bonds with a reductive glucopyranose moiety at the end of the molecule. In some cases, such as Dahlia (*Dahlia coccinea* Cav.) or Chicory (*Cichorium intybus* L.) they present branches with $\beta(2\rightarrow6)$ linkages so they are also considered as levans. Besides, due to one or more of the fructosyl-fructose molecules they are also considered as fructans (Stevens, Meriggi, & Booten, 2001). The importance of the inulin lies in its contribution as a part of the dietary fiber and because it is considered as a prebiotic compound (Glenn R Gibson, Probert, Loo, Rastall, & Roberfroid, 2004b; Kolida & Gibson, 2007). Many other health issues related to these polysaccharides include the prevention of diabetes and cancer, the improvement of lipids' metabolism, the reduction of serum cholesterol, and the increase of calcium and magnesium absorption, among others (Lou *et al.*, 2009; Pejin *et al.*, 2013). Inulin-type fructans do not present antioxidant activity although these molecules could be combined, by grafting, with phenolic compounds in order to synthesize redox systems with a stress regulating capacity by decreasing oxygen reactive species (ROS) (Bolouri-Moghaddam, Le Roy, Xiang, Rolland, & Van den Ende, 2010; Hernandez-Marin & Martinez, 2012). In some cases inulin has been grafted in order to obtain flocculants for coal washery effluent treatments (Rahul, Jha, Sen, & Mishra, 2014).

Molecular grafting is a polymeric material modification method (Spizzirri, *et al.*, 2011; Spizzirri, *et al.*, 2009; Cirillo, *et al.*, 2010; Curcio, *et al.*, 2009) involving reactions requiring a reductive compound as initiator, for example, potassium persulfate, ceric ammonium nitrate and ammonium persulfate, that generate a free radical after heating to 40°C (Spizzirri, *et al.*, 2009). Nowadays there have been developed new techniques that allow the grafting of simple

molecules onto biopolymeric structures in a single step at room temperature without the production of toxic by-products. Kitagawa & Tokiwa (2006) evaluated the polymerization of sugar esters using ascorbic acid (AA) (10 mM) and hydrogen peroxide (H_2O_2) (7mM) as oxydizing agents. This method has also been succesfully applied by Curcio, *et al.* (2009), Spizzirri, *et al.* (2009), Cirillo, *et al.*, (2010) and Spizzirri, *et al.*, (2011) in chitosan, gelatin and Dahlia's inulin grafted conjugates.

The hydroxyl radical ($\bullet OH$) that initiates the reaction is formed when H_2O_2 oxidizes AA. In this step the ascorbate radical is also formed. After that, the grafting process of the molecule takes place in two steps. The first one consists in generating a macro-radical in the polysaccharide chain through the reaction with the free radical obtained between AA and H_2O_2 . In the second step, the antioxidant molecule links to the recently formed macro-radical through a covalent bond (Curcio *et al.*, 2009; Spizzirri *et al.*, 2010; Spizzirri *et al.*, 2009; Toti & Aminabhavi, 2004).

This grafting method has been evaluated using different H_2O_2/AA ratios and different biopolymers. Some grafted biopolymer-antioxidant conjugates of gelatin-gallic acid and gelatin-catechin using a molar ratio of 5.0/1.4 H_2O_2/AA as the initiator system produced a new polymer containing, respectively, 0.7 and 0.9 mg of antioxidant molecules per gram. Gelatin-phenolic compounds conjugates obtained showed a reduction of *Electrophorus electricus*' acetylcholinesterase and α -amylase enzymatic activity, being suitable to apply in Alzheimer disease and diabetes treatment (Cirillo *et al.*, 2010; Spizzirri *et al.*, 2009). Using a molar ratio of 1.0/0.307 of H_2O_2/AA , phenolic groups linked to chitosan-gallic acid, chitosan-catechin and inulin-catechin conjugates produced 0.7, 0.4 and 4.85 mg/g of dry polymer respectively being those new polymers suitable to be used in the pharmaceutical and

food industry as new packaging materials (Curcio *et al.*, 2009; Spizzirri *et al.*, 2011). Besides, the effect of the concentration of the phenolic acid used in the second step of the reaction has been evaluated by Cho, Kim, Ahn, & Je, (2011). These authors reported that the content of GA in chitosan-GA grafted conjugates was higher as the phenolic acid concentration was increased in the reaction mixture; although the efficiency of the linkage between the phenolic acid and the polymer was reduced. The optimal molar ratio to synthesize the grafting polymer was 0.1 of GA per chitosan's glucose monomer showing, at the same time, the ability to reduce cellular oxidative damage. Meanwhile, Liu, Lu, Kan, Tang, & Jin, (2013b) and Liu, Lu, Kan, Wen, & Jin (2014b) mixed 0.1 g of AA (0.1135 M) and 2 ml of H₂O₂ (5 M) in a reaction flask containing 0.8 g of a phenolic acid (gallic acid, catechin or ferulic acid) in 50 ml of an acetic acid solution (0.5%). The reaction took place under an inert atmosphere. Assays about antioxidant activity suggest that antioxidant properties of carboxymethyl-chitosan grafted conjugates decrease when molecules are grafted with gallic acid > catechin > ferulic acid. The inulin grafted with catechin fractions using the same method showed 128.3 mg catechin equivalents/g of dry polymer. This new polymer showed some anti-diabetic properties during *in vitro* assays to inhibit α -glucosidase activity (Liu, Lu, Kan, & Jin, 2013b).

Although the grafting efficiency has been observed to vary with the type of macromolecule to be grafted, to the antioxidant compound to be linked, and to the H₂O₂/AA molar ratio, the polymer oxidation process through the action of the H₂O₂/AA system has not been evaluated considering it as the first step in the grafting reaction. It is not clear until now which functional groups of the polymer are susceptible to become free radicals, explaining the efficiency of the molecular grafting, and the influence of H₂O₂/AA ratio in this oxidation

process. Thus, the aim of the present study was to describe the synthesis of the radical initiator in the grafting reaction through the reaction between H_2O_2 and AA, then, to evaluate the radicalization of the inulin with different $\text{H}_2\text{O}_2/\text{AA}$ molar ratios using Electron Paramagnetic Resonance (EPR). Finally, an additional objective was to characterize the structure of the resulting inulin-gallic acid conjugate verifying the presence of phenolic groups linked to the inulin biopolymer.

The EPR is a highly sensitive tool in the detection of molecules with unpaired electrons such as the free radicals. Although short lifetime free radicals, as ROS, hydroxyl radical, superoxide radical or carbon based radicals could only be detected by spin trap methods because of their high reactivity (Faure *et al.*, 2012). Spin traps are diamagnetic compounds that in the presence of ROS and carbon radicals react fast with them forming adducts which stability allows its detection. The 5,5-dimethyl-1-pyrroline N-oxide (DMPO) is the most used spin trap compound and has been employed in the detection of polysaccharides' free radicals formed through the oxidation of hydroxyl radical ($\bullet\text{OH}$) (Pejin *et al.*, 2013; Yamaguchi *et al.*, 1999).

2. Materials and methods

2.1. Materials

Chicory inulin with an average molecular weight (MW) 5000 and purity >99%; fructose (purity>99%), hydrogen peroxide (H_2O_2), ascorbic acid (AA), gallic acid (GA), Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA) and 5,5-di-methyl-1-pyrroline-1-oxide (DMPO) from Dojindo, (Rockville, MN, USA) to be used as the spin trap.

2.2. EPR Equipment

Electron Paramagnetic Resonance Spectroscopy (EPR) determinations were conducted in an EPR spectrometer (Jeol JES-TE300), operated at X-Band fashions at 100 KHz modulation frequency with a cylindrical cavity (TE₀₁₁ mode), individual samples were placed in a quartz flat cell (synthetic quartz, Wilmad Glass Company) with a path length of 0.2 mm. The external calibration of the magnetic field was conducted using a JEOL ES-FC5 precision gaussmeter. The acquisition and manipulation of spectra were performed using the ES-IPRIT/TE program. The settings used were as follows: microwave power 12 mW, magnetic field 3350 G \pm 5, microwave frequency 9.4396 GHz, modulation amplitude 2500 G, sweep time 64 s, accumulations 3. In order to characterize the grafted molecules, the following assays were conducted: Fourier Transformed Infrared Spectroscopy (FTIR) were obtained using a Perkin-Elmer ATR-FTIR. X Ray Diffraction patterns were collected by using a Bruker D8 Advance, Cu-k α at 1.5404 Å at 2 θ 5-60° at 30 kV and 20 mA. UV-Vis spectra were performed in a Genesys, 10_{UV} Scanning Spectrometer (Thermo Spectronic, NY, USA).

2.3. H₂O₂/AA oxidation system radical formation: EPR assay

Ascorbate radical (Asc^{•-}) EPR spectra from the H₂O₂/AA initiator redox pair were obtained directly (*i.e.* in the absence of the DMPO spin trap) and the concentration of the radical was measured as the peak height of the first peak in the doublet of the resulting spectra. The effect of H₂O₂ (5.88, 7.84 and 9.80 M), ascorbic acid (0.119, 0.213 and 0.307 M) and time (15, 30 and 45 min) on the increase of the radical's concentration was evaluated through a Box-Behnken response surface factorial design. Individual effect and interactions were evaluated through an ANOVA and a regression analysis.

2.4. Inulin and fructose oxidation *via* H₂O₂/AA: Radicals assays

Inulin and fructose (50 mg) were hydrated with 500 μ L of deionized water and then 400 μ L of DMPO (0.05mM) were added. After that, 100 μ L of 9:1, 20:1, 39:1 and 49:1 of H₂O₂/AA (molar ratios) were added as the oxidizing reagent. These molar ratios represent roughly, in the Box-Behnken design, the central part of the response surface (Table 1). After 1 min, the reaction mixture was transferred to a quartz cuvette (Synthetic quartz, Wilman Glass Company) to run the assay. Controls with DMPO where EPR analyzed in the absence of inulin and H₂O₂.

Table 1. Molar ratios of H₂O₂ per Ascorbic Acid in the initiator redox pair.

Sample	H ₂ O ₂ (M)	AA (M)	Molar Ratio (H ₂ O ₂ /AA)
IGA-1	5.88	0.68	9
IGA-2	3.92	0.20	20
IGA-3	7.84	0.20	39
IGA-4	5.88	0.12	49

2.5. Inulin-gallic Acid grafts synthesis

Inulin-gallic acid (IGA) conjugates synthesis using H₂O₂/AA as grafting initiator redox pair took place according to Spizzirri *et al.*, (2011) with some modifications. A sample of 0.04 g of inulin were dissolved in 10 mL of deionized water in a reaction flask. Subsequently, 0.05 g of gallic acid and 1 mL of the initiator redox pair was added according to concentrations shown in Table 1. Resulting polymers were named according to their molar ratio as follows: IGA-1, IGA-2, IGA-3 and IGA-4. Samples were incubated at 25 °C for 24 h with constant stirred. Inulin samples were dialyzed in distilled water using a 10000 Da membrane during

48 h in order to eliminate the not reacting gallic acid. All samples were lyophilized and kept for further analysis. Control Inulin was prepared in the same conditions but in the absence of gallic-acid.

2.6. Total phenolic compounds determination

Total phenolic equivalents were calculated using Folin-Ciocalteu reagent according to Spizzirri *et al.*, (2009) with some modifications. 5 mg of each modified polymer were dissolved in 1 mL distilled water. Subsequently, a 120 μ L aliquot and 47.5 μ L of Folin-Ciocalteu reagent were mixed in a 2.5 mL cuvette. After 15 min, 300 μ L of Na₂CO₃ (15%) and 1080 μ L deionized water were added, mixed and let rest for 2 h. Absorbance was measured at 760 nm and total phenolic equivalents in IGA conjugates were expressed as gallic acid equivalents.

2.7. Experimental method and statistical analysis

Statistical analyses were carried out with Statgraphics 4.1 (Manugistics Corp., Rockville, MD, USA). Molar ratio effect on the grafted efficiency was analyzed with ANOVA and multiple linear regressions. Analyses were done in triplicate.

