UNIVERSIDAD AUTÓNOMA DE MADRID

Facultad de Ciencias

Departamento de Química Física Aplicada

OBTENCIÓN Y CARACTERIZACIÓN DE COMPUESTOS BIOACTIVOS PROCEDENTES DE TUBÉRCULOS ANDINOS Y DE SUBPRODUCTOS DE LA INDUSTRIA AGROALIMENTARIA

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Memoria presentada por

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Para optar al grado de

Doctor

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Instituto de Investigación en Ciencias de la Alimentación

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CERTIFICAN: Que el presente trabajo titulado: **"Obtención y caracterización de compuestos bioactivos procedentes de tubérculos andinos y de subproductos de la industria agroalimentaria"**, y que constituye la memoria que presenta Dña. María Teresa Pacheco Tigselema para optar al grado de Doctor por la Universidad Autónoma de Madrid, ha sido realizado en el Departamento de Bioactividad y Análisis de los Alimentos del Instituto de Investigación en Ciencias de la Alimentación, bajo su dirección.

Y para que así conste firman el presente certificado en Madrid, a 6 de marzo del 2019.

Fdo. Dr. Fco. Javier Moreno Andújar Fdo. Dra.. Mª del Mar Villamiel Guerra

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Abreviaturas y Acrónimos

Abreviaturas / Abbreviations

(Th) 17	:	T helper cells 17. Cells that help the activity of other immune cells by releasing T cell cytokines. (Th) 17 produce IL-17 which is a proinflammatory substance.
°Brix	:	percentage of dissolved solids
2-FM-AA	:	2-furoylmethyl amino acids
2-furoylmethyl-lysine, 2-FM-Lys	:	furosine
a*	:	redness
AC	:	capacidad antioxidante
AEP	:	pectina solubilizada en ácido
AF	:	alimento para animales
AGN	:	División de Nutrición y Protección al Consumidor de la FAO
AGP	:	División de Producción y Protección Vegetal de la FAO
AM	:	acid method
AOAC	:	Association of Official Analytical Chemists
AS	:	available starch
Aw	:	water activity
b*	:	yellowness
BSA	:	bovine serum albumin
C*	:	chroma
Caco-2	:	heterogeneous human epithelial colorectal adenocarcinoma cells
CAE	:	ácido clorogénico
CCR	:	cáncer de colon rectal
CD	:	Crohn's disease
cDNA	:	complementary DNA
CP	:	citrus pectin
DAI	:	Disease Activity Index
DE	:	grado de esterificación
DES	:	dietary energy supply
DM	:	dry matter
DM	:	degree of methoxylation
DNS	:	3,5-dinitrosalicylic acid
DOR	:	residuo de naranja seco
DP	:	degree of polymerization
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
DSC	:	differential scanning calorimetry

DSS	:	sodium dextran sulfate
DW	:	dry weight
EAI	:	Emulsifying Activity Index
ECU	:	Ecuador
EM	:	enzymatic
FA	:	ácido ferúlico
FAO	:	Organización de las Naciones Unidas para la Alimentación y la Agricultura
FOR	:	residuo de naranja fresco
FOS	:	fructooligosacáridos
FTIR	:	Fourier transform infrared spectroscopy
GAE	:	equivalentes de ácido gálico
GalA	:	ácido galacturónico
GAPDH	:	glyceraldehyde-3-phosphate dehydrogenase
GC-FID	:	gas chromatography with flame-ionization detection
GI	:	glycemic index
h°	:	huge angle
HDL	:	lipoproteínas de alta densidad
H_{gel}	:	gelatinization enthalpy
HHP	:	alta presión hidrostática
HMDS	:	hexamethyldisylazane
HPLC-DAD-ESI/MS^n	:	cromatografía líquida de alta resolución acoplada a espectrometría de masas con ionización electrospray y detección de diodo array
HVED	:	descargas eléctricas de alto voltaje
IB	:	pardeamiento interno
IBD	:	inflammatory bowel disease
ICAM I	:	intercellular adhesion molecule 1
ICP-MS	:	inductively coupled plasma mass spectrometry
IDF	:	insoluble dietary fiber
IL-1β	:	interleukin-1 *β*. Proinflammatory cytokine.
IL-6	:	interleukin-6. Proinflammatory cytokine.
INIAP	:	Instituto Nacional de Investigaciones Agropecuarias de Ecuador
iNOS	:	inducible nitric oxide synthase
L*	:	lightness
LES	:	extracción sólido-líquido
MAE	:	extracción asistida por microondas
MF	:	multi-fibre mix

MHG	:	hidrodifusión y gravedad por microondas
MR	:	Maillard reaction
MRPs	:	Maillard reaction products
MS	:	materia seca
MS-ESI	:	espectrometría de masas con ionización electrospray
MUC-3	:	mucin 3. Protective molecules of the intestinal mucosa.
Mw	:	peso molecular
n	:	flow behavior index
NCR	:	National Research Council
Occludin (OCLN)	:	Transmembrane proteins associated to tight junction in IBD
OEP	:	pectina extraída con oxalato
OL	:	licor de naranja
ONU	:	Organización de las Naciones Unidas
PCA	:	Programa de Cultivos Andinos de la FAO
PCs	:	compuestos fenólicos
PLE	:	extracción con líquidos presurizados
PPO	:	polifenol oxidasa
P-SBP-D	:	pectin from sugar beet pulp dried
P-SBP-E	:	pectin from sugar beet pulp ensiled
P-SBP-P	:	pectin from sugar beet pulp pressed
q-PCR	:	quantitative polymerase chain reaction
RNA	:	ribonucleic acid
RNS	:	reactive nitrogen species
ROS	:	especies reactivas de oxígeno
RRS	:	retrograded resistant starch
RS	:	almidón resistente
RS1	:	physically inaccessible starch
RS2	:	ungelatinized starch
RS3	:	retrograded starch
RS4	:	chemically modified starch
RS5	:	amylose-lipid complex
SBP	:	pulpa de remolacha azucarera
SBP-D	:	pulpa de remolacha seca
SBP-E	:	pulpa de remolacha ensilada
SBP-P	:	pulpa de remolacha prensada
SCFA	:	ácidos grasos de cadena corta
SD	:	standard deviation
SDS	:	almidón de lenta digestión

SDS	:	sodium dodecyl sulphate
SEC	:	size exclusion chromatography
SEM	:	microscopía electrónica de barrido
SEM	:	scanning electron microscopy
SFE	:	extracción con fluidos supercríticos
SIdI-UAM	:	Interdepartmental Research Service - Universidad Autónoma de Madrid
SPSS	:	Statistical Product and Service Solutions
TAC	:	total anthocyanins content
Tc	:	conclusion temperature
TCC	:	total carotenoids content
TDF	:	total dietary fiber
TDF	:	soluble dietary fiber
TE	:	trolox equivalente
TEAC	:	trolox equivalent antioxidant capacity
TFA	:	trifluoroacetic acid
TGA	:	thermogravimetric analysis
T_m	:	gelatinization temperature
TMS-oximes	:	trimethylsilylated oximes
TNF-α	:	tumor necrosis factor-α. Proinflammatory cytokine.
To	:	onset temperature
TP	:	fenoles totales
Tp	:	peak temperature
TPC	:	total phenolic content
TR	:	time of resolution
Treg	:	T regulatory cells. Cells that modulate the immune system, maintain tolerance to self-antigens, and prevent autoimmune disease.
UC	:	ulcerative colitis
UFC	:	unidades formadoras de colonias
UPLC-QTOF-MS^n	:	cromatografía líquida de ultra alta resolución, acoplada a espectrometría de masas y cuadrupolo de tiempo de vuelo.
USDA	:	Departamento de Agricultura de los Estados Unidos
WHO	:	World Health Organization
WL	:	weight loss
ZO-1	:	zonula occludens-1 (Tight junction protein 1). Peripheral membrane protein.
$\dot{\gamma}$	:	shear rate

ΔE	:	total colour differences
ΔH	:	enthalpy
Δm	:	weight loss
$\Delta\Delta Ct$	:	comparative CT (cycle threshold) method
σ	:	shear stress
σ_0	:	yield stress
k	:	consistency index
η	:	*apparent viscosity*
ζ	:	zeta potential

Resumen

Resumen

Este trabajo se ha enfocado en la obtención y caracterización de compuestos bioactivos procedentes de tubérculos andinos y de subproductos de la industria agroalimentaria. El yacón destacó por su alto contenido de fructooligosacáridos y compuestos fenólicos; la mashua y el melloco presentaron una alta concentración de carotenos y vitamina C, mientras que la zanahoria blanca mostró el mayor rendimiento de almidón, y el camote morado un elevado contenido de almidón rico en amilosa. Los extractos metanólicos de los tubérculos revelaron la presencia de flavonoles, derivados hidroxicinámicos, flavan-3-oles, flavanoles y antocianos, con importantes propiedades antioxidantes. Los almidones de mashua y melloco mostraron mayor capacidad estabilizante y mejores propiedades reológicas que el almidón de patata, resultado atribuido principalmente a su contenido de amilosa y mayor tamaño de partícula. El método enzimático para la extracción de pectina a partir del subproducto de remolacha azucarera ensilado permitió obtener pectina de alto contenido en ácido galacturónico, mientras que el método ácido facilitó la extracción de pectina con mayor rendimiento y estabilidad en solución acuosa e índice de actividad emulsionante. Los subproductos de la extracción de zumo de naranja también mostraron diferente composición química como resultado del proceso aplicado en la industria. Mediante ensayos *in vivo* realizados con ratones a los que se había inducido la enfermedad inflamatoria intestinal, se observó una menor severidad en la sintomatología y mejores indicadores bioquímicos tras el consumo del residuo fresco, pienso y pectina cítrica. Dichos efectos podrían estar relacionados con el contenido en fenoles, ácido galacturónico y *N*-ε-fructosil-lisina. Este es el primer estudio de caracterización y aprovechamiento de tubérculos andinos inexplorados y subproductos industriales de remolacha azucarera y naranja. Los resultados obtenidos representan un aporte valioso para su uso como fuentes de compuestos bioactivos no convencionales, promoviendo el crecimiento de pequeños agricultores y contribuyendo al desarrollo sostenible dentro de los nuevos retos sobre la alimentación para las próximas décadas.

Abstract

This work has focused on the obtainment and characterization of bioactive compounds from Andean tubers and by-products of the agri-food industry. The yacon stood out for its high content of fructooligosaccharides and phenolic compounds; mashua and melloco showed a high concentration of carotenes and vitamin C, while white carrot showed the highest starch yield, and purple sweet potato exhibited a high content of starch rich in amylose. The methanolic extracts of the tubers revealed the presence of flavonols, hydroxycinnamic derivatives, flavan-3-ols, flavanols and anthocyanins, with important antioxidant properties. The starches of mashua and melloco showed greater stabilizing capacity and better rheological properties than potato starch, a result attributed mainly to their amylose content and larger particle size. The enzymatic method for extracting pectin from the silage sugar beet by-product was useful to obtain pectin with a high galacturonic acid content, while the acid method facilitated the extraction of pectin with higher yield and stability in aqueous solution and emulsifying activity index. The by-products of the extraction of orange juice also showed different chemical composition as a result of the process applied in the industry. By means of *in vivo* tests carried out in mice, with induced inflammatory bowel disease, a lower severity of symptoms and better biochemical indicators were observed after the consumption of fresh residue, animal feed and citrus pectin. These effects could be related to the content of phenols, galacturonic acid and *N*-ε-fructosyl-lysine. This is the first study of characterization and exploitation of unexplored Andean tubers and industrial by-products of sugar beet and orange. The results obtained represent a valuable contribution for their use as sources of non-conventional bioactive compounds, promoting the progress of small farmers, and contributing to sustainable development, inside the new challenges on food for the coming decades.

Estructura de la Memoria

Estructura de la Memoria

Esta memoria está estructurada en seis secciones que se indican a continuación:

a. **Introducción general:** donde se introduce al lector en la composición e importancia de compuestos bioactivos de los tubérculos andinos y los subproductos de la industria agroalimentaria, describiendo antecedentes relacionados con los objetivos planteados para esta Memoria.

b. **Justificación y objetivos:** donde se justifica la importancia del tema de estudio, su contexto mundial y/o nacional actual, sus objetivos generales y parciales.

c. **Plan de trabajo:** donde se explica de forma global y esquematizada cómo se ha abordado el trabajo para alcanzar los objetivos prefijados.

d. **Resultados:** esta sección está dividida en dos subsecciones. En cada una se exponen los trabajos científicos generados (publicados o en revisión), en lengua inglesa y de acuerdo al formato de las publicaciones científicas (Resumen, Introducción, Materiales y Métodos, Resultados y Discusión, Conclusiones).

   La **sección 4.1** se centra en la caracterización fisicoquímica y aprovechamiento de tubérculos andinos, determinación de perfil fenólico y propiedades morfológicas y funcionales del almidón. Los resultados de esta sección han permitido elaborar 3 artículos científicos (2 enviados y uno en revisión).

   Y, la **sección 4.2** se enfoca en la composición fisicoquímica de residuos de remolacha azucarera y de la extracción de zumo de naranja. Se aplican diferentes métodos para la obtención de pectina y se analizan sus propiedades. Adicionalmente, se evalúa el efecto del consumo de subproductos de naranja sobre indicadores de inflamación intestinal en ratones. Esta sección ha dado lugar a otros 3 artículos científicos (publicados).

e. **Discusión general:** donde se persigue unificar e interrelacionar los resultados conseguidos en cada una de las secciones de esta Memoria. De modo general se discuten los resultados obtenidos por otros investigadores y la importancia de nuestros resultados, destacando el potencial de obtener compuestos bioactivos a partir de tubérculos andinos y de residuos de la industria alimentaria.

f. **Conclusiones generales:** donde se presentan las conclusiones más relevantes de todos los trabajos expuestos en la sección de Resultados.

1. Introducción General

1. INTRODUCCIÓN GENERAL

La población mundial actual (7.600 millones de personas) alcanzará los 8.600 millones para el año 2030 y 9.800 millones para el 2050, siendo los países menos desarrollados, los responsables del crecimiento demográfico del siglo XXI (ONU-DAES, 2017). África pasará de 1.000 a 2.000 millones, Asia deberá seguir albergando entre el 50 y el 60% de la población, mientras que Europa verá cómo su población desciende de 730 a 660 millones de habitantes. No obstante, de manera global, hasta el 2100, se prevé que la población mundial aumentará en un 47 % (FAO, 2017). En este escenario, satisfacer las necesidades de los seres humanos a un nivel aceptable es uno de los retos y desafíos más grandes que las sociedades deberán resolver en los próximos decenios (FAO, 2016).

Según el Informe de la FAO-ONU (FAO-ONU, 2017), alrededor de un tercio de la producción de los alimentos destinados al consumo humano se pierde o desperdicia en todo el mundo, lo que equivale a, aproximadamente, 1.300 millones de toneladas al año; sin embargo, aún existen 821 millones de personas que sufren de hambre y desnutrición.

En los países en desarrollo, más del 40% de estas pérdidas se produce en las etapas de post cosecha y procesamiento, mientras que en países industrializados más del 40% de la pérdida de alimentos se produce en la venta minorista y el consumo. Estos residuos suelen ser depositados en vertederos, ríos o lagos, siendo un peligro para la salud y con un elevado impacto medioambiental debido a la emisión de aproximadamente 3.300 millones de toneladas métricas de gases de efecto invernadero.

En el caso de raíces y tubérculos, alimentos básicos de países en desarrollo, se pierde hasta un 45 % de su volumen producido que, junto con los residuos agroindustriales y la pérdida de otro tipo de alimentos, agrava enormemente el problema del hambre y de la contaminación ambiental.

En este sentido, el aprovechamiento de estos recursos mediante procesos sostenibles podría ayudar a reducir las pérdidas de alimentos y constituirse como una medida eficaz para paliar, en parte, el déficit de alimentos al que nos

enfrentaremos en los próximos decenios y apoyar al desarrollo económico de países subdesarrollados y desarrollados (FAO-ONU, 2017).

Hará falta también movilizar importantes recursos y unir esfuerzos para incrementar la producción de alimentos tradicionales y buscar nuevas fuentes menos exploradas de una forma sostenible y sustentable. No obstante, las comunidades locales dependen de la agricultura para la generación de ingresos, sin embargo, ésta no se puede desarrollar fácilmente, debido al desgaste biofísico de las tierras y escasa accesibilidad a recursos hídricos. Considerando además los efectos del cambio climático, se tendrán que hallar nuevas alternativas de cultivos saludables, rentables y resistentes a plagas, que puedan servir de sustento, motivando al campesino a permanecer en el campo evitando acrecentar el hacinamiento urbano.

Sin las suficientes oportunidades de empleo en el campo, la tendencia de la población rural, sobre todo de países subdesarrollados, será acrecentar la emigración. Entre tanto, otras regiones deberán adaptarse a una población que envejece rápidamente, característica que también afectará a los países con bajos ingresos (FAO, 2017).

Ante esta problemática, la FAO a través de los programas de División de Producción y Protección Vegetal (AGP) y de División de Nutrición y Protección al Consumidor (AGN), ha venido realizando diversas actividades de apoyo a la producción y consumo de los cultivos andinos subutilizados (Tapia & Fries, 2007). Sin embargo, aún es insuficiente la información científica que se posee sobre los beneficios derivados de su consumo, así como sobre su composición química y propiedades tecnológicas, por lo cual, continúan siendo especies infrautilizadas.

1.1. Tubérculos andinos

1.1.1. Importancia

Muchos de los productores desfavorecidos y los hogares subnutridos del mundo dependen del cultivo de raíces y tubérculos (Scott, Rosegrant, y Ringer, 2000), entre

los que destacan la yuca (*Manihot esculenta*), la papa (*Solanum tuberosum*), la batata (*Ipomoea batatas*) y el ñame (*Dioscorea spp*.), siendo considerados por la FAO como raíces y tubérculos de importancia mundial (Alexandratos & Bruinsma, 2012), esenciales, especialmente, en Asia, África, América Latina y el Caribe (Okogbenin et al., 2013).

En América Latina, se estima que alrededor de 500.000 familias campesinas tienen parcelas con estos cultivos destinados para el autoconsumo y, ocasionalmente, para la venta de sus excedentes (Condori et al., 2008). La importancia de estos cultivos en la seguridad familiar y la nutrición radica en que:

- Aumentan la variedad de alimentos ricos en carbohidratos.
- Son resistentes a la sequía, no requieren elevados gastos y son de fácil almacenamiento, permitiendo evitar los períodos de escasez estacional.
- Aumentan la productividad de otros cultivos, ya que conservan el suelo y elevan su fertilidad.
- Son resistentes a las plagas y cuando se intercalan con otros cultivos (ej. leguminosas o cereales) actúan como barrera ecológica para las enfermedades.

En el ámbito nacional de los países andinos que los cultivan, contribuyen a reducir las importaciones de productos similares, pudiendo convertirse en una importante fuente de divisas, derivada de su exportación (Morón, 1999).

En los Andes, en general, se cultivan cuatro tubérculos diferentes, que se utilizan sobre todo a nivel local. Sin embargo, en la región de los Andes Centrales (Ecuador a Bolivia) (**Figura 1**), donde la agricultura de montaña se ubica desde los 1.500 hasta más de 4.000 metros sobre el nivel del mar (m.s.n.m.), la población está eminentemente conformada por campesinos que poseen conocimientos tradicionales, existiendo la mayor variabilidad de especies, características que hacen de los Andes Centrales una región única (Tapia et al., 2007).

Figura 1. Andes Centrales (Ecuador, Perú y Bolivia) (Tomada de Francou & Pouyaud, 2008).

Por otro lado, Ecuador es considerado uno de los diez países con mayor biodiversidad del mundo (Tene et al., 2007). Las posibilidades de obtener beneficios sostenidos de su vegetación, para la alimentación e industria, son enormes, pero aún no han sido apropiadamente aprovechados (MAGAP, 2016).

En el caso de tubérculos con estas características, sobresalen, concretamente, algunas variedades de yacón, mashua, melloco, camote y zanahoria blanca.

1.1.2. Especies

1.1.2.1. Yacón (*Smallanthus sonchifolius*)

El yacón es una raíz comestible de sabor dulce, originaria de la zona andina en la época preincaica. Se conoce en el norte de Perú como “yacón” o “llacón”, en Bolivia como “lacjon” o “yakuma”, y en Ecuador como “jícama” o “jiquima”. Se consume como fruta cruda, cocida u horneada (Velezmoro, 2004).

El cultivo de yacón en Ecuador es rústico y puede llegar a tener altos rendimientos (30 ton/ha). Se espera que, en los próximos años, la industria requiera mayor cantidad de producto fresco para elaborar jarabe, hojuelas, harina, etc.; pero pese a la importancia creciente de este cultivo, la información existente sobre su manejo técnico y composición específica según la variedad es bastante escasa. Se puede cultivar todo el año, y de preferencia a inicios de septiembre y octubre, o entre de julio y agosto, para evitar las heladas (Barrera et al., 2003).

1.1.2.2. Mashua (*Tropaeolum tuberosum*)

La mashua es originaria de los Andes centrales y su cultivo se extendió por migraciones hasta Colombia, el norte de Argentina y Chile. A pesar de su rusticidad no existen referencias de introducción en otros países de América, posiblemente porque el sabor del tubérculo resulta poco agradable para quien lo prueba por primera vez. Sin embargo, este cultivo se ha introducido con éxito en Nueva Zelanda (Roca & Manrique, 2005).

A este tubérculo se le atribuyen propiedades anafrodisíacas desde la época de los incas (Panel on the Lost Crops of the Incas-National Research Council, 1989), que concuerda con estudios realizados en ratas macho alimentadas con *T. tuberosum,* en los cuales se ha observado una caída del 45% en los niveles de testosterona y dihidrotestosterona en la sangre, efecto que parece estar relacionado con la presencia de isotiocianatos (John et al., 1982). Por otro lado, algunos investigadores sostienen que la presencia de glucosinolatos en este tubérculo podría tener efectos beneficiosos sobre el sistema inmunológico y que podrían proteger contra el cáncer (Traka & Mithen, 2009).

Se adapta a suelos muy pobres y su época de siembra mayor es de octubre a diciembre, y siembra menor en mayo y junio, para evitar heladas (Barrera et al., 2003).

1.1.2.3. Melloco (*Ullucus tuberosus*)

El Melloco posee un origen aún no bien definido, ya que se han observado plantas silvestres en el Cuzco-Perú de tubérculos similares pero amargos no comestibles, y también se cree que variedades encontradas en Colombia pueden ser las más antiguas. En ambos casos, restos arqueológicos demuestran que fue domesticado en los Andes, alrededor del 5.500 a.C. (Vimos et al., 1993).

En el Ecuador es el segundo tubérculo en importancia tras la patata (*Solanum tuberosum*) y sus principales centros de producción se encuentran en las provincias de Carchi, Imbabura, Pichincha, Cotopaxi, Tungurahua, Chimborazo y Cañar. En las restantes provincias el cultivo casi ha desaparecido, o se produce en parcelas

pequeñas de autoconsumo (Suquilanda, 2007). En trabajos realizados por el Programa de Cultivos Andinos (PCA) se encontró que algunos clones de melloco presentaron una buena capacidad de rebrote tras tres heladas sucesivas, lo que constituye una ventaja frente a otros tubérculos como la patata, junto con su mayor rendimiento en número de tubérculos por planta (Basantes, 2015). Otra característica peculiar del melloco es el contenido de mucílago que tiende a limitar su consumo. Sin embargo, en el PCA se han identificado varios clones de bajo contenido de mucílago. Se consume en ensalada y sopas, y en varias localidades alto-andinas se utiliza para curar traumatismos internos y reducir procesos inflamatorios (Vimos et al., 1993).

En el norte de la sierra ecuatoriana, su siembra se realiza durante todo el año, siendo importante sembrarlo antes de la patata debido a su largo período vegetativo (Basantes, 2015).

1.1.2.4. Camote o batata (*Ipomoea batatas*)

El camote, batata o patata dulce, es una planta dicotiledónea originaria de los valles calientes de la costa andina o la Amazonía (Barrera et al., 2003). En países en desarrollo ayuda sobre todo a prevenir la desnutrición por su gran capacidad de adaptación y aporte de energía (Bovell-Benjamin, 2007). En China este cultivo se halla en auge, disponiendo actualmente de más de 2.000 variedades (Zhang et al., 2018).

Su importancia en el mundo puede compararse con la de arroz, trigo o maíz, ya que tiene escasas exigencias ambientales y puede dar buenos rendimientos, aún en terrenos de mediana calidad. Como es una planta tropical, no tolera las heladas, pero es sumamente rústica, resistente a las sequías y con gran capacidad de emitir raíces (Daryanto et al., 2016).

Distintos centros de investigación trabajan para incrementar la producción, adaptar las variedades a determinadas regiones y conferirles resistencia a plagas y a enfermedades, al punto de que nuevos cultivares comerciales de batata dotados de esas características han ido desplazando a los tradicionales. En Japón se han

desarrollado clones con la pulpa enteramente morada, como la variedad llamada Ayamurasaki, mientras que en los Estados Unidos se prefieren los de pulpa anaranjada y textura húmeda, como Beauregard y recientemente Covington. Se consume asada o frita y a base de este tubérculo se elabora el *shochu* o *soju* en los Estados Unidos, bebida alcohólica tradicional del Japón, obtenida por destilación (Martí, 2014).

En Ecuador se cultiva en los valles subtropicales, templados interandinos y en la costa (principalmente Guayas y Manabí). Se siembra y cosecha en cualquier momento del año (Barrera et al., 2003).

1.1.2.5. Zanahoria blanca (*Arracacia xhanthorrrhiza*)

La zanahoria blanca, conocida también como arracacha, se cultiva desde Venezuela hasta el norte de Chile y noroeste de Argentina, siendo probablemente una de las plantas andinas más antiguas y más cultivadas en la etapa preincaica, cuya domesticación precedió a la patata y el maíz. Hay quienes manifiestan que la arracacha es originaria de Jamaica, sin embargo, hay otros investigadores que sostienen que el área de origen de esta planta está ubicada en los Andes del norte, porque allí están la mayoría de las especies de este género (Hermann, 1997).

La zanahoria blanca se cultiva principalmente debido al sabor agradable de su raíz. En algunos sectores, se consume en sopas, guisos y tortas (Barrera et al., 2003). Los limitantes para su cultivo son la alta perecibilidad de las raíces, el periodo vegetativo largo de (10-12 meses) y la lignificación de las raíces en la madurez.

La **Tabla 1** resume las condiciones de siembra y características agrícolas más importantes de estos cultivos.

Tabla 1. Principales condiciones de cultivo y ventajas agrícolas del yacón, mashua, melloco, camote y zanahoria blanca.

Tubérculo	Tipo de suelo, pH	Altura óptima de cultivo	Precipitación lluviosa y temperatura	Resistencia			Referencias
				A plagas	A las heladas	A la sequía	
Yacón (*Smallanthus sonchifolius*)	Suelos franco-arenosos, bien drenados, con pHs ácidos a ligeramente alcalinos (6-7,5)	En los Andes en general: entre los 900 y 2.750 m.s.n.m. En Ecuador: hasta una altitud de 3.500 m.s.n.m. También a menores altitudes, pero con menor tuberización En Nueva Zelanda y Estados Unidos: a nivel del mar	550 a 1.000 mm de lluvia anuales (550 a 1.000 L/m^2/año) Temperatura: 14 a 20 °C Susceptible a fuertes vientos	Moderada	Moderada Inicialmente se ve afectado, pero luego muestra gran capacidad de rebrote	Alta Gracias a su follaje y tallos perennes, aunque con menor productividad	Barrera et al. (2003) Comision de Codex Alimentarius (2012) Seminario et al. (2003) Tapia et al. (2007)
Mashua (*Tropaeolum tuberosum*)	Suelos negro-andinos; aunque también crece en suelos pobres, sin uso de fertilizantes ni pesticidas químicos, duplicando aún en estas condiciones, su rendimiento en comparación al de la papa	2.400 a 3.700 m.s.n.m.	Alrededor de 700 a 1.200 mm de lluvia anuales Temperatura: 6° a 14 °C (igual que para la papa)	Alta Poder nematicida e insecticida	Moderada	Alta	Barrera et al. (2003) FAO et al. (2007)

Tubérculo	Tipo de suelo, pH	Altura óptima de cultivo	Precipitación lluviosa y temperatura	Resistencia			Referencias
				A plagas	A las heladas	A la sequía	
Melloco (*Ullucus tuberosus*)	Suelos marginales de baja fertilidad, pH ligeramente ácido y alto	2400 a 3700 m.s.n.m.	800 a 1.400 mm de lluvia anuales. Temperatura: 8 y 14 °C	Alta	Alta	Moderada	Basantes (2015) Vimos et al. (1993)
Camote o batata (*Ipomoea batatas*)	Francos a franco arenosos con buena nivelación y bien drenados, pH de 6 a 6.5	100 a 1000 m.s.n.m. A medida que aumenta la altura las cosechas se retrasan	400 a 1400 mm de lluvia anuales. Temperatura: 12 a 30 °C	Moderada. En el caso de nuevos clones desarrollados	Nula	Alta	Daryanto et al. (2016) Barrera et al. (2003)
Zanahoria blanca (*Arracacia xhanthorrrhiza*)	Suelos profundos con buena materia orgánica, bien drenados, con un pH entre 5 y 6	Se puede cultivar desde 200 m a 3600 m.s.n.m., pero se desarrolla mejor entre 1800 a 2500 m.s.n.m.	780 a 1200 mm de lluvia anuales. Temperatura: 15 a 20 °C	Moderada	Nula	Alta	FAO et al. (2007)

1.1.3. Composición y propiedades biológicas

Como se ha indicado anteriormente, los estudios realizados sobre estos tubérculos son escasos. En relación a su composición quizás los fenoles, fructooligosacáridos y almidón son los constituyentes que han despertado un mayor interés.

1.1.3.1. Perfil fenólico

Los fenoles o compuestos fenólicos (PCs) son metabolitos secundarios de las plantas, conformados por al menos un grupo fenol que consiste en un anillo aromático unido, como mínimo, a un grupo hidroxilo (Velderrain- Rodríguez et al., 2014). Los PCs son biosintetizados por la ruta del ácido shikímico, vía del ácido malónico, o por las dos (Crozier et al., 2006). La **Figura 2** muestra los PCs más observados en alimentos.

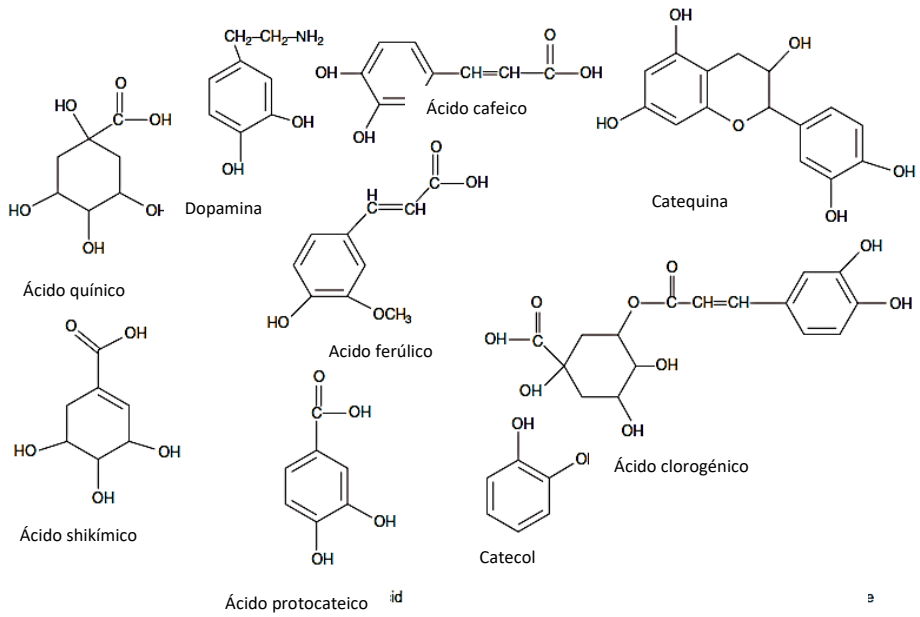

Figura 2. Compuestos fenólicos más comunes en alimentos (adaptado de Corzo-Martínez et al., 2012).

Los PCs actúan como defensa ante herbívoros y patógenos, otros atraen polinizadores y algunos de ellos absorben la radiación ultravioleta, o actúan como agentes alelopáticos (reducen el crecimiento de plantas competidoras que estén cerca) (Dai & Mumper, 2010). Debido a sus propiedades antioxidantes, pueden tener efectos beneficios para la salud actuando como antibacterianos, antivirales, antiinflamatorios o vasodilatadores (Qin et al., 2015).

En tubérculos como la patata, el fenol más abundante es el ácido clorogénico, pero se han descrito más de 10.000 PCs y en los tubérculos andinos mencionados, el perfil fenólico aún no se ha elucidado completamente.

La **Figura 3** muestra de forma esquemática la clasificación de antioxidantes fenólicos.

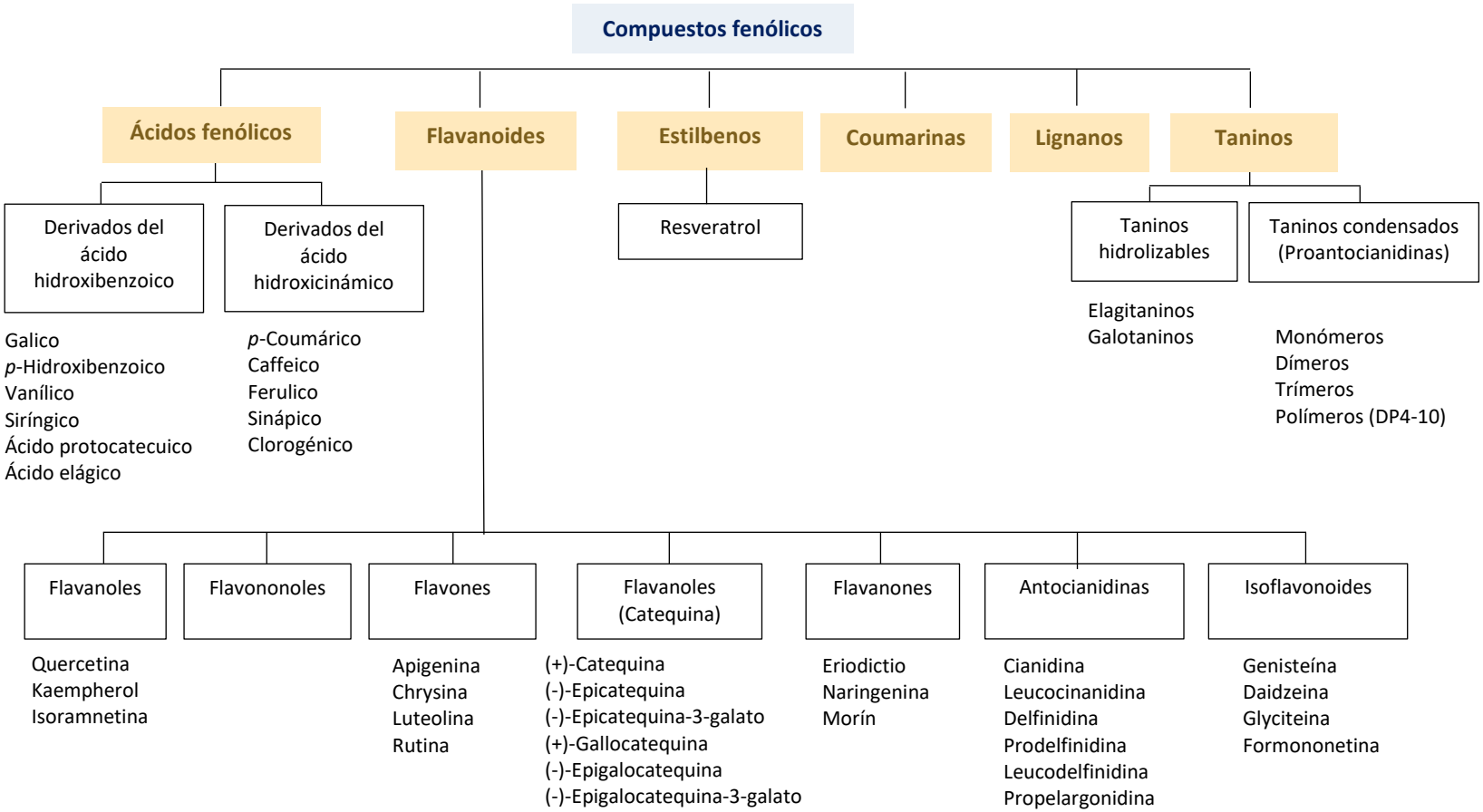

Figura 3. Clasificación de los compuestos fenólicos (adaptado de Shahidi & Ambigaipalan, 2015).

Los pocos estudios realizados sobre estos tubérculos, referidos a compuestos fenólicos y su actividad biológica, se resumen a continuación.

En cinco genotipos de yacón de Nueva Zelanda, Alemania, Ecuador y Bolivia, cultivados en Praga, se ha determinado el contenido de polifenoles totales (TP), inulina y fructosa. El genotipo de Nueva Zelanda con pulpa de color blanco (NZLw) y genotipos de Ecuador (ECU) y Bolivia (BOL) se han distinguido por su alto contenido de fenoles totales (TP) en rizomas y hojas de color púrpura. Los genotipos de Ecuador (ECU) y Bolivia (BOL) han mostrado mayor contenido de inulina en comparación con los genotipos de Alemania (DEU) y Nueva Zelanda (NZLy) (Lachman et al., 2007). También se ha analizado treinta y cinco accesiones diferentes de yacón procedente del Perú, observando contenidos de 7.9–30.8 mg de ácido clorogénico (CAE)/g de DM y una capacidad antioxidante (AC) de 23–136 mol trolox equivalente (TE)/g de DM, pudiendo identificar cultivares de yacón con capacidad antioxidante (AC) para aplicaciones nutracéuticas (Campos et al., 2012). Recientemente, mediante cromatografía líquida de ultra alto rendimiento, acoplada a espectrometría de masas con cuadrupolo tiempo de vuelo (UPLC-QTOF-MS^E) se han observado en jarabe de yacón 25 compuestos fenólicos, siendo los principales, ácido clorogénico y derivados de ácidos quínicos y trans-cinámicos (Gomes da Silva et al., 2018).

En tres genotipos de mashua de diferentes colores cultivada en Perú, mediante cromatografía líquida de alta resolución con detección de diodo array (HPLC-DAD) se ha observado la presencia de ácido gálico, gallocatequina, procianidina B2, epigalocatequina; derivados de epicatequina, hidroxicinámico, ácido hidroxibenzoico, ácido hidroxicinámico, hidroxibenzoico y proantocianidinas (Chirinos et al., 2008a). Además, se ha observado que extractos fenólicos de mashua son capaces de eliminar los radicales peroxilo en ensayos *in vitro,* y que los extractos de mashua de diferentes genotipos inhiben la hemólisis de eritrocitos dentro del rango 20.8-25.1%, pero también se ha señalado que este efecto no guarda correlación con los compuestos fenólicos hallados, pudiendo obedecer a la presencia de otro tipo de fenoles aún no determinados (Chirinos et al., 2008b).

El melloco analizado en un estudio sobre la capacidad antioxidante y metabolitos secundarios de algunos tubérculos endémicos de los Andes, se ha destacado por ser el único cultivo que contiene betalaínas (22–96 µg/g) y betacianinas (64 µg/g), señalando su importancia para uso medicinal (Campos et al., 2006). También, los extractos de melloco se han evaluado a fin de obtener colorantes naturales, observando cambios de color al modificar el pH y la temperatura. El extracto de melloco ajustado a pH 4 mantiene la estabilidad del tono rojo, mientras que a pHs ácidos, reducen el contenido de betalaínas amarillas (Cejudo-Basantes, 2014). Más recientemente, se ha observado que los extractos de melloco generan un aumento en la proliferación y migración de fibroblastos dérmicos humanos y la producción de pro-colágeno, concluyendo que *U. tuberosus* es un candidato prometedor para ayudar a la regeneración de tejido sin cicatrices (Heil et al., 2017); pero, no se describen las causas de dicho efecto, asociadas a la composición química del tubérculo.