3. Results and Discussion

3.1. Synthesis of radical from the redox pair H₂O₂/AA as initiator system

In the spectra obtained from the EPR analysis of the initiator redox pair it was observed the formation of a stable free radical revealed by a doublet with gyromagnetic constant (g) and hyperfine coupling constant A_H, equal to 2.0058 and 1.798 G, respectively. This signal has been assigned to the ascorbate radical (Asc^{•-}) (Yamaguchi *et al.*, 1999) and the concentration

of this radical has been calculated as the height of the first peak. Besides, such concentration has been considered as the dependent variable. The concentration of H₂O₂, AA and the reaction time were considered as independent variables in the ANOVA analysis. ANOVA's results and the estimated coefficients of the surface response model are shown in Table 2, where it can be seen that only AA and reaction time showed a significant effect.

Table 2. Regression coefficients and ANOVA for the ascorbate radical (Asc^{•-}) concentration in the H₂O₂/AA reaction.

Source	Sum of Squares	Df	Mean Square	F-Ratio	p-Value	Parameter Estimates
A: H ₂ O ₂	1849.00	1	1849.00	0.98	0.3607	-26.68
B: AA	13514.10	1	13514.10	7.16	0.0368	2176.61
C: Time	36005.10	1	36005.10	19.06	0.0047	-5.46
AA	488.28	1	488.28	0.26	0.6293	2.03
AB	1352.00	1	1352.00	0.72	0.4300	-70.56
AC	144.50	1	144.50	0.08	0.7914	0.14
BB	3140.28	1	3140.28	1.66	0.2447	-2242.25
BC	2278.13	1	2278.13	1.21	0.3142	-11.97
CC	1554.03	1	1554.03	0.82	0.3993	0.06
Blocks	3894.03	1	3894.03	2.06	0.2010	
Lack-of-fit	41488.60	15	2765.91	1.46	0.3337	
Pure error	11332.00	6	1888.67			
Total (corr.)	117040.00	31			Constant	260.24

Figure 1(A) shows the individual effects of the three factors and Figure 1(B) the joint effect of significant factors on the production of ascorbate radical at intermediate concentration of H₂O₂ (7.84 M). Ascorbate radical concentration increases as AA concentration increases in the reaction media at constant reaction time and at constant H₂O₂ concentration. This effect was not linear, an inflection point was observed at a given concentration, and after that, an

opposite tendency was observed probably due to the effect of H_2O_2 as the limiting reagent on ascorbate radical production. Besides, maintaining H_2O_2 and AA at constant concentration, ascorbate radical presence decreases linearly with the increase of the reaction time. Increasing the concentration of H_2O_2 generates a higher amount of $\bullet\text{OH}$ and $\text{Asc}^{\cdot-}$ radicals (Kitagawa & Tokiwa, 2006). Moreover, reaction time between H_2O_2 and AA affects the concentration of both radicals due to the simultaneous reaction between $\bullet\text{OH}$ radical and $\text{Asc}^{\cdot-}$ being able to degrade AA to Dehydroascorbic acid (Du, Cullen, & Buettner, 2012).

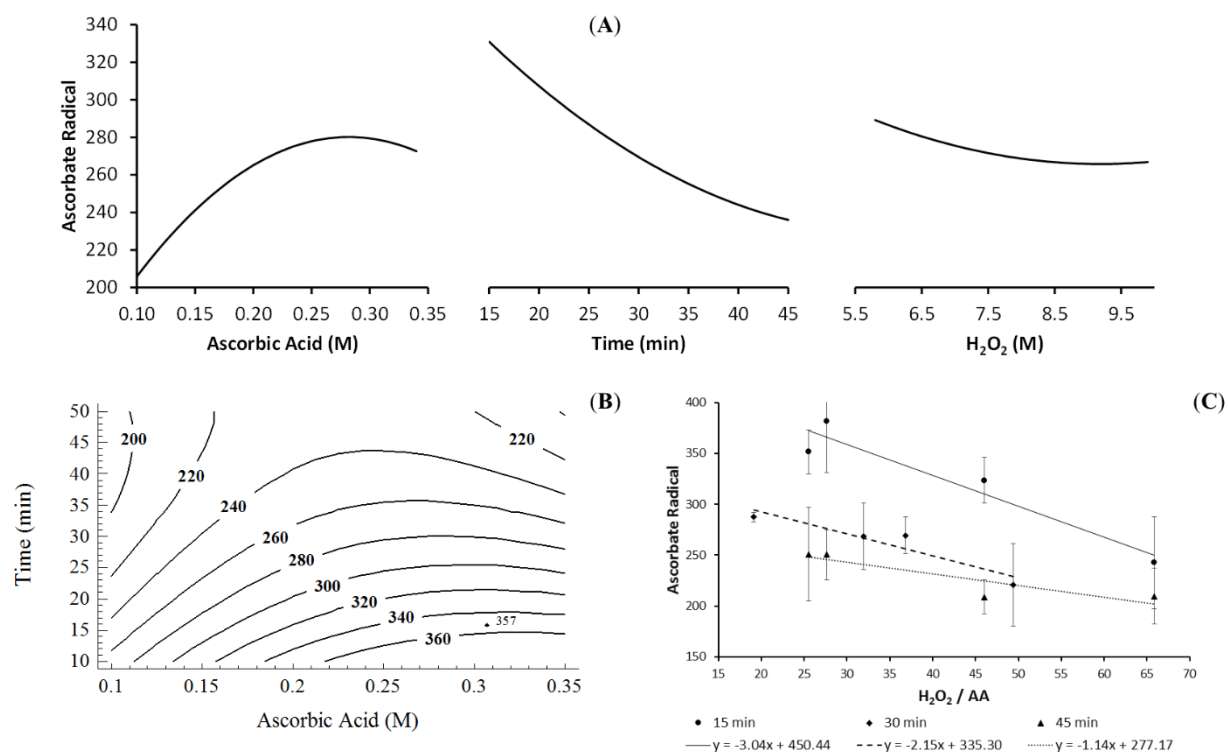


Figure 1. Individual effects (A), *Iso*-response curves estimated at a H_2O_2 concentration of 7.84 M (B) and effect of the molar ratio $\text{H}_2\text{O}_2/\text{AA}$ for the $\text{Asc}^{\cdot-}$ radical concentration formation (C).

Although H_2O_2 has not yielded a significant effect, this compound is critical to generate the redox pair, therefore it must be part of a specific molar ratio regardless of its absolute

concentration. Thus Figure 1(C) shows the effect of the molar ratio H_2O_2/AA on the same dependent variable at the three reaction times studied, where it can be seen that when this ratio increases the ascorbate radical decreases. At the same time, the radicals' loss gradient is significantly higher when measured at 15 min of the reaction time. At 45 min the radical amount is significantly lower than the other two times measured and the gradient loss is lower. This effect could be due to the conversion of the radical ascorbate into dehydroascorbic acid, as previously mentioned. It is possible that an optimal molar ratio could have as a consequence a better grafting capacity between the antioxidant molecules onto the macromolecule. Hence, it was observed the radical highest concentration point a peak of 388 units at the lowest reaction time, with the minimum concentration of H_2O_2 and the maximum concentration of AA, implying a MR of 19.15.

3.2. Inulin and fructose oxidation through the H_2O_2/AA redox pair

DMPO (0.05 mM) was used as the spin trap to evaluate the adduct radicals synthesized in the inulin and fructose oxidation reaction through the H_2O_2/AA redox pair. One minute after the reaction started, spectra have been recovered and their simulation has been taken place by means of the ES-IPRIT/TE software. In the spectra obtained (Figure 2) besides to identifying the formation of radical ascorbate ($Asc^{\cdot-}$), it has been detected the DMPO's adduct as a six-split with a $g = 2.0061$ and hyperfine coupling constants A_H and A_N equal to 23.05 G and 16.02 G, respectively. This signal has been assigned to the carbon radical-DMPO adduct agreeing with results obtained by Yamaguchi *et al.* (1999). The concentration of each radical has been measured considering the height of first peak. Control spectrum for AA + Inulin + DMPO, showed in Figure 2, produces a weak signal reflecting a spontaneous oxidation of the AA possibly caused by the environment (light, oxygen, air, etc.). Inulin does

not produce any signal since their monomers have not been radicalized. Moreover, spectrum for AA + H₂O₂+ DMPO shows a strong signal caused by the Asc^{•-}, which was generated by the H₂O₂.

Table 3 shows the results of each molar ratio evaluated. Inulin treated with a molar ratio of 20 and 49 revealed the highest concentration of the carbon radical-DMPO adduct, being of 300.7 and 497 units, respectively. This table also shows the radical ascorbate decreased as increased the MR as discussed above.

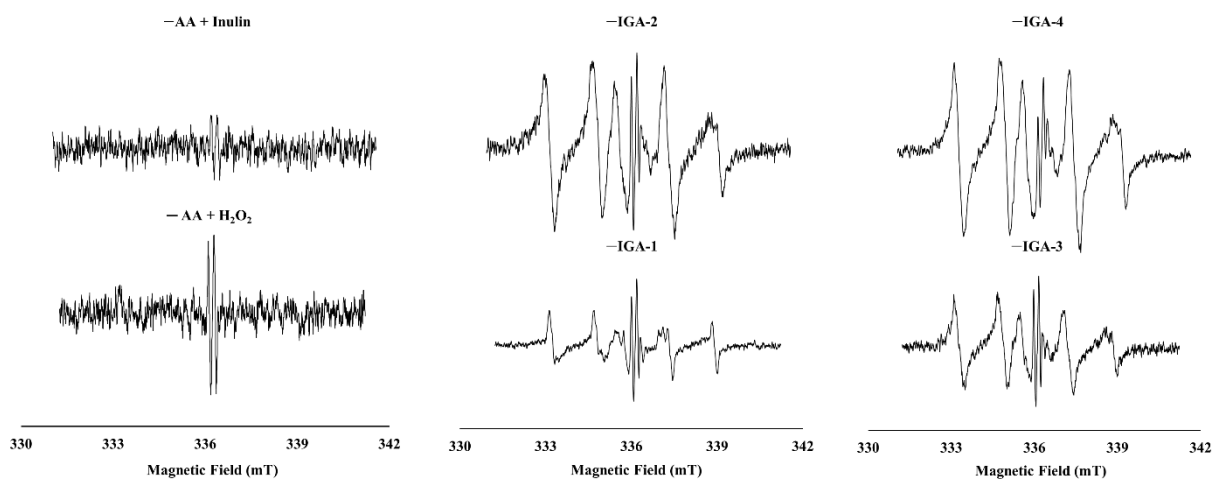


Figure 2. Experimental spectra of inulin-gallic acid (IGA) samples. EPR adducts DMPO-C ($A_H = 23.05$ G; $A_N = 16.02$ G; $g = 2.0061$) and Asc^{•-} ($A_H = 1.798$ G; $g = 2.0058$).

Table 3. Radical concentration (Intensity in arbitrary units) formed in inulin and fructose by the H₂O₂/AA oxidation.

MR	Inulin		Fructose	
	Asc ^{•-}	•C	Asc ^{•-}	•C
9	612.7	235.3	453.0	300.0
20	353.0	300.7	228.3	503.3
39	336.3	212.7	287.7	398.3
49	300.7	497.0	217.7	598.7

Inulin oxidation is the activation step for the grafting of the antioxidant molecule. The proposed reaction mechanism suggests that the oxidation components (•OH and Asc^{•-}) cause a homolytic rupture of a hydrogen atom (H) of α -methyl groups (CH₂) or from hydroxyl (OH) groups the inulin hydroxymethyl chain (Curcio *et al.*, 2009; Toti & Aminabhavi, 2004). Some low molecular weight carbohydrates, like 1-kestose, 6-kestose and nystose, have demonstrated their capacity to donate electrons when reacting with •OH.

The reaction between •OH and carbohydrates takes place when hydrogen atoms are extracted from the C3 or C5 positions in the fructose and C3 or C6 positions in the 6-kestose, which are the highest Gibbs' energy of C-H links (Hernandez-Marin & Martinez, 2012; Pejin *et al.*, 2013). It has also been reported free radical formation over carbon atoms between 1-kestose and nystose with •OH formed through the Fenton reaction (Pejin, *et al.*, 2013). According to these reports, it is possible that the macro-radical in the inulin chain is formed by the subtraction of a hydrogen atom from a methyl group of inulin monomers and their concentration depends on the molar ratio between H₂O₂ and AA. Gallic acid molecule could be inserted in those sites by means of covalent bonds giving rise to the new polymer. A proposed mechanism has been illustrated in Figure 3.

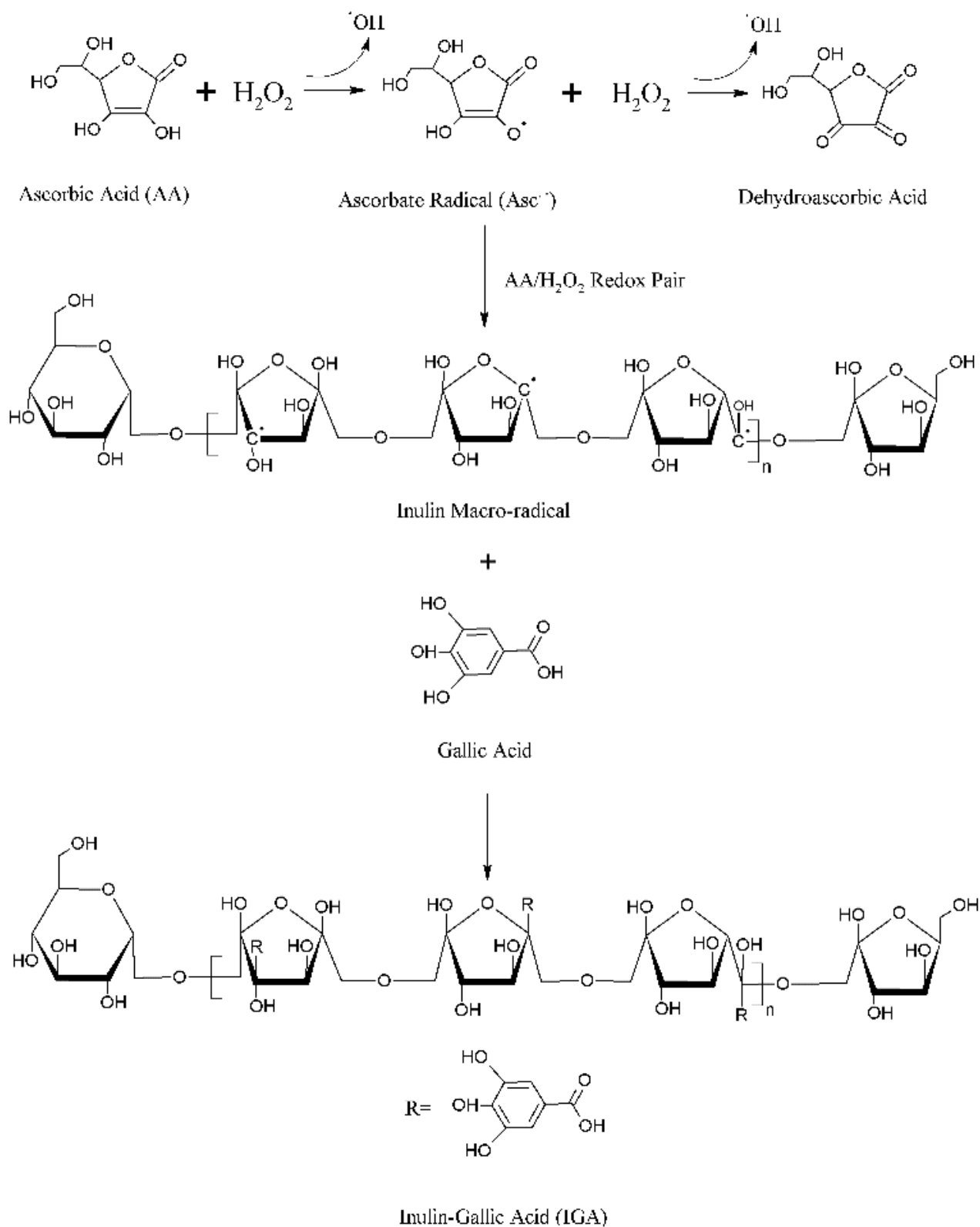


Figure 3. Proposed mechanism of grafted inulin-gallic acid.

The redox pair studied has also been evaluated in fructose to corroborate the formation of the carbon radical-DMPO adduct on the inulin's monomer. Figure 4 shows the experimental spectra of inulin and fructose treated with a RM equal to 49. Both inulin and fructose, the radical formation by the oxidation reaction takes place on the carbon atom and the molar ratio effect is similar in both carbohydrates. Hernandez-Marin & Martinez, (2012) point out that fructo-oligosaccharides (FOS) present their lowest dissociation energy in C-H bonds of C3, C5 and C6 positions. Those positions seem to be responsible of losing a hydrogen atom giving rise to the formation of inulin's macro-radical, as observed in the present study.

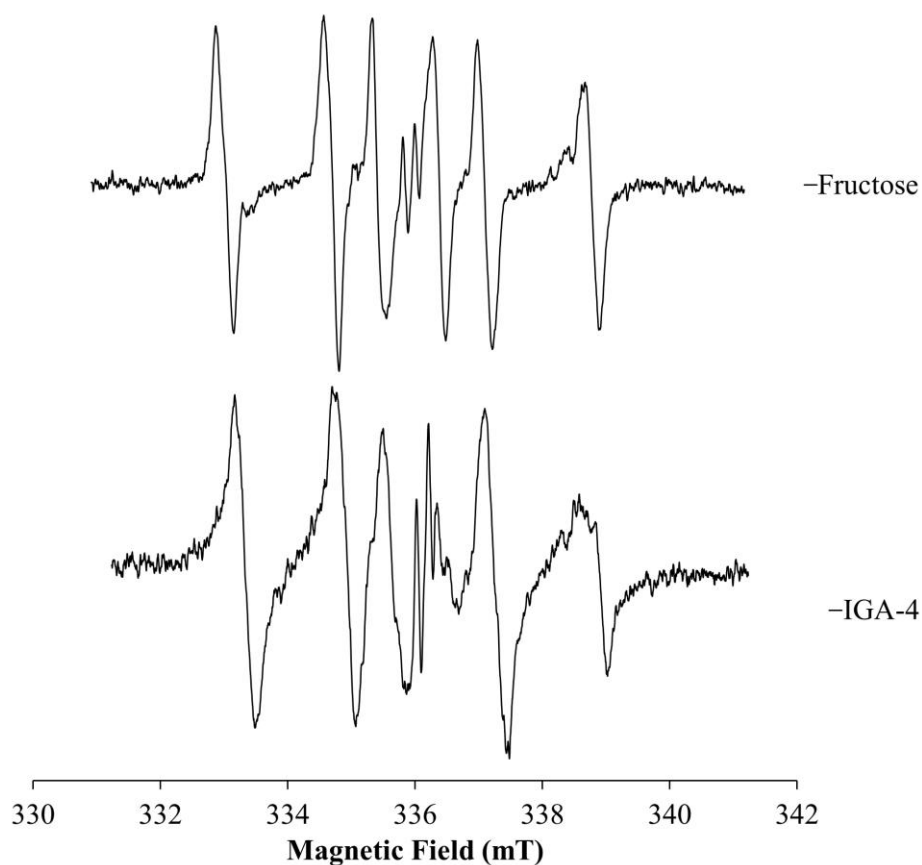


Figure 4. Adduct radical DMPO•C generated by the initiator redox pair, H_2O_2/AA , in fructose and inulin ($A_H = 23.05$ G; $A_N = 16.02$ G; $g = de 2.0061$).

3.3. Inulin-gallic acid conjugates characterization

3.3.1. UV-Vis spectra

Gallic acid spectrum shows two peaks in the aromatic region (212 and 262 nm) (Liu *et al.*, 2013a). Baseline corresponds to a non-modified inulin previously dissolved in water. Inulin-gallic acid polymers spectra show absorption bands in 213 and 263-269 nm, related to the covalent bond between gallic acid and polymer (Cirillo *et al.*, 2010; Spizzirri *et al.*, 2009). The peaks within the intervals observed towards the red wavelength could be attributed to the low energy of $n-\pi^*$ and $\pi-\pi^*$ on the covalent bond of the GA in the inulin molecule (Liu *et al.*, 2013a) (Figure 5).

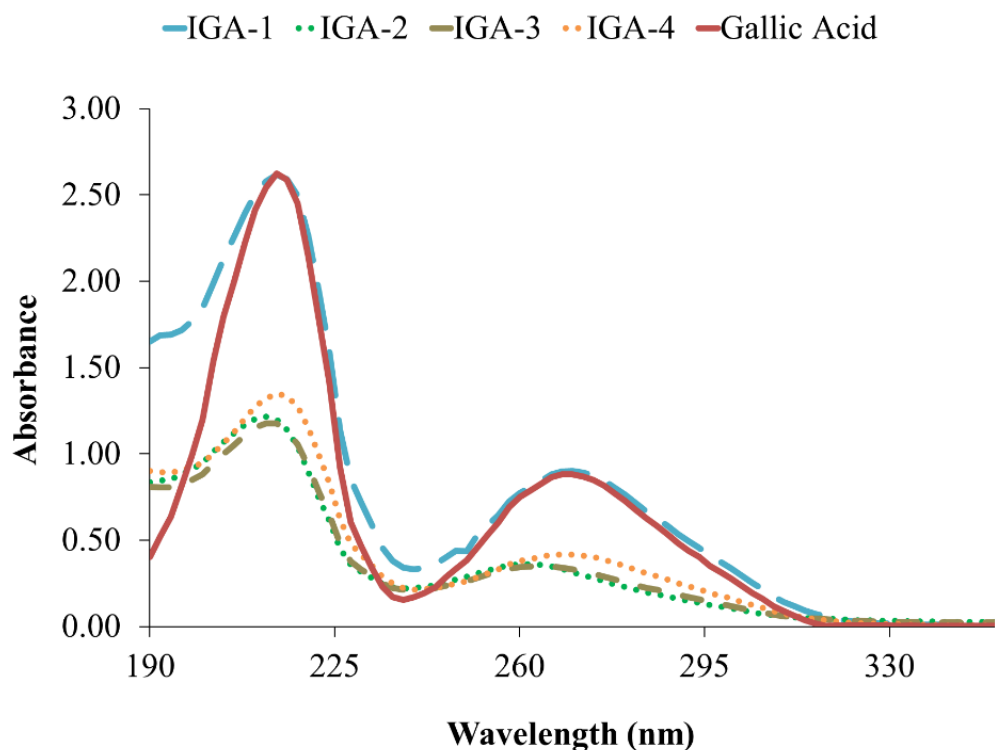


Figure 5. UV-Vis spectra of gallic acid and inulin-gallic acid grafts (IGA).

3.3.1. FT-IR spectra

FT-IR analysis was used to characterize the Inulin-gallic acid conjugates and the control at different molar ratios (Figure 6). Inulin spectrum shows typical carbohydrates bands at 900 and 1200 cm^{-1} related to stretching vibrations of C-C and C-O bonds. Besides, it shows at 2840 and 2975 cm^{-1} bands related to stretching vibrations of C-H and CH_2 and at 1605 and 1650 cm^{-1} the stretching vibrations of C=C of the alkenes. Rahul *et al.* (2014) found similar values in inulin samples. GA shows bands at 1205 and 1265 cm^{-1} related to the stretching vibrations of C-O-C and at 1425 and 1475 cm^{-1} related to the torsion of C-H of CH_2/CH_3 groups. Moreover, it shows a signal at 1750 and 1730 cm^{-1} related to the stretching of C=O of the carbonyl group. Gallic acid-polymer conjugates show new bands at 1800 and 1000 cm^{-1} . The signal at 1740 cm^{-1} corresponds to the C=O vibration of the carbonyl group in ketones. This supports the hypothesis that the covalent bond between the GA and the inulin is carbon-centered. It is important to notice that those new peaks are not visible in the control polymer, suggesting that the inulin has been efficiently grafted. Similar results have been reported by Liu *et al.*, (2014b), Liu *et al.*, (2013b), Cirillo *et al.*, (2010), and Spizzirri *et al.*, (2010).