En el caso del camote, batatas moradas de tres procedencias analizadas mediante HPLC-DAD-MS^n han mostrado gran variación de contenido antociánico total e individual, y al adicionar ácido clorogénico, rosmarínico, extractos de romero y manzana, se ha logrado variar el pk del extracto entre 3,28 a 4,71, ampliando su rango de aplicación entre pH 2,6 a 4,6, pudiendo observar colores: azul violáceo, rosa claro, magenta, rojos ladrillo e intensos (Gras et al., 2017). Adicionalmente, mediante espectrometría de masas por ionización electrospray (ESI-MS), se han analizado los perfiles metabólicos de cinco variedades de batatas de diferente color de pulpa cultivadas en China, e identificado 29 flavonoides y 27 ácidos fenólicos. En dicho trabajo, se logró determinar que además de las antocianinas, los ácidos quínico y ferúlico, el *O*-hexósido de quercetina y el crioseriol, fueron los flavonoides prominentes, indicando que existen diferencias en los perfiles de metabolitos entre batatas con diferentes colores de pulpa (Wang et al., 2018).

En cuanto a la zanahoria blanca, Pedreschi et al. (2011) observaron en las raíces de arracacha una pérdida significativa de compuestos bioactivos fenólicos (ácidos cafeico, clorogénido y derivados) durante el calentamiento con aire. Además de este

estudio, no se ha encontrado más información hasta el momento, referida al tipo y cuantificación de fenoles individuales presentes en este tubérculo, crudo o cocido.

1.1.3.2. Fructooligosacáridos y almidón

Los tubérculos en general son ricos en almidón (Hoover, 2001), aunque algunos, dependiendo del tipo y estado de madurez, pueden contener otros carbohidratos como son los fructooligosacáridos (FOS).

El almidón es un importante ingrediente alimentario debido a su aplicabilidad, abundancia y bajo coste. Se agrega principalmente para proporcionar textura, viscosidad, consistencia y apariencia grasa. A pesar de que la naturaleza presenta varias fuentes de almidón, solo cuatro almidones están disponibles comercialmente para satisfacer las demandas del mercado (arroz, patata, yuca y maíz) (Vilpoux et al., 2019).

Desde el punto de vista químico, el almidón es un polisacárido compuesto por amilosa y amilopectina, que representan el 98-99% del peso seco (Tester et al., 2004). Tanto la amilosa como la amilopectina son polímeros de glucosa, unidos mediante enlaces α-1,4 (amilosa), y ramificaciones α-1,6 (amilopectina) (Ao et al., 2007). La amilopectina tiene una mayor tasa de digestión, debido a que las enzimas digestivas (**Figura 4**) alcanzan múltiples extremos reductores, mientras que la amilosa tiende a formar agregados semicristalinos insolubles durante el procesamiento y es menos digerible (Copeland et al., 2009).

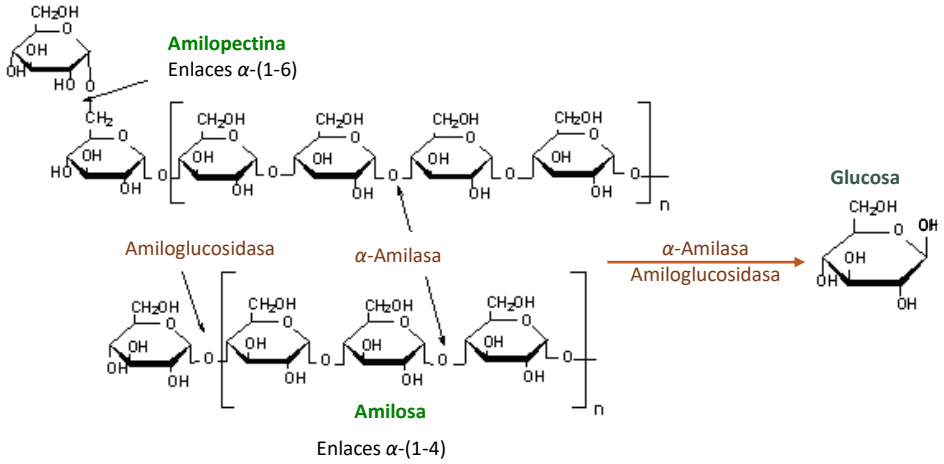

Figura 4. Hidrólisis de almidón llevada a cabo mediante enzimas digestivas (Adaptada de Simic & Maffezzoli, 2018).

Los FOS son oligosacáridos lineales naturalmente presentes en muchas plantas formados por monómeros de fructosa unidos por enlaces β-(1→2) que pueden contener una molécula inicial de glucosa producidos por una amplia gama de bacterias, levaduras u hongos (**Figura 5**). Los FOS en las plantas, después del almidón y la sacarosa, son los carbohidratos de almacenamiento más abundantes, y están presentes en, aproximadamente, el 15% de las plantas sin flor (Holck et al., 2014), aunque su rendimiento puede verse limitado por restricciones estacionales o despolimerización enzimática en la cosecha (van Arkel et al., 2013).

Su función contra el estrés por la sequía y el frío, así como su papel prebiótico, otorga gran importancia a estos compuestos (Vaňková et al., 2008; Livingston et al., 2009). Su actividad prebiótica es debida a su capacidad para escapar de la digestión y absorción en la parte superior del intestino y alcanzar el colon para inducir la estimulación del crecimiento y/o actividad de bacterias intestinales beneficiosas (Qiang et al., 2009).

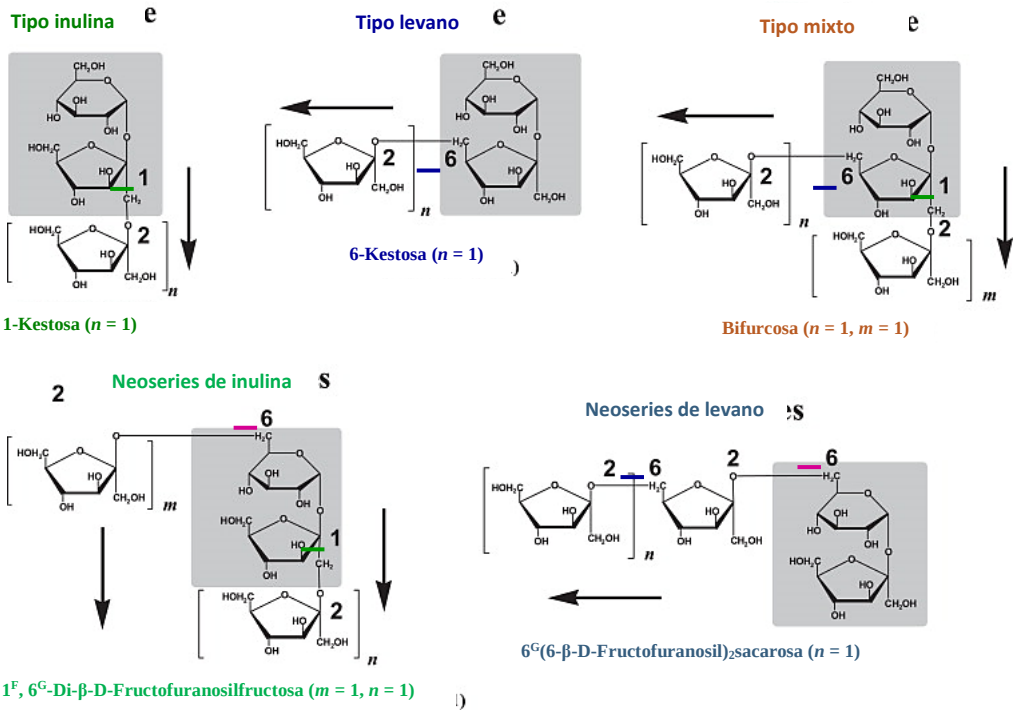

Figura 5. Estructuras moleculares de los diferentes tipos de fructooligosacáridos encontrados en plantas superiores (adaptado de Benkeblia, 2013).

A continuación, se resumen los estudios más importantes, documentados hasta el momento, sobre determinación de almidón y/o FOS en el yacón, mashua, melloco, camote y zanahoria blanca.

Grancieri et al. (2017) han investigado los efectos intestinales de la alimentación con FOS de harina de yacón al 7.5%, durante 8 semanas, en ratas Wistar macho con cáncer de colon rectal (CCR) inducido por 1,2-dimetilhidracina (25 mg/kg/peso corporal), observando reducción de criptas aberrantes, menor permeabilidad intestinal y concentraciones más altas de ácidos grasos de cadena corta (SCFA). Estos resultados les permitió concluir que la harina de yacón promueve efectos beneficiosos sobre la salud intestinal de animales con CCR inducido. En un estudio más reciente, se ha observado que el yacón consumido por adultos sanos (21 g de harina de yacón/350 ml ⇔ 7.4 g de FOS) en dos días no consecutivos, no afectó a la respuesta glucémica, ni al apetito o la ingesta de alimentos, señalando que quizá podrían verse efectos mayores después de su consumo crónico, y que hacen falta

estudios para evaluar el efecto de su consumo a largo plazo sobre la respuesta de la glucosa y el peso corporal (Usuda Prado Rocha et al., 2018). También se ha determinado que extractos acuosos de yacón están constituidos por FOS lineales conformados casi exclusivamente por unidades de *β*-fructofuranosilo unidas por enlace (2 → 1) a unidades terminales de *α*-glucopiranosilo y *β*-fructofuranosilo, y que el efecto de los FOS lineales del yacón sobre la actividad inmune no específica de células THP-1 fue más efectivo que el de los FOS ramificados, reforzando el uso de los lineales como inmunomoduladores (Paredes et al., 2018). En ratas Wistar, Fabersani et al. (2018) han observado que la ingesta de un yogurt de cabra + 7% de harina de yacón durante 30 días tras una dieta rica en grasa, dio como resultado menor peso corporal, índice de masa corporal, nivel de glucosa en ayunas, e índice aterogénico (colesterol total/lipoproteínas de alta densidad HDL) (p <0.05). Estos autores concluyeron que el yogur de cabra + yacón es un excelente alimento funcional que evita el impacto metabólico del alto consumo de grasa.

En el almidón de mashua y melloco cultivado en el Perú, se ha observado, mediante microscopía electrónica de barrido (SEM), gránulos de hasta 16.29 µm y mayores a 32.09 µm, con contenidos de amilosa del 27.44% y 26.49%, respectivamente. Se obtuvieron geles opacos y poco firmes y se concluyó que puede ser usado en formulaciones que requieran temperaturas de procesamiento suaves (Valcárcel Yamani et al., 2013). Empleando almidón de melloco cultivado en Colombia, se han observado características satisfactorias en películas comestibles (solubilidad, permeabilidad al vapor de agua, resistencia a la tracción, fuerzas de rotura y transparencia), señalando que este tipo de almidón tiene un enorme potencial para reemplazar el uso de envases plásticos no biodegradables (Daza et al., 2018). En otro ámbito, Mosso et al. (2018) han indicado que el melloco podría utilizarse como nuevo sustrato para el crecimiento de cepas productoras de folato con alto potencial probiótico (supervivencia tras digestión simulada: 8.0 log UFC/mL), siendo *Lactobacillus sakei* (CRL 2209 y CRL 2210) las bacterias que produjeron las concentraciones más altas de esta vitamina (730-1484 ng/g, en 24 h) de entre 5 cepas ensayadas.

En el camote, durante el tratamiento con alta presión hidrostática (HHP), se ha observado que la concentración de azúcares reductores aumenta a medida que se incrementa la presión y la temperatura (100 a 500 MPa y 60 a 70 °C / 10 min), y que la gelatinización y la sacarificación enzimática del almidón tiene lugar a una temperatura inferior a 80 °C a presión atmosférica (Shigematsu et al., 2017). También se ha estudiado el mecanismo de su pardeamiento interno (IB), determinando que este proceso comienza en las raíces desde aproximadamente 90 días después del trasplante y aumenta significativamente con el tiempo, existiendo mayor cantidad de almidón y altos niveles de azúcares reductores, actividad de polifenol oxidasa (PPO), ácido clorogénico y peróxido de hidrógeno (H_2O_2), en las regiones afectadas. Estos resultados indicaron que el IB en el camote depende en gran medida de la generación excesiva de especies reactivas de oxígeno (ROS) alrededor de los tejidos vasculares secundarios, debido a la abundante acumulación de azúcar y/o almidón durante el período de maduración de la raíz (Fukuoka et al., 2018). También se ha estudiado la modificación de almidón de camote, mediante adición de ácido cítrico y succinilación de octenilo, observando correlación entre el nivel de sustitución frente a las propiedades fisicoquímicas y digestibilidad *in vitro*, así como un aumento significativo en el contenido de almidón resistente (RS) y almidón de lenta digestión (SDS) en comparación con el almidón nativo (Remya et al., 2018b).

El almidón de zanahoria blanca, según ensayos *in vitro*, presenta un alto contenido de RS (14-56%, base seca) a diferencia del almidón de yuca (Lovera et al., 2017). El almidón de zanahoria blanca (*Arracacia xanthorrhiza* Bancroft) cultivada en el Perú está constituido por gránulos redondos o poligonales, con un diámetro medio de ~20 µm, ricos en amilopectina, de baja temperatura y entalpía de gelatinización, y con tendencia a la retrogradación incluso a temperaturas moderadas (60 °C), formando geles claros, lisos y elásticos. Estos resultados apuntan al uso potencial de estas raíces como alternativa para la extracción de almidón (Castanha et al., 2018).

1.2. Subproductos de la industria agroalimentaria

Cuando los residuos de la agroindustria son aprovechados, habitualmente se usan para la obtención de compost, piensos para animales o biomasa para la generación de combustibles (Soratheme, 2018). Sin embargo, una alternativa novedosa y de mayor beneficio para el ser humano, en términos de preservar su salud, es la obtención de compuestos bioactivos, útiles para la industria química, biomédica y de alimentos funcionales. Los *compuestos bioactivos* pueden ser definidos como *"*componentes de los alimentos que influyen en las actividades fisiológicas o celulares de los animales o seres humanos que los consumen" (Wang et al., 2016).

Cuando se selecciona la alternativa más apropiada para el aprovechamiento de un residuo agroindustrial, es necesario conocer su composición y tener en cuenta que, de este proceso, se puede producir un residuo más agotado, que quizá puede tener otra aplicación o convertirse en otro desecho (Saval, 2012).

Un 45 % de raíces y tubérculos y un 45 % de frutas y hortalizas se desperdician y, aproximadamente, el 50 % de estas pérdidas tienen lugar durante el procesamiento, distribución y consumo (Asambla, 2018). Entre los tubérculos y frutas de mayor importancia debido a su uso industrial y volumen de residuos, podemos mencionar la remolacha azucarera y la naranja, cuyos subproductos merecen especial atención a fin de lograr un adecuado aprovechamiento.

1.2.1. Subproductos del procesado de remolacha azucarera

El consumo anual de azúcar en el mundo ronda los 120 millones de toneladas y aumenta a un ritmo de 2 millones de toneladas al año, siendo la remolacha azucarera (*Beta vulgaris* L. subsp. *vulgaris* var. *altissima* Döll) fuente de casi el 30% de esta producción (Dohm et al., 2014; Nieberl et al., 2017).

Europa es uno de los principales productores de remolacha azucarera, cuyo cultivo equivale al 50% de toda la producción a nivel mundial generando un volumen de residuos que alcanza los 111,6 millones de toneladas al año (Eurostat, 2017). En España es la única

materia prima a partir de la cual se obtiene azúcar, proceso que genera 3.000 toneladas de residuos anuales (Smit et al., 2017) (**Figura 6**).

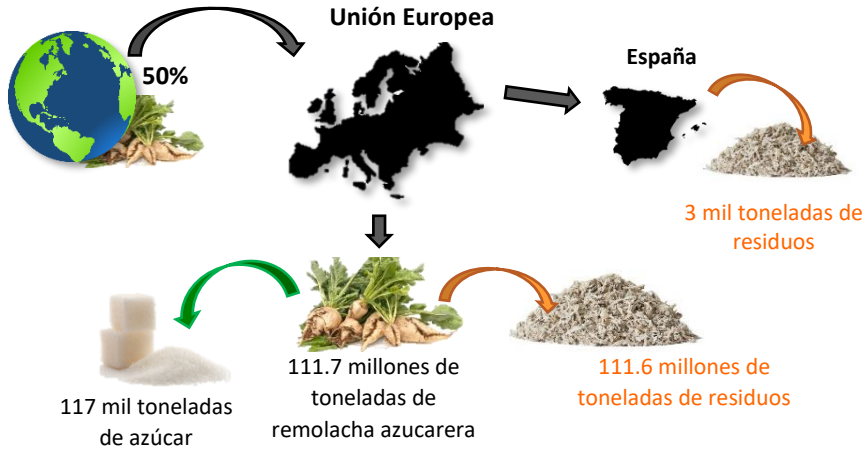

Figura 6. Volumen de residuos de remolacha azucarera generados al año en Europa y España (Datos tomados de Eurostat, 2017) (Elaboración propia).

1.2.1.1. Proceso de extracción de azúcar a partir de remolacha azucarera

El proceso de extracción de azúcar a partir de remolacha genera como subproductos primarios la melaza agotada y la pulpa de remolacha azucarera (SBP), que suele ensilarse y secarse. La SBP, a pesar de su deficiencia en nitrógeno, presenta gran interés para la generación de biogás debido a su alto contenido de material rico en carbono como hemicelulosa, celulosa, lignina y azúcares solubles (Aboudi et al., 2016). La melaza agotada y la pulpa seca se mezclan y se usan, normalmente, como un suplemento para la alimentación animal (Hutnan et al., 2000), mientras que la pulpa prensada, fresca, ensilada o seca, generalmente, suelen destinarse a venta directa, siendo importantes subproductos agroindustriales, aún muy poco estudiados.

Durante el ensilado es esencial asegurar una buena fermentación que permita inhibir el desarrollo de microorganismos nocivos. En los países desarrollados, es el método más empleado para la conservación de forrajes. No obstante, en países subdesarrollados, muchos alimentos y subproductos agroindustriales son

desperdiciados (FAO, 2018) quizá, en gran parte, debido al desconocimiento del manejo del proceso de ensilado y de los cambios químicos que pueden presentarse sobre los productos almacenados. Pese a su importancia, el ensilaje de subproductos ha recibido poca atención por parte de investigadores y organismos agrícolas gubernamentales (FAO-Eurostat, 2018).

1.2.1.2. Aprovechamiento de subproductos de remolacha azucarera

Los residuos de la remolacha azucarera han sido empleados en la producción de metano para compensar el consumo de energía empleado en la extracción de azúcar (Bevill, 2008). Por otro lado, varios autores han estudiado el fraccionamiento de SBP para obtener ácido ferúlico (Bonnin et al., 2002; Saulnier & Thibault, 1999), arabinoxilanos y/o sustancias pécticas (Leijdekkers et al., 2013; Spagnuolo et al., 2000).

La *pectina* es un importante heteropolisacárido aniónico, existente en las paredes celulares de las plantas dicotiledóneas (Agoda-Tandjawa et al., 2012), que comprende polímeros conformados por unidades de ácido galacturónico (GalA) enlazados en las posiciones α-1,4; que conforman estructuras conocidas como homogalacturonano (HG ≈ 65%), ramnogalacturonano I (RG-I ≈ 20-35%), ramnogalacturonano II (RG-II ≈ 2-10%) y xilogalacturonano (XG ≈ 2-8%) (Holck et al., 2014; Noreen et al., 2017) (**Figura 7**).

En la industria alimentaria, la pectina se usa generalmente como estabilizante, espesante y gelificante (Kaya et al., 2014). Sin embargo, estudios *in vitro* e *in vivo* con animales han demostrado que puede ejercer actividades biológicas relevantes (Park et al., 2017). Sus propiedades tecnológicas y/o funcionales pueden depender, principalmente, de características como el contenido de azúcares neutros, el grado de esterificación (DE) (Fishman et al., 2008; Siew & Williams, 2008), tipo de carga (Mandala & Bayas, 2004) y peso molecular (Mw) (Schmidt et al., 2015).

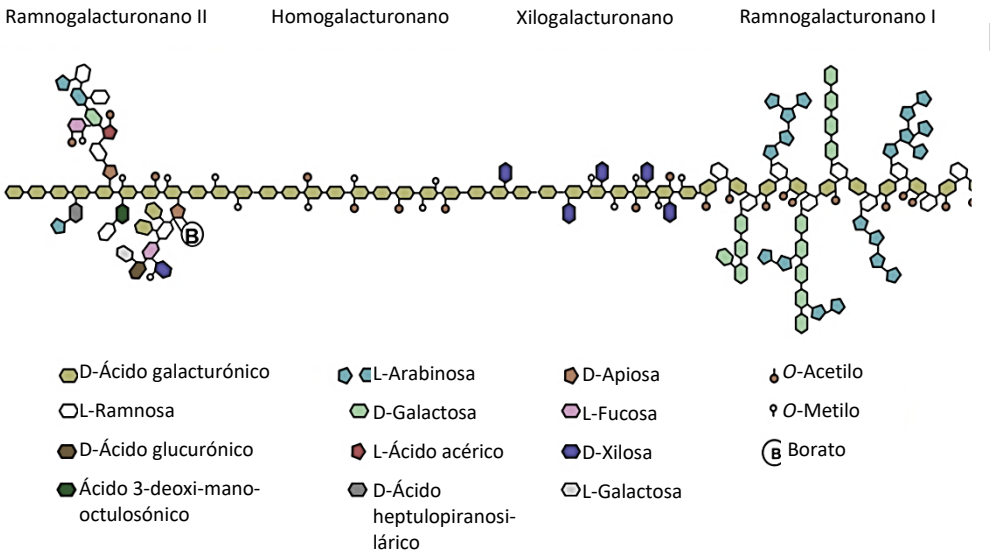

Figura 7. Estructura esquemática de la pectina (Adaptada de Harholt et al., 2010).

La **Tabla 2** resume los estudios más importantes llevados a cabo en las últimas décadas, acerca de extracción de pectina a partir de remolacha azucarera.

Tabla 2. Estudios más relevantes documentados hasta el momento, sobre extracción de pectina de remolacha azucarera.

Materia prima	Objetivo	Método de extracción	Resultados	Referencia
SBP prensada. La pulpa se secó en horno a 40 °C durante 24 h (H_2O 2%)	Extraer pectina de SBP utilizando agua subcrítica combinada con un tratamiento asistido por ultrasonidos; utilizando la metodología de superficie de respuesta	Pretratamiento con ultrasonidos (frecuencia 25 kHz, sonicación 10 min; Intensidad de 1 W/cm^2) y extracción con agua subcrítica	Las condiciones óptimas de modificación fueron relación líquido/sólido 44,03, temperatura de extracción 120,72 °C, tiempo de extracción 30,49 min y presión de extracción 10.,0 MPa; el rendimiento máximo fue de 24,63% y los contenidos de ácido galacturónico y arabinosa fueron 59,12% y 21,66%, respectivamente	Chen et al. (2015)
SBP secada en estufa a 45 °C por 24 h	Investigar el efecto de la temperatura (75 y 85 ° C), el tiempo (2 y 3 h) y el pH inicial (1,5 y 2,0) sobre la composición molecular de la pectina de remolacha azucarera extraída con ácido cítrico	Ácido. pH 1,5 o 2,0 con ácido cítrico. Temperatura ambiente y luego a 75 °C u 85 °C por 2 a 3 h	El pH de la solución no fue constante durante la extracción de pectina. El pH inicial fue el factor más significativo en la composición de la pectina, como la arabinosa, los azúcares neutros totales y el grado de acetilación, mientras que la temperatura influyó más sobre el rendimiento, ácido galacturónico, ramnosa, galactosa y peso. El grado de metoxilación se vió significativamente afectado por el tiempo de extracción. Las propiedades de emulsión de la pectina extraída también se vieron afectadas significativamente por las condiciones de extracción	Guo Xiaobing et al. (2017)

Materia prima	Objetivo	Método de extracción	Resultados
SBP en forma de pellets (8% H_2O)	Evaluar la posibilidad de extracción de pectina utilizando descargas eléctricas de alto voltaje (HVED), como tecnología de tratamiento previo	Ácido con pretratamiento (HVED). pH 1,5 a 7 con HCl (1 M). 60 a 90 °C	Se variaron los parámetros de HVED (amplitud de pulso U y número de pulsos n) y las mejores condiciones de tratamiento previo fueron U = 40 kV y n = 100. Después de este tratamiento, se llevó a cabo una extracción de pectina con agua acidificada, variando el pH y la temperatura; observando un aumento del rendimiento de pectina de 42,6% a 53,4% al aplicar HVED a 90 °C, pH 2 y duración de una hora. La pectina obtenida con o sin pretratamiento, mostró una composición química similar
SBP secada con aire caliente (40, 50, 60 °C), al vacío (40, 50, 60 °C), por congelación, y por aspersión (160, 190, 220 °C)	Investigar el efecto de diferentes condiciones de secado de pulpa de remolacha azucarera, sobre las características de la pectina extraída	Ácido. pH 1.2 con HCl (12 M) 90°C	Las condiciones de secado afectaron la viscosidad aparente, la energía de activación y las propiedades emulsionantes de todas las muestras de pectina. El potencial zeta y la conductividad para diferentes muestras secas fueron de −47,9 a −55,6 mV y de 0,0079 a 0,0095 mS/cm, respectivamente

Materia prima	Objetivo	Método de extracción	Resultados
SBP	Evaluar el efecto del tamaño de partícula sobre el rendimiento y las propiedades de la pectina extraída	Ácido. pH 1,5 con HCl (12 M). 90°C	Al disminuir el tamaño de partícula de 406,33 a 24,93 µm, el rendimiento de extracción aumentó de 15,81% a 20,50% y el contenido de ácido galacturónico aumentó de 38,51% a 59,97%; pero, a mayor tamaño de partícula, la viscosidad, y el peso molecular de la pectina SBP aumentaron significativamente
SBP secada a 45 °C durante 24 h	Estudiar el efecto del tipo de extractante y los pasos de extracción sobre las propiedades estructurales y emulsionantes de las pectinas de remolacha azucarera	Extracción con oxalato de amonio y ácido sulfúrico	Dos pectinas extraídas con oxalato (denotadas OEP1 y OEP2) y cuatro pectinas solubilizadas en ácido (AEP1, AEP2, AEP3 y AEP4) se extrajeron secuencialmente y se caracterizaron. Las seis pectinas tuvieron un alto contenido de ácido galacturónico y un bajo grado de metilación, sin embargo, OEP1 y OEP2 no solo tenían cantidades más bajas de ácido ferúlico (FA) y proteína, sino que sus tamaños moleculares también fueron más pequeños en comparación con el resto. Además, las pectinas extraídas con oxalato tuvieron diferente composición de aminoácidos en comparación con las pectinas extraídas con ácido

1.2.2. Subproductos de la elaboración de zumo de cítricos/naranja

En 2016 la producción mundial de cítricos alcanzó 124,2 millones de toneladas (FAO, 2016), siendo China el mayor productor (30,2 millones de toneladas), seguido de la región mediterránea, en la que España se caracteriza por ser el mayor productor de cítricos de Europa y el sexto a nivel mundial (7 millones de toneladas; 2016-2017) (Statista, 2018).

La distribución geográfica de la producción de cítricos varía en los diferentes países (**Figura 8**), siendo la de naranja la más elevada a nivel mundial. Brasil es el productor dominante de naranja dulce y los países de la Unión Europea son importantes productores de naranjas dulces, limones y mandarinas (Kimball, 2012).

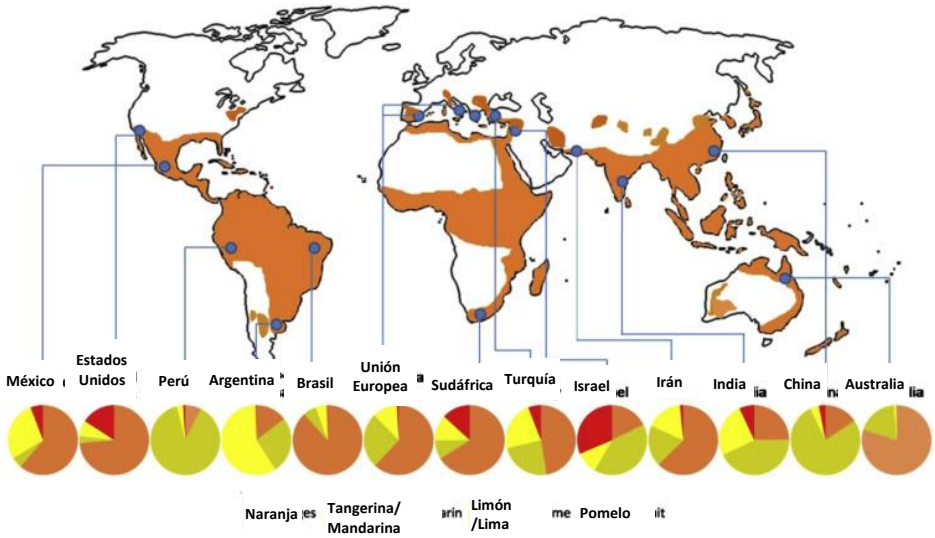

Figura 8. Distribución geográfica de la producción de los principales cítricos (2007-2016). (Adaptada de Sharma et al., 2017).

La producción mundial de naranjas se acerca a los 50 millones de toneladas, de los cuales, aproximadamente, el 40% es utilizado por la agroindustria para extraer cerca de la mitad del peso de la fruta como zumo. El resto se convierte en residuo, constituyendo un volumen estimado de 10 millones de ton/año. De modo que, tan solo en España, la cantidad de residuos derivados de la extracción de zumo de naranja asciende a 600.000 ton/año (FAO, 2012) (**Figura 9**).

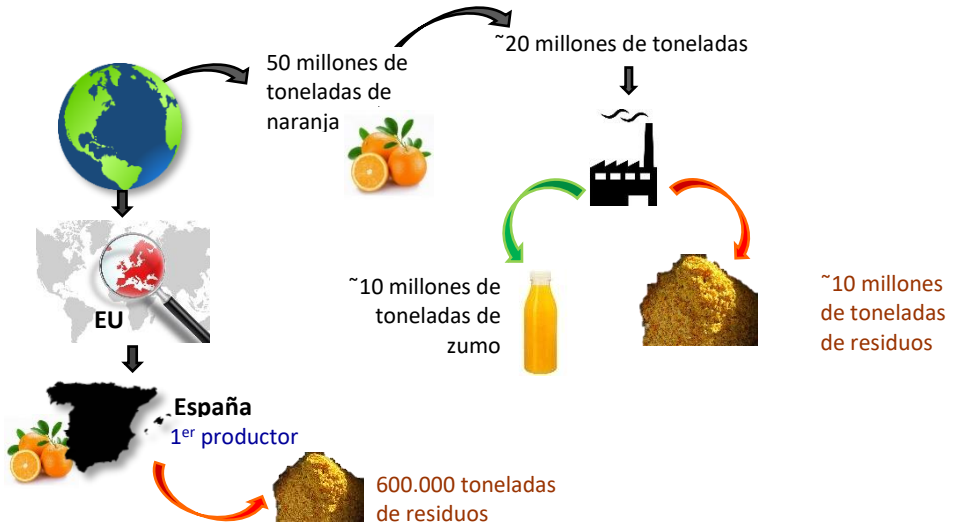

Figura 9. Producción de naranja y volumen de residuos anual, generado en el proceso de extracción de zumo (FAO 2012) (Elaboración propia).

1.2.2.1. Proceso de extracción de zumo de naranja

El proceso industrial de extracción de zumo de naranja genera varios subproductos, que en el mejor de los casos, son transformados en alimento para animales. Cuando esto sucede, se obtiene un residuo fresco, la melaza o licor de naranja, un residuo seco y el pienso de naranja. La aplicación de altas temperaturas durante la transformación de estos subproductos puede alterar la composición química de los mismos, siendo un factor importante a tener en cuenta al momento de evaluar su aprovechamiento. La **Tabla 3** resume los estudios más importantes documentados hasta el momento, acerca de cambios o reacciones químicas observados en subproductos de naranja.

1.2.2.2. Aprovechamiento de subproductos de naranja

Algunos de los subproductos industriales de la extracción de zumo de naranja, han sido empleados en las últimas décadas como fuente de obtención de compuestos químicos y/o bioactivos, como se resume en la **Tabla 4**.

Tabla 3. Estudios recientes sobre reacciones químicas y/o cambios observados en subproductos de naranja.

Subproducto	Reacción o proceso estudiado	Resultados	Referencia
Fragmentos y extracto acuoso crudo cáscaras de naranja	Hidrólisis de ésteres de la naranja, por acción de enzimas	La aplicación de enzimas sobre piezas y extractos acuosos de cáscara de naranja permitió la hidrólisis enantioselectiva de alcoholes quirales racémicos con un considerable exceso enantiomérico y buenas tasas de conversión (13 a 81%)	Maia da Silva et al. (2016)
Piel de naranja, banano y papaya	Fermentación abierta de subproductos de naranja	Las actividades más altas de las enzimas pectinasa y lipasa (2.817, 1.870 U/g de sustrato seco) se encontraron al emplear piel de naranja, mientras que las actividades más bajas se observaron al emplear piel de banano y piel de papaya (1.662 U/g y 1.266 U/g, respectivamente); hallazgos que ayudarán a diseñar y desarrollar procesos de producción de enzimas aprovechando residuos de naranja	Sattar Qureshi et al. (2017)
Albedo y flavedo de naranja	Emisión de compuestos orgánicos volátiles (COV), durante el secado de albedo y flavedo de naranja	Los principales compuestos emitidos durante el secado del albedo y flavedo de naranja fueron *d*-limoneno, 3-careno y α-pineno con una tendencia a aumentar la emisión con el aumento de la temperatura. La emisión de COV se produjo por destilación de vapor aplicada entre la temperatura ambiente y 160 °C, y por destilación de vapor seguida de evaporación por efecto de degradación térmica, a temperaturas entre 160 y 250 °C	Pinheiro et al. (2018)

Subproducto	Reacción o proceso estudiado	Resultados
Subproductos de guayaba, naranja y fruta de la pasión	Actividad prebiótica de residuos de guayaba, naranja y fruta de la pasión	Los subproductos de fruta mostraron un contenido total de fibra, compuestos fenólicos y actividad antioxidante que varió de 58,20-89,80%, 253,14-420,89 mg GAE/100 g, y 11,38-17,37 µmolTE/g, respectivamente. La presencia de subproductos de fruta incrementó el tiempo de fermentación de las bebidas de arroz (0,28 a 0,91 h), así como la acidificación a lo largo del almacenamiento; sin embargo, no afectó el recuento de bacterias probióticas. La presencia de subproductos de la naranja y de la fruta de la pasión mejoró la resistencia de los probióticos a condiciones gastrointestinales simuladas y dio como resultado una población de 2 log UFC/ml, más alta que el tratamiento control, señalando que los subproductos analizados pueden considerarse fuentes de compuestos bioactivos útiles para elevar los atributos funcionales de los productos fermentados probióticos

Tabla 4. Estudios recientes sobre el uso de subproductos de naranja en la obtención de compuestos químicos y/o bioactivos.

Materia prima	Objetivo y/o método	Resultados	Compuesto/s obtenidos	Referencia
Residuos de la extracción de jugo de toronja (Ruby y Marsh), limón (Eureka y Fino 49), naranja (Valencia) y manzana Royal Gala (Granny Smith y Liberty)	Evaluar algunas propiedades funcionales de concentrados de fibra a partir de residuos de manzana y cítricos. *Triturado (500-600 µm), lavado y secado (<65°C)	Todos los concentrados de fibra tuvieron un alto contenido en fibra dietética (entre 44,2 y 89,2 g/100 g de MS). Los valores calóricos de los concentrados fueron bajos (50,8-175 kcal/100 g o 213-901 kJ/100 gramos). La textura fue dependiente del tamaño de partícula y del tratamiento térmico. Cada concentrado tuvo características interesantes, sugiriendo posibles usos en el desarrollo de alimentos enriquecidos con fibra	Fibra	Figuerola et al. (2005)
Cáscara de naranja, pulpa y semillas	Estudiar la aplicación de residuos de naranja, como sustituto de grasa en helados. *Obtención de fibra: cortado (1cm^2) desinfección con hipoclorito de sodio (150 mg / L*10 min), secado a 60 ° C/4 h, enfriado y molido. Tamaño <125 µm (malla 115)	Los subproductos F1 (cáscara, pulpa y semillas) y F2 (cáscara) mostraron altos niveles de fibra dietética total y una relación ideal entre fibra soluble e insoluble. Las fibras mostraron una alta retención de agua y aceite, además de alto contenido de compuestos fenólicos y carotenoides. El uso de la fibra de naranja en el helado llevó a una reducción del 70% de la grasa sin causar cambios significativos en el color, el olor y la textura	Fibra	de Moraes Crizel et al. (2013)

Materia prima	Objetivo y/o método	Resultados	Compuesto/s obtenidos	Referencia
Cáscara de naranja agria (*Citrus aurantium* L.)	Obtener pectina a partir de cáscara de naranja agria. *Agua destilada, relación líquido/sólido 20:1, 30:1 y 40:1 (v/w), a 75, 85 y 95 °C, por 30, 60 y 90 min. Purificación con etanol y secado a 50°C/16 h	El mayor rendimiento de extracción (17,95 ± 0,3%) se obtuvo a 95 °C/ 90 min y relación líquido-sólido de 25 (v/w). Se obtuvo pectina de bajo metoxilo (17% - 30,5%), y de una actividad emulsificante del 45%. Las emulsiones fueron estables en un 86,6% a 4 °C y 71,4% a 23 °C, después de 30 días de almacenamiento	Pectina	Hosseini et al. (2016)
Cáscara de naranja desgrasada	Obtener flavonoides no polares a partir de cáscara de naranja desgrasada, mediante extracción con agua subcrítica (SWE). *Temperatura (110 - 150 ° C) y caudal de agua (10 - 30 mL/min).	Se obtuvieron hesperidina y narirutina a 150 °C y 10 mL/min; los cuales representaron aproximadamente el 21% de la cantidad total de flavanonas en los extractos. La SWE se comparó con tres métodos de extracción convencionales, demostrando ser un método altamente eficiente para la recuperación de estos antioxidantes.	Hesperidina, narirutina	Lachos-Pérez et al. (2018)
Pulpa de cítricos prensada	Obtener productos de alto valor agregado a partir del residuo de pulpa de cítricos prensado	Después de aplicar un cóctel de enzimas aislado de *Xanthomonas axonopodis* pv., se obtuvo etanol mixto, con un rendimiento de casi el 100%; hesperidina mediante extracción líquido-sólido y precipitación, con un rendimiento de 1,2% y 92,6% de pureza; y nanocelulosa con más del 98% de pureza.	Etanol, hesperidina, nanocelulosa	Cypriano et al. (2018)

Materia prima	Objetivo y/o método	Resultados	Compuesto/s obtenidos
Piel de naranja	Obtener compuestos volátiles y fenólicos de cáscara de naranja mediante extracción con fluidos supercríticos (SFE) y extracción con líquidos presurizados (PLE). *SFE con CO_2 a 40 °C y 35 MPa. *PLE con etanol absoluto y mezclas de etanol y agua a 45, 55 y 65 °C y presión de 10 MPa	El principal compuesto volátil obtenido mediante SFE fue *α*-terpineol, seguido de d-limoneno, y el principal compuesto fenólico fue la hesperidina, obtenida empleando un 75% de etanol a 65 °C, concluyendo que la PLE a base de agua y etanol se puede aplicar para recuperar compuestos fenólicos de una gran variedad de subproductos de fruta	α-terpineol, d-limoneno, hesperidina
Cáscara de naranja	Investigar la extracción de compuestos polifenólicos de la cáscara de naranja mediante extracción sólido-líquido (LES) usando solventes eutécticos profundos (DES): cloruro de colina, glicerol y etilenglicol	En condiciones óptimas (DES 10% en peso, 333.15 °K, 1:10 sólido/líquido y 100 min), el cloruro de colina superó como extractante al solvente de referencia (etanol acuoso 30% en peso), siendo el ácido ferúlico el compuesto más abundante, seguido del ácido ρ-cumárico y el ácido gálico	Compuestos fenólicos
Cáscaras de naranja dulce (*Citrus sinensis*)	Comparar procesos de extracción asistida por microondas (MAE) y procesos convencionales para la obtención de aceites esenciales de naranja	Se determinó que la hidrodifusión y gravedad por microondas (MHG) sería operacional y rentable para la obtención de aceites esenciales de naranja, señalando que los procesos de extracción por microondas y vapor serían los mejores, seguidos de aquellos solo con microondas	Aceite esencial de naranja

2. Justificación y Objetivos

2. JUSTIFICACION Y OBJETIVOS

En los últimos años se ha observado que, pese a los múltiples esfuerzos de organismos internacionales, el índice mundial de hambre y desnutrición en lugar de disminuir ha ido aumentando, al mismo tiempo que el cambio climático se ha agravado. Esto ha generado la necesidad de buscar nuevas alternativas agrícolas de fácil adaptación a suelos marginales, resistentes a plagas y que, además, representen una potencial fuente de nutrientes y energía, sobre todo en regiones de ingresos medios y bajos.