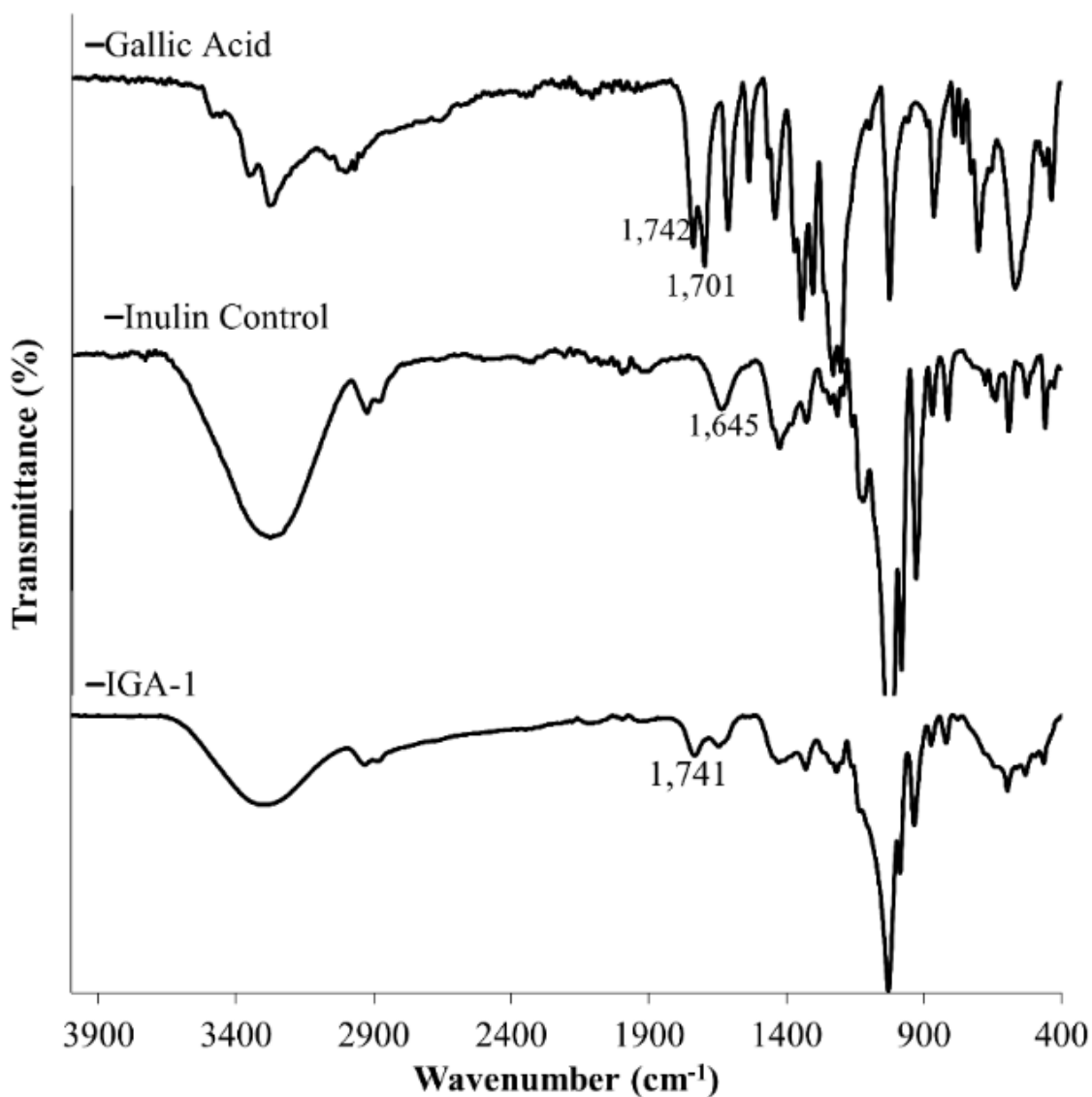


Figure 6. FT-IR spectra for gallic acid, pure inulin and inulin-gallic acid graft (IGA-1).

3.3.3. Powder X-ray diffraction (XRD)

X-ray profile was performed in order to evaluate the change in crystallinity of the modified inulin samples. Figure 7 shows the diffractogram for gallic acid, inulin and inulin-gallic acid conjugates. Gallic acid diffraction profile exhibits well defined peaks in the region between $8^{\circ} < 2\theta < 52^{\circ}$ showing its crystalline nature. Pure inulin shows well defined peaks between $8^{\circ} < 2\theta < 26^{\circ}$, typical of a semi-crystalline molecule. The presence of gallic acid in the inulin

chain and the grafting cause the change in crystallinity of the modified polymers. This behavior was reported by Liu *et al.*, (2013a) who observed changes in the polymer crystallization when modifying the inulin chain with catechin. The loss of crystallinity in the modified polymers suggests that the molecular graft was taken randomly giving rise to a molecule different to the initial material.

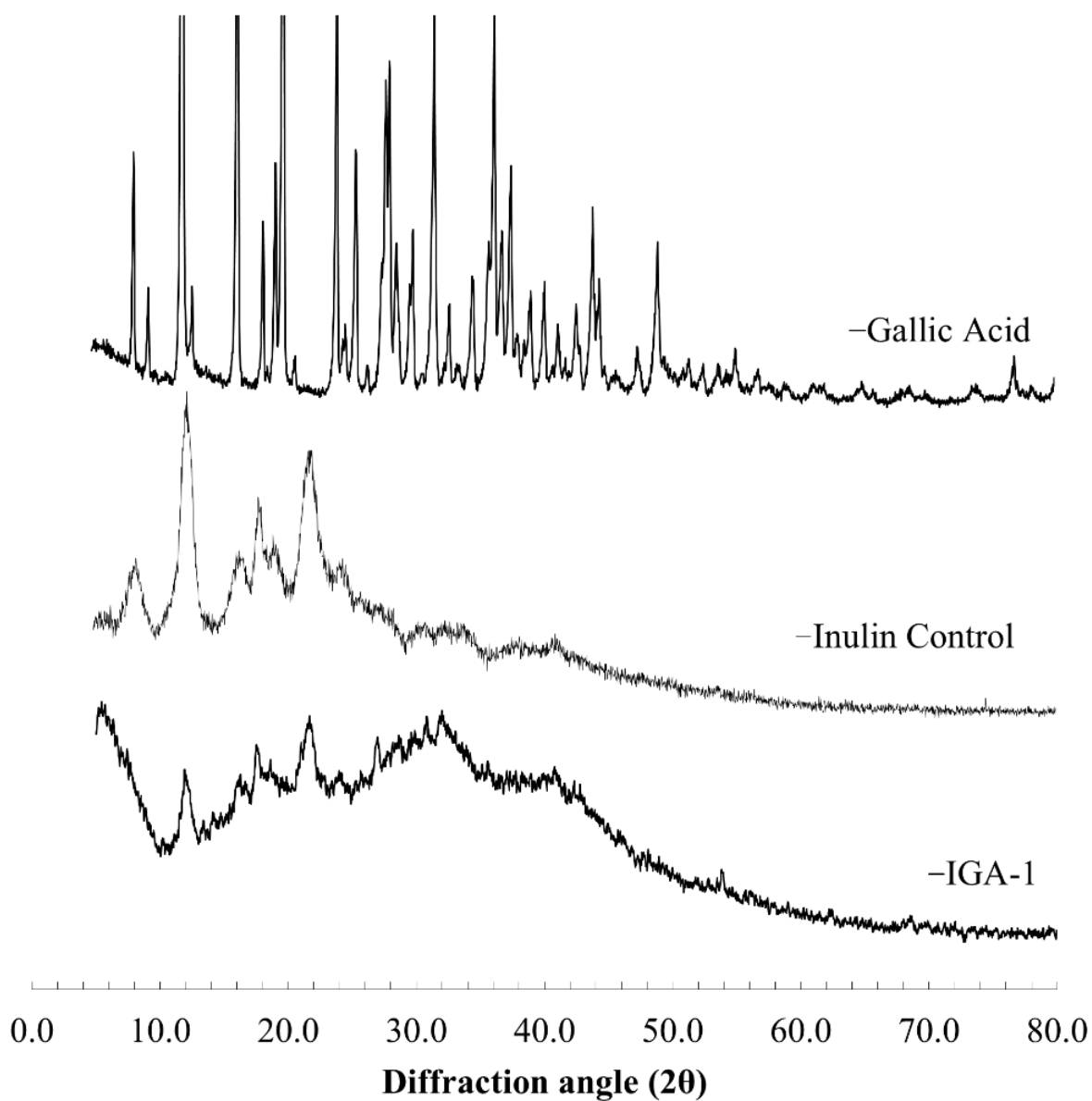


Figure 7. X-Ray Diffraction analysis for gallic acid, inulin and inulin-gallic acid grafts (IGA-1).

3.4. Determination of phenolic compounds in inulin-gallic acid conjugates

Phenolic compounds in inulin-GA samples are reported as mg of gallic acid equivalents (GAE) per g of polymer (Table 4). The best grafting efficiency (30.4 mg GAE/g inulin-GA) was obtained with a molar ratio (H_2O_2/AA) of 9:1. Remaining molar ratios with an average of 16.3 mg GAE/g inulin-GA did not show significant differences between them. Curcio *et al.*, (2009) obtained 7mg GAE/g chitosan-GA, our results are higher using the same method. Besides, Liu *et al.*, (2013a) reported a minimum grafting ratio of 27.4 mg/g chitosan-GA using H_2O_2/AA as initiator under inert atmosphere. The phenolic groups content could be higher due to inert atmosphere, being able to avoid oxidation reactions on the substrate (Liu *et al.*, 2013b).

Table 4. Average content of phenolic compounds in inulin-gallic acid (IGA) samples.

Sample	MR	mg/g	mmol
IGA-1	9	30.36 ± 1.10	0.807 ± 0.029
IGA-2	20	14.62 ± 1.19	0.389 ± 0.032
IGA-3	39	16.22 ± 0.82	0.431 ± 0.022
IGA-4	49	18.05 ± 0.45	0.480 ± 0.012

Phenolic compounds in the grafted polymers depends on the polymer type, antioxidant concentration, reaction time and the inulin radicalization process. Gallic acid has been successfully grafted in gelatin and chitosan, the addition of gallic acid in the same molar ratio of chitosan monomeric units resulted in a higher concentration of gallic acid in the modified polymers (118.92 mg GAE/g dry polymer), nevertheless grafting efficiency was lower (11.9%) (Spizzirri *et al.*, 2009). Cho *et al.*, (2011).

Liu *et al.*, (2013a) reported optimal grafting conditions an input of 16 g/L of gallic acid into 5 g/L of chitosan with a molar ratio of 17.7:1 (H_2O_2/AA). Inulin has been modified through

grafting with catechin using different molar ratios of H₂O₂/AA, varying catechin concentration and reaction time (Liu *et al.*, 2014a; Spizzirri *et al.*, 2010; U.G. Spizzirri *et al.*, 2011). Reports indicate that inulin exhibits antioxidant properties, but none explained the relationship between the redox pair with the radicalization process and with inulin grafting.

Increase in the molar ratio (H₂O₂/AA) helps inulin to form more radicals, making it more susceptible to bond to more GA molecules (Table 3). However, results shown in Table 4 are in contradiction to this assumption. The radical formation increase in the inulin chain does not guarantee that gallic acid could bind to it. This effect could be explained, because the increase of radical species in the reaction mixture could generate simultaneous reactions that deactivate •OH, Asc^{•-} and •CH₂ radicals, due to the presence of electron donor compounds like AA, GA, H₂O₂ or atmospheric oxygen as well.

4. Conclusions

Results reported in this work demonstrate that the concentration of inulin radicals formed by the H₂O₂/AA system is related to the molar ratio, but the efficiency is higher when the molar ratio decreased. The EPR study showed that the macro-radical in the inulin chain is formed on a carbon atom and their concentration depends on the molar ratio between H₂O₂ and AA. The UV-Vis, FT-IR and X-ray analysis of the grafted compounds obtained, indicate that covalent bond between gallic acid and inulin occurred on a methyl group of the fructose monomer. Reaction efficiency is related to the concentration of hydrogen peroxide and ascorbic acid in the redox pair. A simultaneous reaction between all components (H₂O₂, AA, GA, inulin and possibly atmospheric O₂) could participate of inulin-gallic acid grafting efficiency.

Acknowledgements

The authors wish to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT-México) for the scholarship granted to Daniel Arizmendi-Cotero, the Centro Conjunto de Investigación en Química Sustentable UAEM-UNAM, Alejandra Nuñez Pineda and Lizbeth Triana Cruz for their assistance in the laboratory of X-Ray Diffraction analysis and in the characterization and analysis of the IR spectra.

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Table and figure captions

Table 1. Molar ratios of H₂O₂ per Ascorbic Acid in the initiator redox pair.

Table 2. Regression coefficients and ANOVA for the ascorbate radical (Asc^{•-}) concentration in the H₂O₂/AA reaction.

Table 3. Radical concentration (Intensity in arbitrary units) formed in inulin and fructose by the H₂O₂/AA oxidation.

Table 4. Average content of phenolic compounds in inulin-gallic acid (IGA) samples.

Figure 1. Individual effects (A), *Iso*-response curves estimated at a H₂O₂ concentration of 7.84 M (B) and effect of the molar ratio H₂O₂/AA for the Asc^{•-} radical concentration formation (C).

Figure 2. Experimental spectra of inulin-gallic acid (IGA) samples. EPR adducts DMPO-C (A_H = 23.05 G; A_N = 16.02 G; g = de 2.0061) and Asc^{•-} (A_H = 1.798 G; g = 2.0058).

Figure 3. Proposed mechanism of grafted inulin-gallic acid.

Figure 4. Adduct radical DMPO-•C generated by the initiator redox pair, H₂O₂/AA, in fructose and inulin (A_H = 23.05 G; A_N = 16.02 G; g = de 2.0061).

Figure 5. UV-Vis spectra of gallic acid and inulin-gallic acid grafts (IGA).

Figure 6. FT-IR spectra for gallic acid, pure inulin and inulin-gallic acid graft (IGA-1).

Figure 7. X-Ray Diffraction analysis for gallic acid, inulin and inulin-gallic acid grafts (IGA-1).

5.2. Radical scavenging activity of an inulin-gallic acid graft and its prebiotic effect on *Lactobacillus acidophilus* In vitro growth

Full Length Article

Dear Dr. Aurelio Dominguez-Lopez,

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Radical scavenging activity of an inulin-gallic acid graft and its prebiotic effect on

***Lactobacillus acidophilus* In vitro growth**

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Abstract

In order to generate a new antioxidant and prebiotic dietary fibre, gallic acid (GA) was grafted onto native inulin. Inulin-gallic acid (IGA) graft was confirmed by UV and infrared spectroscopy (FT-IR). The antioxidant activity was evaluated by spectroscopic methods and the prebiotic activity of IGA was determined by *In-Vitro* growth of *Lactobacillus acidophilus*. UV spectra show absorbance peaks at 214 and 266-268 nm showing aromatic ring presence in the IGA graft and FT-IR spectra showed a band at 1743 cm^{-1} , confirming the covalent bond between the polymer and GA. GA provides a significant antioxidant capacity to IGA graft. Inulin shows a significant capacity to stimulate the growth of *L. acidophilus* and GA grafted onto inulin (16.3 mg/g polymer) does not interfere with its prebiotic capacity. It is possible to provide radical-scavenging capacity to fructo-oligosaccharides avoiding the decrease of its prebiotic properties, which could extend their potential use as functional foods.

Keywords

Inulin; Molecular grafting, Prebiotics; Radical scavenging activity.

1. Introduction

Dietary fibre contained in some vegetable food products can be associated with phenolic compounds and therefore exhibits a significant antioxidant activity (Saura-Calixto, Pérez-Jiménez, & Goñi, 2009). This kind of fibre is known as antioxidant dietary fibre (ADF) and because of its polymer structure it could show a considerable prebiotic capacity as well. Some studies have suggested that polyphenols associated with this dietary fibre could be released in the gastrointestinal tract during digestion (Mercado-Mercado *et al.*, 2015). Hence, besides its prebiotic activity ADF could increase the radical scavenging activity of the human gut environment.

Recent research has been directed to the synthesis of ADF through the molecular grafting of polymeric materials such as phenolic derivatives onto several biopolymers. Molecular grafting is a polymeric material modification method involving reactions that require a reductive compound as initiator generating a free radical, par example ascorbic acid /hydrogen peroxide (AA/H₂O₂) redox pair (Cirillo *et al.*, 2010; Curcio *et al.*, 2009; Spizzirri *et al.*, 2009, 2011). The hydroxyl radical (\bullet OH) that initiates the reaction is formed when H₂O₂ oxidizes AA. After that, the grafting process of the molecule takes place in two steps. The first one consists in generating a macro-radical in the biopolymer chain through the reaction with the free radical obtained between AA and H₂O₂. In the second step, the antioxidant molecule links to the recently formed macro-radical through a covalent bond (Curcio *et al.*, 2009; Spizzirri *et al.*, 2009, 2010; Toti & Aminabhavi, 2004). In the last years several phenolic compounds-biopolymers grafted conjugates with diverse food applications related to their antioxidant activity have been studied: catechin-alginate and catechin-inulin (Spizzirri, *et al.*, 2010); gallic acid-chitosans (Cho, Kim, Ahn, & Je, 2011; Woranuch,

Yoksan, & Akashi, 2015), gallic acid and catechin-gelatin (Cirillo, *et al.* 2010; Spizzirri, *et al.*, 2009), etc. Since some of these biopolymers, prior to being grafted, have significant prebiotic activity, the novel conjugates could have a potential use as prebiotic and antioxidant compounds (Arizmendi-Cotero, Gómez-Espinosa, Dublán-García, Gómez-Vidales, & Dominguez-Lopez, 2016).

According to Saura-Calixto, (2011) around 50% of the total dietary antioxidants, mainly phenolics pass through the small intestine linked to dietary fibre. They are released from the fibre matrix in the colon by the action of the bacterial microbiota, producing metabolites and an antioxidant environment. Consequently the transportation of dietary antioxidants through the gastrointestinal tract may be an essential function of dietary fibre. However, in food (particularly vegetables), both dietary fibre and antioxidant compounds are a complex group of substances with a wide range of molecular mass. The inulin-gallic acid conjugate synthesized by Arizmendi-Cotero *et al.* (2016) or those obtained by others (Liu, Wen, Lu, Kan, & Jin, 2014; Zhang, Geng, Jiang, Li, & Huang, 2015) could be a suitable and relatively simple model to study the antioxidant and prebiotic effect of dietary fibre naturally associated with polyphenols. Thus, the goals of this study were (i) to measure the antioxidant activity of an inulin-gallic acid conjugate obtained by molecular grafting and (ii) to evaluate the conjugate's *in vitro* prebiotic capacity on a *Lactobacillus acidophilus* culture.

2. Materials and methods

2.1. Materials

Native inulin (inulin) was purchased from Frutafit (IQ VA Mexico S.A. C.V.); Hydrogen peroxide (H₂O₂), ascorbic acid (AA), gallic acid (GA), pyrogallol, Folin-Ciocalteu's

reagent, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2,6,6-tetra-methylpiperidine (TEMP, 99%), 2,2,6,6-tetramethylpiperidine-1-oxil (TEMPO, 99%) and hematoporphyrin (HP, 50%) were purchased from Sigma-Aldrich (Sigma Chemical Co., St. Louis, MO, USA); 5,5-dimethyl-1-pyrroline-1-oxide (DMPO, ultra-high purity) was purchased from Dojindo; 2-tiobarbituric acid (TBA, 99%) from ICN Biomedical, Inc. (Ohio); trichloro acetic acid (TCA, 99%) from Fulka; iron (II) sulfate heptahydrate (99%), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA, 98.5%) from Sigma Chemical Co.; ethyl alcohol absolute (99.95%) from J.T. Baker; and deionized water.

2.2. Equipment

Electron Paramagnetic Resonance Spectroscopy (EPR) determinations were conducted in an EPR spectrometer (Jeol JES-TE300), operated at X-Band fashions at 100 kHz modulation frequency with a cylindrical cavity (TE011 mode). Individual samples were placed in a quartz flat cell (synthetic quartz, Wilmad Glass Company) with a path length of 0.2 mm. The external calibration of the magnetic field was conducted using a JEOL ES-FC5 precision gauss-meter. The acquisition and manipulation of spectra were performed using the ES-IPRIT/TE program. In order to characterize the grafted molecules, the following assays were conducted: Fourier Transformed Infrared Spectroscopy (FT-IR) which were obtained using a Perkin-Elmer ATR-FT-IR; UV-Vis spectra which were performed in a Genesys, 10 UV Scanning Spectrometer (Thermo Spectronic, NY, USA).

2.3. Inulin-gallic acid grafts' synthesis

Inulin-gallic acid (IGA) conjugates' synthesis, using H_2O_2/AA as the grafting initiator redox pair, took place according to Arizmendi-Cotero *et al.* (2016) with some modifications. A

sample of 0.8 g of inulin was dissolved in 10 mL of deionized water in a reaction flask. Subsequently, 0.05 g of gallic acid and 1 mL of the initiator redox pair (5.88 M H₂O₂/0.68 M AA) was added. Samples were incubated at 25° C for 24 h with constant stirring. Inulin samples were dialyzed in distilled water using a 3500 Da membrane during 48 h in order to eliminate the non-reacting gallic acid. IGA conjugates were lyophilized and kept in the freezer for further analysis. Inulin control sample was prepared in the same conditions but in the absence of gallic-acid. The three samples (IGA, Inulin and GA) were characterized via UV-Vis and FTIR to make sure that gallic acid-inulin grafting was successful.

2.4. Antioxidant activity of inulin-gallic acid grafts

2.4.1. DPPH radical-scavenging assay by EPR spin-trap

DPPH radical scavenging activity was measured using the method described by Gómez-Vidales, Granados-Oliveros, Nieto-Camacho, Reyes-Solís, & Jiménez-Estrada (2014). In all cases, the liquid medium used was a mixture of ethanol-water (85:15 v/v). 200 µL of Inulin and IGA graft solutions at a concentration of 18, 31, 56, 100, 178 and 317 µg/mL and GA solutions at 0.10, 0.18, 0.31, 0.56, 1.00, 1.78, 3.16, 5.62 µg/mL were mixed with 100 µL DPPH solution (DPPH 0.3 mM,). Each mixture was held at 37°C for 15 min before collecting EPR spectra. Individual samples were placed in the quartz flat cell of the EPR-spectrometer. Measurement conditions were as follows: central field, 3550 ± 40 G; modulation frequency, 100 kHz; modulation amplitude, 2500 G; microwave power, 8 mW and sweep time 2 min. Relative percentage of DPPH scavenging capacity was calculated according to the following equation where H_c and H₀ are the middle peak's heights (DPPH spectrum) with and without antioxidants, respectively:

$$\text{Scavenging capacity} = (H_0 - H_c) / H_0 * 100 \quad (1)$$

2.4.2. $^1\text{O}_2$ formation and scavenging capacity by EPR spin-trap

Singlet oxygen's ($^1\text{O}_2$) measurement was performed in order to determine the role of IGA graft and GA as scavengers. The method, reported by Gómez-Vidales *et al.* (2014) is briefly described as follows: the $^1\text{O}_2$ oxygen intermediate was generated in presence of hematoporphyrin (a photosensitizer) under visible light irradiation: a sample of hematoporphyrin (HP) in an air-equilibrated ethanol-water (85:15 v/v) solution (10.7 mM) and in the presence of TEMP (30 mM) was irradiated for up to 10 min with $\lambda > 400$ nm, generating a TEMPO signal in the EPR spectrometer. Immediately, samples of GA and IGA (10, 100 and 317 $\mu\text{g/mL}$) in an air-equilibrated ethanol-water (85:15 v/v) solution with an amount of TEMP (30 mM) were irradiated for up to 20 min with UV-Vis light ($\lambda > 400$ nm), generating a TEMPO signal that indicated the photo-production of $^1\text{O}_2$. To determine the inhibition of $^1\text{O}_2$, the drop of the peak- height central peak of TEMPO was measured. EPR parameters were as follows: center field, 3345 ± 40 G; microwave frequency, 9.43 GHz; modulation width, 7.9 ± 1 G; time constant, 0.1 s; amplitude, 200. In each case, EPR parameters were held constant, as it also was the concentration of TEMP; samples were irradiated directly within the EPR cavity.

2.4.3. Inhibition effect of Lipid peroxidation

The lipid peroxidation (LP) inhibition effect was determined by the method described by Granados-Oliveros *et al.* (2013). Using an ice bath, 375 μL of the protein solution (2.66 mg protein/mL) and 50 μL of 20 μM EDTA were mixed in 1.5 mL microtubes. Then, 25 μL of GA, IGA and Inulin at adjusted concentrations to obtain final concentrations of 1, 10 and 100

$\mu\text{g/mL}$ were added to the microtubes. Samples were incubated during 30 min at 37°C , after that, $50\ \mu\text{L}$ of $100\ \mu\text{M}$ FeSO_4 were added and incubated at the same temperature for 60 min more. Control experiments to test the induction of LP were conducted in the presence of (i) protein solution ($2.66\ \text{mg protein/mL}$) and (ii) FeSO_4 ($10\ \mu\text{M}$). In order to measure lipid peroxidation, $0.5\ \text{mL}$ of TBA reagent ($1\% \text{ w/v}$ TBA in $0.05\ \text{N}$ NaOH mixed with $30\% \text{ w/v}$ TCA in a 1:1 proportion) was added to each microtube. The tubes were cooled on ice for 10 min, centrifuged at $13000\ \text{g}$ for 5 min, and finally heated at $95\ ^\circ\text{C}$ for 30 min. The tubes were allowed to reach ambient temperature and finally $200\ \mu\text{L}$ of the supernatant solution were separated for analysis. The content of TBARS in all solutions was determined by optical density at $\lambda = 540\ \text{nm}$ using a Bio-Tek ELx 808 microplate reader.