Por otro lado, también se ha incrementado el interés de la población hacia el consumo de alimentos que, además de proporcionar componentes básicos necesarios para nutrirnos y desarrollarnos adecuadamente, promuevan el cuidado de la salud, respecto a enfermedades crónicas de alta incidencia a nivel mundial.

En este sentido, la Región Andina ofrece un sinnúmero de opciones agrícolas aún no exploradas o subexplotadas, como son los tubérculos andinos (yacón, mashua, melloco, camote y zanahoria blanca) que, pese a las valiosas ventajas de cultivo que poseen, en algunos sectores se cultivan apenas como cultivos de subsistencia, debido al desconocimiento de su potencial alimentario y como fuente de compuestos bioactivos. A esta falta de estudios pormenorizados sobre su composición química, se une que, como en otros vegetales, dicha composición puede variar mucho dependiendo del origen, condiciones de cultivo incluyendo tipo de terreno y altitud y de la variedad. Por todo ello, resulta necesario llevar a cabo una exhaustiva caracterización con el fin de conocer si son fuentes de nutrientes y componentes bioactivos o compuestos interesantes desde el punto de vista tecnológico, tales como los fructooligosacáridos, el almidón y los compuestos fenólicos.

Al mismo tiempo, como parte del rápido crecimiento agroindustrial, millones de toneladas de residuos orgánicos son desechados al medio ambiente causando un daño

irreparable, o desaprovechando recursos que quizá podrían usarse como materia prima para la extracción de compuestos con propiedades importantes.

Estos subproductos, según su proceso de transformación en la industria, que puede involucrar procesos de fermentación como sucede en el caso del ensilado y/o degradación de nutrientes ante altas temperaturas durante procesos de secado, pueden mostrar cambios en su composición química, siendo importante evaluar el efecto en estos procesos sobre la calidad de los compuestos extraídos. Estudios sobre el aprovechamiento de subproductos de remolacha azucarera, solo han considerado, hasta el momento, el residuo fresco prensado, obteniendo pectina generalmente por el método ácido, el cual presenta una serie de desventajas operacionales y de calidad sobre el producto final.

Respecto al aprovechamiento de residuos de naranja, los estudios realizados se han centrado en la obtención de etanol, compuestos fenólicos y pectina a partir de cortezas y/o sus extractos frescos, pero no se ha prestado atención al efecto del tratamiento térmico de estos subproductos sobre su calidad final, incluyendo su potencial en la extracción de pectina.

De modo que, en este trabajo, encuadrado en el Proyecto MINECO-Spain (AGL2014-53445-R) se ha planteado estudiar:

i) composición y las propiedades de cinco tubérculos andinos (yacón, mashua, melloco, camote y zanahoria blanca) infravalorados y aún muy poco conocidos, con el objetivo final de revalorizar productos autóctonos de los Andes para mejorar las condiciones de vida de las poblaciones encargadas de su cultivo;

ii) dos tipos de residuos agroindustriales de gran importancia a nivel mundial, como son la pulpa de remolacha y los residuos de extracción de zumo de naranja, con el fin último de ampliar y diversificar las opciones del consumidor actual, preocupado cada vez más por mantener y mejorar su estado de salud.

Para alcanzar estos objetivos generales, se han trazado los objetivos específicos y el plan de trabajo que se muestran a continuación:

- Determinar la composición química, fisicoquímica, nutricional y funcional de tubérculos andinos cultivados en Ecuador.

- Caracterizar la composición fenólica de tubérculos andinos cultivados en Ecuador.

- Evaluar las propiedades morfológicas, tecnológicas y nutricionales de harinas y almidones de mashua (*Tropaeolum tuberosum*) y melloco (*Ullucus tuberosus*) cultivados en Ecuador.

- Estudiar las propiedades estructurales y reológicas de pectina extraída a partir de subproductos de la industria de remolacha azucarera.

- Determinar la composición fisicoquímica y química de subproductos de la extracción de zumo de naranja y su potencial para la obtención de pectina.

- Evaluar el efecto antiinflamatorio intestinal del consumo de subproductos de la elaboración de zumo de naranja.

3. Plan de Trabajo

Tubérculos andinos			Subproductos de la industria alimentaria		
Yacón (*Smallantus sonchifolius,* var. INIAP-ECU-1247) Mashua (*Tropaeolum tuberosum,* var. INIAP-ECU-Izaño) Melloco (*Ullucus tuberosus,* var. INIAP-ECU-amarillo-rosa) Camote (*Ipomea batatas,* var. INIAP-ECU-morado) Zanahoria blanca (*Arracacia xanthorrhiza,* var. INIAP-ECU-blanca)			**Residuos del procesamiento de remolacha azucarera** SBP: Pulpa de remolacha azucarera	**Residuos de la extracción industrial de zumo de naranja**	
Caracterización fisicoquímica, nutricional y funcional	***Determinación del perfil fenólico***	***Caracterización morfológica, tecnológicas y nutricional de harina y almidón***	***Caracterización de subproductos y extracción de pectina por método ácido y enzimático***	***Caracterización de subproductos y extracción de pectina por método enzimático***	***Evaluación del efecto antiinflamatorio intestinal ←→ modelo DSS en ratones***
• Yacón • Mashua • Melloco • Camote • Zanahoria blanca	• Yacón • Mashua • Melloco • Camote	• Mashua • Melloco (harina y almidón)	• Pulpa prensada (SBP-P) • Pulpa ensilada (SBP-E) • Pulpa seca (SBP-D)	• Residuo de naranja fresco (FOR) • Residuo de naranja seco (DOR) • Licor de naranja (OL) • Alimento para animales (AF)	
Determinaciones: Análisis proximal Carbohidratos solubles en agua Carotenos Vitamina C Almidón	Determinaciones: Identificación y cuantificación de compuestos fenólicos por HPLC–DAD–ESI/MS^n	Determinaciones: Extracción de almidón Estabilidad en dispersión Propiedades reológicas y térmicas Digestibilidad	Determinaciones: Propiedades estructurales Propiedades reológicas	Determinaciones: Análisis proximal Carbohidratos solubles en agua Composición química de la pectina extraída	Determinaciones: Indicadores macroscópicos y bioquímicos (citoquinas pro y antiinflamatorias)
Sección 4.1. Obtención y caracterización de compuestos/ingredientes funcionales a partir de tubérculos andinos			**Sección 4.2. Obtención y caracterización de compuestos/ingredientes funcionales a partir de subproductos de la industria agroalimentaria**		
Artículo 1	**Artículo 2**	**Artículo 3**	**Artículo 4**	**Artículo 5**	**Artículo 6**

Figura 10. Plan de trabajo aplicado para la consecución de los objetivos de la investigación.

4. Resultados

4. RESULTADOS

En esta sección se muestran los resultados más importantes, obtenidos durante el desarrollo de la presente Tesis Doctoral, que se recogen en 6 artículos de investigación, distribuidos en dos subsecciones:

4.1. Obtención y caracterización de compuestos/ingredientes funcionales a partir de tubérculos andinos

Se inició el estudio caracterizando fisicoquímicamente 5 especies de tubérculos andinos ancestrales, cultivados en Ecuador, hasta el momento muy poco estudiados y de gran potencial productivo debido a su facilidad de cultivo, adaptación y resistencia: yacón (*Smallantus sonchifolius*, var. INIAP-ECU-1247), mashua (*Tropaeolum tuberosum*, var. INIAP-ECU-Izaño), melloco (*Ullucus tuberosus*, var. INIAP-ECU-amarillo-rosa), camote (*Ipomea batatas*, var. INIAP-ECU-morado) y zanahoria blanca (*Arracacia xanthorrhiza*, var. INIAP-ECU-blanca). Tras determinar su composición proximal, cantidad de azúcares reductores, fenoles totales, actividad antioxidante y carbohidratos solubles en agua, se extrajo el almidón y se procedió a su caracterización tecnológica. Se pudo observar, principalmente, presencia de compuestos fenólicos y FOS en el yacón, carotenos y vitamina C en mashua y melloco, antocianos en el camote, y almidón en todos los tubérculos analizados excepto en el yacón, sobresaliendo los almidones de camote, zanahoria blanca, mashua y melloco por su alto rendimiento y alto contenido de amilosa. Estos datos indicaron que dichos tubérculos pueden considerarse nuevas fuentes de

compuestos bioactivos de gran interés para la industria farmacológica y alimentaria **(Artículo I).**

Dado que se observó un considerable contenido fenólico en los tubérculos andinos se llevó a cabo la determinación del perfil fenólico del yacón, mashua, melloco y camote, de las variedades señaladas, mediante HPLC-DAD-ESI-MS^n, durante una estancia predoctoral realizada en la Universidad de Salamanca**.** Los resultados se recogen en un segundo artículo científico **(Artículo II)**.

El almidón de mashua y melloco había sido escasamente estudiado hasta el momento, lo que motivó un tercer tema de investigación **(Artículo III)**. Las harinas y almidones de estos tubérculos fueron caracterizados morfológica, nutricional y tecnológicamente, observando un tamaño de gránulo de almidón mucho más grande que el de almidón de patata, que junto con la cantidad de amilosa cuantificada en el estudio previo, influyó sobre las propiedades reológicas de estos polisacáridos, confiriendo mayor resistencia térmica, menor temperatura de gelificación y mayor viscosidad. Tanto los almidones como las harinas mostraron una alta tasa de digestión, con excepción de la harina de mashua, efecto atribuido a la presencia de compuestos fenólicos inhibidores de la α-amilasa. Los resultados indicaron que estos tubérculos pueden considerarse alimentos nutritivos y fuentes económicas y no convencionales de almidón de propiedades tecnológicas mejores que el almidón de patata.

Los artículos científicos mencionados son los que se indican a continuación:

4.1.1. Artículo I. Tubérculos andinos inexplorados cultivados en Ecuador como nuevas fuentes de ingredientes funcionales.

4.1.2. Artículo II. Determinación mediante HPLC-DAD-ESI/MS^n de compuestos fenólicos en tubérculos andinos cultivados en Ecuador.

4.1.3. Artículo III. Propiedades morfológicas, tecnológicas y nutricionales de harinas y almidones de mashua (*Tropaeolum tuberosum*) y melloco (*Ullucus tuberosus*) cultivados en Ecuador.

4.1.1. Artículo I. Tubérculos andinos inexplorados cultivados en Ecuador como nuevas fuentes de ingredientes funcionales

Unexplored Andean tubers grown in Ecuador: New sources of functional ingredients

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Food Bioscience (under review)

Abstract

This article describes the comprehensive compositional analysis of five unexplored Andean tubers grown in Ecuador. Yacon (*Smallanthus sonchifolius,* var. INIAP-ECU-1247) was characterised by a high level of prebiotic inulin-type fructooligosaccharides (FOS) with a degree of polymerisation from 2 to 6 and the highest content of phenolic compounds. Mashua (*Tropaeolum tuberosum,* var. INIAP-ECU-Izaño) presented great antioxidant capacity, attributed to its high carotenoid and vitamin C content. Melloco (*Ullucus tuberosus,* var. INIAP-ECU-amarillo-rosa) had the highest content of fibre and protein, while purple sweet potato (*Ipomea batatas,* var. INIAP-ECU-morado) and white carrot (*Arracacia xanthorrhiza,* var. INIAP-ECU-blanca) showed a high yield of starch rich in amylose, with possible better functional properties than potato starch. Fructose, glucose and sucrose were found in all samples. To the best of our knowledge, this is the first report dealing with the chemical, physicochemical and technological characterisation of these Andean tubers that may contribute to the valorisation of the production of Ecuadorian ancestral Andean crops. These tubers can be useful as non-traditional sources of bioactive compounds with applications in the development of biomaterials, pharmacological products or new functional ingredients.

Introduction

According to the FAO's baseline projections, in the year 2050, the world population will reach 9.1 billion people, with it being necessary to increase the agricultural production of nutritious food (United Nations Department of Economic and Social Affairs, 2017; Food and Agriculture Organization, 2009). In addition, at least half of the world's population cannot obtain essential health services (Ghebreyesus & World Health Organization, 2018), for which it is important to emphasise the development of functional foods that can contribute to improving health and well-being. Moreover, the exploration of new sources of bioactive compounds is an intense field of research (Ibañez & Cifuentes, 2012).

In this regard, tubers domesticated 8,000 years ago in the Middle East, India, China, Mesoamerica and the Central Andes (Iriarte, 2006; Amat Olazával, 2014) represent many agricultural advantages with medical and technological potential, although they are still very little exploited (Pochettino, 2015). Among these, *Smallantus sonchifolius, Ullucus tuberosus, Tropaeolum tuberosum, Ipomea batatas,* and *Arracacia xanthorrhiza* stand out for being resistant to drought and pests, for the low cost of agricultural inputs, and for providing protection against diseases when they are interspersed with legumes (Morón, 1999).

In addition, in *S. sonchifolius* Grancieri et al. (2017), Paredes et al. (2018) and Fabersani et al. (2018) have observed the presence of fructooligosaccharides (FOS) with prebiotic, immunomodulatory, hypoglycaemic and hypolipidemic activity. *U. tuberosus* has been used to treat internal trauma and reduce inflammatory processes, and its extract has shown the regenerative properties of procollagen (Vimos et al. 1993,

Heil et al. 2017). *T. tuberosum* presents isothiocyanates derived from glucosinolates, which can have effects on the immune system and could protect against cancer, as well as carotenoids of known antioxidant activity (Johns et al. 1982; Traka & Mithen, 2009; Campos et al., 2018). *I. batatas* and *A. xanthorrhiza* have been shown to have high levels of resistant starch (RS), which exhibits strong resistance to hydrolysis by human digestive enzymes and is fermented by beneficial gut bacteria, helping to reduce the risk of colon cancer and type 2 diabetes (Lehmann & Robin, 2007; Sajilata et al., 2006).

Chandrasekara and Joshepkumar (2016) have pointed out that the nutritional value of roots and tubers varies with the variety, location, soil type and agricultural practices, among other factors. In the Central Andes region (Ecuador to Bolivia), where mountain agriculture is located from 1,500 to more than 4,000 metres above sea level (m.a.s.l.), there is the greatest variability of species (Tapia et al., 2007), with Ecuador being considered one of the ten countries with the greatest biodiversity in the world (Tene et al., 2007). Yacon (*Smallanthus sonchifolius,* var. INIAP-ECU-1247), mashua (*Tropaeolum tuberosum,* var. INIAP-ECU-Izaño), melloco (*Ullucus tuberosus,* var. INIAP-ECU-amarillo-rosa), sweet potato (*Ipomea batatas,* var. INIAP-ECU-morado) and white carrot (*Arracacia xanthorrhiza,* var. INIAP-ECU-blanca) are varieties of Andean tubers, with little information about their composition and potential industrial use. Research on undervalued crops for the development of new functional foods represents a strategy to improve regional economies and preserve biodiversity. Based on the aforementioned, the objective of this study was to carry out an exhaustive characterisation of these five Andean tubers from Ecuador, paying special attention to their chemical and physicochemical composition, as well as their possible technological applications.

Material and methods

Samples and reactives

Yacón (*Smallanthus sonchifolius,* var. INIAP-ECU-1247), mashua (*Tropaeolum tuberosum,* var. INIAP-ECU-Izaño), melloco (*Ullucus tuberosus,* var. INIAP-ECU-amarillo-rosa), sweet potato (*Ipomea batatas,* var. INIAP-ECU-morado) and white carrot (*Arracacia xanthorrhiza,* var. INIAP-ECU-blanca) grown in the province of Cotopaxi-Ecuador (2,800-3,600 m.a.s.l.) and harvested in mid-April 2017 (**Figure 1**) were immediately cleaned, lyophilised, ground, sieved through 250 µm mesh and stored at -20°C until analysis. Analytical reference substances such as sucrose, D-arabinose, L-rhamnose, D-galactose, D-mannose, D-glucose, D-fructose, galacturonic acid, ascorbic acid and β-phenyl-glucoside were purchased from Sigma Aldrich (Steinheim, Germany), and the FOS standard mixture was acquired from FUJIFILM Wako Pure Chemical Corporation (Europe). Chemicals were reagent grade from J.T. Baker (Phillipsburg, NJ), and enzymes were from Sigma (Sigma Co., St. Louis, MO, USA).

Figure 1. Ecuadorian Andean tubers analyzed.

Overall characterization

°Brix, pH, activity water (Aw), dry matter (DM), total fat, protein, and ash of Andean tubers were determined following the AOAC methods described by Megías-Pérez et al. (2014). Total and reducing carbohydrates were measured by phenol-sulfuric, and by 3,5-dinitrosalicylic acid (DNS) method, respectively; fiber content was determined by the modified enzymatic–gravimetric method purposed by Prosky et al. (1988). Mineral content was analysed by ICP-MS, in the Service Interdepartmental Research (SIdI-UAM) (Madrid). Total phenolic content was determined by Folin-Ciocalteu method, and the antioxidant activity was measured by the assay 2,2-diphenyl-1-picrylhydrazyl *(*DPPH*),* as described by Pacheco et al. (2018).

Phytochemicals and vitamin C

Carotenoids

Carotenoids extraction was carried out following the method described by Sanusi and Adebiyi (2009) with some modifications. Briefly, 0.5 g of tuber flour was diluted with 5 mL of ethanolic butylated hydroxyl toluene (ethanol/BHT – 100:1, v/w). The mixture was placed in a water bath at 85°C for 5 min, followed by the addition of 0.5 mL of 80% w/v KOH for the saponification and properly vortexed before putting it back to 85°C for 10 min. Then it was added 3 mL of deionised water and cooled down in an ice-water bath. Three mL of *n*-hexane were added to the mixture before centrifugation at 7,500 rpm for 5 min for the separation of two layers. The upper yellow layer was transferred and collected. This procedure was repeated four times. Therefore, a total of 12 mL of hexane

were placed into each centrifuge tube and the final volume of each tube was recorded. The samples were read using a Spectrophotometer VIS (UNIC, S-1200-E) at 450 nm and 503 nm against the hexane as the blank. The total carotenoid content (TCC) in µg/mL was expressed by the Eq. (1).

$$Total\ carotene = 4.642 * A_{450} - 3.091 * A_{503} \quad (1)$$

Anthocyanins

Total anthocyanins content (TAC) was determined by using the pH differential method, which allows the detection and quantification of anthocyanins both, in the form of a flavonoid cation (orange to purple, pH = 1.0), and in the form of a hemiacetal (colorless, pH = 4.5). According to this, the absorbance was measured at 520 and 700 nm in pH 1.0 buffer (KCl 0.025M) and 4.5 pH buffer (CH_3CO_2Na 0.4M), using a molar extinction coefficient of 26900 l cm^{-1} mol^{-1} and a molecular weight of 449.2 Da for the anthocyanin calculation. Results were expressed as mg of cyanidin 3-glucoside equivalents (CGE)/g DM.

Vitamin C

Total vitamin C (ascorbic acid plus dehydroascorbic acid) was determined by the reduction of dehydroascorbic acid to ascorbic acid, using D,L-dithiothreitol (DTT) as reducing reagent (Gamboa-Santos et al., 2013). Tuber extracts were prepared by adding 12.5 mL of oxalic acid (0.4% w/v) to 0.25 g of tuber flour samples, for inhibit further degradation of vitamin C. Mixtures were homogenised for 1 min at 13500 rpm using an Ultra-Turrax T-25 homogeniser (IKA Labortechnik,

Janke & Kunkel, Saufen, Germany). After addition of DTT, the supernatants were filtered through 0.45 µm syringe filters.

Total vitamin C content was determined by RP-HPLC-DAD on an Agilent Technologies 1220 Infinity LC System – 1260 DAD (Boeblingen, Germany) at wavelength of 260 nm. The separation of vitamin C was carried out with an ACE 5 C_{18} column (ACE®, UK) (250 mm, 4.6 mm i.d., 5 µm) thermostated at 25°C, using 5 mM KH_2PO_4 at pH 3.0 as the mobile phase. Elution was done under isocratic conditions at a flow rate of 1 mL/min for 10 min. Data acquisition was performed using the Agilent ChemStation software (Agilent Technologies, Germany).

Quantitation was carried out using the external standard method with ascorbic acid. Results were expressed as mg of vitamin C/100 g DM.

Water soluble carbohydrates

Soluble carbohydrates were determined by gas chromatography following the method reported by Soria et al. (2010), with some modifications. Ethanolic extracts of 30 mg of powder tuber were evaporated under vacuum at 43°C. The dried mixtures were treated with 250 µL hydroxylamine chloride in pyridine (2.5% w/v) and incubated at 70°C during 30 min; subsequently, the samples were persilylated by the addition of 250 µL of hexamethyldisilazane and 25 µL of trifluoroacetic acid followed by an incubation at 45°C during 30 min. Reaction mixtures were centrifuged, and supernatants were analysed in an Agilent Technologies 7890A gas chromatograph coupled to a flame-ionization detector (Agilent Technologies, Santa Clara, California, USA) equipped with an DB-5HT column (15 m x 0.32 mm x 0.10 µm) (J & W Scientific, Folsom, California, USA).

The oven temperature rose from 150°C to 165°C at a heating rate of 1°C min^{-1} and rose again to 380°C at 10°C min^{-1}, with a maintenance time of 2 min at this temperature. The injector and detector temperatures were 280 and 350°C, respectively. Injections were made in the split mode (1:20). Data acquisition and integration were performed using Agilent ChemStation Rev. B.03.01 software (Wilmington, DE, USA). Solutions of water-soluble carbohydrates were prepared in concentrations of 0.01-5 mg/mL to calculate the response factor of each of these sugars relative to the internal standard (*β*-phenyl glucoside -0.5 mg/mL). The identification of carbohydrates was done by comparison of retention times with the respective standards.

Starch analysis

Starch extraction

For the starch extraction, samples of fresh tuber (100 g) were ground and the porridge was rinsed on a 250-mesh sieve with five times its volume of deionized water to remove the fibre. The filtered liquid was allowed to settle, and the supernatant was decanted. The starch was suspended in water and repeatedly washed by centrifugation at 10,000 *g* for 15 min. After each centrifugation, the supernatant was decanted and a brownish band of materials from the top layer of the settled starch was scraped off. The resulting starch was lyophilized, pulverized and stored in airtight containers. Starch extraction yield was calculated according to Eq. (2).

$$Starch\ extraction\ yield = \frac{g\ of\ powder\ starch}{g\ of\ flour\ or\ powder\ tuber} * 100\ \%\quad (2)$$

Total starch

Total starch (TS) was analysed by the enzymatic/colorimetric method described by Tovar et al. (1990), with some modifications. Flours and starches samples (250 mg) were suspended in 5 mL of deionised water and 5 mL of KOH solution (4 M) were added. The mixture was kept for 30 min at room temperature and then neutralized (pH 6.5-7) with 5 M HCl, followed by an incubation with 50 µL of Termamyl (Novozyme) for 30 min at 98°C and then, with amyloglucosidase at 60°C (30 min). The glucose concentration was measure using the combined glucose oxidase/peroxidase colorimetric assay. Additionally, the starch extraction efficiency was estimated according to Eq. (3).

$$Starch\ extraction\ efficiency = \frac{Starch\ extraction\ yield}{Total\ starch\ of\ flour} * 100\ \%\quad (3)$$

Amylose content

Amylose content of the starch samples was determined by the colorimetric method described by Williams et al. (1970). A portion (20 mg) of extracted starch was mixed with 10 mL of 0.5 M KOH and the suspension was vortexed thoroughly. The dispersed sample was transferred to a 100 mL volumetric flask and the volume was made up with deionised water. An aliquot of the test starch solution (10 mL) was pipetted into a 50 mL volumetric flask and 5 mL of 0.1 M aqueous HCl was added followed by 0.5 mL of iodine reagent. The volume was diluted to 50 mL and allowed to stand for 5 min. The absorbance was measured at 625 nm (UV

Spectrophotometer, U-2900, Hitachi, Tokyo, Japan). The content of amylose was determined from a standard curve developed using standard amylose from potato starch.

Swelling power and water solubility

According to the method optimized by Konik-Rose et al., (2001), samples of flour or starch (40 mg) of known DM were put into a pre-weighed micro-centrifuge tube (1.5 ml), mixed with water (1 mL, vortex mix), and held in an Eppendorf thermomixer (R Mixer 5355, Marshall Scientific, 1.5 ml Block) for 30 min at 92.5°C and 750 rpm. Samples were then cooled to room temperature by being placed in a cool (20°C) water bath for 3 min, with two gentle inversions initially and after 1.5 min. The tube was then centrifuged (10 min, 17,000 *g*). The supernatant was removed by suction and the weight of the residue used to calculate the total starch swelling power by Eq. (4).

$$Swelling\ power = weight\ of\ residue/dry\ weight\ of\ sample \quad (4)$$

For water solubility, the supernatant obtained was filtered and dried during 4 h at 90°C, and the value was calculated through the Eq. (5).

$$Solubility = (weight\ of\ solubles/dry\ weight\ of\ sample) * 100\ \% \quad (5)$$

Statistical analysis

A triplicate of each analysis was carried out, and data were expressed as the mean ± standard deviation. Statistical analysis of data was carried out using the

Tukey's tests ($p < 0.05$), using the program SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA).

Results

General composition

Table 1 shows the chemical composition of the Ecuadorian Andean tubers analysed. Yacon presented the highest water activity (Aw), fat, Na and total phenolic content (TPC) ($p < 0.05$). Nevertheless, carbohydrates were the major components in purple sweet potato, in a similar way to that observed in white carrot ($p < 0.05$).

Mashua showed the lowest content of total carbohydrates, and the highest °Brix, reducing carbohydrates, ash, K, Mg, P, Ca and antioxidant capacity (AC), and, similar to yacon, its fat and Na contents were also high ($p < 0.05$). K was the major mineral detected in all the tubers analysed, with amounts in the range of 1215-3254 mg/100 g DM. The presence of this mineral had not been reported so far for mashua. In this regard, it is important to consider that the micronutrient content could vary depending on the fertilisation methods.

The level of P, K, Mg, S, Fe, Mn and Cu are often higher in tubers grown using organic systems, while N and Mn content is higher in tubers farmed with conventional methods (Sawicka et al., 2016), and in addition, higher levels of irrigation may increase the P content, while reducing the concentration of Ca and Mg (Ierna et al., 2011). These effects are due to the result of complex interactions between minerals in the soil and in the tissues throughout the plant; so, enabling

the mineral profiling of tubers can therefore be used to verify the origin of the products (Sawicka et al., 2016).

Table 1. Chemical composition of Andean tubers (g/100g DM).

Parameter	Yacon *(S. Sonchifolius,* var. INIAP-ECU-1247*)*	Mashua *(T. tuberosum,* var. INIAP-ECU-Izaño*)*	Melloco *(U. tuberosus,* var. INIAP-ECU-amarillo-rosa*)*	Purple sweet potato *(I. batatas,* var. INIAP-ECU-morado*)*	White carrot *(A. xanthorrhiza,* var. INIAP-ECU-blanca*)*
°Brix	12.5±0.21c	13.1±0.28d	7.4±0.07b	12.3±0.14c	4.5±0.07a
pH	6.1±0.10a	6.7±0.10c	6.5±0.05b	6.8±0.07cd	6.8±0.02cd
Aw	0.97±0.05cd	0.93±0.01b	0.95±0.00bc	0.88±0.01a	0.88±0.02a
DM (g/100g FM)	12.0±0.23a	13.8±0.28c	13.0±0.14b	29.1±0.42de	28.6±0.12d
Total fat	1.5±0.03de	1.1±0.02d	0.4±0.06ab	0.3±0.05a	0.5±0.01abc
Protein	4.2±0.05a	11.7±0.40d	13.4±0.60e	4.5±0.07ab	6.2±0.11c
Total carbohydrates	84.6±0.06bc	77.9±0.50a	83.2±0.43b	91.4±0.80e	87.0±0.72d
Reducing carbohydrates	8.8±0.06c	39.3±0.12e	10.3±0.01d	1.8±0.13a	5.1±0.07b
TDF	10.3±0.20d	6.8±0.07b	20.6±0.35e	8.3±0.09c	3.2±0.13a
IDF	8.8±0.11d	4.8±0.05c	14.2±0.09e	3.0±0.04ab	2.8±0.04a
SDF	1.5±0.03b	2.0±0.02bc	6.4±0.26e	5.3±0.05d	0.4±0.09a
Ash	2.9d	3.7e	2.3c	1.4a	1.5ab
Na (mg/100g DM)	16.73de	15.78d	0.87ab	13.77c	0.14a
Mg (mg/100g DM)	80.40cd	163.93e	77.33c	60.43b	40.33a
P (mg/100g DM)	92.62d	160.59e	77.69c	36.80a	37.25ab
K (mg/100g DM)	2644.12cd	3254.52e	2137.53c	1215.57a	1324.62ab
Ca (mg/100g DM)	70.67c	90.28e	10.59a	62.32b	82.04d
Fe (mg/100g DM)	1.31ab	2.75d	3.27e	1.78c	1.14a
TPC (mg GAE/100g DM)	3384.4±0.01e	1001.6±0.02d	260.1±0.01ab	384.8±0.01c	196.3±0.01a
AC DPPH (mM de trolox/100g DM)	4.4±0.00d	6.0±0.02e	2.4±0.01ab	3.9±0.02c	2.2±0.02a
TCC (mg/100g DM)	0.135±0.021c	1.445±0.151d	0.004±0.001b	0.001±0.000a	0.002±0.000a
TAC (mg/100g DM)	13.86±1.09a	52.89±0.54b	115.40±0.33d	1561.83±68.51e	57.05±2.29bc
Vitamin C (mg/100g DM)	55.47±0.16b	81.70±0.29c	32.46±0.36a	n.d.	n.d.

Aw: activity water. DM: dry matter. FM: fresh matter. TDF: total dietary fiber. IDF: insoluble dietary fiber. SDF: soluble dietary fiber. TPC: total phenolic content. AC: antioxidant capacity. TCC: Total carotenoids content. TAC: Total anthocyanins content. n.d.: not detected. Means with different letters a-e denote significant difference ($p < 0.05$) in the same row.

Melloco presented the highest quantity of protein, total dietary fibre (TDF), insoluble dietary fibre (IDF), soluble dietary fibre (SDF) and Fe.

Purple sweet potato exhibited the lowest Aw, fat, reducing carbohydrates, ash, P and K content, and the highest total carbohydrate content; mashua followed by white carrot showed the highest content of Ca.

Phytochemicals and vitamin C

Total carotenoid content (TCC), total anthocyanin content (TAC) and vitamin C are displayed in **Table 1.**

Carotenoids are strongly hydrophobic compounds with a tetraterpenoid structure that contains multiple conjugated double bonds (Mariutti et al., 2018), that are claimed to exhibit numerous health benefits when consumed at sufficiently high levels, including enhancing immune system functions, protecting cells from free radicals and reducing the risk of age-related functional decline (Rodriguez-Concepcion et al., 2018, Leermakers et al., 2016). The TCC was observed in the range of 0.001-1.445 mg/100 g DM, and mashua presented the highest value of TCC, followed distantly by yacon ($p < 0.05$). No significant presence of these compounds was found in the other tubers.

Vitamin C was also found, mainly in mashua (81.7 mg/100 g DM) followed by yacon and melloco ($p < 0.05$), which suggests that the TCC and vitamin C content of mashua are directly related to its greater antioxidant capacity (AC).

In purple sweet potato and white carrot, vitamin C was not detected; however the TAC in these tubers was 1561.83 mg/100 g DM and 57.05 mg/100 g DM.

Water soluble carbohydrates

As observed in **Table 2**, all Andean tubers studied presented fructose, glucose and sucrose at different levels. Mashua was the tuber with the highest amount of fructose, glucose and sucrose (total 53.2 g/100 g DM) ($p < 0.05$), while white carrot had the highest amount of sucrose content. Fructose and glucose contents in yacon, mashua, purple sweet potato and white carrot followed the same trend as the amount of reducing sugars determined by 3, 5-dinitrosalicylic acid (**Table 1**).

Table 2. Water soluble carbohydrates present in Andean tubers (g/100g DM) determined by GC-FID.

Carbohydrate	Yacon	Mashua	Melloco	Purple sweet potato	White carrot
Fructose	4.66±0.23c	19.73±0.39e	6.53±0.18d	0.45±0.00a	3.49±0.04b
Glucose	1.80±0.03b	25.05±0.72e	11.71±0.05d	0.78±0.02a	3.71±0.09c
Sucrose	7.48±0.21b	8.47±0.48cd	0.07±0.00a	8.42±0.24c	11.26±0.09e
*Kestose	17.34±0.16	n.d.	n.d.	n.d.	n.d.
*Nystose	16.25±0.36	n.d.	n.d.	n.d.	n.d.
*Fructosyl-nystose	8.59±0.20	n.d.	n.d.	n.d.	n.d.
*Difructosyl-nystose	1.64±0.07	n.d.	n.d.	n.d.	n.d.
Total:	56.12d	53.25e	18.31b	9.65a	18.46bc
*Total FOS:	43.82	---	---	---	---

Additionally, as can be observed in **Figure 2**, the GC-FID profile of the water-soluble fraction of yacon was dominated by inulin-type fructooligosaccharides (FOS) (kestose, nystose, fructosyl-nystose and difructosyl-nystose). Remarkably, the total FOS content in yacon comprised 78% of the water-soluble carbohydrate fraction as determined by GC-FID (**Table 2**).

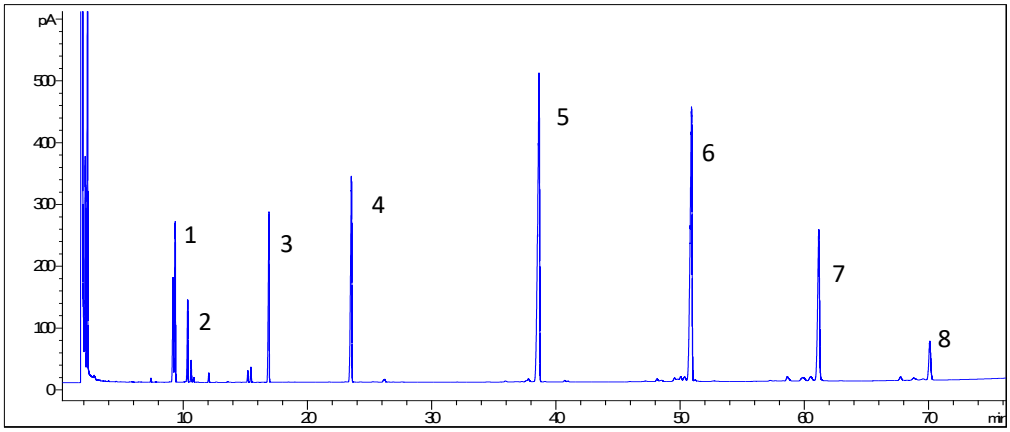

Figure 2. Chromatographic profile obtained by GC-FID of TMS-oximes of soluble carbohydrates present in yacon (*S. Sonchifolius,* var. INIAP-ECU-1247): 1: fructose, 2: glucose, 3: Internal standard, 4: sucrose, 5: kestose, 6: nystose, 7: fructosyl-nystose, 8: difructosyl-nystose.

Starch

The starch extraction yields from the powder samples (flours) of mashua, melloco, purple sweet potato and white carrot were 55.13, 39.34, 68.72, and 70.04 g/100 g DM, respectively (**Table 3**). White carrot and purple sweet potato flours had a noticeable amount of starch ($p < 0.05$), and yacon tuber did not present starch, unlike the other tubers analysed.

Total starch

Total starch analysed in the extracted product (starch) was in all cases higher than 89 g/100 g DM (**Table 3**). The starches extracted from white carrot and purple sweet potato were the products with the highest purity (96.77 and 95.05 g/100 g DM) ($p < 0.05$).

Melloco starch was the product with the lowest purity (89.91 g/100 g DM) ($p < 0.05$), probably due to the presence of fibre and/or mucilage that hindered starch separation.

Amylose content

Starch consists of two main biopolymers: linear amylose (20–30%) and highly branched amylopectin (70–80%). Amylose is related to the presence of resistant starch (RS), rheological and technological properties of starch (Lehmann & Robin, 2007; Xie et al., 2009). Amylose content in the tubers analysed had values from 29.55 to 72.26% (**Table 3**).

The highest value was found in purple sweet potato starch, followed by melloco and white carrot starch, which exhibited similar amounts of amylose (51.70 and 53.03%, respectively) ($p < 0.05$); whereas mashua starch presented the lowest amylose content.

Swelling power and water solubility

The extent of granular swelling can be quantified as swelling power (SP) and the solubilisation of starch components as water solubility (WS) (%) during heating. SP and WS of flours and starches of tubers determined at 95°C are summarised in **Table 3**.

Table 3. Main technological properties of Andean tubers and starches.

Parameter	Mashua		Melloco		Purple sweet potato		White carrot	
	Flour	Starch	Flour	Starch	Flour	Starch	Flour	Starch
Starch extraction yield (g/100g DM)	55.13±1.02b	…..	39.34±0.75a	….	68.72±1.40c	….	70.04±1.86cd	….
Total starch (g/100g DM)	66.37±1.23b	92.42±1.87b	52.39±0.41a	89.91±0.53a	69.89±1.71c	95.05±0.33c	72.30±1.99d	96.77±1.97d
Starch extraction efficiency (%)	n.m.	83.07±0.46b	n.m.	75.09±0.27a	n.m.	98.32±0.75d	n.m.	96.87±0.68c
Amylose content (g/100g starch)	n.m.	29.55±0.02a	n.m.	51.70±0.10b	n.m.	72.26±0.15d	n.m.	53.03±0.24bc
Swelling power (SP) (g/g)	5.44±0.04c	9.89±0.11a	13.16±0.32d	23.21±0.14c	4.91±0.05b	17.53±0.36b	4.00±0.04a	24.40±0.01cd
Water solubility (WS) (%)	31.20±2.32b	4.77±0.38b	3.97±0.47a	n.d.	42.37±3.36c	2.12±0.17a	47.21±2.71cd	n.d.

DM: dry matter. Means with different letters a-e denote significant difference ($p < 0.05$) in the same file between flours or starches. n.m.: not measurable. n.d.: not detected.

In the case of flours, melloco flour presented the highest SP (13.16 g/g) and, among the starches, white carrot (24.40 g/g), melloco (23.21 g/g) and purple sweet potato starches (17.53 g/g) had the highest values of this parameter ($p < 0.05$). The swelling properties of starch can depend on the crop's physiological maturity, the water stresses on the crop at both initial and later stages, the freshness of the root, the amount of protein associated to the granule and the applied heat treatment (Zhu, 2015). Starch isolated from fresh roots can present lower SP than that extracted from dried roots (Abera & Rakshit, 2004), and heat treatment in excess of water can induce the redistribution of granule-associated proteins and the formation of the protein envelope, which can retain the granular content during swelling, increasing the swelling strength and reducing solubility (Israkarn et al., 2007).

On the other hand, the highest WS was observed in white carrot and purple sweet potato flours (47.21% and 42.37%, respectively). And, among the starches, mashua starch presented the highest value (4.77%), followed by purple sweet potato starch (2.12%) ($p < 0.05$). Melloco flour presented the lowest WS ($p < 0.05$), and in melloco and white carrot starches, WS could not be quantified, due to its high SP (**Table 3**).

Discussion

The highest total phenolic content (TPC) and antioxidant capacity (AC) observed in yacon and mashua (3384.4 mg GAE/100 g DM and 6.0 mM trolox/100 g DM, respectively) (**Table 1**) were approximately around 3-fold greater than

those observed in the yacon and mashua cultivated in Junín (Peru) (1020 mg GAE/100 g DM and 2.32 mM trolox/100g DM) (Chirinos et al., 2013).

Phenolic compounds can reduce the risk of health problems such as diabetes, cancer, cardiovascular disease, inflammation and Alzheimer's disease (Russo, 2007; Xu et al., 2019). In addition, Duque et al. (2017) have highlighted the ability of *S. sonchifolius* extracts to protect the main components of the extracellular matrix of human dermal fibroblasts from UVB-irradiation-induced damage, and Chirinos et al. (2008) have observed that purified mashua extracts of different genotypes inhibit the haemolysis of erythrocytes within the range of 20.8-25.1%. The presence of phenolic compounds in the tubers analysed and the antioxidant potential of this type of extract point to the importance of identifying and quantifying these compounds individually in a future study.

The contents of total carotenoid (TCC) and vitamin C observed in mashua (1.45 mg/100 g DM and 81.70 mg/100 g DM, respectively) (**Table 1**) were similar to those reported by Campos et al. (2018) for Peruvian mashua (0.10-2.36 mg/100 g DM and 77.5 mg/100 g DM, respectively), who pointed out the relevance of the tuber due to its carotenoid content. The amount of vitamin C in mashua was inside the range observed for 25 potato varieties (*Solanum tuberosum*) grown in three different environments (22.2-121.4 mg/100 g DM) (Burgos et al., 2009). Potato (*S. tuberosum)* is a recognised source of vitamin C (Hamilton et al., 2004), therefore, mashua (*T. tuberosum*, var. INIAP-ECU-Izaño) could also be considered a good source of this vitamin, that acts as an antioxidant and antiallergic, as well as being a crucial factor to delay vision loss and stimulate the immune system; it is also

selectively cytotoxic of different cancer cell lines (Fafunso & Bassir, 1977; Zhao et al., 2018).

On the other hand, mashua could also be a source of fructose and glucose (**Table 2**), exhibiting amounts of these sugars higher than those reported for *S. tuberosum* (fructose: 0.75–7.5 g/100 g DM, glucose: 0.75–7.5 g/100 g DM) (Rady & Guyer, 2015) whereas white carrot could be a good source of sucrose (**Table 2**). No data on soluble carbohydrates have been previously reported for mashua so far.

The fructooligosaccharide (FOS, kestose+nystose+fructosyl-nystose+difructosyl-nystose) concentration in yacon (43.82 g FOS/100 g DM) (**Table 2**) was slightly lower than that observed in yacon cultivated in Brazil (52.20 g FOS/100 g DM) (Grancieri et al., 2017). However, this content is similar to those of conventional sources of FOS, such as Jerusalem artichoke (30-50 g FOS/100 g DM) (Long et al., 2016) and chicory root (35.7-40.6 g FOS/100 g DM) **(**Moshfegh et al., 1999); whereby yacon (*S. sonchifolius,* var. INIAP-ECU-1247) can be considered an excellent source of these important prebiotics.

Regarding melloco, hardly any information is available. Its protein content (13.4 g/100 g DM) (**Table 1**) was higher than that reported for *S. tuberosum* (1.55-2.5 g/100 g DM) (Tian et al., 2016), but its total dietary fibre (TDF) amount was lower than that observed in melloco from New Zealand (20.6 *vs.* 34.2-37.57 g/100g DM) (Busch & Savage, 2000). The presence of mucilage (non-amylaceous polysaccharide) could have hindered the extraction of starch, reducing its purity (**Table 3**); nonetheless, its protein and mucilage content seem to increase the swelling power (SP) (Zhu, 2015) of melloco flour (**Table 3**). This could help to

increase the yield and reduce the costs in the production of foodstuffs. Moreover, the consumption of *U. tuberosus* var. INIAP-ECU-amarillo-rosa (melloco) could help to achieve the minimum daily requirement of fibre (23.5-38.0 g/day) (Suares & Ford, 2011), related to the reduction of the glycaemic response, plasma cholesterol, and protection of the gastrointestinal tract (Elleuch et al., 2011).

Anthocyanins are glycosides with effects that include anti-oxidation, anti-cancer, retinal protection, hypolipidemia, anti-ageing, and improving gut health (Kamiloglu et al., 2015; Olivas-Aguirre et al., 2016; Vendrame & Klimis-Zacas, 2015). *I. batatas,* var. INIAP-ECU-morado (purple sweet potato) cultivated in Ecuador (2800-3600 m.a.s.l.) (**Table 1**) presented a much greater total anthocyanin content (TAC) than that reported in purple sweet potato, var. Covington cultivated in North Carolina (USA) (449 mg/100 g DM) (186 m.a.s.l.) (Grace et al., 2014). This could be ascribed not only to the cultivar but also to the fact that plants that grow in high-UV and UVB radiation environments (i.e. higher altitudes) produce more photoprotective pigments than those endemic to low-UV radiation locations (Cotado et al., 2018).

The calcium content (82.0 mg/100 g DM) (**Table 1**) of white carrot was much higher than that reported by Leterme et al. (2006) for the same tuber var. Brancroft cultivated in the Colombian Andes (36.0 mg/100 g DM), which could confer to *A. xanthorrhiza,* var. INIAP-ECU-blanca (white carrot) a potential use as a source of Ca (Cashman, 2002).

The starch extraction yield in purple sweet potato and white carrot was the highest within the tubers analysed (70.04 and 68.72 g/100 g DM) ($p > 0.05$) (**Table 3**), and similar to that reported for potato (66-80 g/100 g DM), a tuber that is

considered a very good source of this polysaccharide (Bertoft & Blennow, 2016). In addition, the amylose content of purple sweet potato starch (72.26%) was much higher than that reported for high-amylose potato starch (40.8 to 57.1%) (Zhao et al., 2018). Therefore, the starch of *I. batatas,* var. INIAP-ECU-morado (purple sweet potato) could present better behaviour as a gelling agent than high-amylose potato starch.

The high SP observed in white carrot and melloco starches (**Table 3**) indicates that these tubers can be considered sources of starch with high performance as a thickener. It is important to note that only in the purple sweet potato starch, the high amylose content observed (72.26%) seems to be associated with a lower SP (**Table 3**), according to Lai et al. (2016), who indicated that amylose can act as an inhibitor of starch granule swelling by hindering the disruption of amylopectin double helices.

The highest WS of white carrot and purple sweet potato flours (**Table 3**) may be associated with a small granule size (Castanha et al. 2018). In contrast, the lowest values of WS observed in mashua and melloco flours could be related to a large particle and to the presence of carotenes and TDF, respectively (Lai et al. 2016) (**Table 1**). Amongst the starches, mashua starch presented the highest value of WS, while in melloco and white carrot starch the WS cannot be determined due to its elevated SP. It is known that tuber starches show a wide range of applications due to their high solubility, neutral flavour and ability to form translucent gels (Zhao et al., 2018).

Conclusions

The Andean tubers analysed in this work showed a similar or greater content of bioactive compounds as compared to similar varieties grown in other countries and/or regions. Yacon (*S. sonchifolius*, var. INIAP-ECU-1247) can be considered an excellent source of phenolic compounds and FOS. Mashua (*T. tuberosum,* var. INIAP-ECU-Izaño) is a good source of phenols, carotenes, K, Ca, fructose and glucose. Melloco (*U. tuberosus*, var. INIAP-ECU-amarillo-rosa) is an interesting tuber due to its high protein and fibre content; while purple sweet potato (*I. batatas*, var. INIAP-ECU-morado) could be used as an excellent source of anthocyanins and high amylose starch. White carrot *(A. xanthorrhiza*, var. INIAP-ECU-blanca) could also be a good source of high amylose starch, sucrose and Ca.

To conclude, the high content of carbohydrates, phenolic compounds, anthocyanins, carotenoids, starch and minerals of known beneficial activity for health and/or with exceptional technological properties, in the varieties of Ecuadorian tubers analysed, point to their potential as sources of bioactive compounds.

This information will undoubtedly contribute to the preservation of ancient Andean crops, improving the quality of life of small Ecuadorian farmers and contributing to global food security, through their production, commercialisation, consumption and industrial application.

4.1.2. Artículo II. Determinación mediante HPLC-DAD-ESI/MS^n de compuestos fenólicos en tubérculos andinos cultivados en Ecuador

HPLC-DAD-ESI/MS^n determination of phenolic compounds in Andean tubers cultivated in Ecuador

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(En preparación)

Abstract

In this work, the phenolic compounds of four Andean tubers grown in Ecuador, such as yacon (*Smallantus sonchifolius*), mashua (*Tropaeolum tuberosum*), melloco (*Ullucus tuberosus*) and purple-sweet potato (*Ipomea batatas*) were analyzed by HPLC–DAD–ESI/MS^n. Non-flavonoid compounds, such as hydroxycinnamic derivatives were identified in yacon, purple sweet potato and melloco samples, accounting for 100%, 26%, 15% of the total of phenolic compounds, respectively. Mashua sample revealed the presence of flavan-3-ol monomers that were not found in the other samples, being (-)-epicatechin the most abundant (9.22 μg/g DM). Quercetin-3-*O*-rutinoside (40,6 μg/g DM), kaempferol-*O*-dirhamnosil-hexoside (29.5 μg/g DM) and kaempferol-*O*-dihexoside (46.22 μg/g DM) were the main flavonols present in mashua, melloco and purple sweet potato, respectively, being this last specie, which presented the highest concentration in flavonols (89.32 μg/g DM). Purple sweet potato also showed the presence of anthocyanins (157.16 μg/g DM), not detected in the other samples. The presence of these compounds and the consideration of the total

phenolic content suggest the use of these Andean tubers as promising sources of natural antioxidants of wide use in the food industry. Therefore, these tubers could be considered as novel and inexpensive sources of bioactive compounds for their potential use in functional foods and nutraceuticals.

Introduction

The chemical properties and simplicity of use of antioxidants have made them a part of many foodstuffs and pharmacological products. The traditional application of antioxidants is their use as additives to confer taste, colour, or to preserve food for the longest possible time. In addition, it is well known the interest in antioxidants attributed to the extensive evidence on the decrease of health disorders under their consumption. Moreover, in recent years more attention is being paid to the study of natural antioxidants present in unexplored or underutilized plant sources due to the safety concerns and limitations on the use of synthetic antioxidants (Shahidi & Ambigaipalan, 2015; Carocho et al., 2018).

Among underutilized plant sources, Andean tubers are crops that grow at high-elevation and whose chemical composition and biological potential remain largely unexplored (Carrillo-Hormaza, et al., 2015). Phenolic compounds are natural antioxidants present in the vacuoles of vegetables tissues that generally are involved in plants defence against ultraviolet radiation or pathogenic aggression (Manach et al., 2016). In addition, phenolic compounds may possess anti-inflammatory, anti-cancer, anti-proliferative, anti-viral activities, and exert an effect on capillary fragility and against human platelet aggregation (Del Rio et al., 2013).

Particularly in the Central Andes (Ecuador to Bolivia) (1,500 to 4,000 m.s.), the highest variability of Andean species has been observed (Tapia et al., 2007). Particularly, there are unexplored Andean tubers in Ecuador that are produced by small farmers only as subsistence crops, due to the lack of knowledge on their physicochemical properties and potential for industrial application.

For this reason, yacon, mashua, melloco, purple sweet potato and white carrot varieties from Ecuador were analysed in a previous study, observing that they have the same or greater amount of antioxidant compounds compared to species of similar varieties, grown in other countries or regions, being important to identify and quantify their individual phenolic compounds (Pacheco et al., 2019).

In addition, these phenolic compounds have been identified in Andean tubers cultivated mainly in Peru, Czech Republic and China (Shahidi & Ambigaipalan, 2015, Campos et al, 2018). Gomes da Silva et al. (2018) have recently reported the presence of trans-cinnamoylquinic acids derivatives in yacon syrup. In this sense, yacon extracts have been shown to exert photoprotective effect on dermal cells (Duque et al., 2017).

Chirinos et al. (2008) showed that mashua tubers partially inhibit the hemolysis of erytrhocites, which could be due to their composition phenolic compounds. This author described the presence of hydroxycinnamic and hydroxybenzoic acids, as non-flavonoid compounds, and also flavan-3-ols, procyanidins, flavonols and anthocyanins as flavonoid compounds.

In melloco, the presence of betalains and betacyanins have been observed (Campos et al., 2006); and extracts of this tuber have presented collagenase activity, being a promising candidate to help the regeneration of tissue without

scars (Heil et al., 2017). Nevertheless, as far as, we know no studies have described the presence of phenolic compounds in this tuber.

Gras et al. (2017) and Wang et al. (2018) have determined the phenolic profile of some varieties of sweet potato by HPLC-ESI-MS. These authors found the presence of hydroxycinnamic derivatives, flavonols, as quercetin glycosides and anthocyanins, remarking that their content depends considerably on the variety. Thus, the objective of this study was to identify and quantify by HPLC-DAD-ESI/MS^n the main phenolic compounds present in yacon, mashua, melloco, purple sweet potato and white carrot, whose Ecuadorian varieties have not been sufficiently studied to date, in order to elucidate their potential use in the food industry and health care.

Material and methods

Samples

Andean tubers: yacon (*Smallantus sonchifolius*, var. INIAP-ECU-1247), mashua (*Tropaeolum tuberosum*, var. INIAP-ECU-Izaño), melloco (*Ullucus tuberosus*, var. INIAP-ECU-amarillo-rosa), purple sweet potato (*Ipomea batatas*, var. INIAP-ECU-morado) cultivated in Cotopaxi-Ecuador (2800-3600 m.a.s.l.) and harvested in mid-April, were washed, lyophilized, grounded and stored at -20°C until analysis.

Chemicals

Phenolic compound standards were purchased from Sigma-Aldrich (Madrid, Spain) and Extrasynthese (Barcelona, Spain). Acetonitrile, formic acid and ultrapure water were of HPLC grade from Carlo Erba (Rodano, Italy) and Panreac

(Barcelona, Spain). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

Extraction of phenolic compounds

Extraction and analyses of phenolic compounds were performed applying the methods described by Dueñas et al. (2015). Briefly, 2 g of freeze-dried samples were sonicated and macerated in methanol: TFA (10/000)–water (80:20, v:v) at 4°C for 24 h. Subsequently, they were centrifuged at 7000 *g* and 4°C for 30 min in a super-speed centrifuge (Sorval RC 5B). The extraction process was repeated three times. The extracts were combined and concentrated at 30 °C under vacuum for methanol evaporation.

For phenolic analysis, the dry extracts were dissolved in 20 mL of water. For purification, an aliquot (1 or 2 mL) was passed through a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA), previously activated with methanol and water. Sugars and polar substances were removed by passing 10 mL of ultrapure water and phenolic compounds were eluted with methanol. Afterwards, extract was concentrated under vacuum in a rotary evaporator (30 °C) and then dissolved in 0.1% TFA: acetonitrile (90:10, v/v) for phenolic compound analysis.

HPLC–DAD–ESI/MS^n analyses of phenolic compounds

Non-anthocyanin compounds

Samples were analyzed using a Hewlett-Packard 1100MS (Agilent Technologies, Palo Alto, CA) chromatograph equipped with a quaternary pump, diode array detector (DAD) coupled to an HP Chem Station (rev. A.0504) data-

processing station. Solvents used were 0.1% formic acid in water (solvent A), and 100% acetonitrile (solvent B). The elution gradient established was 15% B for 5 min, 15–20% B for 5 min, 20–25% B for 10 min, 25–35% B for 10 min, 35–50% B for 10 min, and re-equilibration of the column. The separation of phenolic compounds was performed in a Spherisorb S3 ODS-2 C_8 column (Waters, Millford, USA) (3 µm, 150 mm × 4.6 mm i.d.) operating at 35 °C and a flow rate of 0.5 mL min^{-1}. Double online detection was carried out in the DAD using 280 nm and 370 nm as preferred wavelengths. Mass spectrometer (MS) connected to the HPLC system via the DAD cell outlet was used and detection was performed in an API 3200 Qtrap (Applied Biosystems, Darmstadt, Germany) equipped with an ESI source, triple quadrupole ion trap mass analyzer and controlled by the Analyst 5.1 software following the method described by Dueñas et al. (2015). The setting parameters were: zero grade air as the nebulizer gas (30 psi), turbo gas for solvent drying (400 °C, 40 psi), nitrogen served as the curtain (20 psi) and collision gas (medium). The quadrupoles were set at unit resolution. The ion spray voltage was set at −4500 V in the negative mode. The MS detector was programmed to perform a series of two consecutive modes: enhanced MS (EMS) and enhanced product ion (EPI) analysis. EMS was employed to show full scan spectra, to give an overview of all the ions in sample. Settings used were: declustering potential (DP) −45 V, entrance potential (EP) −6 V, collision energy (CE) −10 V.

Spectra were recorded in negative ion mode between m/z 100 and 1000. EPI mode was further performed with the aim to obtain the fragmentation pattern of the parent ion(s) of the previous experiment using the following parameters: DP: −50 V, EP: −6 V, CE: −25 V, and collision energy spread (CES) 0 V. The non-

anthocyanin phenolic compounds were characterized according to their UV and mass spectra and retention times, and comparison with authentic standards when available. For quantitative analysis, calibration curves were prepared by injection of known concentrations of different standard compounds. Derivatives of *p*-coumaric, caffeic and ferulic acids were quantified by the curves of the corresponding free acids; flavonols, as derivatives of kaempferol, quercetin, isorhamnetin and myricetin by the curves of kaempferol-3-O-glucoside, quercetin-3-O-glucoside, isorhamnetin 3-O-rutinoside and myricetin 3-O-glucoside, respectively. Results were expressed as µg/g dry matter (DM).

Anthocyanins

Samples were analyzed in an AQUA® HPLC system (Phenomenex) equipped with a reverse phase C_{18} column (5 µm, 150 mm × 4.6 mmi.d) at 35 °C according to García-Marino et al. (2010). Detection was carried out at 520 nm. MS was performed in the same equipment described above.

Zero grade air served as the nebulizer gas (40 psi) and turbo gas (600 °C) for solvent drying (50 psi). Nitrogen served as the curtain (100 psi) and collision gas (high). Both quadrupole units were set at unit resolution. The ion spray voltage was set at 5000 V in the positive mode. EMS and ESI methods were used for acquisition of full scan spectra and fragmentation patterns of the precursor ions, respectively.

Setting parameters used for EMS mode were: declustering potential (DP) 41 V, entrance potential (EP) 7.5 V and collision energy (CE) 10 V. Parameters for EPI mode were: DP 41 V, EP 7.5 V, CE 10 V, and collision energy spread (CES) 0 V. For

the quantitative analysis of anthocyanins, a calibration curve was obtained by injection of different concentrations of cyanidin 3-*O*-glucoside. The results were expressed in μg/g DM.

Statistical analysis

The obtaining of phenolic extracts and analysis of compounds were carried out at least in triplicate. The results were expressed as the mean ± standard deviation and significant differences were observed using the Tukey test at a probability level (p <0.05), using the software IBM *SPSS Statistics V22*.0.

Results and discussion

Identification of phenolic compounds in Andean tubers

Identification and quantification of individual phenolic compounds of different Andean tubers (yacon, mashua, melloco and sweet potato) were carried out by HPLC-DAD-MS^n analysis. **Figures 1-6** show the chromatographic profiles of phenolic compounds recorded at 280 nm, 370 or 520 nm. **Tables 1-5** present the data obtained from HPLC-DAD-MS analysis (retention time, λ_{max}, pseudomolecular ions, MS^2 fragment ions) used for the tentative identification and quantification of phenolic compounds in the four Andean tubers.

Figure 1

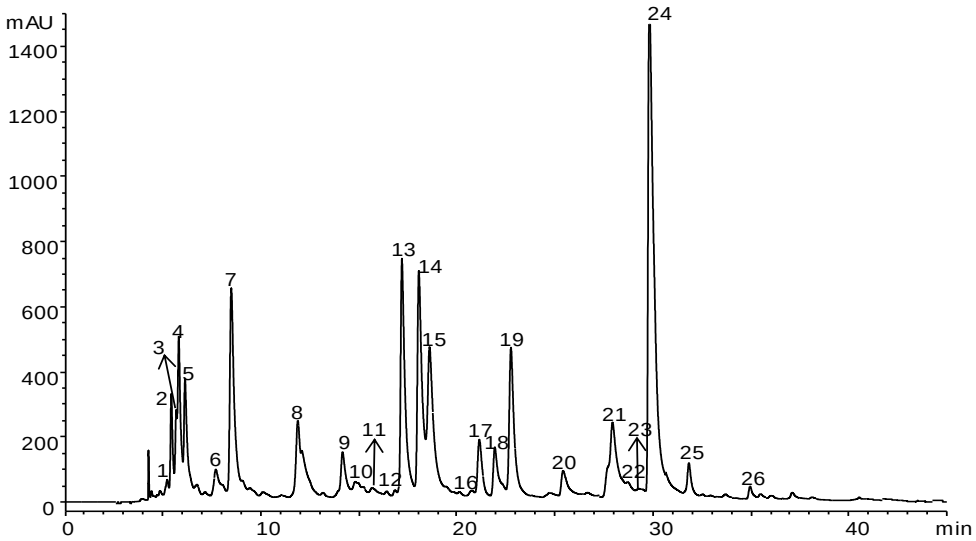
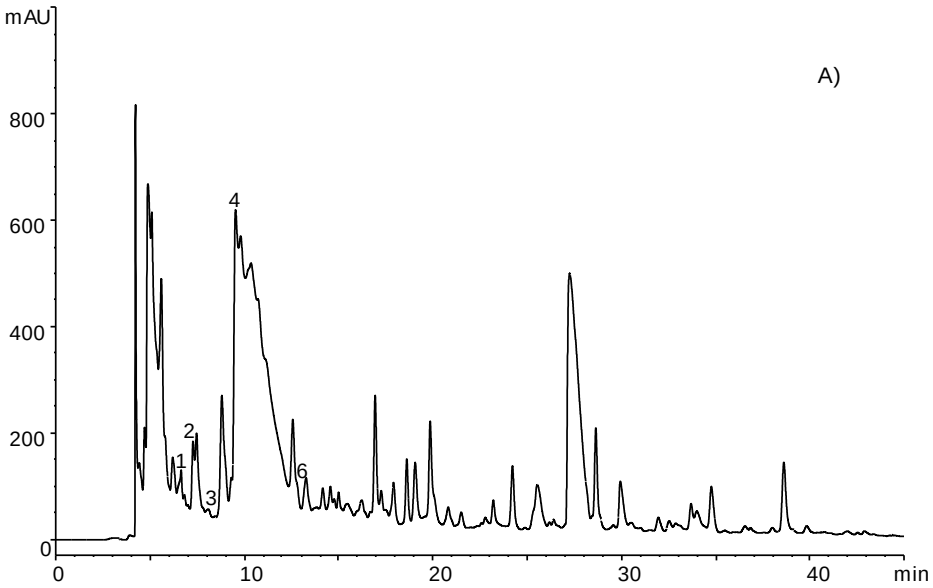


Figure 1. Chromatographic profile of non-anthocyanin compounds observed in yacon recorded at 280 nm. Peaks assignment is exposed in **Table 1**.

Figure 2



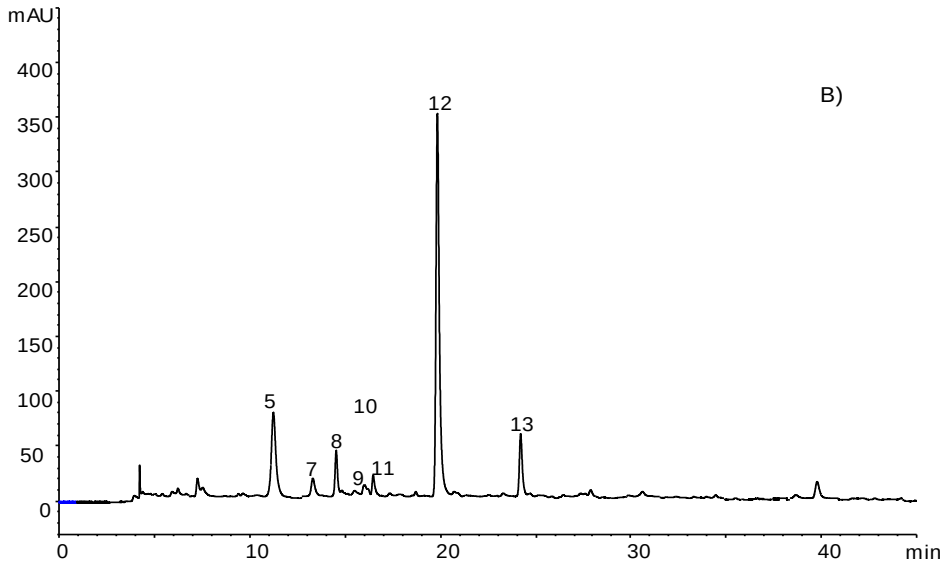
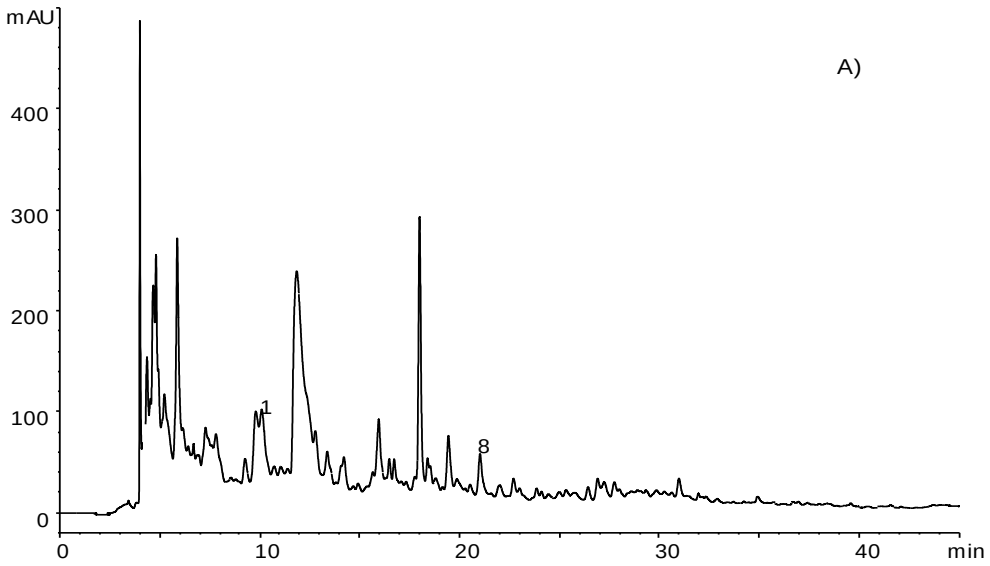


Figure 2. Chromatographic profiles of phenolic compounds determined in mashua recorded at 280 nm (A) and 370 nm (B). Peaks assignment is exposed in **Table 2**.

Figure 3



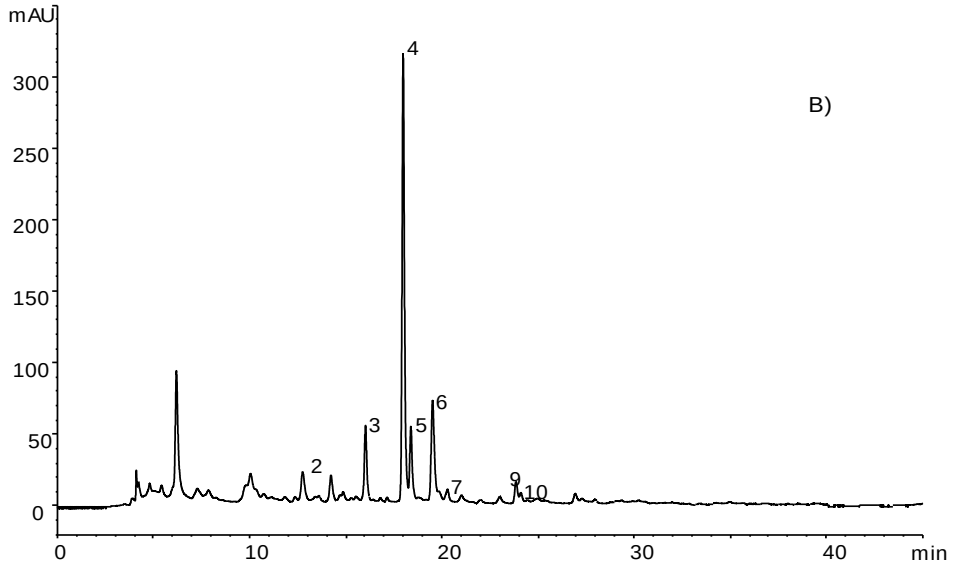
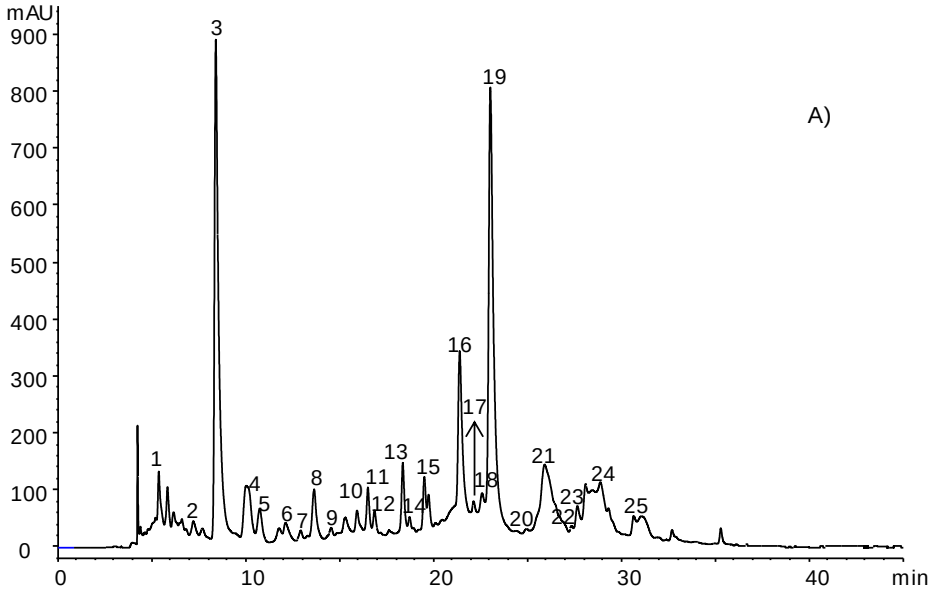


Figure 3. Chromatographic profiles of phenolic compounds determined in melloco recorded at 280 nm (A) and 370 nm (B). Names of peaks are disposed in **Table 3**.

Figure 4



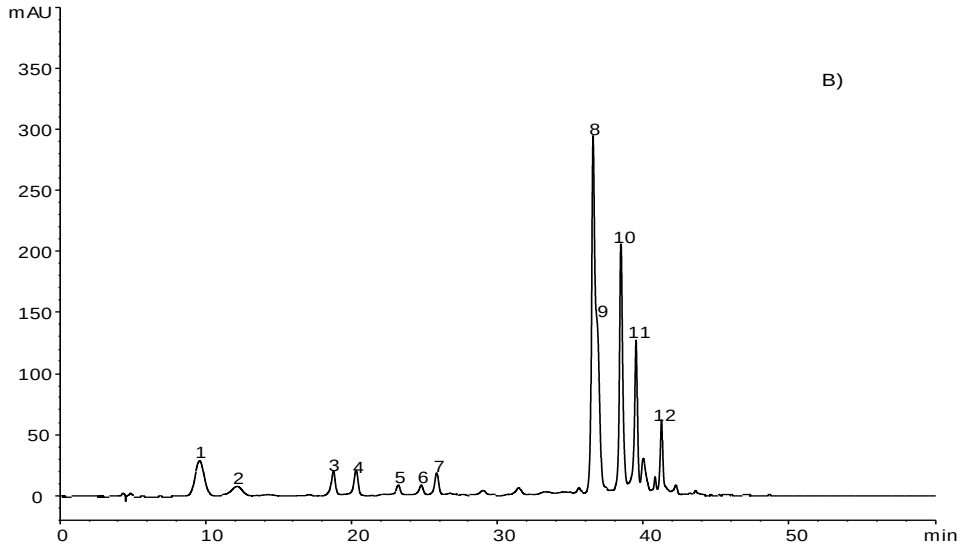


Figure 4. Chromatographic profiles of phenolic compounds observed in purple sweet potato at 280 nm (A) and 520 nm (B). Peaks assignment is exposed in **Tables 4 and 5**.

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (µg/g DM) of non-anthocyanin compounds present in yacon.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]–(m/z)	Main fragments (Da)	Tentative identification	µg/g DM
1	5.1	326	353	191, 179, 173, 161, 135	Caffeoylquinic acid	6.66±0.56
2	5.3	324	353	191, 179, 173, 161, 135	Caffeoylquinic acid	29.15±3.35
3	5.6	328	371	209, 191, 179, 135	Caffeoylglucaric acid	23.18±3.77
4	5.7	328	397	353, 293, 235, 179, 163, 161, 135	4-*O*-caffeoyl-2,7-anhydro-D-glycero-β-D-galacto-oct-2-ulopyranosonic acid isomer 1	53.03±7.93
5	6.0	328	397	353, 293, 235, 179, 163, 161, 135	4-*O*-caffeoyl-2,7-anhydro-D-glycero-β-D-galacto-oct-2-ulopyranosonic acid isomer 2	42.48±1.91
6	7.7	326	353	191, 179, 173, 161, 135, 111	Caffeoylquinic acid	20.17±2.74
7	8.4	326	353	191, 179, 173, 161, 135	Caffeoylquinic acid	180.78±21.10
8	11.7	328	353	191, 179, 173, 155, 135	Caffeoylquinic acid	47.98±3.02
9	14.1	328	533	371, 353, 209, 191, 179, 135	Dicaffeoylaltraric acid isomer 1	46.06±2.68
10	14.7	328	533	371, 353, 209, 191, 179, 136	Dicaffeoylaltraric acid isomer 2	6.46±0.43
11	15.6	330	533	371, 353, 209, 191, 179, 136	Dicaffeoylaltraric acid isomer 3	1.05±0.19
12	16.8	326	533	371, 353, 209, 191, 179, 136	Dicaffeoylaltraric acid isomer 4	0.93±0.17
13	17.1	324	559	397, 293, 235,191, 179, 161, 135	4,5-di-*O*-caffeoyl-2,7-anhydro-D-glycero-*O*-β-galacto-oct-2-ulopyranosonic acid	204.26±12.17
14	18.0	330	533	371, 353, 209, 191, 179, 136	Dicaffeoylaltraric acid isomer 5	257.10±41.52

15	18.6	330	533	371, 353, 209, 191, 179, 137	Dicaffeoylaltraric acid isomer 6	136.68±26.82
16	21.0	328	503	371, 209, 179, 161	Leeaoside [$C_{24}H_{39}O_{11}$]	3.38±0.58
17	21.4	326	515	353, 335, 191, 179, 173, 161, 155, 135	Dicaffeoylquinic acid	39.72±6.14
18	22.1	330	533	371, 353, 209, 191, 179, 136	Dicaffeoylaltraric acid isomer 7	36.23±5.19
19	23.0	324	353	191, 179, 161, 135	Caffeoylquinic acid	178.63±28.03
20	25.7	326	515	353, 335, 191, 179, 173, 161, 155, 135	Dicaffeoylquinic acid	26.96±3.00
21	28.0	328	695	533, 515, 371, 353, 209, 191, 179, 147, 135	Tricaffeoylatraric acid isomer 1	140.98±16.98
22	28.9	330	695	533, 515, 371, 353, 209, 191, 179, 147, 135	Tricaffeoylatraric acid isomer 2	3.41±0.59
23	29.5	330	353	191, 179, 173, 161, 135	Caffeoylquinic acid	1.17±0.18
24	29.8	328	695	533, 515, 371, 353, 209, 191, 179, 147, 135	Tricaffeoylatraric acid isomer 3	649.73±119.40
25	32.1	328	695	533, 515, 371, 353, 209, 191, 179, 147, 136	Tricaffeoylatraric acid isomer 4	27.54±4.68
26	37.4	332	677	515,497,353,335,191, 179,161	Tricaffeoylquinic acid	2.96±0.16
					Total:	2166.66±58.59

Data are the mean ± standard deviation of three replicates.

Table 2. Retention time (Rt), wavelengths of maximum absorption in the visible region (λmax), mass spectral data, tentative identification and quantification (µg/g DM) of non-anthocyanin compounds observed in mashua.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]−(m/z)	Main fragments (Da)	Tentative identification	µg/g DM
1	6.61	272	305	609	(+)-Gallocatechin	t
2	7.28	314	337	191, 173, 163	*p*-coumaroylquinic acid	10.99±0.60
3	8.12	274	305	124.9	(-)-Epigallocatechin	t
4	9.40	280	203	159, 142	Tryptophan	-
5	11.18	354	787	317	Myricetin rhamnosyldihexoside	15.26±1.21
6	12.70	278	289	-	(-)-Epicatechin	9.22±0.80
7	13.26	354	641	317	Myricetin dihexoside	1.81±0.21
8	14.50	354	771	301	Quercetin rhamnosyldihexoside	3.28±0.17
9	15.95	355	829	317	Myricetin acylrhamnosyldihexoside	t
10	16.10	355	625	301	Quercetin dihexoside	t
11	16.40	358	741	609, 301	Quercetin pentosyl rhamnosidehexoside	t
12	19.82	356	609	301	Quercetin 3-rutinoside	40.62±0.25
13	24.27	354	623	315	Isorhamnetin 3-rutinoside	6.98±0.27
					Total:	88.16±3.51

t: traces
Data are the mean ± standard deviation of three replicates.

Table 3. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (µg/g DM) of non-anthocyanin compounds observed in melloco.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]−(m/z)	Main fragments (Da)	Tentative identification	µg/g DM
1	10.09	330	531	193	Ferulic acid derivative	4.31±0.23
2	11.83	280	203	159, 142	Tryptophan	-
3	15.99	352	755	301	Quercetin dirhamnosylhexoside	0.83±0.03
4	17.99	348	739	593, 285	Kaempferol--rhamnoside--rutinoside	29.45±2.26
5	18.41	360	769	315	Isorhamnetin dirhamnosylhexoside	1.23±0.02
6	19.49	350	609	301	Quercetin 3-rutinoside	2.60±0.05
7	20.28	354	593	285	Kaempferol-3-rutinoside	t
8	21.03	322	193	149	Ferulic acid	1.39±0.05
9	23.86	356	635	285	Kaempferol acetylrhamnosylhexoside	0.85±0.05
10	24.12	330	623	315	Isorhamnetin 3-rutinoside	0.14±0.01
					Total:	40.80±1.95

t: traces
Data are the mean ± standard deviation of three replicates.