2.5. *In vitro* prebiotic effect of the IGA conjugates

In order to determine the prebiotic effect of the IGA conjugates, MRS carbohydrate-free broth was used, according to Adebola, Corcoran, & Morgan (2014). Briefly, the media contained the following components (g/L): peptone (10.0), yeast (5.0), Na-acetate.3H₂O (5.0), K₂HPO₄.3H₂O (2.0), (NH₄)₃C₆H₅O₇.2H₂O (2.0), MgSO₄.7H₂O (0.2), MnSO₄.4H₂O (0.05) and Tween 80 (1 mL). The pH was adjusted to 6.2, and the medium was sterilized at $121\ ^\circ\text{C}$ for 15 min. Glucose (MRS-G), native inulin (MRS-Inulin) and IGA (MRS-IGA) were added up to final concentrations of 1% each. This concentration is recommended as the minimum to ensure the stimulating effect of a carbohydrate on the growth of bacteria on a basal MRS medium (Rubel, Pérez, Genovese, & Manrique, 2014). A negative control deprived of a carbon source was included as well.

Lactobacillus acidophilus (LA) were incubated in MRS broth for 24 h and these cultures were used as starters in subsequent fermentations, according to the methodology proposed by Corral, Valdivieso-Ugarte, Ferna, Adrio, & Velasco, (2008). Cells were collected by centrifugation, and suspended in sterile distilled water. Bottles containing 95 mL of MRS-G, MRS-Inulin and MRS-IGA medium were inoculated with 5 mL of each cell suspension. Then, each culture was distributed in several aliquots of 10 mL, using sterile tubes, and incubated at 37 °C. The OD₆₀₀ was taken every 3 h or 5 h, each tube was centrifuged at 1610 g during 15 minutes. The precipitate was suspended in 10 mL of distilled water and the pH was also recorded as an indirect parameter of growth and sugar metabolism. All measurements were performed in triplicate in two independent experiments.

3. Results and discussion

3.1. Inulin-gallic acid conjugate characterization

3.1.1. UV spectra

Figure 1 shows the UV spectra of gallic acid, pyrogallol and the inulin-gallic acid conjugate (IGA) where baseline corresponds to the previously dissolved in water unmodified inulin. The UV spectra of aromatic hydrocarbons, such as gallic acid and pyrogallol, are characterized by three peaks with origin in the $\pi \rightarrow \pi^*$ transitions (Liu, Lu, Kan, & Jin, 2013). The aromatic ring is a chromophore which has three maximum absorbances at wavelengths neighbouring 184 (not shown), 204 and 256 nm. Hydroxyl groups (-OH) have an auxochromic effect on this chromophore, particularly on the peak at 256 nm, and their presence causes a displacement of these peaks toward longer wavelengths (bathochromic effect). Therefore, in the case of gallic acid and pyrogallol they were 208-214 and 266-268

nm by effect of the three hydroxyl groups bonded to these compounds. The spectrum of the Inulin-gallic acid conjugate was similar to aromatic hydrocarbons which suggest that the grafting was done between gallic acid's carboxylic group and inulin (200-210 nm) (Cirillo *et al.*, 2010; Spizzirri *et al.*, 2009).

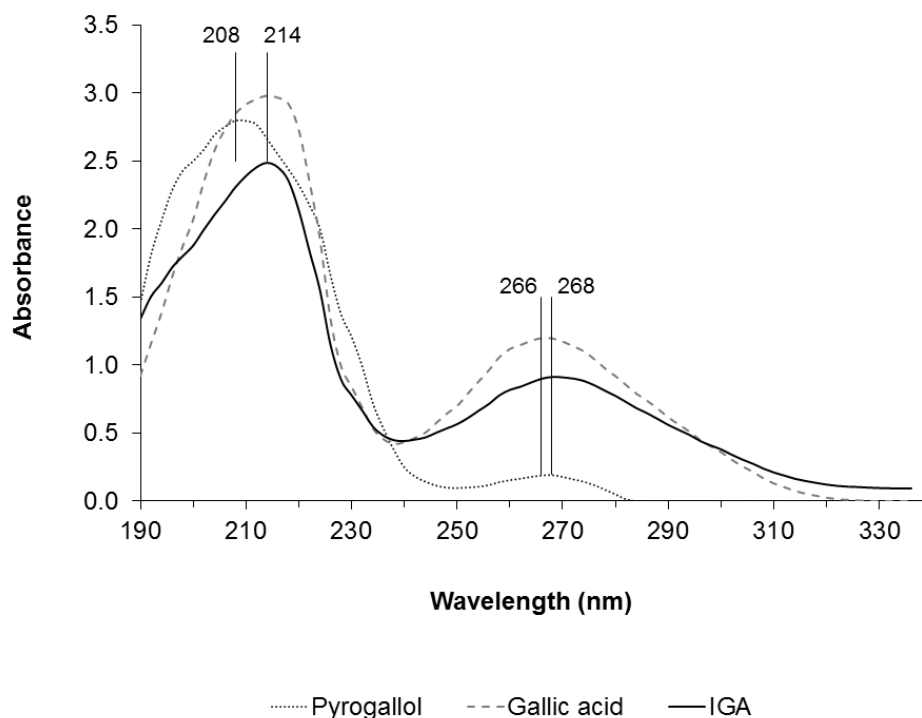


Figure 1. UV spectrum of gallic acid, pyrogallol and inulin-gallic acid conjugate (IGA).

3.1.2. FT-IR spectra

FT-IR analysis (Figure 2) was performed to characterize the IGA conjugate, unmodified inulin and gallic acid. Unmodified Inulin spectrum shows typical carbohydrate bands at 950 and 1100 cm^{-1} related to stretching vibrations of C-C and C-O bonds. Besides, it shows at 2800 and 3000 cm^{-1} bands related to stretching vibrations of C-H and CH_2 and at 1425 and 1475 cm^{-1} related to the torsion of C-H of CH_2/CH_3 groups of the alkenes. Grube, Bekers, Upite, & Kaminska (2002), found similar values in native inulin samples with different

polymerization rates. GA shows bands at 1500 and 1600 cm^{-1} associated to the substitutions on the aromatic ring. Moreover, it shows a signal at 1720 and 1760 cm^{-1} linked to the stretching of C=O of the carbonyl group. Gallic acid-polymer conjugates show new bands at 1800 and 1600 cm^{-1} . The signal at 1760 and 1720 cm^{-1} corresponds to the C=O vibration of the carbonyl group in esters. This supports the hypothesis that the covalent bond between the GA and the inulin is an ester. It is important to notice that those new peaks are not visible in the control polymer, suggesting that the inulin has been efficiently grafted (as an ester link). Similar results have been reported by Liu, *et al.*, (2014b), Cirillo *et al.* (2010), and Spizzirri *et al.* (2010).

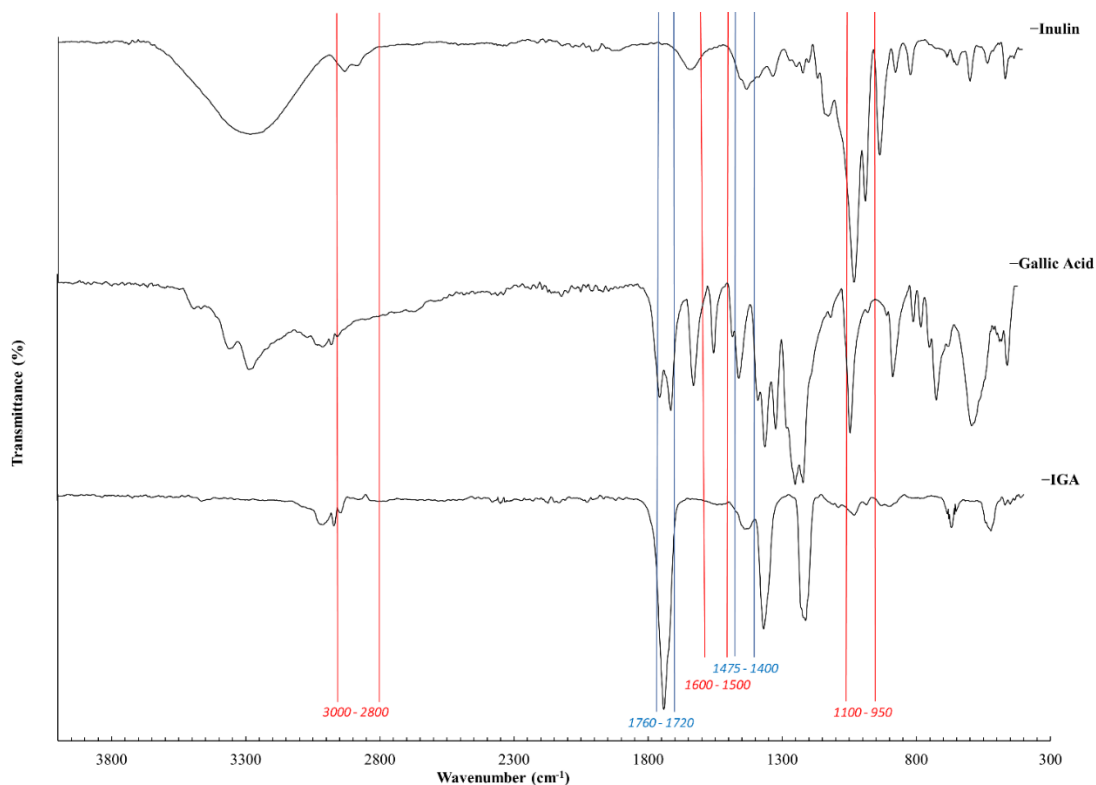


Figure 2. FT-IR spectra for gallic acid (GA), native inulin (Inulin) and inulin-gallic acid conjugate (IGA).

3.2. Antioxidant activity of IGA graft

3.2.1. DPPH radical-scavenging assay by EPR spin-trap

DPPH is a relatively stable free radical and its use in the EPR spectroscopy is a valuable and practical approach to evaluate the antioxidant potential of grafted polymer molecular fractions of antioxidants (Pasanphan, Buettner, & Chirachanchai, 2010; Pasanphan & Chirachanchai, 2008). IGA's DPPH radical scavenging activity was investigated through the reduction of EPR's signal spectra and the relative percent of DPPH scavenging capacity was calculated according to equation 1. Inulin and GA were used as negative and positive controls, respectively. Figure 3a shows DPPH's EPR spectrum and the resulting spectra of this radical in the presence of GA and IGA graft. The inhibition of the signal's intensity caused by GA and IGA graft is evident as the wave amplitude of the radical spectra decreased significantly until it has almost been extinguished. Both GA and IGA graft showed a significant capacity to reduce DPPH radical as a function of their concentration in the liquid medium. As showed in Figure 3b, GA exhibited the greatest activity to scavenge DPPH radical because the concentration required to reduce by 50% the signal amplitude (IC_{50}) was $0.72 \pm 0.05 \mu\text{g/ml}$ in contrast to the IGA graft which had a mean value of $77.5 \pm 10.6 \mu\text{g/ml}$.

Table 1 shows the concentration of GA, IGA graft and inulin at which the highest DPPH signal inhibition was achieved, where inulin reflects a nonsignificant inhibition of DPPH radical (7.48%). Some oligosaccharides associated with fructans have a considerable capacity to reduce DPPH depending on its concentration in the liquid medium (Je, Park, & Kim, 2004; Yang, Prasad, Xie, Lin, & Jiang, 2011; Zhong, Lin, Wang, & Zhou, 2012). However, when the molecular weight of these oligosaccharides increases, its capacity to

reduce DPPH decreases (Je, *et al.*, 2004; Park, Je, & Kim, 2003). This may explain the nonsignificant DPPH radical inhibition of Inulin (MW > 3 kDa).