Table 4. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (µg/g DM) of non-anthocyanins compounds present in purple sweet potato.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]−(m/z)	Main fragments (Da)	Tentative identification	µg/g DM
1	5.38	326	353	191, 179, 173, 161, 135	Caffeoylquinic acid isomer 1	t
2	7.70	324	465	303, 193	Ferulic hexoside derivative	t
3	8.41	326	353	191,179, 161,135	Caffeoylquinic acid isomer 2	32.64±4.52
4	10.20	324	353	191,179, 161,135	Caffeoylquinic acid isomer 3	5.88±0.02
5	10.77	288, 338	449	287	Eriodictyol hexoside	t
6	12.12	278	203	159, 142	Tryptophan	-
7	12.95	288, 338	449	287	Eriodictyol 7-glucoside	t
8	13.69	312	337	193, 191, 173, 163, 145	*p*-coumaroyl quinic acid isomer 1	t
9	14.55	314	338	193, 191, 173, 163, 146	*p*-coumaroyl quinic acid isomer 2	t
10	15.95	350	625	301	Quercetin dihexoside	t
11	16.55	326	367	191, 191, 161, 135	Feruloylquinic acid isomer1	1.83±0.23
12	16.89	332	367	193, 191, 191, 161, 135	Feruloylquinic acid isomer 2	t
13	18.41	346	609	285	Kaempferol dihexoside	46.22±0.07
14	19.55	326	651	285	Kaempferol acyldihexoside	12.84±0.45
15	19.79	354	681	315	Isorhamnetin acyldihexoside	28.46±0.06
16	21.45	324	515	353, 335, 191, 179, 173, 161, 155,135	Dicaffeoylquinic acid isomer 1	6.67±0.65
17	22.19	324	515	353, 335, 191, 179, 173, 161, 155,136	Dicaffeoylquinic acid isomer 2	t

18	22.64	324	515	353, 335, 191, 179, 173, 161, 155,137	Dicaffeoylquinic acid isomer 3	t
19	23.08	324	353	191,179, 161,135	Caffeoylquinic acid isomer 4	36.07±4.19
20	24.87	346	447	285	Kaempferol 3-glucoside	2.02±0.26
21	25.88	324	515	353, 335, 191, 179, 173, 161, 155,137	Dicaffeoylquinic acid isomer 4	2.14±0.06
22	26.88	332	771	609.285	Kaempferol trihexoside	0.18±0.02
23	27.63	318	529	367, 355, 179, 173, 161, 135	Feruloylquinic-hexoside acid isomer 1	t
24	28.86	328	529	367, 355, 179, 173, 161, 135	Feruloylquinic-hexoside acid isomer 2	t
25	30.64	328	529	367, 355, 179, 173, 161, 136	Feruloylquinic-hexoside acid isomer 3	t
					Total:	174.94±12.54

t: traces. Data are the mean ± standard deviation of three replicates.

Table 5. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data, tentative identification and quantification (µg/g DM) of anthocyanins compounds present in purple sweet potato.

Peak	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]−(m/z)	Main fragments (Da)	Tentative identification	µg/g DM
1	9.60	514	773	611, 449, 287	Cyanidin 3-dihexoside-5-hexose	13.08±0.51
2	12.15	500	757	595, 433, 271	Pelargonidin 3-dihexoside-5-hexoside	6.15±0.31
3	18.74	520	893	731, 449, 287	Cyanidin 3-p-hidroxybenzoilhexoside-5-hexoside	4.18±0.06
4	20.31	522	935	773, 449, 287	Cyanidin 3-cafeoyldihexoside-5-hexoside	3.91±0.06
5	23.19	504	877	715, 433, 271	Pelargonidin 3-p-hydroxybenzoilhexoside-5-hexoside	2.20±0.04
6	24.77	506	919	757, 433, 271	Pelargonidin 3-caffeoyldihexoside-5-hexoside	2.17±0.05
7	25.87	522	949	787, 449, 287	Cyanidin 3-feruloyldihexoside-5-hexoside	3.32±0.03
8	36.57	530	935	773, 449, 287	Cyanidin 3-dicafeoyldihexoside-5-hexoside	44.78±1.15
9	36.88	520	893	731, 449, 287	Cyanidin 3-p-hydroxybenzoildihexoside-5-hexoside	20.28±0.57
10	38.50	524	919	757, 433, 271	Pelargonidin 3-caffeoyldihexoside-5-hexoside	30.84±1.13
11	39.48	512	877	715, 433, 271	Pelargonidin 3-*p*-hydroxybenzoildihexoside-5-hexoside	18.81±0.85
12	41.25	528	933	771, 433, 271	Pelargonidin 3-feruloyldihexoside-5-hexoside	7.46±0.12
					Total:	157.16±4.18

Data are the mean ± standard deviation of three replicates.

Hydroxicinnamic compounds

Hydroxycinnamic acid derivatives were found in all studied Andean tubers with exception of mashua (*Tropaeolum tuberosum*). Diverse hydroxycinnamic acid derivatives were identified in Yacon (*Smallantus sonchifolius),* Melloco (*Ullucus tuberosus*) and purple sweet potato (*Ipomea batatas).*

Ferulic acid (peak 8 in melloco) was completely identified according to their retention time, mass and UV-vis characteristics by comparison with commercial standard.

Peaks 1, 2, 6, 7, 8, 19 and 23 in yacon and peaks 1, 3, 4 and 19 in purple sweet potato showed an UV-vis spectrum similar to caffeic acid with λ_{max} 324-330 nm, but eluted at different retention times. They presented a precursor ion $[M-H]^-$ at *m/z* 353 and fragments at *m/z* 191, due to the deprotonated quinic acid, 179 [caffeic acid-H]$^-$, 173 and 135, characteristics of caffeoylquinic acid according to their characteristic fragmentation pattern (Clifford et al., 2005). Similarly, peaks 17, 20 and 26 in yacon and peaks 16-18 and 21 in purple sweet potato were tentatively assigned as dicaffeoylquinic acid, and tricaffeoylquinic acid, respectively, based on their fragmentation patterns described by Clifford et al. (2005). Peak 3 was tentatively detected as aldaric acid (glucaric acid) derivative according to its UV-vis spectra (λ_{max} 328 nm) and precursor ion $[M-H]^-$ at *m/z* 371 and fragment ion at m/z 209, corresponding to an aldaric acid residue.

Peaks 4 and 5 presented a precursor ion $[M-H]^-$ at *m/z* 397, and fragment ions at *m/z* 235, due to the loss of caffeoyl moiety, 179 [caffeoyl acid –H]$^-$ and 135 [caffeoyl acid – carboxylic group]$^-$. These compounds were tentatively identified as 4-*O*-caffeoyl-2,7-anhydro-D-glycero-β-D-galactooct-2-ulopyranosonic acid

isomers. Thus, peak 13 showed an additional caffeoyl group with respect to previous compounds (peak 4 and 5), with $[M-H]^-$ at *m/z* 559, and fragments ions at *m/z* 397 and 235 was putatively identified as 4,5-di-*O*-caffeoyl-2,7-anhydro-D-glycero-*β*-D-galactooct-2-ulopyranosonic acid. These three compounds were previously isolated in yacon (*Smallanthus sonchifolius*) and confirmed their identity by NMR (Takenaka, & Ono, 2003).

Peaks 9-12, 14, 15 and 18 showed a precursor ion $[M-H]^-$ at *m/z* 533, and fragments at *m/z* 371 and 209, due to losses of one and two caffeoyl moieties, respectively. These compounds were tentatively identified as dicaffeoylaltraric acid isomers. Similarly, peaks 21, 22, 24 and 25 presented one additional caffeoyl group with respect to previous compounds, with a precursor ion $[M-H]^-$ at *m/z* 695 and fragments at *m/z* 533, 371 and 209. These compounds were tentatively identified as tricaffeoylaltraric acid isomers.

Peak 16 showed an UV-vis spectrum similar to caffeic acid with λ_{max} 328 nm. It presented precursor ion $[M-H]^-$ at *m/z* 503 and fragment ions at *m/z* 371 corresponding to the loss of a pentoside moiety $[M-132]^-$, at *m/z* 209 due to the losses of one pentoside and one hexoside $[M-132-162]^-$ and *m/z* 179 corresponding to caffeoyl moiety. This compound was tentatively identified as leeaoside.

Peak 2 in mashua, and peaks 8, 9 in purple sweet potato were identified as *p*-coumaroylquinic acid; and peaks 11 and 12 in purple sweet potato were identified as feruloylquinic acid. These compounds showed a pseudomolecular ion $[M-H]^-$ at *m/z* 337 and 367, and fragment ions at *m/z* 191 due to the deprotonated quinic

acid, and 163 and 193 corresponding to [*p*-coumaroyl –H]$^-$ and [feruloyl –H]$^-$, respectively.

Peaks 23-25 in purple sweet potato showed typical UV-vis spectra of ferulic acid. These compounds were identified as feruloylquinic-hexoside acid according to their pseudomolecular ion [M-H]$^-$ at *m/z* 529, and fragments characteristic of a feruloylquinic acid, the MS^2 spectrum of these compounds produced fragments of 367 Da ([M-H]-162$^-$, due to the loss of a hexose residue) and 355 Da ([M-H]-174$^-$, due to the loss of a moiety of quinic acid).

To sum up, chlorogenic acids, esters formed between certain trans cinnamic acids, most commonly caffeic, *p*-coumaric and ferulic acid and quinic acid (Clifford et al., 2003; Clifford et al., 2005; Clifford et al., 2006; Clifford et al., 2007) were found in yacon and purple sweet potato.

The presence of caffeoyl derivatives in the yacon variety analyzed in our study was in agreement with the previous studies carried out by UPLC-ESI-Q-TOF-MSE in yacon coming from Brazil (Gómez da Silva, et al., 2018). The presence of ferulic and caffeic acid in yacon (*S. sonchifolius*, var. INIAP-ECU-1247) has been also reported to yacon coming from Ecuador (*S. sonchifolius*, Asteraceae), cultivated in the Czech Republic and analysed by HPLC/MS (Simonovska et al., 2003).

The presence of ferulic and quinic acid in purple sweet potato (*I. batatas,* var. INIAP-ECU-morado) has also been observed in five varieties of purple sweet potato grown in China (Wang et al., 2018).

In mashua cultivated in Peru, Chirinos et al. (2008) also observed the presence of *p*-coumaroylquinic acid but they did not find feruloylquinic acid, as observed in the present study for mashua (*Tropaeolum tuberosum*, var. INIAP-ECU-Izaño). The

differences between the type and content of phenolic compounds identified can be due to the cultivar and growing conditions, type of solvent, the pH level, the water-solvent ratio and the extraction time (Chirinos et al., 2007).

Flavan-3-ols

Flavan-3-ols monomers were only identified in mashua sample. (+)-Gallocatechin (peak 1), (-)-epigallocatech (peak 3) and (-)-epicatechin (peak 6) were identified according to their retention time and mass and UV-vis characteristics by comparison with commercial standards.

These results are related to those obtained by Chirinos et al. (2008), who characterized non-anthocyanin phenolic compounds in Peruvian mashua genotypes by HPLC-DAD, revealing the presence of gallocatechin, procyanidin B2, epigallocatechin and (-)-epicatechin.

Flavonols

Flavonols were the main flavonoids detected in all studied samples with the exception of yacon sample, being quercetin, kaempferol, myricetin and isorhamnetin derivatives found mostly in mashua, melloco and purple sweet potato.

Quercetin 3-rutinoside and isorhamnetin 3-rutinoside were found in mashua, melloco samples, while kaempferol 3-rutinoside was only found melloco and kaempferol 3-glucoside was only found in purple sweet potato. These compounds were positively identified according to their retention time and mass and UV-vis characteristics by comparison with commercial standards.

Myricetin derivatives (peaks 5, 7 and 9) were only identified in mashua sample. These compounds presented similar UV-vis spectra to myricetin glycoside and fragment ion at *m/z* 317. Myricetin rhamnosyl-dihexoside (peak 5) ($[M-H]^-$ at *m/z* 787), myricetin dihexoside (peak 7) ($[M-H]^-$ at *m/z* 641) and myricetin acetylrhamnosyl-dihexoside (peak 9) ($[M-H]^-$ at *m/z* 829) released fragments corresponding to the losses of rhamnosyl moiety (-146 amu), two hexosides units (-324 amu) and acetyl residue (-42 amu). In none of them the identity of the sugar and positions of location of the substituents could be established.

Other quercetin derivatives were peaks 8, 10 and 11 in mashua sample and peak 3 in melloco. These compounds were assigned to a quercetin rhamnosyldihexoside ($[M-H]^-$ at *m/z* 771) (peak 10), quercetin dihexoside ($[M-H]^-$ at *m/z* 625) (peak 11), quercetin pentosyl rhamnosilhexoside ($[M-H]^-$ at *m/z* 741) (peak 3 in melloco), quercetin dirhamnosylhexoside ($[M-H]^-$ at *m/z* 755), according to their precursor ions and MS^2 spectra, releasing fragments corresponding to the losses of rhamnosyl moieties (-146 amu), two hexosides units (-324 amu) and one pentosyl residue (-132 amu). In none of them the identity of the sugar and positions of location of the substituents could be established.

Kaempferol derivatives (λ_{max} around 347 nm, and unique fragment ion at *m/z* 285) were also identified in melloco and purple sweet potato. Kaempferol rhamnosyl-rhamnosylhexoside (peak 4) was identified in melloco sample, showing precursor ions $[M-H]^-$ at *m/z* 739 and fragment ions at *m/z* 593 ($[M-146]^-$, loss of a rhamnoside moiety) and at *m/z* 285 ($[M-308]^-$, loss of a rhamnoside-hexoside moiety). A kaempferol acetyl-rhamnosylhexoside (peak 9) was also detected in melloco sample, showing precursor ions $[M-H]^-$ at *m/z* 635, releasing MS^2

fragment at *m/z* 285 ([M-42-146-162]$^-$, loss of acetyl+rhamnosyl-hexoside residue).

Kaempferol dihexoside (peak 13), kaempferol acetyl-dihexoside (peak 14) and kaempferol trihexoside (peak 22) were tentatively identified in purple sweet potato. These compounds were identified acording to [M-H]$^-$ at *m/z* 609, 651 and 771, respectively. Fragment ion at m/z 285 corresponded to kaempferol due to losses of 324 amu (two hexoside units), loss of 42 amu (one acetyl residue) and 486 (three hexoside units).

Another group of detected flavonols were isorhamnetin derivatives according to their UV-vis and mass spectra. All of them released a fragment ion at *m/z* 315 and they were tentatively identified in melloco (peak 10) and sweet purple potato (peak 15). The first peak was positively identified as isorhamnetin 3-rutinoside by comparison with standard, while peak 15 in sweet purple potato was assigned to isorhamnetin acetyldihexoside from the loss of 366 amu (-42-324 amu, corresponding to acetyl+dihexosyl residues).

The Ecuadorian yacon var. INIAP-ECU-1247 analyzed in the present study differs from the Ecuadorian yacon var. Asteraceae analyzed by Simonovska et al. (2003), in which it was observed the presence of quercetin.

Purple sweet potato var. INIAP-ECU-purple presented kaempferol and isorhamnetin derivatives as the main flavonols; compounds not observed in five varieties of purple sweet potato grown in China, which have only shown the presence of *O*-hexoside of quercetin as flavonol compound (Wang et al., 2018).

To the best of our knowledge, the presence of flavonols had not been reported for mashua or melloco to date.

Flavanones

This flavonoid group was only identified in purple sweet potato. Peak 7 was identified as eriodyctiol 7-glucoside by comparison with a commercial standard. Peak 5 was assigned to eriodyctiol hexoside according to its $[M-H]^-$ at 449 and fragment ion corresponding to the loss of hexosyl moiety (-162 amu). This type of compound has not been reported for purple sweet potato in other studies.

Anthocyanins

Twelve anthocyanin pigments were only detected in purple sweet potato sample (**Table 5**). Pigments were cyaniding (Cy) and pelargonidin (Pg) derivatives, as demonstrated for their UV-vis spectra and mass spectral data.

Peaks 1 and 2 corresponded to derivatives of cyanidin and pelargonidin. Peak 1 showed an UV-vis spectra λ_{max} 514 nm and precursor ion at *m/z* 773, releasing a fragment ions at *m/z* 611 ($[M-162]^+$ due to the loss of a hexoside moiety, at *m/z* 449 ($[M-162-162]^+$ derived from the losses of two successive hexoside moieties, and 287 ($[M-162-162-162])^+$ corresponding to cyanidin. This peak was tentatively identified as cyanidin 3-dihexoside-5-hexoside. Similarly, peak 2 presented the same fragmentation pattern as peak 1, which was tentatively assigned as pelargonidin 3-dihexoside-5-hexoside, based on the fragment ion at *m/z* 271 corresponding to pelargonidin.

Peaks 3-12 corresponded to acyl derivatives of cyanidin and pelargonidin. Peaks 3 and 9 showed the molecular ions at *m/z* 893 that release three fragments MS^2 at *m/z* 731, (-162 amu, loss of one hexoside moiety), *m/z* 449 (-162-120 amu, loss of hexoside-p-hydroxybenzoyl moieties), *m/z* 287 (cyanidin) corresponded to

cyanidin-3-*p*-hydroxybenzoylhexoside-5-hexoside (peak 3) and cyanidin-3-*p*-hydroxybenzoyldihexoside-5-hexoside (peak 9).

Peaks 5 and 11 showed a similar fragmentation pattern, so that it can be assigned to pelargonidin-3-*p*-hydroxybenzoylhexoside-5-hexoside (peak 3) and pelargonidin-3-*p*-hydroxybenzoyldihexoside-5-hexoside (peak 9).

Peaks 4, 6, 8 and 10 were observed around λ_{max} 506-530 nm, with a UV-visible spectra characteristic of acyl anthocyanin derivatives. These compounds presented precursor ions at *m/z* 935 and 919, releasing three fragment ions at *m/z* 773 and 757 ($[M-162]^+$ due to the loss of a hexoside moiety), *m/z* 499 and 433 ($[M-162-162]^+$ due to the loss of a caffeoyl+hexoside residues), and *m/z* 287 (cyanidin) and 271 (pelargonidin) corresponded to cyanidin-3-caffeoyldihexoside-5-hexoside (peaks 4 and 8), and pelargonidin-3-caffeoyldihexoside-5-hexoside (peaks 6 and 10).

Peaks 7 and 12 showed their maximum absorption to λ_{max} 522 and 528 nm, respectively, with a UV-visible spectra characteristic of acyl anthocyanin derivatives. These compounds had precursor ions at *m/z* 949 and 933, releasing three fragment ions at *m/z* 787 and 771 ($[M-162]^+$ due to the loss of a hexoside moiety), *m/z* 499 and 433 ($[M-162-176]^+$ due to the loss of a feruloyl+hexoside residues), and *m/z* 287 (cyanidin) and 271 (pelargonidin) corresponded to cyanidin-3-feruloyldihexoside-5-hexoside (peak 7) and pelargonidin-3-feruloyldihexoside-5-hexoside (peak 12).

The presence of anthocyanins has been reported in different varieties of purple sweet potato (Gras et al., 2017; Wang et al., 2018; Sun et al., 2018). The presence of cyanidin derivatives has also been observed in some varieties of purple sweet

potato grown in Japan, which have also shown presence of peonidin (Cuevas Montilla et al., 2011).

Others non-phenolic compounds

Tryptophan, an aromatic amino acid was found in mashua (peak 4), melloco (peak 2) and purple sweet potato (peak 6). This compound showed a pseudomolecular ion $[M-H]^-$ at *m/z* 203. Its identity was confirmed by comparison with retention time and UV-vis characteristics with commercial standard.
The detection of this amino acid in mashua is in accordance with the observed by Grau et al. (2003); while in melloco and purple sweet potato, tryptophan has not been reported so far.

Content of phenolic compounds in Andean tubers

Table 6 shows the total content of phenolic compounds identified. According to this, yacon has a very high phenolic content followed by purple sweet potato, whereas mashua and melloco exhibited the lowest phenolic content (p <0.05).

Table 6. Total content of phenolic compounds identified (µg/g DM) by HPLC–DAD–ESI/MS^n in Andean tubers.

Tuber	Total
Yacon	2166.66±58.59 d
Mashua	88.16±3.51 ab
Melloco	40.80±1.95 a
Purple sweet potato	332.10±8.36 c

DM: dry matter. Means with different letters a-d denote significant difference (p < 0.05) in the same column.

Figure 5 shows the contribution of each phenolic group with respect to the total of phenolic compounds detected by HPLC–DAD–ESI/MS^n in the Andean tubers analyzed. Thus, 100% of phenolic compounds present in yacon correspond to hydroxycinnamic derivatives, while in melloco and purple sweet potato only 15.42 and 25.82% of phenolic compounds are constituted by this type of compounds. Melloco, instead, showed the greatest presence of flavonols equivalent to 84.58%, also present in mashua and purple sweet potato but in a proportion of 68.82 and 26.57%, respectively. Flavan-3-ols were detected only in mashua (31.18% of total phenolic compounds), and anthocyanins were only present in purple sweet potato in a proportion of 47.61%.

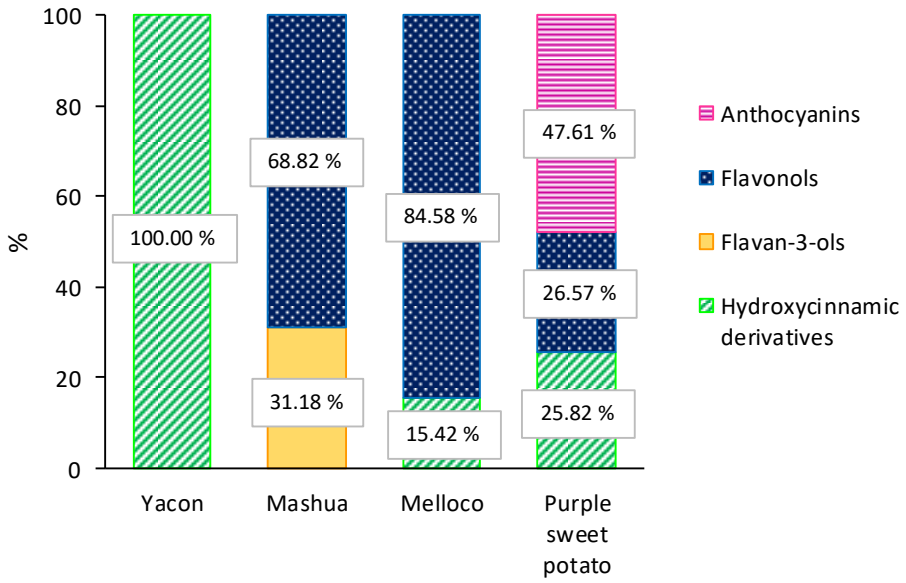


Figure 5. Percentage of distribution of different phenolic groups to the total phenolic content.

Hydroxycinnamic acid derivatives and flavanoids (eg. flavonols, flavanols and anthocyanins) have revealed anti-proliferative activities in numerous cancer cell lines and inhibit tumour growth in some animal models (Roleira et al., 2015).

Non-anthocyanin compounds

Yacon showed mainly hydroxycinnamic quinic acid derivatives in a total concentration of 2166.66 µg/g DM (**Table 1**). Chlorogenic acids observed are hydroxycinnamic esters (isomers of quinic acid), that can reduce the risk of developing metabolic syndrome by reducing the risk of type 2 diabetes and decreasing triglyceride concentrations. These effects of chlorogenic acid consumption have been attributed to its antioxidant and anti-inflammatory properties (Tajik et al., 2017).

The phenolic total concentration in mashua (88.16 µg/g DM) was constituted by quercetin 3-rutinoside as the most abundant compound (40.62 µg/g DM) (**Table 2**). This glycoside has shown effective protection against mortality induced by radiation in animal models, related to the inhibition of oxidative stress and apoptosis (Bansal et al, 2012).

The total phenolic content observed in melloco (40.8 µg/g DM) was constituted by kaempferol-*O*-rhamnoside-*O*-rutinoside as the most abundant phenolic compound (29.45 µg/g DM) followed by ferulic acid derivative (4.31 µg/g DM), and quercetin rutinoside (2.60 µg/g DM) (**Table 3**). This polyphenol has exerted anti-inflammatory and anti-asthmatic effects in mice model (Chung et al., 2015). Ferulic acid in recent years have been deeply studied on its potential effect in cancer, cardiovascular diseases, diabetes mellitus, skin disease and Alzheimer's

disease (Shahabadi et al., 2017; Sgarbossa et al., 2015); whist quercetin 3-O-rutinoside has shown protective effect against atherosclerosis, oxidative stress, cardiotoxicity, endothelial cell dysfunction and heart failure (Patel et al., 2018). However, it has been mentioned that very few plants have the ability to store rutin in great quantities (Habtemariam, & Varghese, 2015).

The analysis by HPLC–DAD–ESI/MS^n applied on purple sweet potato samples, allowed to identify 25 peaks of non-anthocyanin compounds, constituted by flavonols and total hydroxycinnamic acid derivatives, almost in similar proportion (89.72 *vs.* 85.23 µg/g DM), resulting a total of 174.95 µg/g DM (**Table 4**). The majority compounds were caffeoylquinic acids, kaempferol dihexoside, isorhamnetin, and kaempferol acyldihexoside. Caffeoylquinic acids and di-caffeoylquinic acids are the main derivatives of chlorogenic acid, which is found in plant extracts (eg., coffee). Chlorogenic acid derivatives reduces the risk of cardiovascular disease and type 2 diabetes mellitus (Ranheim, & Halvorsen, 2005; Salazar-Martinez et al., 2004), antibacterial activity (Almeida et al., 2006) and anti-inflammatory effects (Santos et al. al., 2006), as mentioned in previous lines.

Anthocyanins

Anthocyanin compounds were observed only in purple sweet potato, in a concentration of 157.16 µg/g DM, being the majority compounds: cyanidin 3-dicafeoyldihexoside-5-hexoside, followed by pelargonidin 3-caffeoyldihexoside-5-hexoside, and cyanidin 3-p-hydroxybenzoildihexoside-5-hexoside (**Table 5**).

The type and concentration of anthocyanin compounds not only provides information on antioxidant potential, but also gives an idea on the color of the vegetable pulp. In the case of the purple sweet potato, when the ratio

peonidin/cyanidin is superior to 1 the color is more red, and when the ratio peonidin/cyanidin is inferior to 1, the color is more blue (Cuevas Montilla et al., 2011).

The absence of peonidin in the sweet potato analyzed and the presence of cyanidin (cyanidin/peonidin < 1), indicates a pulp of intense blue color, in accordance with the levels observed in the analyzed samples.

Conclusion

The phenolic composition of four undervalued Andean tubers from Ecuador, yacon, mashua, melloco and purple sweet potato were analysed by HPLC-DAD-ESI/MS. Notable qualitative and quantitative differences were observed among them, due to genetic differences and/or origin of the crop. The identification of phenolic compounds has been described for the first time in these Andean tubers. Non-flavonoid compounds, such as hydroxycinnamic derivatives were identified in yacon, purple sweet potato samples and melloco, accounting for 100%, 26%, 15% of the total of phenolic compounds, respectively, being yacon the tuber with the highest total phenolic content. The presence of flavonoid, such as flavan-3-ols was only shown in mashua sample, accounting for 31% of the total of phenolic compounds. Flavonols were the majority group in mashua and melloco, presenting 69% and 85% of total of phenolic compounds. Purple sweet potato also showed the unique presence of anthocyanins (157.16 μg/g DM). The phenolic profiles and phenolic content observed especially in yacon and in purple sweet potato, point out their great potential as novel raw materials, for antioxidant compound sources of known therapeutic activity and, therefore, of great

importance for health care, with promising perspectives to be used in the pharmacological field and/or in the development of new functional foods.

4.1.3. Artículo III. Propiedades morfológicas, tecnológicas y nutricionales de harinas y almidones de mashua (*Tropaeolum tuberosum*) y melloco (*Ullucus tuberosus*) cultivados en Ecuador

Morphological, technological and nutritional properties of flours and starches from mashua (*Tropaeolum tuberosum*) and melloco (*Ullucus tuberosus*) cultivated in Ecuador

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Food Chemistry (submited)

Abstract

Morphological, technological and nutritional analyses were done in two unexplored starches from Andean tubers (mashua and melloco). The average particle size of both starch samples was significantly higher than that observed for potato starch, which could explain the high zeta potential of mashua and melloco starches in cold dispersions at a wide range of pH, indicating a better behaviour as stabilizer than potato starch. During heating, mashua and melloco starches presented much higher viscosity than potato starch. DSC and TGA analyses indicated that melloco starch had the highest gelatinization enthalpy H_{gel} (12.32 Jg^{-1}) and degradation temperature (270 °C), which are indicators of its high thermal resistance. Consequently, extracted mashua and melloco starches could be excellent and cost-effective thickening or gelling agents in both foods and a wide range of biomaterials. Mashua and melloco starches also exhibited a digestion rate close to 80%, which agreed with the low resistant starch content.

Introduction

Starch is the most important food polysaccharide, maize, cassava, potato, wheat and rice being the most important sources for obtaining the commercially available starch. The global production of starch reached more than 340.1 million Tons in 2017; however, there are 795 million people who still suffer from hunger, with more than two billion people undergoing micronutrient deficiencies or forms of over-nourishment (FAO, 2017). The hunger situation is exacerbated by other global issues, such as uneven demographic expansion, climate change, intensification of natural disasters, upsurges in pests and diseases, and the need to adjust to major changes of the global food systems (Ojuederie & Ogunsola, 2017). In addition, despite the fact that global food production has increased, the Dietary Energy Supply (DES) in middle- and low-income countries (e.g. some of the nations of Latin America and the Caribbean) remains well/quite below than the DES observed in high-income countries (FAO, 2017b). These trends and challenges have spurred the interest in seeking new sources of nutrients, that can contribute to a healthier lifestyle, which, in turn, can promote social and economic development.

The Andean region is known for its great diversity of roots and tubers that are consumed by the rural population as staple foods because the high amount of carbohydrates in them, especially starch. In tuberous plants, on average, starch accounts for no more than 16–24% of their weight, the rest being water and other non-starchy components (Hammes et al., 2005). However, some Andean tubers, such as mashua (*Tropaeolum tuberosum*) and melloco (*Ullucus tuberosus*), have showed to possess more than 35% of starch, and in addition, high resistance to

drought, low temperatures and insects, nematodes and fungi attack (Campos et al., 2018; Morón, 1999). Mashua and melloco cultivated in Ecuador (at 2800-3600 metres above the sea level, m.a.s.l.) constitute two underutilised sources of starch whose thermal, technological, functional properties and applications are still scarcely described.

Starch granules are formed by two polymers: amylose and amylopectin. Amylose is often slightly branched and amylopectin is highly branched. Amylose constitutes 20–30% of the starch granule. It is made of long chains of α-(1,4)-linked D-glucose units with a degree of polymerization ranging between 3×10^2 and 1×10^4. By contrast, amylopectin has α-(1,4)-linked glucose chains, joined by α-(1,6)-linkages with a degree of polymerization of approximately 10^8 (Castro et al., 2005).

Starches can be classified into three main groups according to their digestibility properties. These categories are: rapidly digestible, slowly digestible and resistant starches. Although the largest portion of starch is digested in the human small intestine, a substantial part may reach the large bowel. Resistant starch (RS) is defined as the portion of starch that is not digested in the small intestine but reaches the large intestine in its intact form (Lovegrove et al., 2017). Recently, RS has gained more importance because of its positive physiological benefits including the prebiotic effect, improvement of cholesterol metabolism, and reduction of the risks of ulcerative colitis and colon cancer (Shi & Maningat, 2017). RS can be classified into five types: physically inaccessible starch (RS1); ungelatinized starch (RS2); retrograded starch (RS3); chemically modified starch (RS4); and amylose-lipid complex (RS5). RS3 has been particularly studied because

of its beneficial functions in cereal products, such as thermal stability, high gelatinization temperature, low water holding properties, improved texture, appearance and organoleptic properties (Shi & Maningat, 2017).

A fundamental characteristic of native starches from different vegetal sources is that their granular and molecular structures influence their physicochemical and functional properties. The thickening, binding and emulsifying properties of starches make them useful in the food industry, cosmetics manufacturing and in the development of biomaterials (Godbillot et al., 2006). Therefore, although several studies have examined the different applications of starch (Ogunsona et al., 2018; Vilpoux et al., 2019), it deems valuable to evaluate new starch sources with specific characteristics to explore possibilities for new industrial applications across sectors (Turola Barbi et al., 2018). In consequence, the aim of this work was to analyze the morphological, technological and nutritional properties of flours and starches extracted from mashua (*Tropaeolum tuberosum,* var. INIAP-ECU-Izaño) and melloco (*Ullucus tuberosus,* var. INIAP-ECU-amarillo-rosa) cultivated in Ecuador to determine their possible industrial applications.

Materials and methods

Mashua (*Tropaeolum tuberosum,* var. INIAP-ECU-Izaño) and melloco (*Ullucus tuberosus,* var. INIAP-ECU-amarillo-rosa) were cultivated in Cotopaxi-Ecuador (2800-3600 m.a.s.l.) and harvested in mid-April. The tubers were maintained in a cool and dry environment, and they were cleaned, lyophilized, and ground to obtain the flours. Starch was obtained grinding the fresh tubers until obtaining a porridge that was rinsed through a 250 µm-mesh sieve with deionized water (1:5

v/v). After decanting the filtered liquid, the precipitate was resuspended in water and washed again several times by centrifugation at 10,000 x*g* for 15 min, separating the brownish layer from the upper part. Flours and starches were preserved at -20 °C until the analysis. Potato starch and corn flakes, used as standard of resistant starch, were purchased from Sigma-Aldrich Ltd (Switzerland) and Kellogg Company (Svendborg, Denmark), respectively. Chemicals were reagent grade from J.T. Baker (Phillipsburg, NJ) and enzymes were from Sigma (Sigma Co., St. Louis, MO, USA).

Morphological analysis and particle properties

Scanning electron microscopy (SEM)

Morphological observations of flours and starches were performed using scanning electron microscopy (SEM). Samples were mounted on aluminium stubs with sticky double-sided conductive metal tape and vacuum-metalized with gold-palladium. Microscopic images were elucidated using a DSM 950 scanning electron microscope (Zeiss Iberia, Madrid, Spain) at 7 kV accelerating voltage, a magnification of 1,000x and a distance of 11 mm. Carbon adhesive tabs and aluminium mount were acquired from Aname (Madrid, Spain).

Particle size distribution

Particle size distribution of starch samples was determined at room temperature in water dispersion using a laser scattering analyser (Malvern Instruments, Ltd, UK, Model Master Sizer S), and a refractive index (n_D) of 1.53 (20 °C). The method used in this instrument relies on the fact that the diffraction angle

is inversely proportional to the particle size. The size distribution was described in terms of d (0.1), d (0.5) and d (0.9) that correspond respectively to 10, 50 and 90% of particles that are smaller than the reported size.

Stability of starches in aqueous dispersion

Sedimentation

Sedimentation of each starch was analysed using a Turbiscan MA 2000 (AGS Instrument). Glass tube was filled with starch dilution 8% w/v of deionized water up to a height of 6.5 mL, and it was immediately placed inside the device. The transmittance and backscattering were measured every 2 min, during 1 h, at 20 °C.

Zeta potential (ζ)

The zeta potential (ξ) was analysed using a Malvern Zeta sizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). A suspension was first prepared by dispersing 250 mg of starch in 250 mL of KCl 0.1 M (mixture 1). Mixture 1 was agitated, sonicated during 1 min, and its pH was measured. Then, 10 mL of mixture 1 and 90 mL of KCl 0.1 M were agitated, sonicated during 1 min, and its pH was recorded (mixture 2). Then, the cell of the equipment was filled with mixture 2, using a syringe, and the zeta potential was measured. The procedure for the preparation of mixtures was repeated, in order to obtain dilutions of mixture 1, at different pH (from 2 to 10), by adding of HCl 0.1 M or KOH 0.1 M drops, and their respective ζ values were measured.

Cold flow curve

Flow curves were monitored only for starches, since flours are usually used to prepare food with heat, in order to confer consistency, rather than a gelling ingredient. Flow curves of 8% w/v of starch dispersions in water (23 °C) were recorded by performing hysteresis loop tests using a Rheometer (Modular Advanced Rheometer system MARS, Thermo Haake, Germany). Shear rate was continually increased from 0 to 1000 s^{-1}, maintained constant at 1000 s^{-1} and decreased to 0 s^{-1}; the duration of each step was 300, 60 and 300 s, respectively. The measuring geometry used was a double gap cylinder system DG41.

Behaviour during heating

Viscosity during heating

The applied methodology has been previously described by de Souza Gomes et al. (2018). Briefly, the gelation temperature during heating was determined using the rheometer described above. Starch dispersions were prepared in water to a concentration of 8% w/v and stabilized at 50 °C for 1 min and then heated from 50 °C to 90 °C, at a heating rate of 6 °C min^{-1}, maintaining a shear rate of 50 s^{-1}.

Differential scanning calorimetry (DSC)

The thermal properties were measured using a differential scanning calorimeter (Discovery DSC T. A. Instruments). Certified indium standard was used for calibration of the temperature. The samples (3 mg) and 7 mg of Milli-Q water were placed in a DSC hermetic aluminium pan. The sample was sealed, and maintained at room temperature during 4 h, then heated from 20 °C to 95 °C (10

°C/min). An empty pan was used as a reference. From the thermogram, the onset (*To*), peak (*Tp*), conclusion (*Tc*) and enthalpy (*ΔH*) were calculated.

Thermogravimetric analysis (TGA)

Thermogravimetric analyses of flours and starches were carried out with a Thermobalance TGA Q 500 TA Instrument (USA). Analyses were conducted under a nitrogen flow of 10 and 90 mL/min for the protective and the purge gas, respectively. The assays were carried out in platinum pans without lids. Thermograms were obtained over a temperature range comprised between 20 to 900 °C with a heating rate of 10 °C/min. The non-oxidative combustion of starch granules was evaluated directly by the mass loss between 235 °C and 500 °C. To characterize the temperature range and the peak of combustion, the first derivative of mass loss according to the combustion temperature was used.

Nutritional properties

Starch content and available starch (AS)

The starch content of flours was expressed as the extraction yield, determined by the ratio between the final weight of the extracted starch, over the initial weight of the powder tuber sample. The available starch (AS) content of flours and starches was determined by the method described by Holm, Björck, Asp and Lundquist (1985), heating the diluted sample with *α*-amylase from *Bacillus licheniformis* (Termamyl® Novozymes) at 90 °C for 20 min and then the resulting digesta were subjected to a second digestion with 25 µL of amyloglucosidase from *Aspergillus niger* (~14 units/mg protein) diluted in sodium acetate buffer 0.1 M pH 4.75, (1:2.5), at 60 °C for 30 min. Released glucose was quantified with the

reaction kit of glucose oxidase/peroxidase (Nzytech genes & enzymes, Portugal) at 510 nm. Available starch was expressed as g of available starch/100 g of pure starch.

In vitro starch digestion rate

In vitro starch digestion rate was analysed following the method proposed by Holm et al. (1986) with slight modifications. Initially the equivalent of 100 mg of available starch was diluted with 15 mL of 0.1 M sodium/potassium phosphate buffer pH 6.9. The samples were gelatinized at 95 °C for 20 min. Then, the samples were placed in a bath at 37 °C and once the temperature was stable, one aliquot of 20 µL was taken (time 0). Then 250 µL of *α*-amylase from hog pancreas (~50 U/mg) prepared in sodium/potassium phosphate buffer pH 6.9 (2.3 mg/mL) was added and aliquots of 20 µL were then collected at 5, 15, 30 and 60 min. All the aliquots were placed in tubes containing 80 µL of Milli-Q water and 100 µL of 3,5-dinitrosalicylic acid (DNS). Mixtures were boiled for 10 min and cooled in an ice-water bath. After adding 1.5 mL of Milli-Q water, the absorbances were read at 530 nm. The hydrolysis rate was expressed as mg of maltose/100 mg of pure starch.

Resistant starch (RS)

The RS content was measured in raw and gelatinized samples using the methodology proposed by Goñi et al. (1996) with slight modifications. Samples (50 mg) were diluted in 5 mL of 0.2 M KCl-HCl buffer pH 1.5 and incubated with 100 µL of pepsin from the pig gastric mucosa (~2500 units/mg protein) (0.1 g/mL

buffer KCl-HCl) at 40 °C for 60 min. Then, 4.5 mL of 0.1 M Tris-maleate buffer pH 6.9 was added and the pH was adjusted to 6.9, followed by the addition of 500 µL of *α*-amylase from hog pancreas (~50 U/mg) (40 mg/mL Tris buffer). The samples were incubated at 37 °C during 16 h in a thermostatic bath with constant agitation. Later, the samples were centrifuged (3,000 x*g*/15 min), the supernatants were removed, and the precipitate was washed with 5 mL of water and centrifuged again. A volume of 1.5 mL of water was added and stirred, followed by 1.5 mL of 4 M KOH; and the mixture was vigorously stirred at room temperature for 30 min. Then, 2.75 mL of 2 M HCl and 1.5 mL of 0.4 M sodium acetate buffer were added, the pH was adjusted to 4.75 and 40 µL of amyloglucosidase from *Aspergillus niger* (~14 units/mg protein) (5 mg/mL of sodium acetate buffer) was added and incubated at 60 °C for 45 min with continuous stirring. The mixtures were centrifuged (3,000 x*g*/15 min), 5 mL of Milli-Q water were added on the residues, and the centrifugation was repeated collecting the supernatants in 25 mL volumetric flasks. Finally, the glucose content was measured, with the reaction kit of glucose oxidase/peroxidase (Nzytech genes & enzymes) at 510 nm. Resistant starch values were calculated using the following formula:

$$\%\ Resistant\ starch = \frac{glucose\ \left(\frac{ug}{mL}\right) * volume * 100 * 0.9\ (\frac{ug}{mL})}{1000 * dry\ sample\ weight}$$

Retrograded resistant starch (RRS)

RRS was determined in gelatinized and cooled samples, according to the method purposed by Saura-Calixto et al. (1993) with small modifications. After the

gelatinization of 50 mg of sample, and successive incubations with 5 µL of *α*-amylase thermostable from *Bacillus licheniformis* (≥500 units/mg protein; 4 µL/mL of potassium phosphate buffer pH 6.9), 50 µL of pepsin (pig gastric mucosa - ~2500 units/mg protein; 4 µL/mL of potassium phosphate buffer pH 6.9) and 30 µL of amyloglucosidase (*Aspergillus niger* - ~14 units/mg protein), the supernatant was disregarded by centrifugation (3000 x*g*/15 min) and the residue was washed with Milli-Q water, ethanol and acetone, through a centrifugation. A volume of 1.5 mL of water was added to each residue, followed by the addition of 3 mL of 4 M KOH. The mixtures were stirred vigorously during 30 min at room temperature and then the pH was adjusted to 4.75 and 30 µL of amyloglucosidase (*Aspergillus niger* - ~14 units/mg protein) were added. Mixtures were incubated at 60 °C for 30 min, and centrifuged (3,000 x*g*/15 min), collecting the supernatants in volumetric flasks and rinsing with water at least once. Flasks were brought to volume and resistant starch values were estimated by the equation displayed above.

Statistical analysis

All the analyses were made in triplicate and the data were expressed as mean ± standard deviation. The Tukey's test was used to compare the sample means at the 95% confidence level ($p < 0.05$) using the SPSS 22.0 software.

Results and discussion

Morphological analysis and particles properties

Particle size and SEM morphology

Figure 1 presents the scanning electron microscope (SEM) images of starches and flours from mashua and melloco. The more abundant particles corresponded to the starch granules. The non-starchy components, present in both flours could be mainly fiber and/or protein, generally present in these types of Andean tubers (Campos et al., 2018; Pacheco et al., 2019). **Figure 1B** points out that mashua starch presented three shapes of particles: spherical small, spherical-truncated medium and ellipsoidal-truncated large, while melloco starch (**Figure 1D**) presented spherical small and oval medium and large particles. The electron micrographs in **Figure 1B** and **Figure 1D** of starch preparations showed individual granules in the range of 5 - 30 µm, which were not as large as those estimated by light scattering (**Table 1**). Nevertheless, the lower left side of **Figure 1D** provided an explanation by showing a large number of starch granules agglomerated together with thin sheet-like material which is likely derived from tuber cell walls. This finding is supported by the fact that the total starch contents determined enzymatically in the samples of extracted starch were 92.42 g/100 g DM of mashua starch and 89.91 g/100 g DM of melloco starch (**Table 3**) (Pacheco et al., 2019). Therefore, around a 10 % of the extracted product could correspond, as above indicated, to other non-starch compounds that could be responsible for fusing/agglomerating the individual starch granules into larger particles.

The determination of particle size performed by laser scattering (**Table 1**) allowed the definition of three different size distributions of particles. The average size distribution (<50%) observed for potato starch (control standard) was 46.9 µm, similar value to that reported by Choi, Baik and Kim (2017). In contrast, the average particle size distribution (<50%) observed in the mashua starch sample

was higher to than observed in the melloco starch sample (190.2 *vs.* 146.0 µm); however, in the fraction (<90%) the sample of starch extracted from melloco presented the greater mean particle size (576.6 µm) followed closely by the mean particle size of the mashua starch sample (560.2 µm).

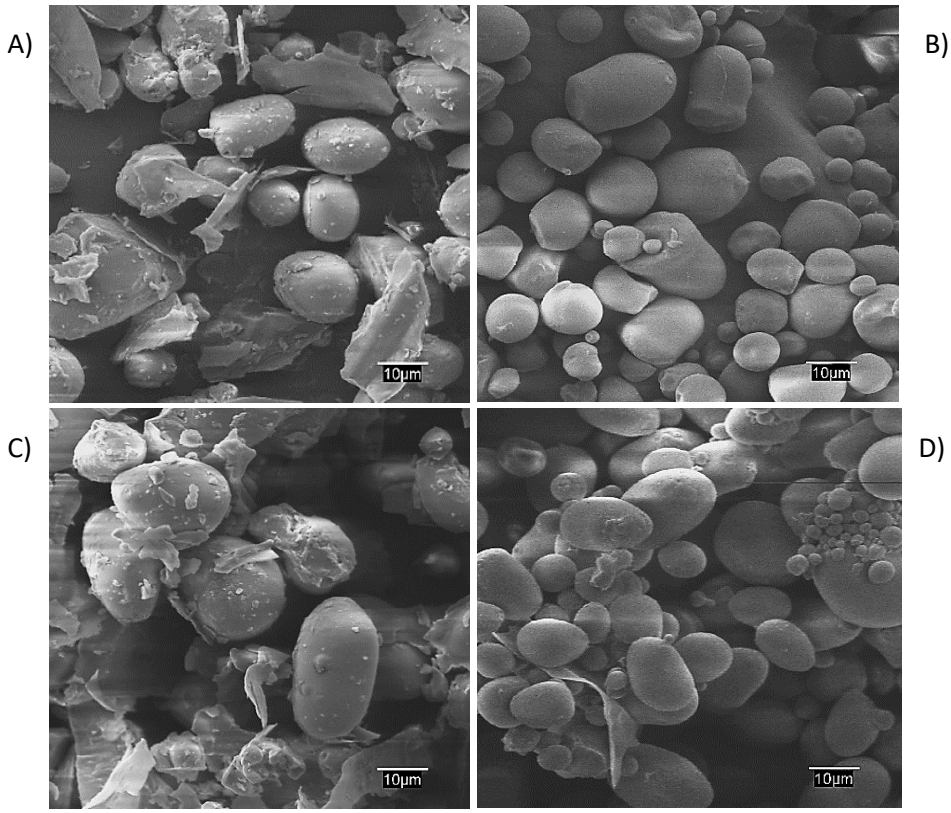


Figure 1. Scanning electron micrographs of: mashua flour (1A), mashua starch (1B), melloco flour (1C), melloco starch (1D).

The particle size and nature of the native starch granules derived from mashua and melloco can dramatically influence the behavior of the extracted starch during processing, as thermoplastic, stabilizing and/or gelling agent. In general, the larger

the particle size the better technological and/or functional properties can be found (Alcázar-Alay & Meireles, 2015).

Stability of starches in aqueous dispersion

Sedimentation and Zeta potential

The variation of light transmittance in the dispersions (8% w/v) showed the sedimentation values for potato, mashua and melloco starches, which indicated a greater stability of mashua and melloco starches in aqueous dispersion as compared to potato starch ($p < 0.05$). This could be possibly due to their higher particle size (**Table 1**) and its correlation with a greater repulsive force between the particles (Mandala & Bayas, 2004).

Table 1. Particle size distribution (µm) and sedimentation values (mm/min) of mashua and melloco starches.

Sample	<10%	<50%	<90%	mm/min
Potato starch	23.7±0.3^{c}	46.9±0.4^{a}	72.0±0.3^{a}	1.970^{b}
Mashua starch	9.6±0.2^{a}	190.2±1.4^{c}	560.2±0.9^{b}	0.105^{a}
Melloco starch	11.0±0.1^{b}	146.0±0.1^{b}	576.6±0.5^{c}	0.119^{a}

Different superscripts denote significant differences between values in the same column ($p < 0.05$). Values expressed as means ± SD ($n = 3$).

The electrostatic repulsive force (Zeta potential) could predict the potential stability of the hydrocolloids system and also help to provide stable formulations. If the particles have large negative or positive zeta potential values (that is, from -30 to -60 mV or from 30 to 60 mV), the system is considered stable and anti-cohesive (Genovese & Lozano, 2001). The zeta potential values obtained in this

study indicated that potato starch in aqueous dispersion is more stable at pH values above 7.0 (**Figure 2**). This great stability found in potato starch dispersion at high pH can be related to the smaller particle size of potato starch in comparison with melloco and mashua starches. In consequence, the number of particles per volume unit is higher, which might generate a higher electrostatic repulsion force between the particles (Mandala & Bayas, 2004). Nevertheless, the majority of foods have a pH lower than 7.0 (Badui, 2006), and at pH values from 6.5 to 3.0, mashua and melloco starches formed aqueous dispersions more stable than those obtained with potato starch ($p < 0.5$) (**Figure 2**).

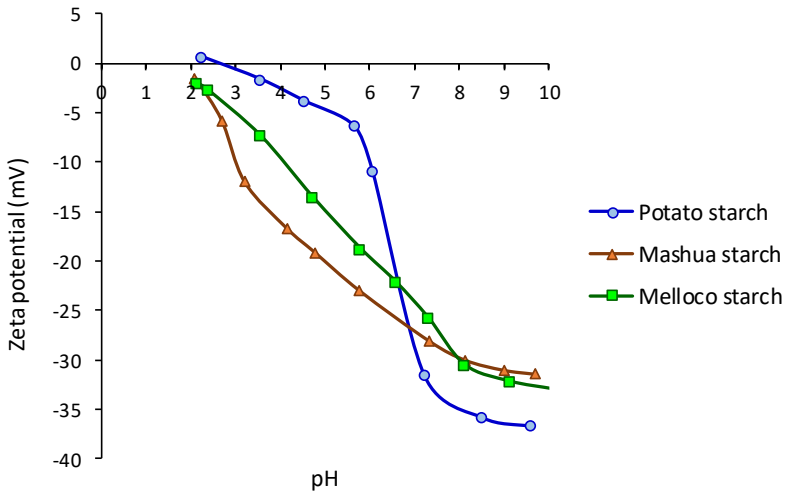


Figure 2. Zeta potential (ζ) of mashua and melloco starch dispersions at different pH.

On the other hand, when starch particles present greater electronegativity, they also can exhibit greater facility to retrogradation (Genovese & Lozano, 2001). Therefore, mashua and melloco starches could have high potential to retrograde at the mentioned pH acquiring higher resistance to enzymatic digestion. In

addition, greater stability of cold aqueous dispersions of starch could be achieved by combination with stabilizers that inhibit the destruction of starch granule and the leaching of amylase, increasing the electrostatic repulsion between particles (Cai et al., 2011).

According to the obtained results, mashua and melloco starches as produced in the present study could be better food stabilizers of cold mixtures than potato starch, even at low pH (*e.g.,* some sausages, jams and fruit drinks), with the possibility to retrograde after the pasteurization process and cold storage.

Cold flow curve

Shear stress and viscosity variations as a function of shear rate (flow and viscosity curves, respectively) were recorded in a controlled rate mode up to a shear rate of 1000 s^{-1} using the ramp mentioned before. **Figure 3** shows the viscosity curves of 8% (w/v) aqueous dispersions of potato, mashua and melloco starches. The viscosity of the dispersions was high at low shear rate and decreases with increasing shear rate, remaining constant for the three types of starches at 8% (w/v) when increasing the shear rate above around 50 s^{-1}.

The final viscosity was greater for melloco and mashua starches than for potato starch, which could be associated with the larger average particle size of the former. Therefore, the starches extracted from mashua and melloco could provide better behaviour than potato starch, as thickener of foodstuffs prepared in cold, such as soups, juices, sausages, films, and/or in pharmacology and cosmetics products that require a certain and durable consistency at room or cold temperature.

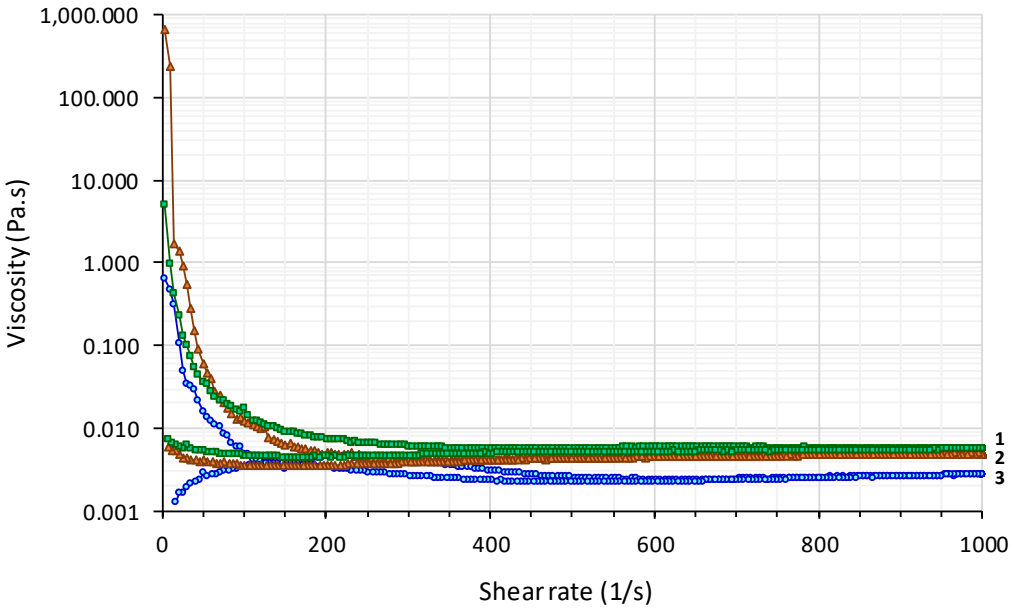


Figure 3. Viscosity curves of 8% aqueous dispersions of native starches. 1: melloco starch, 2: mashua starch, 3: potato starch.

Heat behaviour

Viscosity during heating

Starches from mashua and melloco, at a shear rate of 50 s^{-1}, displayed a typical viscosity pattern based on a sharp increase of viscosity followed by a more gradual drop of viscosity under agitation and heating (**Figure 4**). Melloco starch showed the highest viscosity peak, reaching values of 17830 m Pa.s at 66.1 °C, followed by mashua starch with a viscosity of 12220 m Pa.s at 68.3 °C ($p < 0.05$). Likewise, melloco flour presented a better behaviour during heating than mashua flour, showing a viscosity value of 3678 mPa.s *vs.*247 mPa.s ($p < 0.05$). Recently, Pacheco et al. (2019) found a higher amylose content in melloco starch (51.70 %) in comparison with potato starch (18-29%) (Vamadevan & Bertoft, 2015). This high amylose content and the high average particle size of melloco starch (**Table 1**) can

be related to its high viscosity at low gelatinization temperature. In the case of mashua starch, these properties seem to be related mainly to the larger average particle size in comparison with potato starch, since both tubers have shown similar amounts of amylose (29.55% *vs.* 18.00-29.00% of amylose) (Pacheco et al., 2019).

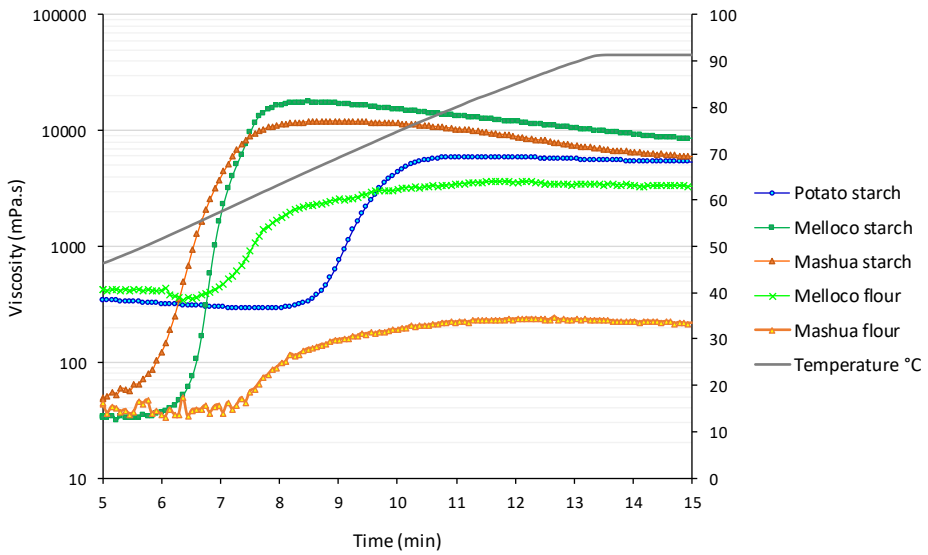


Figure 4. Viscosity during heating of starches and flours from mashua and melloco.

Based on these results, the starches extracted in the present study, that is, mashua starch, and especially, melloco starch, could confer higher viscosity than potato starch in foods prepared by heating, which could allow the use of less amount of starch, to achieve the same thickener effect in foods, and consequently, having lower caloric value.

Differential scanning calorimetry (DSC)

The results of DSC analysis shown in **Table 2** indicate a range of gelatinization temperature (T_o-T_c) of 46.4-60.7 °C and melting or gelatinization peak temperatures (T_m) of 52.3-54.9 °C for mashua and melloco starches. These ranges were low as compared to those reported for starch from potato (T_o-T_c: 58.06-67.27 °C, and T_m: 62.56 °C) (Fonseca-Florido et al., 2017). The differences among ranges of gelatinization temperatures can vary according to the botanical species. Lower gelatinization temperatures may suggest higher crystalline perfection and/or higher amylose content (Tian et al., 2016). At the same time, a higher *gelatinization enthalpy* value (ΔH_{gel}) could be observed for melloco starch as compared to mashua starch (12.3 *vs.* 4.3 J g-1) ($p < 0.05$), which indicated that melloco starch had a greater number of double helix areas and required more energy to the rupture of the H-bonds between glucan strands (Cooke & Gidley, 1992).

Table 2. DSC results for starch and flour from mashua and melloco.

Sample		T_o (°C)	T_m (°C)	T_c (°C)	ΔH_{gel} (J g^{-1})
Mashua	Starch	48.7±0.3^{b}	54.9±0.2^{b}	60.7±0.6^{b}	4.3±0.1^{b}
	Flour	58.4±0.2^{d}	63.4±0.3^{cd}	69.3±1.1^{cd}	1.2±0.1^{a}
Melloco	Starch	46.4±0.1^{a}	52.3±0.2^{a}	57.5±0.3^{a}	12.3±0.4^{d}
	Flour	56.5±0.2^{c}	62.3±0.3^{c}	67.9±1.0^{c}	9.0±0.3^{c}

Different superscripts denote significant differences between values in the same column ($p < 0.05$). Values expressed as means ± SD (n = 3). T_o: 'onset' initial temperature, T_m: 'melting' peak temperature, *Tc*: 'conclusion' or final temperature, ΔH_{gel}: gelatinization enthalpy.

The higher T_m values for melloco and mashua flours as compared to the starch samples ($p < 0.05$) can be due to the presence of compounds such as proteins, lipids and fiber that are degraded at higher temperatures but with less energy consumption (ΔH_{gel}) (Alcázar-Alay & Meireles, 2015).

Thermogravimetric analysis (TGA)

The TGA curves (**Figure 5**) showed the occurrence of three mass losses. The first loss was related to dehydration (20-110 °C). After that mass loss, the weight loss was stable, which is a characteristic behaviour for starches. The second and third mass losses were observed, which are related to the degradation due to carbonization of organic matter followed by oxidation (Hornung et al., 2017). In the second step the greatest weight loss (Δm) was observed in melloco starch followed by mashua starch ($p < 0.05$); however, the weight loss of melloco starch occurred at 270 °C (T_p), while in mashua starch was degraded at 210 °C. The mashua and melloco flours were more resistant to thermal degradation, exhibiting a greater residue (21.04% and 21.64%) than their respective starches at the final of the heating process (15.80% and 8.94%), in accordance with the higher degradation temperature of other components presents in mashua and melloco roots (eg. lipids, protein, fiber, ash) (Campos et al., 2018; Pacheco et al., 2019).

The observed thermal behaviour in the mashua and melloco starches pointed out that they could be used in cooking processes requiring high temperatures (<210 and <270 °C, respectively) without organic matter degradation (carbonization). Mashua and melloco flours, instead, could be heated without organic degradation at lower temperatures (<125 and 130 °C, respectively).

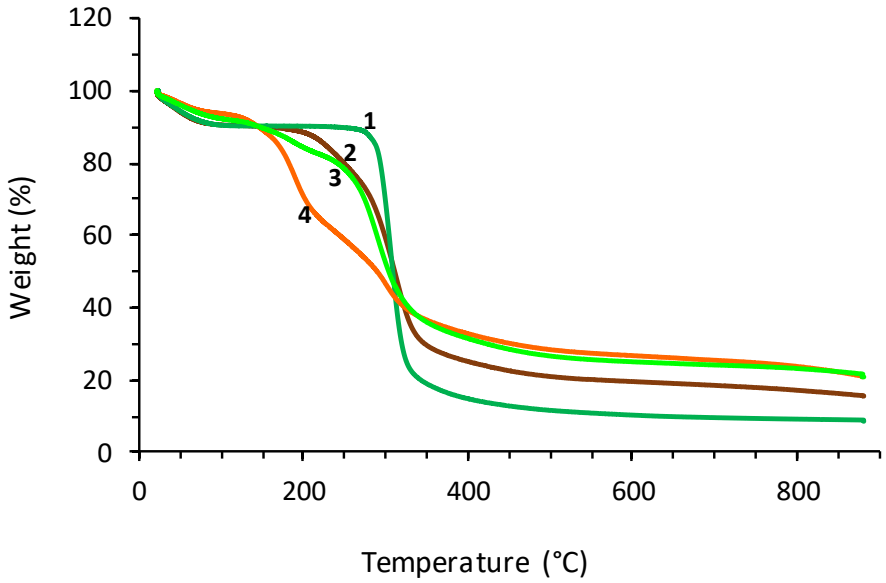


Figure 5. Comparison of the TGA curves of starches and flours from mashua and melloco. 1: Melloco starch, 2: Mashua starch, 3: Melloco flour, 4: Mashua flour.

Nutritional properties

Starch content and available starch (AS)

Table 3 shows the starch content of flours and the available starch (*AS*) of mashua and melloco samples (flour and starch). In general, melloco starch and flour presented higher *AS* quantities than those observed in mashua starch and flour ($p < 0.05$). Differences observed between the AS contents could be due to the botanical origin (Tovar, 2001). Mashua flour presented the lowest *AS* content (20.91%) in comparation with melloco flour (60.95%), despite its higher starch content ($p < 0.05$). The presence of phenolic compounds in mashua samples, which are well-known as α-amylase inhibitors (Hernández-Uribe et al., 2007), could explain the low activity of enzymatic hydrolysis, and therefore a low amount

of AS. Melloco flour, instead, showed acceptable values for available starch (60.95%).

Table 3. Starch content (g/100 g DM), available starch (AS) and resistant starch (RS) (%) of mashua and melloco flours and starches.

Tuber	Sample	Starch content	AS	RS Raw	RS Cooked	RRS Cooked and cooled
Mashua	Flour	56.22±1.05 [b]	20.91±0.45[a]	10.0±0.02[d]	4.70±0.01[d]	5.23±0.01[d]
	Starch	*92.42±1.87[cd]	78.57±2.59[c]	0.62±0.03[a]	0.27±0.02[a]	0.51±0.02[a]
Melloco	Flour	41.07±0.90 [a]	60.95±0.87[b]	7.73±0.06[c]	0.63±0.02[bc]	1.83±0.03b[bc]
	Starch	*89.91±0.53[c]	85.83±1.64[d]	5.45±0.18[b]	0.52±0.03[b]	1.72±0.02[b]

Values expressed as means ± SD (n=3). RS Raw: Resistant starch in raw samples, RS Cooked: Resistant starch in cooked samples, RRS: Retrograded resistant starch: Resistant starch in cooked and cooled samples. Different letters superscripts denote significant differences between values of the same column ($p < 0.05$).
* Pacheco et al., 2019.

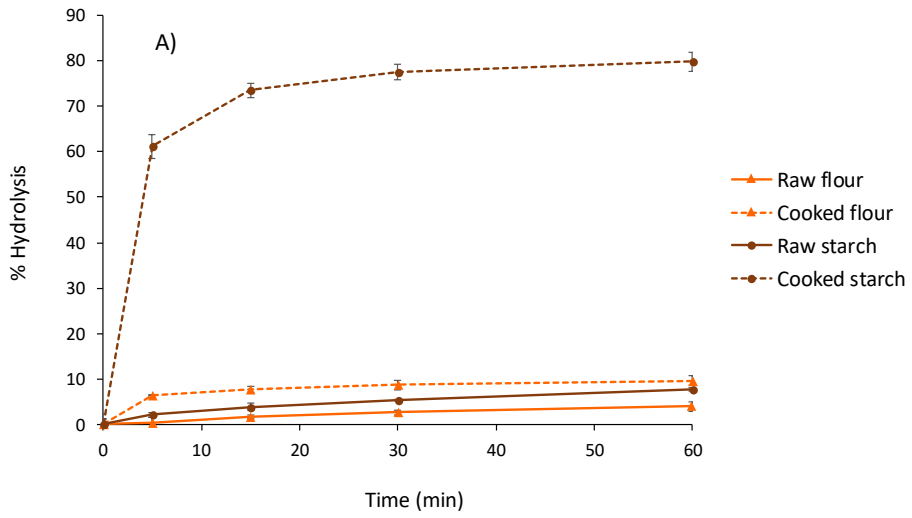
Flour and starch digestion rate in vitro

Figure 6 shows the *in vitro* digestion rate of flour and starch samples of mashua and melloco. Either flours or starches from both Andean tubers in raw state presented low digestion rates because their crystalline structure was not destroyed (Ao et al., 2012). This compact structure limits the accessibility of digestive enzymes, thus explaining the resistance of raw samples. Consequently, this type of starch is very slowly and incompletely digested during its passing through the small intestine (Shi & Maningat, 2017).

Gelatinized mashua and melloco starches presented a digestion rate close to 80%. In contrast, the corresponding cooked flour samples (after adjustment to the

same amount of available starch) showed a much lesser digestion rate. This behaviour could be attributed to the presence of fiber and/or protein and other food components that could prevent effective diffusion and adsorption of *α*-amylase, as well as to the presence of inhibitors of *α*-amylase as phenolic compounds (Hernández-Uribe et al., 2007). Remarkably, the digestion rate of cooked mashua flour was lower than that of melloco flour ($p < 0.05$), which is in line with its low AS content (**Table 3**) and it could also be attributed to the presence of the inhibitor compounds already mentioned.

Considering that the lower the availability of starch, the lower the digestibility and consequent conversion to glucose (Sáyago-Ayerdi et al., 2005), the low starch *in vitro* digestion rate observed in mashua cooked flour could point out its application as a promising candidate to reduce the glycemic index (GI), as long as the ingredient has a low content of low molecular-weight carbohydrates.



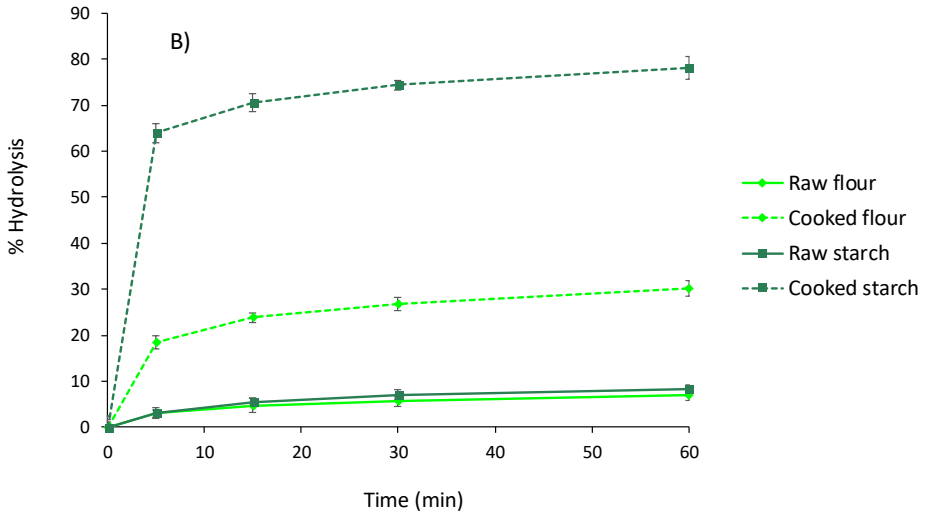


Figure 6. Starch digestion rate in vitro of flour and starch from mashua (6A) and melloco (6B). A triplicate of the test was performed for all the samples. Error bars indicate the variation of hydrolysis percentage observed between the different repetitions.

Resistant starch (RS)

The resistant starch (RS) content was higher in melloco starch than in the mashua starch ($p < 0.05$) (**Table 3**). In contrast, the RS observed in the raw melloco starch (5.45 %) was much lower than the RS reported for raw potato starch (75.0%) (Raigond et al., 2015). Starch resistance does not depend on any single factor but on a large number of factors, including the botanical origin, size of granules, shape of granule surface, amylose content, starch crystallinity and size of pores in starch granules (Leszczynski, 2004).

In the case of flours, the value of RS determined in mashua flour was higher than the measured in melloco flour ($p < 0.05$), confirming the presence of possible inhibitors of α-amylase, *e.g.* phenolic compounds, as already mentioned above.

Some characteristics such as molecular weight, number and position of substitution and glycosylation of flavonoids seem to be related to the inhibitory effect of phenolic compounds (Martinez-Gonzalez et al., 2017). In a recent study, mashua flour presented a total phenolic content of 1001.6 mg GAE/100 g DM *vs.* 260.1 mg GAE/100 g DM observed in melloco flour (Pacheco et al., 2019). This reported difference seems to be consistent with the observed inhibition effect.

Overall, flours presented greater amount of RS than starches, possibly due to its low rate of hydrolysis, as mentioned in the previous section.

Retrograded resistant starch (RRS)

An increase of RS after cooking (99 °C/20 minutes) and cooling (4 °C/24 h) was observed in all samples (**Table 3**), as a result of the rearrangement of amylose chains around the remnants of amylopectin during cooling, forming new crystalline structures that confer resistance to digestion. The tubers may present lower resistance to enzymatic digestion following a loss of crystallinity in starch granules and the change of water distribution. With subsequent cooling, the disrupted amylose and amylopectin chains gradually re-associated and aggregated, a process known as retrogradation. The reorganized structure showed an increased transition temperature and relative crystallinity by comparing to freshly gelatinized starch (Tian et al., 2016). As a result, the digestibility of retrograded starch decreases because the aggregation of melted amylose and amylopectin upon cooling and storage, which make them less accessible to digestive enzymes (Xie et al. 2014).

Melloco starch showed a higher content of retrograded resistant starch (RRS) as compared to mashua starch (1.72% *vs.* 0.51%, respectively). Nevertheless, this content is still low as compared to that observed, for instance, in improved genetically-modified lines of potato rich in amylose and RS. The observed resistance could be improved by increasing the cooling time or genetic modification of the granular structure (Chen et al. 2018).

Conclusions

Our data highlight the potential role of two underexplored Andean tubers as sources of flours and starches with peculiar technological and nutritional properties due to the presence of proteins and/or mucilages or other non-amylaceous components. According to the morphological characterization and stability studies on cold and heated aqueous dispersions, the starches extracted from mashua and melloco in the present study showed better behaviour as stabilizers than potato starch, indicating their possible application as excellent and cost-effective thickening or gelling agents not only in foods but also in a wide range of biomaterials, such as paper, textiles, plastics, cosmetic or pharmacological goods.

The nutritional analysis revealed that mashua and melloco starches were highly available, with a high digestion rate. Moreover, the levels of resistant starch in mashua and melloco flours and starches were low, regardless of the state of the sample (raw, cooked, or cooked and cooled). Therefore, mashua and melloco tubers could represent a novel source of flours and starches with nutritional and physical characteristics different from the conventional botanical sources.

4.2. Obtención y caracterización de compuestos/ingredientes funcionales a partir de subproductos de la industria agroalimentaria

Con el objetivo de diversificar las fuentes a partir de las cuales se puedan obtener ingredientes que ayuden a paliar las necesidades nutricionales de los próximos decenios, y considerando las ingentes cantidades de residuos de las industrias agroalimentarias, se plantearon dos estudios. Uno de ellos enfocado al aprovechamiento de subproductos de la industria azucarera y otro referente a subproductos de la industria elaboradora de zumo de naranja. En ambos casos, se contó con muestras muy valiosas procedentes de empresas punteras del sector como Azucarera y García-Carrión, respectivamente.

Para determinar las posibilidades de aprovechamiento de subproductos de la remolacha azucarera, se caracterizó física- y químicamente el residuo prensado, sugar y deshidratado. Se observaron diferencias en cuanto a composición química, debido al proceso tecnológico aplicado en la industria, y a transformaciones bioquímicas de fermentación en el caso del residuo ensilado. Tras la aplicación de un método enzimático, a partir de dicho residuo se logró obtener pectina con alto contenido de ácido galacturónico, mientras que con un método químico, basado en la utilización de ácido, se extrajo pectina con un mayor rendimiento y con una mejor estabilidad en solución acuosa y mayor actividad emulsionante **(Artículo IV).**

En el caso de los subproductos de la extracción de zumo de naranja, se realizó en inicio una caracterización fisicoquímica, observando, principalmente, la presencia de compuestos fenólicos en el residuo fresco, azúcares reductores y *N*-

ε-fructosil-lisina en el residuo seco, licor de naranja y pienso para animales, y ácido galacturónico en los residuos sólidos. Se consiguió extraer pectina de la cáscara fresca, seca y pienso para animales con un contenido de ácido galacturónico mayor al 65 %, y debido al contenido de compuestos bioactivos observados en todos los subproductos, se concluyó que era conveniente investigar sobre el efecto bioactivo del consumo de los subproductos analizados **(Artículo V).**

Durante una estancia predoctoral realizada en la Universidad de Granada, se pudo estudiar el efecto del consumo de subproductos de naranja sobre indicadores de la enfermedad inflamatoria intestinal inducida mediante la administración de dextrano sulfato sódico (DSS) empleando un modelo animal basado en ratones macho C57BL/6J. El consumo de la cáscara fresca, pienso de naranja y pectina cítrica permitieron mejorar los síntomas de la enfermedad, debiendo, presumiblemente, su efecto al contenido de compuestos fenólicos, ácido galacturónico y *N*-ε-fructosil-lisina. Estos resultados fueron objeto de una sexta publicación **(Artículo VI).**

El trabajo referido a subproductos de la industria agroalimentaria se expone en los siguientes artículos:

4.2.1. Artículo IV. Propiedades estructurales y reológicas de pectinas extraídas a partir de subproductos industriales de remolacha azucarera.

4.2.2. Artículo V. Caracterización química y fisicoquímica de subproductos de naranja derivados de la industria.

4.2.3. Artículo VI. Efecto antiinflamatorio intestinal del consumo de subproductos industriales de naranja en ratones tratados con DSS.

4.2.1. Artículo IV. Propiedades estructurales y reológicas de pectinas extraídas a partir de subproductos industriales de remolacha azucarera

Structural and rheological properties of pectins extracted from industrial sugar beet by-products

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Abstract

In this work, the efficient extraction of pectin from sugar beet by-products (pressed, ensiled and dried pulp), by using an acid method or a commercial cellulose, is accomplished. The extraction method had an impact on the pectin monomeric composition, mainly in xylose, arabinose, and galacturonic acid content, as determined by GC-FID. FTIR and SEC analyses allowed the determination of similar degrees of methoxylation and molecular weights, respectively, in the extracted pectins. The acid extraction of pectin in the ensiled by-product led to the highest yield (19%) with a galacturonic acid content of 46%, whereas the application of the enzymatic extraction method resulted in a lower yield (13%) but higher galacturonic acid content (72%). Moreover, the stability in aqueous solution as well as the emulsifying activity index was higher for pectin extracted by the acid method, whereas the viscosity was higher in pectin extracted by the enzymatic method. To the best of our knowledge, this is the first study analyzing the physicochemical properties and exploring the potential reuse of ensiled and dried by-products from sugar beet industry for the extraction of pectin to be further used in the food and pharmaceutical areas.

Introduction

In 2016, the largest area of root crops (1.7 million hectares) in the European Union (EU) was occupied by potatoes closely followed by sugar beet (*Beta vulgaris L.* subsp. *vulgaris* var. altissima Döll) (1.5 million hectares) (Eurostat, 2017). These values point out the EU as the leading producer of sugar beet, providing approximately 50% of the global production, whose process generates a volume waste of 111.6 million tons per year. In addition, in Spain sugar beet is the only source of sugar, producing 3000 tons of residues per year.

Only 30% of the world's sugar production comes from sugar beet, whereas the rest is derived from cane (Eurostat, 2017); however, the obtainment of sugar from beet generates a significant volume of wastes each year, which is considered of great importance in terms of underexploited opportunities and generated levels (RedCorn et al., 2018). When the sugar beet residues are exploited, habitually they are used as lignocellulosic material for the ethanol obtaining and pectin extraction (Maravić et al., 2018).

Pectin, an important anionic heteropolysaccharide, exists in the cell walls of dicotyledonous plants (Agoda-Tandjawa et al., 2012), and over the last years, pectin has gained increasing interest as thickening or gelling agent for chemical and food industry (Kaya et al., 2014). Furthermore, pectin has been described as an emerging prebiotic with ability to modulate the bacterial composition of the colon microbiota (Ferreira-Lazarte et al., 2018), being able to exert beneficial effects on health.

Sugar beet pectin (SBP), compared to the main sources of pectin which are citrus and apple, has poorer gelling properties due to its higher content of neutral sugars, low presence of acetyl groups (4-5%) (Leroux et al., 2003), content of ferulic acid, higher protein content (Jankovská et al., 2001) and/or its relatively low molecular mass, but in contrast, these molecular characteristics give the SBP better emulsifying properties (Levigne et al., 2002; Siew & Williams, 2008).

However, depending on the applied extraction method, the structure and technological properties of SBP can vary widely. A large number of studies have addressed the extraction and properties of pectin from sugar beet pulp pressed (SBP-P) in recent years, and most of the studies have been focused on the effect of extractants and extraction conditions on pectin yield, chemical composition and technological behavior (Lv et al., 2013; Guo et al., 2017; X. Huang et al., 2018). However, there is an excess of other underutilized industrial sugar beet by-products, such as ensiled sugar beet (SBP-E) and dried sugar beet pulp (SBP-D) whose potential as raw materials for obtaining similar compounds has not yet been addressed.

Therefore, the main objective of this work was to explore the potential use of different physico-chemically characterized sugar beet by-products (pressed, ensiled and dried pulp) as efficient and alternative sources of pectin following its extraction by acid or enzymatic methods. Likewise, the potential of the extracted pectins as thickening or gelling agents is investigated through their rheological characterization.