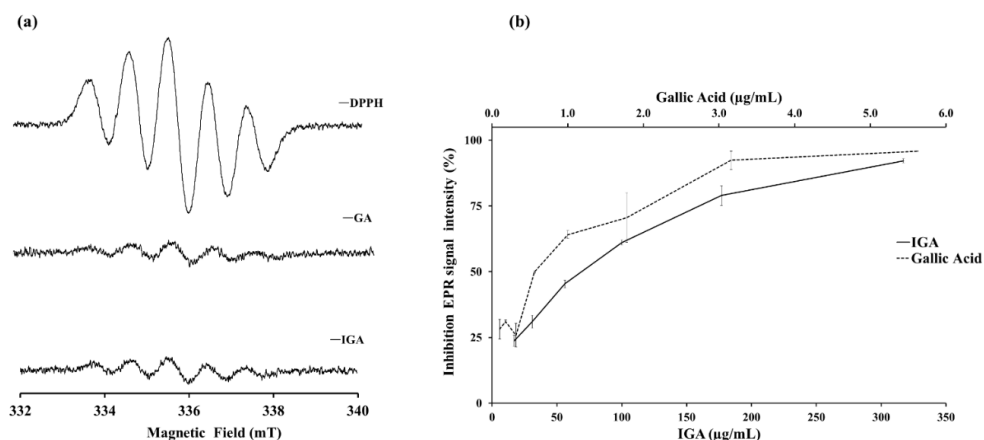


Figure 3. DPPH radical-scavenging measured by EPR spin-trap of gallic acid (GA) and inulin-gallic acid graft (IGA). a) DPPH experimental spectra and (b) Inhibition of intensity of DPPH-signal]. ¹O₂ scavenging [(c) TEMP experimental spectra and (d) Inhibition of intensity of TEMP-signal].

Table 1. Antioxidant activity of gallic acid, IGA graft and inulin samples.

Sample	Radical	Concentration (µg/ml)	GA equivalents (µg)	Inhibition of the radical signal (%)
Gallic Acid	DPPH	5.6	5.60	95.8 ± 0.02 ¹
	¹ O ₂	10	10.00	32.3 ± 0.80 ²
	TBARS	1	1.00	19.0 ± 1.40
IGA	DPPH	317	5.20	92.2 ± 0.92
	¹ O ₂	317	5.20	29.8 ± 1.30
	TBARS	100	1.63	19.6 ± 0.70
Inulin	DPPH	317	0.00	7.48 ± 2.90
	¹ O ₂	317	0.00	0.00 ³
	TBARS	100	0.00	-0.76 ± 1.80

¹. Mean values ± standard deviation of three replicates.

². Results after eight minutes of irradiation

³. No activity.

Grafting antioxidant molecules onto the backbone of oligosaccharides gives to the new polymer a significant antioxidant capacity (Liu, et. al, 2013a; Liu, Lu, Kan, Tang, & Jin, 2013; Liu, Lu, Kan, Wen & Jim, 2014; Pasanphan *et al.*, 2010), likewise GA grafted onto inulin acquires the capacity to scavenge DPPH radical. Table 1 shows 92.2% of DPPH's radical inhibition at 1.63 µg/100 µg of GA grafted onto inulin backbone (5.2 g gallic acid onto 317 g of inulin), compared to 95.8% of inhibition of 5.6 g of gallic acid in its pure state. Grafting of other molecules have successfully showed antioxidant activity (Table 2) coinciding with the results showed in the present study.

Table 2. Inhibition of the DPPH signal as a function of the concentration according to the data of several authors.

Authors	Inhibition of the radical signal (%)	Polymer concentration (µg/ml)
Spizzirri <i>et al.</i> , 2010	74.0	2500
Spizzirri <i>et al.</i> , 2011	80.0	2500
Curcio <i>et al.</i> , 2009	92.0	2000
Pasanphan <i>et al.</i> , 2010	87.0	230
Liu <i>et al.</i> , 2013b	74.5	30
Cho <i>et al.</i> , 2011	92.3	50

3.2.2. ¹O₂ scavenging effect by EPR spin-trap

¹O₂ is generated within cells by exposure to light. This radical compound can induce oxidative damage of lipids, amino acids and nucleic acids (Gómez-Vidales, *et al.*, 2014). An EPR's inhibition intensity signal-time curve was obtained (Figure 4) and results are expressed as the percentage of EPR's inhibition signal of the irradiated TEMP solution. Figure 4a shows TEMP's EPR spectrum and the resulting spectra of this radical in the

presence of GA and IGA. The signal's inhibition intensity caused by GA and IGA graft is evident as the wave amplitude of the radical spectrum decreased. GA and to a lesser extent IGA graft showed a significant capacity to reduce TEMP radical depending on their concentration in the liquid medium and on the irradiation time. After 5 and 8 minutes of irradiation, GA (100 µg/ml) caused $89 \pm 3\%$ and $94 \pm 1\%$ inhibition of $^1\text{O}_2$, respectively (Figure 4b). Remaining samples (GA and IGA graft at other concentration) showed nonsignificant differences ($P > 0.05$) at any time of irradiation. As showed in Table 1, IGA graft exhibited a low capacity to inhibit $^1\text{O}_2$, about 18%. In general, phenolic compounds have a reduced capacity to extinguish the $^1\text{O}_2$ radical. Wang & Jiao (2000) assessed the antioxidant activity of fruit juices rich in phenolic compounds and reported an inhibition of $^1\text{O}_2$ from 6.3 to 17.4%. (Wang & Jiao, 2000), several molecules have different antioxidant capacity to inhibit $^1\text{O}_2$, for example β -carotene (35.3%), α -tocopherol (22.5%), glutathione (22.5%), ascorbic acid (6.18%) and chlorogenic acid (0.44%).

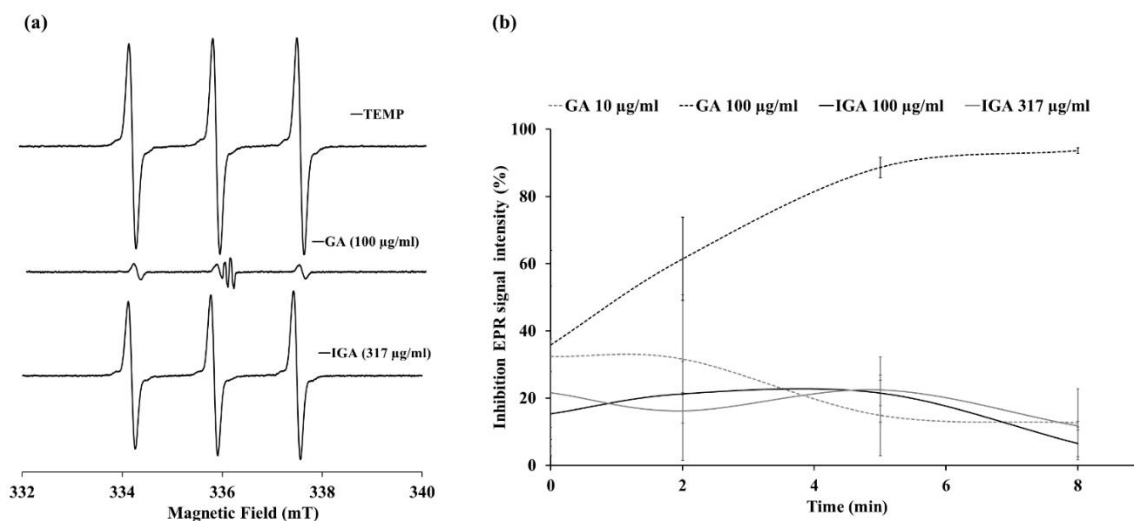


Figure 4. $^1\text{O}_2$ scavenging measured by EPR spin-trap of gallic acid (GA) and inulin-gallic acid graft (IGA). (a): TEMP experimental spectra and (b): Inhibition of intensity of TEMP-signal.

The efficiency of the chemical reaction to scavenge $^1\text{O}_2$, depends on the presence of hydroxyl groups at the $\text{C}_2=\text{C}_3$ position of the flavonoids' C ring (Tournaire *et al.*, 1993). Furthermore, when the hydroxyl group is glycosylated its capacity to scavenge $^1\text{O}_2$ decreases (Majer, Neugart, Krumbein, Schreiner, & Hideg, 2014). In phenolic compounds and according to Tournaire *et al.* (1993), the absence of a carbonyl group in the C ring leads to a flat molecular structure, that is why catechin is the flavonoid with the highest efficiency to extinguish the $^1\text{O}_2$ radical ($5.8 \times 10^6 \text{ Lmol}^{-1}\text{s}^{-1}$). This may explain the significant capacity of GA and the low reducing power of IGA to scavenge $^1\text{O}_2$.

3.2.3. Inhibition effect of lipid peroxidation

Lipid peroxidation (LP) includes a series of chain reactions where a free radical will cause oxidation of unsaturated fatty acids and to produce a large number of degradation products [$\text{LH} + \text{R}\cdot \rightarrow \text{RH} + \text{L}\cdot + \text{O}_2 \rightarrow \text{LOO}\cdot + \text{LH} \rightarrow \text{L}\cdot + \text{LOOH}$] (Abuja & Albertini, 2001). The method to measure LP is based on the reaction of TBARS which yields some compounds formed in the course of the reaction. After reacting with TBA these compounds yield a pinkish red chromogen with the maximum absorbance at 540 nm, whose concentration is related to the extent of lipid peroxidation. Figure 5 shows the effect of IGA, inulin and GA on the production of TBARS where native inulin was used as a negative control. GA showed a significant effect ($P < 0.05$) on the inhibition of TBARS depending on its concentration (100 $\mu\text{g/ml}$) causing an inhibition of 92% (Table 1). IGA showed a significant effect only at a high concentration (100 $\mu\text{g/ml}$). Grafting polymers with some type of phenolic compounds increase their capacity to reduce LP, which depends on the number of grafted moieties in the polymer (Liu, *et al.*, 2013b; Liu, *et al.*, 2014b; Parisi, *et al.*, 2010). In the present study and according to Table 1, the low capacity of IGA to inhibit LP (19.6% at 100 $\mu\text{g/ml}$) could be

due to the low availability of the grafted gallic acid (16.3 mg/g inulin) either to steric hindrance caused by the substitution of hydroxyl groups in the structure of GA (Tournaire, *et al.*, 1993). Nevertheless, the increase in the grafted polymer concentration increases the antioxidant capacity of the medium (Liu, *et al.*, 2013a).

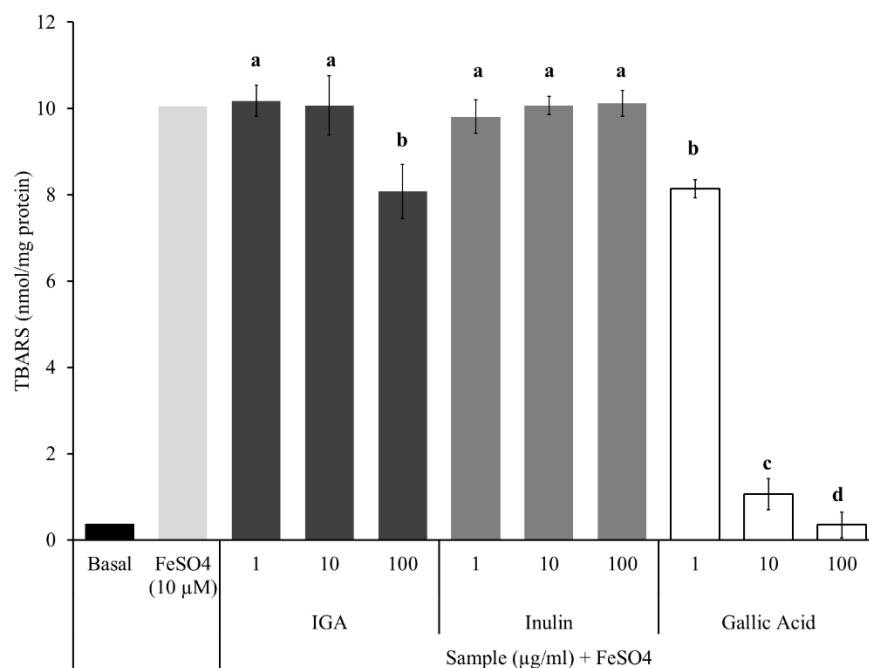


Figure 5. Effect of inulin-gallic acid graft (IGA), inulin and gallic acid concentrations on TBARS formation.