Materials and Methods

Samples

Commercial citrus pectin was purchased from Acofarma (Barcelona, Spain). Industrial sugar beet by-products were provided by Azucarera Ebro (Madrid, Spain). **Figure 1** shows the industrial process of sugar extraction and derived by-products. Briefly, sugar beet is washed, disinfected with hot water and grinded to obtain small particles named cossettes. Then, sugar is extracted from cossettes by a diffusion process with water heated at 70 °C. Water and sucrose are concentrated and dried, and the cossettes with a 7-8% of dry weight (DW) are pressed to extract more sugar, obtaining the Sugar Beet Pulp Pressed (SBP-P),

with a DW of 28-29%. This residue is stored in silos for 7-8 months obtaining the Sugar Beet Pulp Ensiled (SBP-E), and, then, it can be dried by sun (3 days), or with boiler gas caldera 2-3 h, until 88-96% DW obtaining the Sugar Beet Pulp Dried (SBP-D). Those residues are destined to the direct sell or used in the production of animal feed. SBP-P, SBP-E and SBP-D were selected performing a simple, non-stratified random sampling. The beet used in the extraction process came from different cultivars of *Beta vulgaris* var. *altissima* Döll, harvested in early-January, in Spain. All samples were lyophilized, ground, sieved thought 250 μm mesh, and maintained at -20 °C until analysis.

Physicochemical characterization of sugar beet by-products

°Brix, pH, water activity (a_w), dry weight (DW) and protein content were determined according to the AOAC methods described by Megías-Pérez et al., (2014). Fat content was determined by the soxhlet method using propanol during 2 h of heating. Minerals content was determined in the Interdepartmental Research Service (SIdI-UAM) (Madrid, Spain), by ICP-MS in an Elan 6000 Perkin-Elmer Sciex instrument (Concord, Canada).

Total carbohydrates were determined according to the phenol sulfuric method described by Masuko et al. (2005). Working inside a fume hood, 278 μL of aqueous dilutions of the samples (70 μg/mL) were disposed in Eppendorf tubes of 2 mL, and 167 μL of phenol sulfuric solution (5% w/v) were added on the dilutions. Tubes were stirred in a vortex, 1 mL of sulfuric acid were carefully added, and the mixture was shaken again and, then, kept for 30 min without agitation. Afterwards, the absorbance was measurement at 480 nm in a Synergy

HT Multi-Mode Microplate reader (BioTek® Instruments, Inc., Winooski, Vermont 05404-0998 USA) (Gen 5 software) and using a calibration curve of galacturonic acid (0-0.2 mg/mL). Results were expressed as total carbohydrates (g/100 g DW).

Reducing carbohydrates were measured using the method described by Sumner and Graham (1921), by adding 100 µL of 3,5-dinitrosalicylic acid (DNS) reactive to 100 µL of the diluted sample previously located in Eppendorf tubes of 1.5 mL. The mixture was stirred and boiled during 5 min and then cooled in an ice bath, and 750 µL of milli-Q water were added. After shaking again, 280 µL of mix were transferred to a multiwell plate and the absorbance was measured at 540 nm. The calibration curve was prepared with GalA (0-0.4 mg/mL), and data were expressed as reducing carbohydrates (g/100 g DW).

Fiber content was determined by the enzymatic-gravimetric method described by McCleary (2010). Samples were milled and sieved through 250 µm mesh. A dilution of 1 g of sample in 50 mL of sodium phosphate buffer 0.08 M pH 6 was prepared, and 100 µL of α-amylase from hog pancreas (Sigma-Aldrich Química SL, Madrid, Spain, ≥5,000 U/mL) was added to remove the starch, heating at 95 °C for 15 min in a water bath with agitation. After cooling down, the pH was adjusted to 7.5 with 0.275 M NaOH, and 5 mg of protease from *Streptomyces griseus* (Sigma Aldrich, ≥3,500 U/g) was added and the mixture was heated (60 °C/ 30 min) to remove the protein.

The pH was adjusted to 4-4.6 with HCl 0.325 N, and 300 µL of amyloglucosidase from *Aspergillus niger* (Sigma Aldrich, 72,500 U/g) (1 mg/mL) (30 min, 60 °C) were added to remove the gelatinized starch. Afterwards, 280 mL of water at 60 °C were added and left to stand for 1 h to precipitate the insoluble fiber. The

precipitate was filtered through a porous crucible of 0.8 µ, washed successively with 60 mL of ethanol 78%, 20 mL of ethanol 95% and 20 mL of acetone. The solid detained was dehydrated for 24 h at 100 °C, and its final weight was corrected depending on the protein and ash value, to obtain the insoluble dietary fiber (IDF) content. Total dietary fiber (TDF) was determined by replacing the 280 mL of water by ethanol 95% and filtering all the precipitate; and soluble dietary fiber (SDT) was calculated by subtracting IDF from TDF values.

Total phenolic content was determined in methanolic extracts of samples by the Folin-Ciocalteu method described by Soria et al. (2010). To obtain the extracts, 0.2 g of powder sample were homogenized in 5 ml of methanol using an Ultra Turrax (IKA Labortechnik, Janke & Kunkel, Staufen, Germany) at 24,000 rpm for 1 min. The homogenates were placed in tubes of 15 mL and stirred at 750 rpm (50 °C/ 20 min), in an Eppendorf ThermoMixer® incubator (15 mL). Mixtures were centrifuged at 2,000x *g* for 15 min and filtered through Acrodisc PVDF syringe filters (0.45 µm, Sigma-Aldrich).

The reaction was carried out, by adding 100 µL of MeOH and 100 µL of Folin-Ciocalteu 2N to 100 µL of the filtered extract, disposed in Eppendorf tubes of 1.5 mL. After 5 min, 700 µL of Na_2CO_3 (75 g/L) were added, and tubes were left in the dark for 20 min. Mixtures were centrifuged at 28,000x *g* for 3 min, and the absorbance was measured in the supernatant at 735 nm using a Synergy HT Multi-Mode Microplate reader (BioTek® Instruments, Inc., Winooski, Vermont 05404-0998 USA) (Gen 5 software). The calibration curve was prepared with gallic acid (0-60 mg/L) and the results were expressed as mg of gallic acid equivalent (GAE)/g DW.

Antioxidant capacity was determined according to the method proposed by Brand-Williams et al. (1995), by the addition of 193 µL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) 2 mM diluted in methanol (1:15) to 7 µL of methanolic extract of powder sample, in Eppendorf tubes of 1.5 mL. Mixture was stirred and transferred to a multiwell cell (280 µL). After 30 min without agitation under dark conditions, the absorbance was measured at 517 nm. The calibration curve was prepared with Trolox (Sigma 648471, 500 mg; ≥98%) (0.25-2.5 mM in methanol). Results were expressed as mM Trolox/100 g DW.

Pectin extraction

Acid method

Pectin was extracted by the traditional acidifying method optimized by Babbar et al. (2016) with slight modifications. The sample was mixed with deionized water (5%, w/v) and the pH was adjusted to 1.2 with HNO_3 (0.4% v/v). The suspended samples were heated at 90 °C with continuous stirring at 200 rpm for 3 h. After the reaction was completed, the resulting slurries were cooled down to 40 °C, and the pH was adjusted to 4.5 with $NH_3.H_2O$ 25% and centrifuged at 2,600x *g* at 4 °C for 10 min, to separate insoluble fiber, protein, and other non-pectin compounds. The supernatant was collected and stored in a refrigerator at 4 °C for subsequent purification. One volume of supernatant was precipitated using two volumes of ethanol 95% for 1 h at room temperature. The centrifugation was repeated and the precipitated was washed three times with ethanol at 70%. After purification, the pectin was dried by lyophilization, and stored until its analysis.

Enzymatic method

According to the method described by Liew et al. (2016), pectin was extracted from sugar beet by-products by dilution of powder samples in buffer sodium citrate 0.05 M at pH 4.5 (1:20 *w/v*) and heating with continuous stirring (125 rpm) with cellulase commercial Celluclast® derived from *Trichoderma reesei* (Novozymes Corp., Bagsvaerd, Denmark. 700 U/g) (1.17 U/g powder sample) at 61 °C during 102 min. Mixtures were left without stirring at room temperature during 24 h, to degrade the cellulose; and then, they were centrifuged at 2,600x *g* at 4 °C for 10 min, to separate insoluble fiber, protein, and other non-pectin compounds. Ethanol 95% was added to the supernatants (2:1 *v/v*), and ethanolic mixtures were kept under dark conditions at 4 °C for 24 h to allow the flotation of pectin. Pectin solutions were centrifuged at 3,400x *g* by 15 min and the precipitate was washed twice with ethanol 70%, mixed and centrifuged before each addition. Finally, pectin was de-colorated by adding acetone drop-by-drop and dried through lyophilization. The pectin yield was calculated by means of Equation (1):

$$\text{Pectin yield } (\%) = \frac{\text{Weight of product obtained (g)}}{\text{Weight of powder sample (g)}} \text{ x } 100 \qquad (1)$$

Pectin characterization

Monomeric composition

Sample was hydrolyzed with trifluoroacetic acid (TFA) 2 M (30 mg/1.5 mL) at 110 °C during 4 h (Garna et al., 2006). Then, 500 µL of hydrolysate were placed in a flask and evaporated under vacuum at 43 °C. 400 µL of phenyl-*β*-D-glucoside

(0.5 mg/mL) (internal standard, I.S.) were added, and the flask was evaporated again. For the oximes formation, 250 µL of hydroxylamine chloride in pyridine (2.5%) were added and the mixture was vortexed and heated at 70 °C during 30 minutes, stirring the sample at the beginning, at the middle, and at the final of the 30 minutes. Samples were persilylated with 250 µL of hexamethyldisylazane (HMDS) and 25 µL of TFA at 50 °C for 30 min, agited, and centrifuged at 10,000x *g* for 2 min.

The released monomers were analyzed by GC-FID (Agilent Technologies 7890A gas chromatograph, Agilent Technologies, Wilmington, DE, USA) using a DB-5HT capillary column (15 m × 0.32 mm × 0.10 µm) (J&W Scientific, Folsom, California, USA). Injector and detector temperatures were 280 and 350 °C, respectively; oven temperature program was increasing from 150 °C to 165 °C at 1 °C/min and up to 300 °C at a heating rate of 10 °C/min. Nitrogen was used as carrier gas, at flow of 1 mL/min, and injections were made in split mode 1:20. Data acquisition was done using a HPChem Station software (Hewlett-Packard, Palo Alto, CA, USA). The response factors were calculated after the analysis of standard solutions (xylose, arabinose, rhamnose, galactose, mannose, glucose, and galacturonic acid), in concentrations of 0.01–2 mg, and 0.2 mg of I.S.

Protein content

Protein content was determined in all the pectin samples following the Bradford assay (Bradford, 1976) using the Bio-Rad protein assay kit, which includes Coomasie Blue and bovine serum albumin (BSA) (0-2 mg/mL) for the calibration curve. The absorbances were measured at 595 nm and protein content was expressed as g/100 g DW.

Degree of methylesterification (DM)

The degree of methylesterification (DM) of extracted pectin was determined by Fourier transform infrared spectroscopy (FTIR) analysis. KBr discs were prepared mixing the pectin with KBr (1:100) and pressed. FTIR spectra Bruker IFS66v (Bruker Optics, Ettlingen, 76275 Germany) were collected at absorbance mode in the frequency range of 400–4000 cm^{-1}, at a resolution of 4 cm^{-1} (mid infrared region) with 250 coadded scans. The DM was expressed as the ratio between the peak area of methylesterified carboxyl groups: $COOCH_3$, measured at 1745 cm^{-1}; and the sum of the peak areas of esterified carboxyl groups: $COOCH_3$ at 1745 and free carboxyl groups COO^- measured at 1608 cm^{-1}; according to the described by Singthong et al. (2004), Equation (2):

$$DM = \frac{Methylesterified\ carboxyl\ groups}{Total\ carboxyl\ groups}\ x\ 100 \qquad (2)$$

Molecular weight (Mw)

The distribution of Mw of pectin samples was determined by Size Exclusion Chromatography (SEC) according to the method described by Muñoz-Almagro et al. (2017) with slight modifications. Dilutions of sample in milli-Q water (1 mg/mL) were eluted with ammonium acetate 0.01 M at a flow rate of 0.5 mL/min for 50 min at 30 °C. The eluent was monitored using a refractive index detector (Boeblingen, Germain) at 30 °C, disposed in a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Boeblingen, Germain), equipped with two consecutive TSK-GEL columns (G5000 PWXL, 7.8 x 300 mm, particle size 10 µm, and G2500 PWXL, 7.8 x 300 mm, particle size 6 µm; Tosoh Bioscience,

Stuttgart, Germany). Calibration curves were prepared using pullulans of Mw 788, 473, 212, 100, 1.3, 0.34 kDa; and, Mw values were the average weight at peak maximum obtained in the analysis by triplicate.

Emulsifying activity

Emulsifying activity was calculated by turbidity according to the method described by Wang et al. (2011) with slight modifications.

A volume of 100 mL of pectin solution in water (20%, w/v), were mixed with 5 g of corn oil using an Ultraturrax at 24,000 rpm for 1 min to obtain an emulsion. The emulsion was diluted 30, 500 and 900-folds with sodium dodecyl sulphate (SDS) (1 g/L). Turbidity of emulsions was measured in a UV spectrophotometer SPECORD®210 and the WinASPECT® PLUS software (Analitik Jena AG, Jena, Germany), at 500 nm, using the SDS solution as blank sample. The turbidity was calculated by Equation (3):

$$T = \frac{2.303\ x\ A\ x\ F}{I} \qquad (3)$$

where T is turbidity of emulsions (m^{-1}), A is the absorbance at 500 nm, F is the dilution factor and I is path length, which is 0.01 m. The emulsion activity index (*EAI*) was calculated using Equation (4):

$$EAI = \frac{2\ x\ T}{\emptyset\ x\ c} \qquad (4)$$

where Ø is the oil volume fraction of the dispersed phase, and c is the concentration of pectin in the emulsion.

Zeta potential (ζ)

Zeta potential (ζ) of pectin in aqueous dilution was determined according to the method described by Falk et al. (2016), using a Malvern Zeta sizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). A volume of 250 mL of pectin solution, was prepared by dissolving the extracted pectin in KCl 0.1 M (1 mg/mL). The solution was agitated, sonicated during 1 min, and its pH was measured (mixture 1). Then, 10 mL of mixture 1 and 90 mL of KCl 0.1 M were agitated, sonicated during 1 min, and its pH was recorded (mixture 2). Briefly, mixture 2 was injected into the clear disposable zeta cell and the ζ was measured. The procedure for the preparation of mixtures was repeated, in order to obtain dilutions of mixture 1, at different pH values (2 to 10) by adding HCl 0.1 M or KOH 0.1 M drops, and their respective ζ values were measured. The measuring cell was carefully washed after each reading, using deionized water and the next dilution, avoiding bubbles inside, to evade measurement errors.

Apparent viscosity

Following the method described by Huang et al. (2017), extracted pectin was dissolved in deionized water (20 mg/mL) using a magnetic stirrer at ambient temperature during 1 h. The apparent viscosity of the sample was determined using a Modular Advanced Rheometer System (MARS) (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). Flow curves over the shear rate (1-100 s^{-1})

were measured at 25 °C. The measuring geometry used was a double-cone and plate system with a truncated cone with an angle of 2° and a diameter pf 60 mm. The apparent viscosity and steady shear rate measurement were fitted to the Herschel-Bulkley model, Equation (5):

$$\sigma = \sigma_0 + k \text{ x } \dot{\gamma}^{n} \qquad (5)$$

where σ is the shear stress (Pa), σ_0 is the yield stress (Pa), k is the consistency index (Pa.s^n), $\dot{\gamma}$ is shear rate (s^{-1}), and n is the flow behavior index.

Statistical analysis

Extractions and analysis were carried out at least in triplicate and means were compared by Tukey's test (p <0.05), using SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA). Differences were expressed as mean ± standard deviation.

Results and discussion

Overall characterization of sugar beet by-products

The results of the physicochemical analysis of the sugar beet by-products reported in **Table 1** show a reduction of °Brix, pH, DW, protein, total carbohydrates, reducing carbohydrates, total dietary fiber, insoluble dietary fiber and Mg when comparing the chemical composition of the sugar beet pulp pressed (SBP-P), with the sugar beet pulp ensiled (SBP-E) (p <0.05).

Table 1. Chemical composition of sugar beet pulp by-products (g/100 g DW).

Parameter	SBP-P	SBP-E	SBP-D
°Brix	5.00±0.21 b	4.60±0.07 a	4.40±0.14 a
pH	4.62±0.08 c	3.51±0.03 a	3.71±0.06 a,b
Aw	0.88±0.01 b	0.90±0.02 b	0.73±0.02 a
DW (%)	91.12±0.16 b	83.41±0.15 a	96.53±0.22 c
Total fat (g/100 g DW)	0.84±0.02 a	1.70±0.04 c	1.33±0.03 b
Protein (g/100 g DW)	10.42±0.52 c	8.30±0.30 a,b	8.01±0.31 a
Total carbohydrates (g/100 g DW)	82.64±0.63 c	70.22±0.32 a	78.14±0.50 b
Reducing carbohydrates (g/100 g DW)	10.40±0.11 c	6.73±0.10 b	4.21±0.08 a
TDF (g/100 g DW)	75.20±0.24 b	64.52±0.07 a	76.84±0.32 b,c
IDF (g/100 g DW)	47.58±0.16 b	34.51±0.09 a	51.32±0.18 b,c
SDF (g/100 g DW)	26.63±0.47 a	30.04±0.50 b,c	29.78±0.34 b
Ash (g/100 g DW)	1.86 a,b	1.87 ab	1.81 a
Na^{+} (mg/100 g DW)	17.66 a,b	26.39 c	16.16 a
Mg^{+2} (mg/100 g DW)	244.97 c	217.16 ab	214.30 a
P^{+3} (mg/100 g DW)	27.51 ab	24.20 a	23.55 a
K^{+} (mg/100 g DW)	184.72 b	189.84 bc	174.68 a
Ca^{+2} (mg/100 g DW)	1327.90 a,b	1332.21 bc	1317.26 a
Fe^{+3} (mg/100 g DW)	54.63 a	84.95 c	62.45 ab
Total phenols (mg GAE / 100 g DW)	0.38±0.05 c	0.29±0.02 b	0.17±0.01 a
Antioxidant capacity (mM Trolox/100 g DW)	2.34±0.14 b,c	2.23±0.09 b	1.16±0.10 a

SBP-P: sugar beet pulp pressed, SBP-E: sugar beet pulp ensiled, SBP-D: sugar beet pulp dried. DW: dry weight. TDF: total dietary fiber. IDF: insoluble dietary fiber. SDF: soluble dietary fiber. Means with different letters a-c denote significant difference (p <0.05) in the same row.

This variation could be due to a fermentation process of DW (20-30%) during the storage time (7-8 months) (**Figure 1**) carried out by saccharolytic and proteolytic bacteria; in a similar way to that observed by Álvarez, Méndez, and Martínez-Fernández (2015) who studied the effect of fermentation during silage of banana by-products. Moreover, the SBP-E showed an increase in the amount of fat and SDF (p <0.05). The higher fat and SDF content may be due to a greater cellular release,

generated by the decrease in pH caused in turn as a result of the transformation of soluble sugars in acetic and lactic acid, as part of the metabolism of anaerobic bacteria (Kung et al., 2018).

On the other hand, the increase of Na and Fe (p <0.05) found in SBP-E could be due to an increase in the solubility and bioavailability of minerals, as an effect of the pH reduction during silage (Hansen & Spears, 2009).

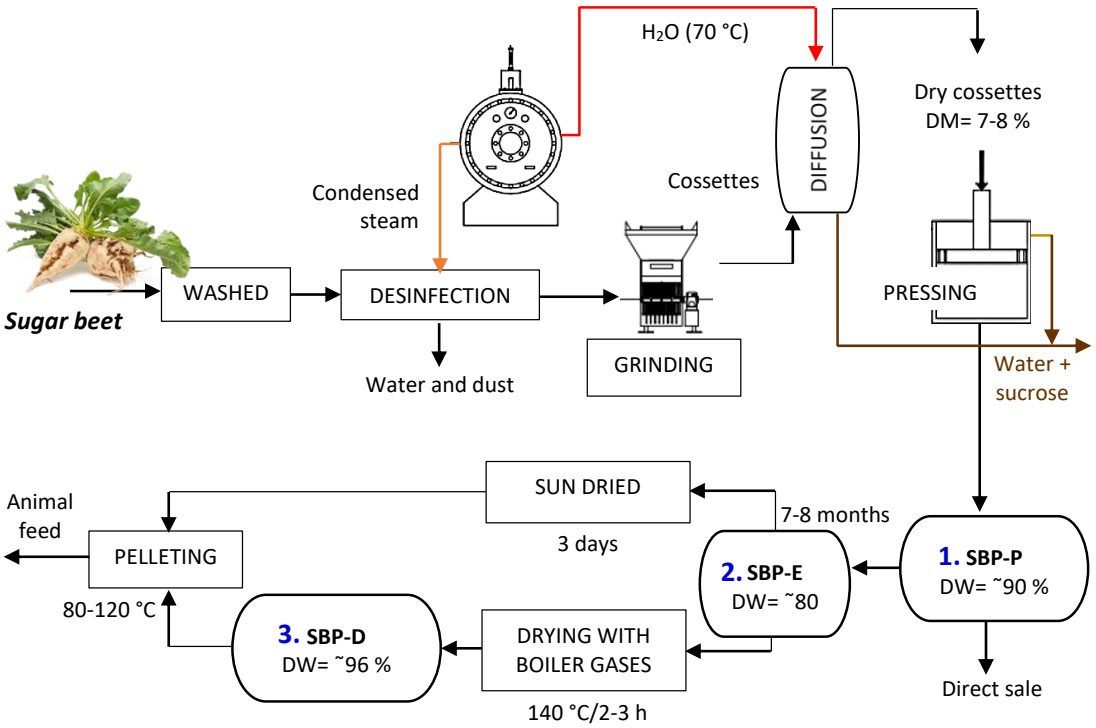

Figure 1. Industrial process of sugar extraction form sugar beet. By-products obtained: 1. SBP-P: sugar beet pulp pressed, 2. SBP-E: sugar beet pulp ensiled, 3. SBP-D: sugar beet pulp dried. DW: dry weight.

Conversely, in the case of the sugar beet pulp dried (SBP-D), an expected reduction of a_w, as well as a decrease in the content of reducing carbohydrates, soluble dietary fiber and antioxidant activity was observed, likely as a

consequence of the heat treatment applied (**Figure 1**) (~100 °C/2-3 h) (Sharma et al., 2015). Lastly, a reduction of K was determined probably due to a lower availability of this mineral for the analysis, as a consequence of the hardening of the sample by the drying effect (p <0.05).

Pectin extraction and characterization

Yield, monomeric composition and protein

The application of the acid method allowed to achieve higher yields in comparison with the enzymatic method, regardless the type of sugar beet waste used (**Table 2**). This result is in line to that reported by Lim et al. (2012) who compared the acid and the enzymatic method to extract pectin from Yuza (*Citrus junos*) pomace. The maximum yield was observed in the case of the pectin extracted from SBP-E followed by SBP-D using acid conditions (18.9 and 16.7%, respectively) (p <0.05), which seems to be related with the high amount of soluble dietary fiber (SDF) observed in the ensiled and dried residue (**Table 1**). Despite yields obtained by the enzymatic method were lower than those observed with the acid method, the sugar beet ensiled residue (P-SBP-E-EM) gave rise to a higher yield (13.4%) in comparison with that reported by Zykwinska et al. (2008) (4.0%) using a similar method of extraction but, instead, starting from fresh sugar beet pulp as raw material.

The analysis by GC-FID of the extracted pectins revealed the presence of xylose, arabinose, rhamnose, galactose, and galacturonic acid, whereas glucose could be derived from the acid hydrolysis of cellulose (Sun et al., 2015), by disruption of β-1,4-glycosidic bonds (Y.-B. Huang & Fu, 2013), and mannose from

mannans and galactomannans (Mayworm et al., 2000; Matsuhiro et al., 2006) (**Table 2**).

Table 2.

Yield extraction (g pectin/100 g DW), monomeric composition and protein of pectin from sugar beet by-products (g/100 g DW).

Pectin	Extraction method	Yield	Xylose	Arabinose	Rhamnose	Galactose	Galacturonic acid	Mannose	Glucose	Protein
P-SBP-P	AM	13.60	33.35±1.11 f	3.60±0.08 ef	9.81±0.31 e	14.50±0.50 d	23.52±0.11 a	6.14±0.18 e	2.01±0.02 b	4.3±0.22 ef
	EM	3.91	6.36±0.19 c	0.10±0.00 a	3.20±0.06 a	8.26±0.27 ab	65.51±0.30 e	4.04±0.03 c	5.22±0.09 d	1.6±0.06 a
P-SBP-E	AM	18.94	25.48±0.69 de	3.22±0.06 e	4.94±0.13 cd	7.60±0.28 a	42.74±0.23 b	2.39±0.04 a	3.30±0.03 c	3.4±0.14 d
	EM	13.40	4.53±0.14 a	0.70±0.01 b	3.50±0.11 ab	9.68±0.33 c	66.98±0.80 ef	5.05±0.15 d	0.76±0.01 a	2.0±0.08 b
P-SBP-D	AM	16.72	21.53±0.85 d	2.82±0.06 d	4.74±0.15 c	8.88±0.24 b	48.92±0.43 d	4.75±0.17 d	0.62±0.01 a	4.1±0.20 e
	EM	7.50	5.22±0.16 ab	0.79±0.02 bc	16.79±0.69 f	8.68±0.26 b	44.80±0.37 bc	3.06±0.09 b	12.57±0.30 e	2.8±0.1 c

DW: dry weight. P-SBP-P: pectin from sugar beet pulp pressed, P-SBP-E: pectin from sugar beet pulp ensiled, P-SBP-D: pectin from sugar beet pulp dried. AM: Acid method. EM: Enzymatic method. Means with different letters a-f denote significant difference (p <0.05) in the same column.

The acid extraction led to high quantities of xylose and arabinose in all cases, whereas the content in galacturonic acid (GalA) was significantly less important which could be attributed to its acid degradation (Wrolstad, 2013). The enzymatic method instead, allowed to obtain pectin with higher amount of galacturonic acid (GalA), and lower amount of xylose and arabinose (p <0.05).

However, it is important to notice that GalA was present in all extracted pectins in the range from 23.52 to 66.98%, having the pectins of sugar beet pulp ensiled (P-SBP-E-EM) and pressed (P-SBP-P-EM), both extracted by the enzymatic method, the highest GalA content (66.98 and 65.51%, respectively). In fact, these values suggest that pectin extracted from these by-products could be considered as food additives, according to the recommendations given by a Joint FAO/WHO Expert Committee on Food Additives, which established that pectin should contain not less than 65% of GalA calculated on the ash-free and dried basis (FAO & WHO, 2009).

The protein content was higher in the case of the pectin extracted by the acid method compared to the pectin extracted by the enzymatic method, possibly due to the severity of the acid method, resulting in a greater amount of protein residues linked to the pectin obtained. The pectin sample with the higher amount of protein was the pectin of sugar beet pulp pressed (P-SBP-P-AM), which is consistent with the greater amount of protein observed in the pressed by-product (SBP-P) (**Table 1**).

Degree of methoxylation (DM) and molecular weight (Mw)

FTIR spectra of pectins extracted from SBP-E by either acid or enzymatic methods are shown in **Figure 2**. The peak between 1052 and 1141 cm^{-1} is assigned to C = C double pectin bond. The absorption peak at 1388 and 1633 cm^{-1} are related to the stretch bands of the pectin COO groups. These results indicate that the final products are true pectin compounds (Shi & Gunasekaran, 2008).

Pectins extracted by the enzymatic method regardless of the type of sugar beet pulp by-product (that is, pressed, silaged or dried) showed larger peaks at 1608 cm^{-1} and 1745 cm^{-1} than those observed at the same wavelengths for pectins extracted by the acid method. However, when the degree of methoxylation (DM) was calculated by

correlating the peak area of the esterified carboxyl groups to the peak area of total carboxyl groups, the DM values of pectin extracted by acid method were statistically similar to the DM of pectins extracted by enzymatic method (p <0.05) (**Table 3**).

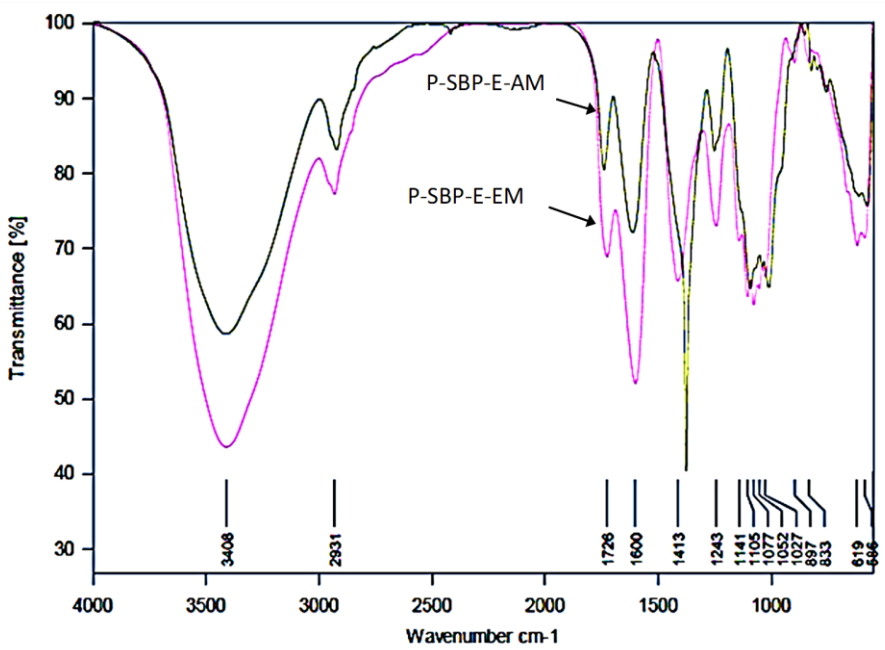

Figure 2. FTIR spectra of sugar beet pulp pectin. P-SBP-E-AM: pectin of sugar beet pulp ensiled, extracted by acid method. P-SBP-E-EM: pectin of sugar beet pulp ensiled, extracted by enzymatic method.

DM values were in the range from 45.2 to 50.1%, which correspond to low-methoxyl pectins (DM < 50%). This type of pectins is known as “slow-gelling” and has the ability to form gels at slightly neutral or basic pH, with maximum consistency in the presence of calcium at concentrations ranging from 20 to 100 mg per gram of pectin, and/or with low amounts of sugar (Yang et al., 2018). Therefore, low-methoxyl pectins are suitable as additives for the development of low-fat products, or foods by diabetic people (Ciriminna et al., 2017).

Table 3 shows the molecular weight (Mw) of the extracted pectins estimated by SEC. This parameter was also statistically similar between the pectins extracted from sugar beet by-products pressed, silage or dried by acid or enzymatic methods (p <0.05). The

determined Mw values were in the range from 303 to 322 kDa, in agreement to the maximum Mw values reported by Zykwinska et al. (2008) for pectin extracted from fresh SBP (310 kDa).

Table 3. Degree of methoxylation (DM) (%), and molecular weight (Mw)(kDa) of pectin extracted from sugar beet by-products.

Pectin	Extraction method	DM	*Mw
P-SBP-P	AM	49.29 a	306±7 a
	EM	47.08 a	311±9 a
P-SBP-E	AM	50.14 a	303±7 a
	EM	48.36 a	322±10 a
P-SBP-D	AM	48.39 a	315±9 a
	EM	45.21 a	319±10 a

P-SBP-P: pectin of sugar beet pulp pressed, P-SBP-E: pectin of sugar beet pulp ensiled, P-SBP-D: pectin of sugar beet pulp dried. AM: Acid method. EM: Enzymatic method. Means with similar letter (a) in the same column do not present significant difference (p <0.05). *Mw was calculated as the average at peak maximum observed in the analysis followed by triplicate.

Emulsifying activity index (EAI)

Figure 3 shows the emulsifying activity index (*EAI*) of the different pectins extracted by acid or enzymatic methods from sugar beet pulp pressed, ensiled or dried, using the commercial citrus pectin as standard. In general, the *EAI* of sugar beet pectins extracted by the acid method were higher than those of the *EAI* of sugar beet pectins extracted by the enzymatic method. Furthermore, all pectin samples extracted from sugar beet residues, excluding P-SBP-P-EM, showed a significant *EAI* higher than that of citrus pectin (p <0.05), according to the observed by Leroux et al. (2003).

Pectin from sugar beet pulp pressed extracted by the acid method (P-SBP-P-AM) and pectin of sugar beet pulp dried extracted by the same method (P-SBP-D-AM), presented the highest *EAI* (73.51, 75.53 m^2/g, respectively) among all the assayed pectins (p <0.05). These values are in the range reported by Huang et al. (2017) (75.3-104.9 m^2/g) for sugar

beet pectin extracted from pulp dried. Remarkably, these authors pointed out that the studied pectin samples exhibited good emulsifying activity.

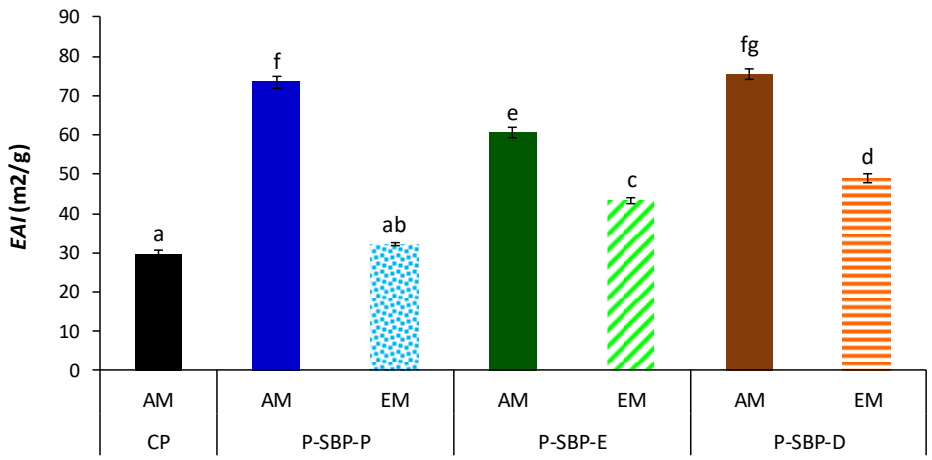

Figure 3. Emulsifying activity index (*EAI*) of pectin obtained from sugar beet by-products by acid or enzymatic methods. CP: citrus pectin. P-SBP-P: pectin of sugar beet pulp pressed, P-SBP-E: pectin of sugar beet pulp ensiled, P-SBP-D: pectin of sugar beet pulp dried. AM: Acid method. EM: Enzymatic method. Different letters (a-d) in the columns denote significant difference (p <0.05).

The difference in the emulsifying activity observed among the analysed pectins can be associated with the different content in protein (**Table 2**), which plays a predominant role in the emulsifying properties of sugar beet pectin (Chen et al., 2016). Thus, it can be observed that pectins with higher *EAI* (P-SBP-P-AM and P-SBP-D-AM) had the highest protein content (4.3 and 4.1 g / 100 g DW) (**Table 2**) and vice versa (p <0.05); effect in turn related to the ability of protein molecules to open in aqueous-lipid media, allowing that their electrically charged outer groups to bind with water molecules, and the internal non-polar amino acids to be released and bound to the oily particles, linking both phases, until forming the stable mixture, called emulsion (Cabra et al., 2008).

Zeta potential (ζ) and apparent viscosity (η)

The zeta potential (ζ) of sugar beet pectins extracted by acid or enzymatic methods from pressed, ensiled and dried residues is presented in **Figure 4**. The isoelectric point was not reached in any of the samples but it should occur at very acidic pHs, 1.5-2.0. Within the range of pH from 4.5 to 9, pectins extracted by the acid method had slightly higher absolute values of ζ (-25 to -34 mV) than pectins extracted by the enzymatic method (-20 to -28 mV) (p <0.05). This indicates that pectin particles extracted by acid method showed higher stability in aqueous dispersion than those obtained by enzymatic method. This behavior is associated with the fact that acidic extraction increases the electronegativity of pectin, which causes the particles move away from each other, and remain suspended in the aqueous medium (Genovese & Lozano, 2001).

Citrus pectin exhibited an intermediate stability between the samples extracted by the enzymatic and the acid method, and lastly, the P-SBP-P-MA showed the highest stability in aqueous solution among all analyzed samples (p <0.05).

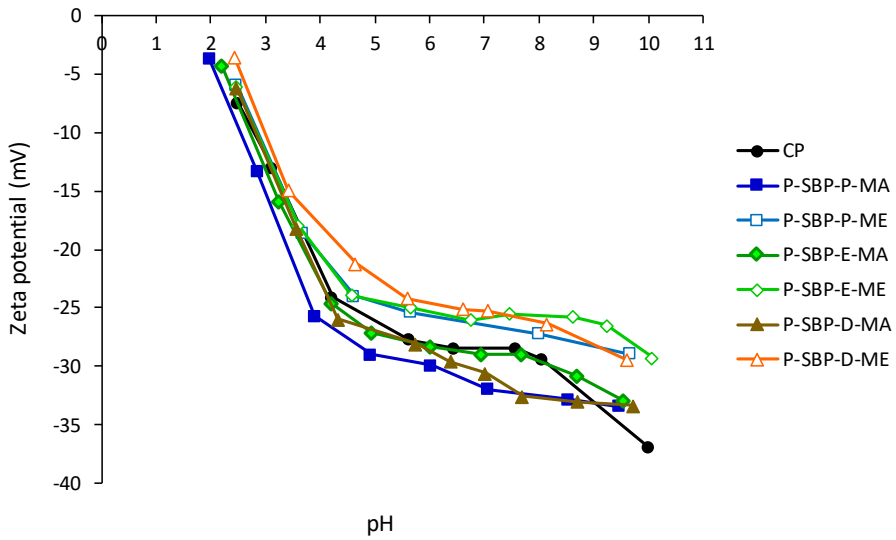

Figure 4. Zeta potential (ζ) curves of pectin. CP: citrus pectin. P-SBP-P-AM: pectin from sugar beet pulp pressed, acid method; P-SBP-P-EM: pectin from sugar beet pulp pressed, enzymatic method; P-SBP-E-AM: pectin from sugar beet pulp ensiled, acid method; P-SBP-E-EM: pectin from sugar beet pulp ensiled, enzymatic method; P-SBP-D-AM: pectin from sugar beet pulp dried, acid method; P-SBP-D-EM: pectin from sugar beet pulp dried, enzymatic method.

Figure 5 shows the apparent viscosity (η) of pectin solutions prepared in water (20 mg/mL). The solutions prepared with all types of sugar beet pectin showed lower viscosity than solutions prepared with commercial citrus pectin; in turn, solutions prepared with sugar beet pectin extracted by the enzymatic method presented higher apparent viscosity values than solutions prepared with sugar beet pectin extracted by the acid method, regardless of the type of by-product used (p <0.05).