3.3. *In vitro* prebiotic effect of IGA

Lactobacilli species have been commonly used as a model to evaluate the prebiotic capacity of fermentable sugars because these lactic acid bacteria, among other characteristics display an observable growth after about 15 hours of incubation (Adebola *et al.*, 2014; Corral *et al.*, 2008). Figure 6 shows the growth of *Lactobacillus acidophilus* in the presence of several carbon sources as a function of time: inulin (MRS-Inulin), IGA (MRS-IGA) and glucose

(MRS-Gluc, positive control) where the higher growth was reached after 14 h of incubation. Furthermore, a trial was performed in the absence of carbon source (negative control) where no growth of the probiotic bacteria was observed. pH values were also determined and its change was considered as an indicator of the fermentative bacterial activity and as an evidence of the prebiotic effect of the inulin incorporated in the culture broth (Madhukumar & Muralikrishna, 2010). In order to get some kinetic parameters summarizing the evolution of the optical density (OD) or the pH of the liquid medium from a time zero to a steady state, the observed data were correlated with a first order kinetic model (Equations 2 and 3).

$$OD_t/OD_{\infty}=1-EXP(-kt) \quad (2)$$

$$pH_t/pH_{\infty}=EXP(-kt) \quad (3)$$

With this model, two parameter estimates were obtained and compared: a rate constant (k), and a maximum value (OD_{∞} or pH_{∞}) considering that at infinite time, the variables reached their equilibrium state (Atkins, 1998). Hence, OD_t or pH_t represent OD or pH units at a t time (in hours).

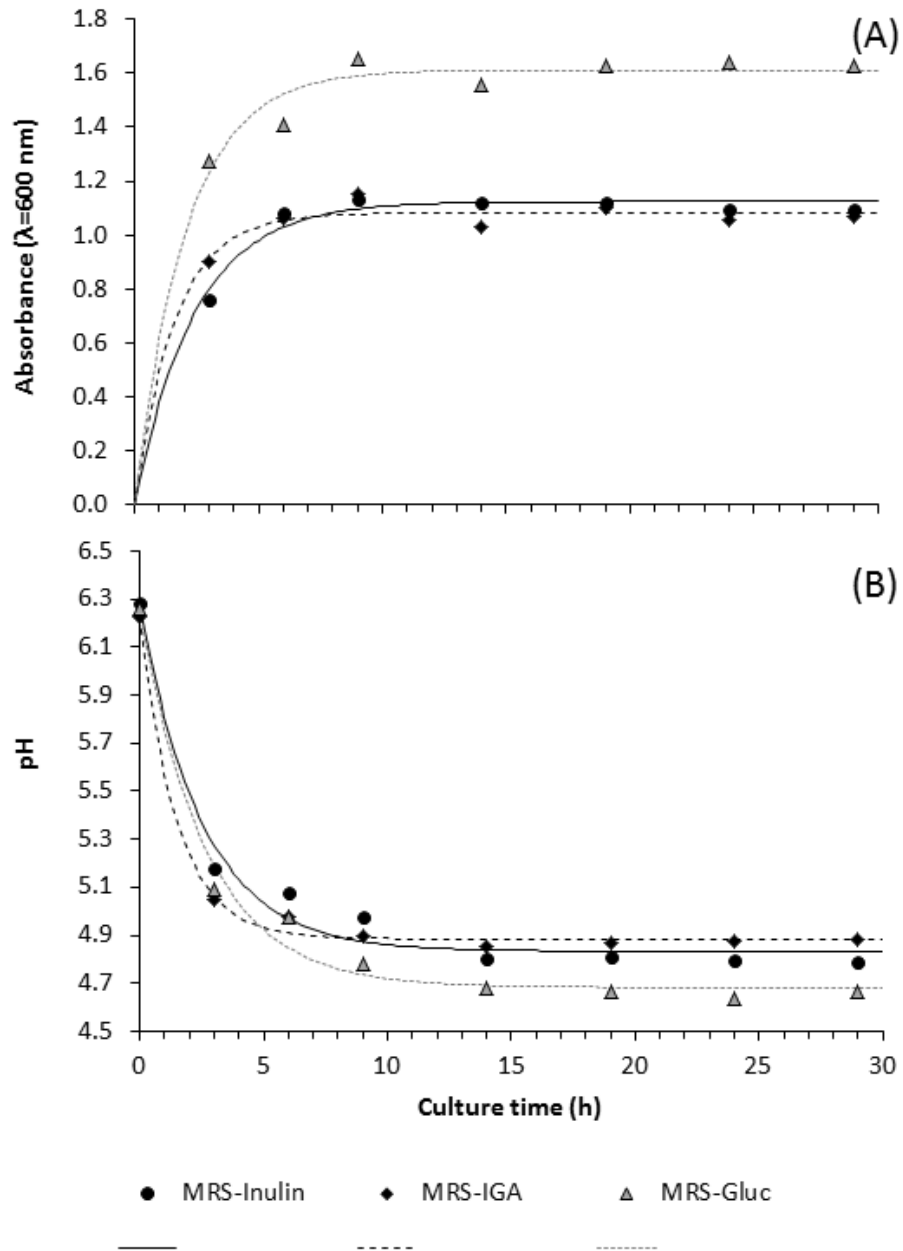


Figure 6. Growth of *Lactobacillus acidophilus* expressed as $OD_{600\text{ nm}}$ and pH decrease. Carbon source: native inulin (MRS-Inulin), inulin-gallic acid graft (MRS-IGA) and glucose (MRS-Gluc).

Table 3 shows the concentration of GA in the MRS-IGA medium (16.3 mg GAE/g), its lack in the MRS-Inulin and MRS-Glucose media, the rate constants and the values at the steady state. According to Figure 6, the growth kinetics of *Lactobacillus acidophilus* responded adequately to the first order kinetic model shown in equations 2 and 3, with coefficients of determination (R^2) higher than 0.90.

Table 3. Total phenolics by carbon source and means values after 14 hours of *L. acidophilus* incubation.

Parameter	MRS-Inulin	MRS-IGA	MRS-Glucose
Total phenolics ¹	0.43 ± 0.320 ¹	16.30 ± 0.420	0.15 ± 0.010
OD _{600 nm} at the steady state	1.12 ± 0.047B ²	1.08 ± 0.043B	1.61 ± 0.077A
OD _{600 nm} rate constant	0.41 ± 0.101A	0.62 ± 0.206A	0.48 ± 0.154A
pH at the steady state	4.83 ± 0.105A	4.88 ± 0.039A	4.68 ± 0.097B
pH rate constant	0.39 ± 0.169B	0.67 ± 0.132A	0.37 ± 0.183B

¹ Total phenolics reported as mg equivalents of gallic acid/g sample.

². Mean values ± 95% Confidence Limits. Values followed by different letters are significantly different at a $p < 0.05$.

According to the obtained parameter estimates showed in Table 3, the rate constants related to the OD were statistically similar suggesting that the rate of bacterial growth was not affected by the carbon source. However, the rate of decrease of the pH in the liquid medium was greater in the presence of IGA which may have been caused by the release of gallic acid after consumption of the inulin fraction by the lactic acid bacteria. Besides, the higher bacterial growth was observed, as expected, in the MRS-Glucose medium because its OD_{600 nm} at the steady state was statistically higher than in the other media. In the same way, the pH at the steady state was the lowest. These results show that the presence of gallic acid grafted in the inulin chain did not affect the growth of the lactic acid bacteria.

The concentration of the carbon source is critical to evaluate the oligosaccharides prebiotic activity. Li, Kim, Jin, & Zhou, (2008) reported that 1% of burdock inulin in the culture medium is an adequate concentration to confirm the growth of bifidobacteria. Corral *et al.* (2008) used 2% chicory inulin and other fructans as carbon source in the growth of *Lactobacillus* strains. Generally, the growth of probiotic bacteria is higher when the sugar concentration is increased in the culture medium (Adebola, *et al.*, 2014; Yang, *et al.*, 2011). Incorporating any type of inulin in the culture media causes a long-term bifidogenic effect on probiotic bacteria which is accompanied by a decrease of pH owing to the production of acetic and lactic acid (López-Molina *et al.*, 2005). The OD curves shown in Figure 6 indicate no difference in the *L. acidophilus* growth modified by the carbon source consumed. However, the pronounced pH drop in the MRS-G medium appears to be an indicator of the increased production of lactic acid caused by *L. acidophilus* which is not reflected in the case of MRS-Inulin and MRS-IGA media. Growth of *L. acidophilus* is not influenced by the presence of gallic acid onto inulin, suggesting that there is not relationship between antioxidant activity and prebiotic activity (Yang *et al.*, 2011).

4. Conclusions

Inulin does not show any antioxidant activity, but grafting gallic acid onto native inulin gives this dietary fibre a significant capacity to scavenge free radicals such as DPPH and $^1\text{O}_2$ and to reduce lipid peroxidation. Moreover, inulin shows a significant capacity to stimulate the growth of *Lactobacillus acidophilus* and gallic acid molecules grafted onto native inulin does not interfere with its prebiotic activity. This work shows that it is possible to provide radical-scavenging ability to the fructo-oligosaccharides avoiding decrease of their prebiotic properties, which could extend its potential as functional foods.

5. Acknowledgements

The authors wish to thank the Consejo Nacional de Ciencia y Tecnología (CONACyT-México) for the scholarship granted to Daniel Arizmendi-Cotero (Grant number: 370573), the Centro Conjunto de Investigación en Química Sustentable UAEM-UNAM, Alejandra Nuñez Pineda and Lizbeth Triana Cruz for their assistance in the characterization and analysis of the IR spectra.

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Table and figure captions

Figure 1. UV spectrum of gallic acid, pyrogallol and inulin-gallic acid conjugate (IGA).

Figure 2. FT-IR spectra for gallic acid (GA), native inulin (Inulin) and inulin-gallic acid conjugate (IGA).

Figure 3. DPPH radical-scavenging measured by EPR spin-trap of gallic acid (GA) and inulin-gallic acid graft (IGA). a) DPPH experimental spectra and (b) Inhibition of intensity of DPPH-signal]. $^1\text{O}_2$ scavenging [(c) TEMP experimental spectra and (d) Inhibition of intensity of TEMP-signal].

Figure 4. $^1\text{O}_2$ scavenging measured by EPR spin-trap of gallic acid (GA) and inulin-gallic acid graft (IGA). (a): TEMP experimental spectra and (b): Inhibition of intensity of TEMP-signal.

Figure 5. Effect of inulin-gallic acid graft (IGA), inulin and gallic acid concentrations on TBARS formation.

Figure 6. Growth of *Lactobacillus acidophilus* expressed as $\text{OD}_{600 \text{ nm}}$ and pH decrease. Carbon source: native inulin (MRS-Inulin), inulin-gallic acid graft (MRS-IGA) and glucose (MRS-Gluc).

Table 1. Antioxidant activity of gallic acid, IGA graft and inulin samples.

Table 2. Inhibition of the DPPH signal as a function of the concentration according to the data of several authors.

Table 3. Total phenolics by carbon source and means values after 14 hours of *L. acidophilus* incubation.

VI. CONCLUSIONES GENERALES

Los resultados expuestos en este trabajo demuestran que la concentración del macro radical formado en la inulina está relacionada positivamente con la relación molar H_2O_2/AA . Sin embargo, la eficiencia del injerto se manifiesta a mayor concentración cuando la relación molar del par *redox* disminuye.

Los ensayos de EPR mostraron que el macro-radical de la cadena de inulina se forma en un átomo de carbono y su concentración depende de la relación molar entre H_2O_2 y AA. Los espectros UV-Vis, FT-IR y análisis de rayos X de los compuestos injertados, confirman el enlace covalente entre el ácido gálico y la inulina.

La eficiencia de la reacción se relaciona con la concentración de peróxido de hidrógeno y ácido ascórbico en el par *redox*. Sin embargo, las reacciones simultáneas entre todos los componentes (H_2O_2 , AA, GA, inulina y posiblemente O_2 atmosférico) podrían intervenir en la eficiencia de injerto inulina-ácido gálico.

El conjugado inulina-ácido gálico exhibe una importante capacidad para eliminar los radicales libres tales como DPPH y 1O_2 y para reducir la peroxidación de lípidos. Además, la inulina muestra una significativa capacidad de estimular el crecimiento de *Lactobacillus acidophilus* y la presencia de moléculas de ácido gálico injertados en la inulina no interfiere con su actividad prebiótica.

Este trabajo demuestra que es posible proporcionar la capacidad de eliminación de radicales libres a los fructo-oligosacáridos evitando disminución de sus propiedades prebióticas, lo que podría extender su potencial como alimentos funcionales.

VII. BIBLIOGRAFÍA GENERAL

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