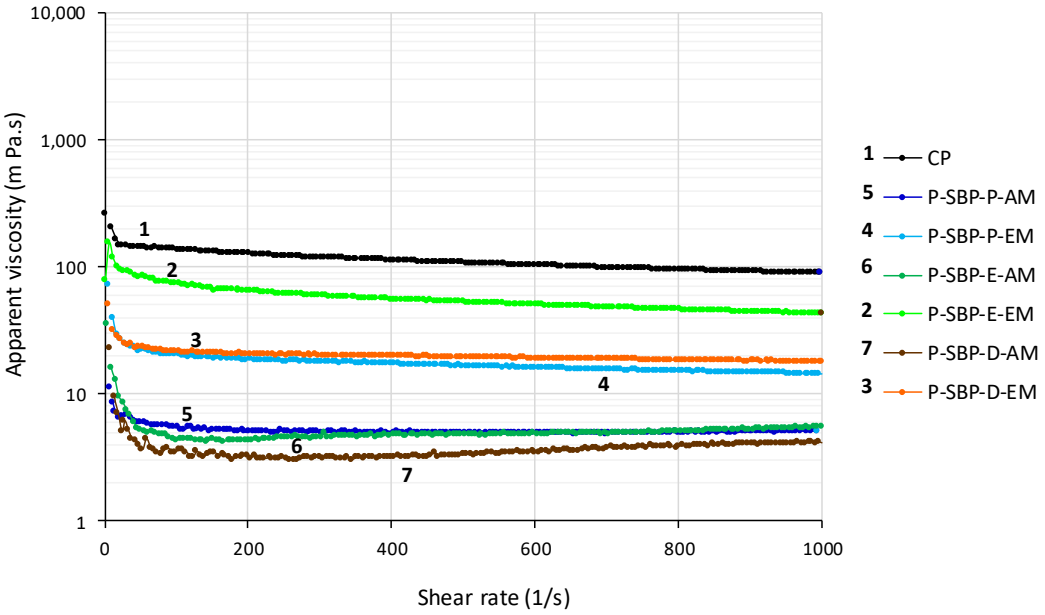

Figure 5. Apparent viscosity of pectin solutions (20 mg/mL). CP: citrus pectin. P-SBP-P-AM: pectin of sugar beet pulp pressed, acid method. P-SBP-P-EM: pectin of sugar beet pulp pressed, enzymatic method. P-SBP-E-AM: pectin of sugar beet pulp ensiled, acid method. P-SBP-E-EM: pectin of sugar beet pulp ensiled, enzymatic method. P-SBP-D-AM: pectin of sugar beet pulp dried, acid method. P-SBP-D-EM: pectin of sugar beet pulp dried, enzymatic method.

The higher viscosity observed in pectin extracted by enzymatic method, could be explained by the presence of polyelectrolytes, since they affect the conformation of the macromolecule and the nature of the counterions, which act as a brake on the flow of polymers (Wyatt et al., 2011). In this sense, the pectin extracted from sugar beet pulp ensiled by the enzymatic method (P-SBP-E-EM) reached the highest final viscosity (40 m Pa.s), followed by pectin of sugar beet pulp dried extracted by the enzymatic method

(P-SBP-D-EM) (18 m Pa.s), while the pectin of sugar beet pulp dried extracted by the acid method (P-SBP-D-AM), exhibited the lowest apparent viscosity value (4 m Pa.s).

The lower viscosity observed in the solutions prepared with pectin obtained from the dried beet residue may be due to the drying processes that can negatively influence on the properties of the rheological properties of gum (Huang et al., 2017). However, the viscosity of the pectin obtained in the present study, by enzymatic method from the dry sugar beet by-product (P-SBP-D-EM) (18 m Pa.s), obtained in the industry by application of 140 °C/2 -3 h with boiler gases (**Figure 1**), was slightly higher than that reported by Huang et al. (2017) for a sugar beet pectin obtained by acid method (12 M HCl), from dehydrated pulp at 40, 50 and 60 °C/8h, in hot air oven (10 m Pa.s); reaffirming the advantage of using the enzymatic method in the extraction process of pectin, in order to obtain high viscosity, since in both cases the waste used presented a moisture content close to 4.6% (~96% DW) **Table 1**.

It should be noted that, the degree of methoxylation (DM) and the molecular weight (Mw) of the extracted pectin (**Table 3**), could not have influenced the viscosity observed in the present study, since both the DM and the Mw did not have a statistically significant difference (p <0.05).

Overall, the apparent viscosity of the pectin solutions decreased when the shear rate increased, which is indicative of a pseudoplastic (shear-thinning) flow behaviour due to a decrease of entanglements of their structure, as is the case of gums (Cui, 2005).

Conclusions

The present study compared the compositional and rheological properties of sugar beet pectin, which was efficiently extracted from pressed, ensiled and dried residues by acid or enzymatic methods. The silage process caused a reduction in the protein and insoluble carbohydrates content of sugar beet pulp, as well as an increase in the fat and soluble dietary fiber amount, likely due to a lactic fermentation process. The drying process instead, caused a reduction in the reducing carbohydrates, soluble fiber and antioxidant capacity. Either the type of sugar beet by-product or the extraction method

had no impact on the degree of methoxylation and molecular weight of extracted pectin. Nevertheless, the enzymatic method allowed the extraction of pectin with a significantly higher content of galacturonic acid as compared to the acid method, due to the milder conditions of the former. The rheological analysis showed that all pectins obtained presented a pseudoplastic flow behavior. Furthermore, the zeta potential and *EAI* values indicated that pectins extracted by the acid method showed good stabilizing behavior in aqueous dispersion and good emulsifying activity, whereas pectins enzymatically extracted had a higher apparent viscosity that was linked to the presence of polyelectrolytes that impede the polymer flow.

To conclude, the information provided in the present work could be very useful for the potential reuse of ensiled and drying by-products from sugar beet industry in the cost-effective production of pectin with different technological properties depending on the applied extraction method. Pectins conveniently characterized and with suitable rheological properties are known to find immediate applications in the pharmaceutical and/or food fields.

4.2.2. Artículo V. Caracterización química y fisicoquímica de subproductos de naranja derivados de la industria

Chemical and physicochemical characterization of orange by-products derived from industry

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Abstract

Industrial extraction of orange juice produces great amounts of wastes that affect the environment and give rise to important economic losses; at the same time, the information about their composition is still limited. The aim of this study was to carry out an exhaustive chemical and physicochemical characterization of these residues, to increase their potential applications for the extraction of functional ingredients.

Four different products (three solids and one liqueur) were provided by the industry. The overall characterization indicated that carbohydrates were the main components. During processing, carbohydrates derivatives were formed such as those corresponding to the initial steps of the Maillard reaction. In this sense, furosine was demonstrated to be a suitable indicator of the process control. Whilst the phenolic content substantially decreased (by up to 57%) as the processing proceeds, the antioxidant capacity was affected to a much lesser extent (~10%). Dehydrated products were rich in galacturonic acid and hardly any change was detected during their elaboration process. The liqueur by-product was found to have a much higher level of fructose than glucose and sucrose.

Orange juice waste obtained industrially under the conditions shown herein could be used as a source of pectic derivatives or fructose in the case of solid or liquid by-products, respectively. The results obtained herein can diversify the present application of these products as a source of food ingredients contributing to the improvement of their utilization.

Introduction

Citrus (*Citrus spp.*) is one of the most important fruit crops worldwide (Bampidis & Robinson, 2006). Between 2013 and 2014 global citrus production was 121.3 million tons (58% corresponds to oranges) reaching in Spain 6.5 million; this country is the largest producer in the European Union and the fifth in the world. Some 40% of the fruit is used by the industry to extract juice, leaving around a half of its weight as waste (mainly peel, seeds and pulp). Citrus wastes reach 24.3 million tons per year from which, 1.3 million tons correspond to Spain (Mamma & Christakopoulos, 2014).

Orange wastes without further treatment can contribute to environmental problems, due to their fermentation implying high chemical and biological oxygen demand (Mamma & Christakopoulos, 2014). Citrus pulp amounts to 50-70 % of the fresh weight of the original fruit and it is contained in the peel (60-65%), internal tissues (30-35%) and seeds (0-10%) (Bampidis & Robinson, 2006). Citrus peel is rich in fibre and polysaccharides with water and oil-holding and cation-exchange capacities, and swelling properties much higher than those of cellulose. Thus, this dietary fibre constitutes a low-calorie bulk ingredient in food applications requiring oil or moisture retention (Chau & Huang, 2003). Over the last decade, orange peel wastes have been reported as an excellent source of essential oils, natural antioxidants, ethanol, organic acids, pectic oligosaccharides and pectin (Mamma & Christakopoulos, 2014; Amaya-Cruz et al., 2015). In this sense, a huge interest in the latter has emerged probably due not only to their well-known technological properties, but also to the benefits for gut microbiota, among others (Cantu-Jungles et al, 2017).

Devatkal, Narsaiah, and Borah (2010) replaced synthetic antioxidants in meat products with powder extract of Kinnow mandarin bark and found that this extract, rich in phenolic compounds with free radical scavenging activity has potential to be used as a safer alternative than synthetic ones. Additionally, Sharma et al. (2017) underlined that citrus wastes can act as potent antioxidants.

Thus, orange waste processing may provide an efficient, inexpensive, and environment-friendly platform for obtaining new nutraceutical products or for improving existing ones (Rafiq et al., 2016).

Among the different citrus by-products applications, their use as animal feed constitutes the cheapest and most realistic option for the food industry. Particularly, fresh citrus pulp is often used locally to feed animals; it has a natural acidity, but it is still a perishable product due to its high content of water and soluble sugars. Dried citrus pulp is used as a cereal substitute due to its high energy content in ruminant species. They can be used to support growth and lactation with good digestibility and fewer negative effects on rumen fermentation than starch rich feeds (Bampidis & Robinson, 2006).

The transformation of raw materials to animal feed can involve treatments at high temperatures to achieve gelatinization of the starch and destruction of microorganisms, which can also give rise to chemical modifications, the Maillard reaction (MR) is one of the most important (Martins et al., 2000). This reaction mainly takes place between amino acids, peptides or proteins and reducing sugars; the advanced stages may favour undesirable colour development together with nutritive losses due to the participation of lysine (Corzo-Martínez et al., 2012). The MR is often used by food manufacturers to develop appealing aromas, colour or texture in food products (cereal based food, coffee, meat…). However, despite some positive aspects, the MR could generate, in advanced stages of the reaction, potentially harmful compounds (e.g. acrylamide, furans, heterocyclic amines) (Rannou et al., 2016). To date, one of the indicators of the MR initial steps are the Amadori and Heyns compounds, which are formed before major subsequent changes in composition and functionality. In this regard, furosine (2-furoylmethyl-lysine, 2-FM-Lys) and other 2-furoylmethyl amino acids (2-FM-AA), which are formed after the acid hydrolysis of the Amadori and Heyns compounds, have been observed as one of the best and most sensitive indicators of the MR's initial steps in products of various origins (Moreno et al., 2006).

Another reaction that can also take place during processing of by-products is the degradation of polyphenols, affecting antioxidant activity (Ancos et al., 2017) due to the action of enzymes and oxygen and the thermal processes used. However, during

certain conditions of processing and storage an increase of phenols can also be observed attributed to the formation of compounds by hydrolysis or polymerization.

Despite these evidences, to the best of our knowledge, no information is available on either the control of the processes involved or on the quality of orange-juice by-products, making it difficult to guarantee their nutritive value and health benefits.

According to this, the objective of this paper was to carry out an exhaustive overall characterization of industrial by-products derived from orange juice extraction, which can afford useful information to retrospectively know the changes taking place during their manufacture, including carbohydrate and antioxidant activity modifications. A complete compositional study of these by-products could diversify the use not only as animal feed but also as potential sources of functional ingredients for human applications.

Materials and methods

Samples

Samples derived from the processing of Valencia late oranges were provided by García Carrión (Daimiel, Spain). **Figure 1** shows the industrial process of orange juice extraction and by-products transformation.

The Fresh Orange Residue (FOR) was pressed with $CaOH_2$ to facilitate the obtainment of the Orange Liqueur (OL). OL was concentrated into molasses, from 10 to 50°Brix (approximately), in a triple-effect evaporator with recirculation, and the pressed orange solid residue was dried. Then, the Dry Orange Residue (DOR) was triturated, mixed with the molasses, and the whole mass was concentrated at 100 °C to obtain the Animal Feed (AF) in form of pellets up to a maximum moisture content of 12%. FOR, DOR, OL and AF were selected performing a simple, non-stratified random sampling. The waste used in the extraction process came from oranges of different cultivars, harvested in early-May.

All samples were lyophilized, ground, sieved and maintained at -20 °C until analysis.

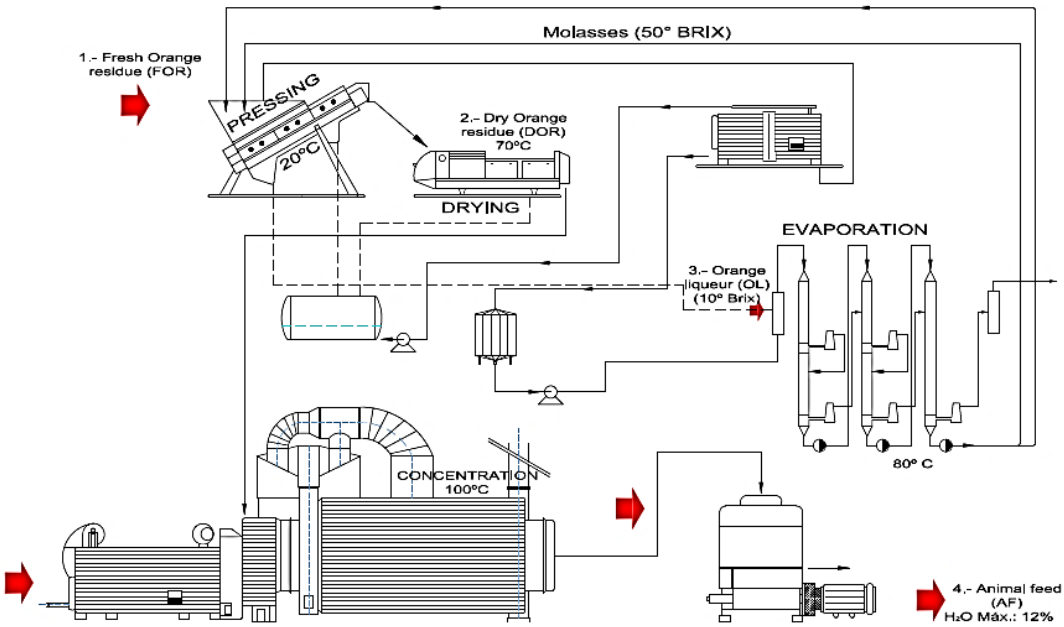


Figure 1. General scheme of the obtainment of by-products from the industrial extraction of orange juice.

Physicochemical characterization

°Brix, pH, water activity (a_w), dry matter (DM), fat and protein content were measured according to the AOAC methods, described by Megías-Pérez et al. (2014). The mineral composition of samples was determined using an ICP-MS Elan 6000 Perkin-Elmer Sciex instrument from the Service Interdepartmental Research (SIdI-UAM) (Madrid). Either a semiquantitative or a quantitative analysis of the elements of interest using the external calibration method, and internal standards to correct instrumental drift, were carried out (Zuluaga et al., 2011).

Total and reducing carbohydrates

Total carbohydrates were determined by the phenol-sulfuric acid method purposed by Dubois et al., (1956), and described by Masuko et al. (2005), with small variations. 278 µL of diluted samples were placed in Eppendorf tubes of 2 mL. Tubes were carried to an extraction sorbonne, and 278 µL of 5% aqueous phenol solution was added. Then of a slight vortex agitation, 1000 µL of sulfuric acid was added carefully, to each tube,

following all chemical safety regulations and using the appropriate protective clothing. Then of 30 min, the absorbance was measured at 480 nm using a BioTek Power Wave XS 201 595 spectrophotometer, equipped with a plate reader (Biotek KcJunior). Galacturonic acid (GalA) (G2125 ≥98% Sigma-Aldrich) was used for the calibration curve (20-100 µg mL^{-1}). Results were expressed as g kg^{-1} of DM.

Reducing sugar determination was done by the 3,5-dinitrosalicylic acid (DNS) method described by Sumner and Graham (1921). The calibration curve was constructed using Galacturonic acid (GalA) (0-5 mg mL^{-1} in methanol). The absorbance was measured at 540 nm.

Dietary fibre

Samples of orange by-products were analysed for total and insoluble fibre by the modified enzymatic–gravimetric method described by McCleary (2010) (AOAC 985.29 and 991.42) (AOAC, 1995). The AOAC method uses a heat-stable, *α*-amylase, amyloglucosidase, and protease treatment. The insoluble dietary fibre (IDF) was determined by weighing the dried residue that remained after the enzymatic treatment. The total dietary fibre (TDF) was determined on a different aliquot by weighing the dry precipitate resulting from the addition of ethyl alcohol to the enzymatically treated samples. The soluble dietary fibre (SDF) values were obtained by the difference between the total and insoluble dietary fibre fractions.

2-Furoylmethyl-Amino Acid (2-FM-AA) content

The determination of these compounds was carried out by ion-pair RP-HPLC-UV analysis (Agilent Technologies 1260 Infinity LC System, Böblingen, Germany) using a C8 column (250 cm x 4.6 mm inside diameter) (Alltech furosine-dedicated, Nicolasville, KY) at 35 °C, a linear binary gradient at a flow rate of 1.2 mL min^{-1} with two mobile phases: A: 0.4% acetic acid and B: 0.34% KCl in phase A, and a UV detector at 280 nm.

Samples (0.25 g) were hydrolysed with 4 mL of 8 M HCl at 110°C for 24 h, filtered (Whatman No. 40) and 0.5 mL of the filtrate was applied to a Sep-Pack C18 cartridge (Millipore, Milford, MA, USA). Furosine was eluted with 3 mL of 3 N HCl and injected

in the system. For quantification, a commercial standard (Neosystem Laboratoire, Strasbourg, France) was used. The content was expressed as mg/100 g protein (Gamboa-Santos et al., 2014).

Total phenolic content

Following the Folin-Ciocalteu method described by Soria et al. (2010) methanolic extracts were obtained by dilution of 0.2 g of powder sample in 5 mL of methanol, trituration in Ultraturrax (24000 rpm for 1 min), heating with continuous stirring (50 °C for 20 min), centrifugation (2000 x *g* for 15 min) and filtration of supernatant (1 mL using Sep-Pak Cartridges of 0.45 µL).

Once obtained the methanolic extracts; 100 µL of methanol, 100 µL of 2 N Folin, and 700 µL of Na_2CO_3 were added on 100 µL of each methanolic extract, and the absorbance was read at 735 nm. The calibration curve was done with gallic acid (GAE) (0-60 mg/L). Results were expressed as mg GAE/100 g DM.

Antioxidant capacity

According to the method proposed by Brand-Williams et al. (1995), 7 µL of methanolic extract obtained as described above and 193 µL of 2 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH) diluted in methanol (1:15), were transferred to a multiwell cell, and kept in the dark for 30 min. The absorbance was read at 517 nm (UV-Vis Perkin Elmer Lambda 40 spectrophotometer). The calibration curve was obtained with trolox (0.25-2.5 mM in methanol). Results were expressed as trolox equivalent antioxidant capacity (TEAC) (mM Trolox/100 g DM).

Colour development

Colour of the samples was measured using a CM-508i colorimeter (Minolta Co. LTD, Japan) and the CIE Lab colour system, where L^* is lightness, a^* is redness and b^* is yellowness (Fernández-Artigas et al. 1999). The chroma (C^*), the huge angle ($h°$) and the total colour differences (ΔE) were calculated by the equations (Holzwarth et al., 2013):

$$C^* = (a^{*2} + b^{*2})^{0.5}$$

$$h^\circ = \arctan(b^*/a^*)$$

$$\Delta E = (\Delta L^{*2} + \Delta a^{*2}\ \Delta b^{*2})^{0.5}$$

Monomeric composition

For this determination a previous hydrolysis of 30 mg of sample was carried out with 1500 µL of 2 M trifluoroacetic acid (TFA) at 110°C during 4 h. The monomers released were analysed by GC-FID (Agilent Technologies 7890A gas chromatograph, Agilent Technologies, Wilmington, DE, USA). 500 µL of hydrolysed samples were evaporated under vacuum and mixed with 400 µL of phenyl-*β*-D-glucoside (0.5 mg mL^{-1}), internal standard. After the formation of oximes (Brobst & Lott, 1966), samples were persilylated with hexamethyldisylazane (HMDS) (250 µL) and TFA (25 µL) at 50 °C for 30 min and centrifuged at 11 200 g for 2 min. Analyses were carried out using a DB-5HT capillary column (15 m × 0.32 mm × 0.10 µm) (J&W Scientific, Folsom, California, USA). Chromatographic conditions were selected according to Muñoz-Almagro et al. (2017).

The carbohydrates with a degree of polymerization (DP) <6 were analyzed by GC-FID as trimethylsilyl derivatives of their oximes. Samples (5 mg) and 400 µl of *β*-phenyl glucoside (0.5 mg mL^{-1}) were evaporated, derivatized and analyzed in a similar way as mentioned above.

Pectin extraction and characterization

Pectin was extracted from by-products with the highest content of GalA, following the enzymatic method of Wikiera et al. (2015), with some modifications. Samples diluted in 0.05 M buffer sodium citrate pH 4.5 were treated with Celluclast 1.5 L (Novozymes Corp., Bagsvaerd, Denmark) (50 µL g^{-1}). After stirring at 50 °C for 18 h, samples were kept at room temperature, and centrifuged. The supernatant was filtered and a double volume of 95% ethanol was added to allow the pectin flotation, which was subsequently filtered, and washed using 70% ethanol and acetone to

remove the colour (Liew et al., 2016; Pinheiro et al., 2008). The resulting pectin was lyophilized and stored at -20 °C. The yield was determined by the equation:

$$Pectin\ yield = \frac{Pectin\ (g)}{Powder\ sample\ (g)} * 100\%$$

Monomeric composition of pectin was determined after acid and enzymatic hydrolysis with 1500 µL of 2 M TFA at 110 °C for 4 h; and 25 µL mL^{-1} of Viscozyme, at 50 °C for 24 h as described by Zhang et al. (2018). The monomers released were analysed as previously described (Brobst & Lott, 1966; Muñoz-Almagro et al., 2017).

Statistical analysis

Data were analysed for comparison of means using SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA). Differences between means were obtained using Tukey's test at α=0.05, and the values were expressed as mean ± standard deviation. Analyses were done at least in duplicate.

Results and discussion

Overall characterization

As shown in **Table 1**, °Brix data are similar in all the samples except in the OL sample that displayed the highest value (39.0 °Brix), probably as a result the extractability of soluble sugars during the pressing of FOR (**Figure 1**). To the best of our knowledge, the value of °Brix of similar by-products obtained from citrus fruit has not been reported. Cane and beet molasses (Sharma et al., 2016; Mladenović et al., 2016) were found to have, respectively, 50.0 and 83.6 °Brix.

Regarding pH, FOR showed the lowest value, probably because of the presence of organic acids that could have been lastly removed or transformed during processing. The pH value of DOR was higher probably due to the addition of $CaOH_2$ during the pressing (**Figure 1**).

Table 1. Chemical and physicochemical composition of orange by-products obtained from the orange juice extraction (g kg^{-1} DM).

Parameter	FOR	DOR	OL	AF
°Brix	29.7±0.00a	29.7±0.00a	39.0±0.15b	29.7±0.00a
pH	3.9±0.01a	6.4±0.01b	5.1±0.01a	5.1±0.01a
Aw	0.3±0.00a	0.5±0.00b	0.9±0.00c	0.5±0.00b
Dry matter (DM)	885.3±0.50c	870.1±0.10b	310.2±0.90a	881.0±0.60b
Total fat	9.0±0.00b	12.4±0.00c	1.1±0.00a	17.3±0.00d
Protein	58.9±0.79a	63.1±0.10c	60.0±0.51b	61.3±0.44b
Na	0.21a	0.25b	0.39d	0.33c
Mg	1.14a	2.60c	2.13b	2.67d
P	0.29a	0.41b	0.51d	0.47c
K	10.87a	12.85b	21.33d	16.09c
Ca	6.09a	33.50d	19.07b	27.95c
Fe	0.02a	0.43d	0.10b	0.26c
Ca/P ratio	21.0:1	81.7:1	37.4:1	59.5:1
Total carbohydrates	798.4±0.70c	742.2±0.20b	206.7±0.30a	753.4±0.60b
Reductor carbohydrates	39.1±0.34a	48.6±0.06b	70.3±0.45c	82.1±0.37d
TDF	460.7±0.19b	674.2±0.02d	20.0±0.25a	576.9±0.93c
IDF	226.0±0.16b	624.8±0.02d	2.5±0.01a	452.2±0.09c
SDF	234.7±0.35c	49.4±0.03b	17.5±0.24a	124.6±1.02c
Furosine (mg furosine / 100 g protein)	9.0±0.14a	182.1±2.35c	19.5±0.35b	455.1±3.07d
Total phenols (mg GAE / 100 g DM)	211.9±0.36d	145.5±0.47c	115.6±0.52b	90.2±0.73a
AC (mM de trolox / 100 g DM)	23.3±0.15d	22.4±0.07c	21.6±0.12b	21.0±0.06a

FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. TDF: Total dietary fiber. IDF: Insoluble dietary fiber. SDF: soluble dietary fiber. AC: Antioxidant capacity. Means with different letters a-d denote significant difference (p <0.05) in the same file.

The a_w of the solid samples (FOR, DOR and AF) had low values, being considered as low moisture products with high stability over the time (Batt & Tortorello, 2014). The highest a_w corresponded to OL. As expected, DM content was opposite to a_w; the highest value was observed in FOR (885.3 g kg^{-1}) and the lowest in OL (310.2 g kg^{-1}) samples.

Fat content was found to be between 9 to 17.3 g kg^{-1} in all solid samples, whereas in the liqueur the value was lower (1.1 g kg^{-1}). Cerón-Salazar and Cardona-Alzate (2011) reported a fat content of 15.5 g kg^{-1} in orange peel wastes. The differences can be due to a different proportion of essential oils depending on the variety and method of extraction. Protein content was similar in all samples analyzed, which varied from 58.9 to 63.1 g kg^{-1}.

Regarding mineral analysis, the most abundant elements were K and Ca ranging from 10.87 to 16.09 g kg^{-1} and 6.09 to 33.50 g kg^{-1}, respectively. Bampidis and Robinson (2006) and the National Research Council (NRC) (2001) reported 11.00 and 7.10 g kg^{-1} of K and Ca, respectively, for dried citrus pulp. The DOR sample presented the highest calcium content (33.50 g kg^{-1}) followed by AF (27.95 g kg^{-1}) and OL (19.07 g kg^{-1}) due to the addition of $CaOH_2$ during the pressing and concentration (**Figure 1**). NRC (1988) reported a Ca content of 17.2 g kg^{-1} for citrus molasses, similar to the value shown in the present study for the OL sample.

In animal feed, the determination of the Ca/P ratio is important since a value of 2:1 is recommended to avoid modifications in the nutrient uptake for ruminants (Cherdthong et al., 2014). This ratio in AF was 59.5:1, which could be improved by the addition of P, or substitution of $Ca(OH)_2$ by enzymes with a similar effect to alkaline hydrolysis, employed to dissolve lignin during the degradation process of agro wastes (Vadivel, Moncalvo, Dordoni, & Spigno, 2016).

The DM of this kind of samples consists mostly of total carbohydrates (**Table 1**). OL was the by-product with the lowest content, due to the fact that solid residue retains a large part of insoluble carbohydrates. The highest total carbohydrate content was observed in FOR, compared to DOR, and AF, possibly because the fresh

residue is more porous, which could facilitate hydrolysis during the application of the phenol-sulfuric method.

The content of reducing sugars observed in the fresh (39.1 g kg^{-1}) and dry (48.6 g kg^{-1}) residue was in the range reported for orange peel (23.2 to 59.9 g kg^{-1}) (Cortés Ortiz et al., 2015). The liquor showed a higher content (70.3 g kg^{-1}) because, during the pressing of the fresh residue, reducing sugars may have easily migrated to the soluble phase. In the final product (AF) the reducing carbohydrates were higher (82.1 g kg^{-1}) than expected, most likely ascribed to the hydrolysis of other non-reducing carbohydrates like sucrose, during the heat treatment (**Figure 1**).

DOR presented the highest content of TDF (674.2 g kg^{-1}) and IDF (624.8 g kg^{-1}). Its content of TDF was similar to that reported by Figuerola et al. (2005). for orange peel (643.0 g kg^{-1}) and the SDF content of FOR (234.7 g kg^{-1}) was higher than that reported for peel of *Citrus sinensis* L. cv. *Liucheng* (94.1 g kg^{-1}) (Chau & Huang, 2003). The mixture of DOR and OL led to lower fibre content in the final product (AF) (576.9 g kg^{-1}); however, this quantity is well suited to the needs of cattle. This product is classified as effective fibre to maintain the optimum ruminal pH (6.2 to 6.6) (Dairy Australia, 2013).

The SDF was high in FOR (234.7 g kg^{-1}) and remained mostly in this fraction during the pressing, thus a very small amount appeared in OL (17.5 g kg^{-1}). In DOR, the small amount of SDF (49.4 g kg^{-1}) could be related to an increase in the degree of sample hardness during drying (**Figure 1**), which apparently affects the hydrolysis of the sample by the enzymatic method applied for the SDF determination. Conversely, AF had a higher SDF content (124.6 g kg^{-1}), probably because it was made from the crushed dry residue, which facilitates the extraction of SDF.

Assessment of initial steps of Maillard reaction

The initial steps of MR were assessed by the formation of furosine. The FOR sample presented 9 mg of furosine/100 g of protein, and higher values were observed for DOR, OL and AF (182.1, 19.5, 455.1 mg of furosine/100 g of protein, respectively), mainly due to the heat treatment applied at the industry (**Figure 1**) and the composition of

each by-product (**Table 1**). The higher pH values of DOR, OL and AF, as compared to FOR, could also have favoured the MR. The highest value of furosine in AF could also be due to the intensity of the process that can increase the hydrolysis of non-reducing sugars, thus, increasing the amount of reducing sugars able to participate in the MR. No data on the formation of furosine in these types of products have been reported to date. Megías-Pérez et al. (2014) found joint levels of furosine and 2-FM-Arg ranging from 38.4 to 3766.3 mg/100 g protein in dehydrated fruits.

Total phenolic content and antioxidant capacity

The total phenolic content of FOR (211.9 mg GAE/100 g DM) was according to the results reported by Ghasemi et al. (2009) for the *Citrus sinensis* var. Washington Navel (peel 160.3 mg GAE/100 g DM and tissues 232.5 mg GAE/100 g DM). During processing, this parameter decreased with the intensity of the treatment (**Table 1**). Comparing the phenolic content between FOR and AF, a significant reduction was observed.

The antioxidant capacity of citrus by-product in the initial sample (23.3 mM trolox/100 g) was lower than the value reported by Escobedo-Avellaneda et al. (2014) for flavedo of Valencia orange (46.4 mM trolox/100 g), probably ascribed to the presence of other non-antioxidant vegetal tissues (e.g. rag) in FOR (Fancello et al., 2016) or different cultivar, among other factors.

The antioxidant capacity decreased significantly from the initial to the final product, but to a much lower extent (10%) than the total polyphenol content (57%). This could be due to the formation of antioxidant compounds during the progress of the MR, due to the intensity of processing (Yu et al., 2012). Ghasemi et al. (2009) did not find any correlation between the total phenolic content and antioxidant activity in tissues or peels of 13 citrus species.

Colour changes

L^* decreased significantly (p <0.05) during processing. The greatest decrease was between FOR and DOR (**Table 2**), which could be due to the loss of water and MR progress, related to the browning (<L^*).

The tone *h*° was low in all by-products and hardly any difference was detected. The redness *a** was also low in all the by-products analysed which might be due to the low amount of anthocyanins in orange wastes. *a**, *b** and *C** parameters presented a similar trend with the highest change occurring after the first step of drying to obtain DOR. These parameters were higher in the liquor due to the presence of coloured soluble compounds. When OL was added to DOR to obtain AF, intermediate values of *a**, *b** and *C** were observed. Therefore, redness, yellowness and the chroma decreased with the reduction of humidity and intensity of heating. The parameter *C**, that correlates redness (*a**), yellowing (*b**) and luminosity (*L**), can be an indicator of non-enzymatic browning; the lower the value, the greater the non-enzymatic browning (**Tables 1-2**).

Table 2. Colour parameters of orange by-products.

Colour Parameter	FOR	DOR	OL	AF
L*	52.85±0.57d	32.76±0.47b	30.25±0.46a	34.77±0.19c
a*	6.89±0.10c	3.67±0.03a	9.46±0.09d	3.90±0.03b
b*	31.35±0.11c	17.16±0.34a	32.83±0.42d	19.96±0.27b
C*	32.10±0.13b	17.55±0.33a	34.16±0.39c	20.33±0.27b
h°	1.35±0.00b	1.36±0.01b	1.29±0.00a	1.38±0.00c
		FOR and DOR	**FOR and OL**	**FOR and AF**
ΔE		24.81±0.90c	22.79±0.33b	21.58±0.50a

FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. Means with different letters a-d denote significant difference (p <0.05) in the same file.

Soluble fraction of carbohydrates and monomeric composition

The analysis by GC-FID of the samples without previous hydrolysis allowed us to investigate the presence of free carbohydrates of low DP. **Figure 2** shows the presence of fructose, galactose, glucose, sucrose, disaccharides, trisaccharides and tetrasaccharides in FOR.

The most abundant water-soluble carbohydrate (**Table 3**) in FOR was fructose (131.7 g kg^{-1}), a similar value to that reported by Wilkins et al. (2005) for Valencia

orange peel waste (136.6 g Kg^{-1}) hydrolysed enzymatically, followed by glucose (105.3 g kg^{-1}) and sucrose (65.4 g kg^{-1}).

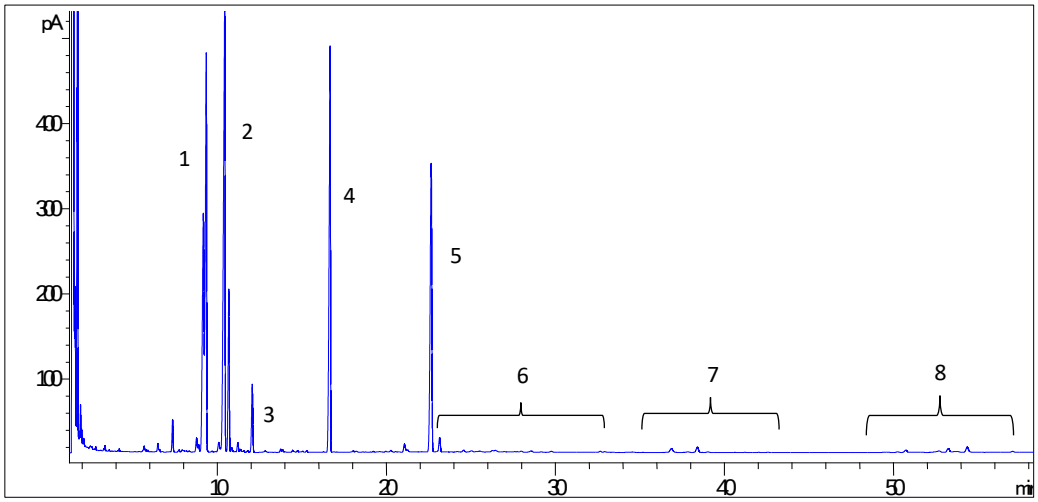


Figure 2. Chromatogram of low molecular weight carbohydrates (water-soluble) (analyzed as their TMS-oximes) present in the fresh orange residue (FOR), determined by GC-FID, without previous hydrolysis with TFA. 1: Fructose; 2: Galactose; 3: Glucose; 4: β-phenyl-glucoside (internal standard); 5: Sucrose; 6: Disaccharides; 7: Trisaccharides; 8: Tetrasaccharides.

Table 3. Carbohydrates of low molecular weight (water-soluble) in orange by-products (g kg^{-1} DM) determined by GC-FID.

Carbohydrates	FOR	DOR	OL	AF
Fructose	131.7±3.13b	91.4±2.28a	154.2±4.12c	92.9±2.20a
Galactose	2.1±0.02a	22.0±0.55b	86.1±3.80d	69.7±0.97c
Glucose	105.3±2.48d	5.2±0.12a	53.3±1.13c	17.9±0.40b
Sucrose	65.4±1.37b	8.1±0.27a	77.2±2.30d	68.7±1.07c
Total disaccharides	7.9±0.11b	8.1±0.12b	16.2±0.38c	6.1±0.14a
Total trisaccharides	5.0±0.11c	4.0±0.07b	4.9±0.11c	3.3±0.08a
Total tetrasaccharides	8.0±0.04d	1.2±0.02b	1.9±0.04c	0.9±0.01a

Results are expressed in dry matter (DM). FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. Means with different lowercase letters indicate a statistically significant difference (p <0.05) in the same row.

In DOR, sucrose could have been hydrolyzed, but fructose and glucose decreased in regard to FOR, probably due to their participation in the MR, in agreement with the

high furosine formation indicated above. Total tetrasaccharides also decreased due to hydrolysis during processing. The most abundant sugar in the liquor was fructose, the content of which was much higher compared to the other by-products. This can probably be ascribed, among other factors, to its higher solubility compared to glucose (Figuerola et al., 2005). It can be inferred that the OL could be an enriched source of fructose in a similar way to cane molasses that are used to extract sucrose. Thus, cane molasses contains 200-300 g kg^{-1} of sucrose, 140-250 g kg^{-1} of glucose, and 150-250 g kg^{-1} of fructose (Sharma et al., 2016), whereas OL contains 77.2 g kg^{-1} of sucrose, 53.3 g kg^{-1} of glucose and 154.2 g kg^{-1} of fructose.

With respect to the final product, AF, the amount of carbohydrates could be explained by the combination of several factors: i) the mixture of DOR and OL; ii) the participation of monosaccharides in the MR and iii) hydrolysis of sucrose, tri- and tetrasaccharides during processing.

When evaluating the monomeric composition after acid hydrolysis (**Figure 3** and **Table 4**), xylose, arabinose, rhamnose, galactose, mannose, glucose and GalA were found in all samples.

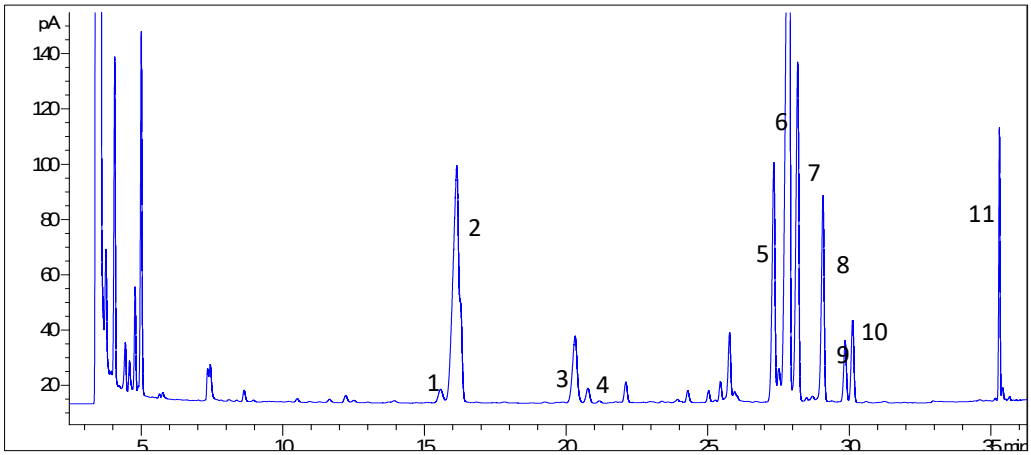


Figure 3. Chromatographic profile. obtained by GC-FID of TMS-oximes of carbohydrates present in the fresh orange residue (FOR) after its hydrolysis with 2 M TFA for 4 h at 110 °C. 1: xylose (Xyl). 2: xylose (Xyl) + arabinose (Ara). 3: rhamnose$_1$ (Rha). 4: rhamnose$_2$ (Rha). 5: galactose (Gal). 6: mannose$_1$ (Man). 7: glucose$_1$ (Glc). 8: galactose$_2$ (Gal) + mannose$_2$ (Man) + glucose$_2$ (Glc). 9: galacturonic acid$_1$ (GalA). 10: galacturonic acid$_2$ (GalA). 11: Internal standard. Sub-indices represent the elution order of compounds.

Apart from glucose and mannose, the other monosaccharides could come mainly from pectic polysaccharides. Glucose could be derived from the acid hydrolysis of cellulose (Sun et al., 2015), by disruption of β-1,4-glycosidic bonds (Huang & Fu, 2013) and mannose from mannans and galactomannans (Mayworm et al., 2000; Matsuhiro et al., 2006). This composition indicates the presence of pectin derivatives in the by-products, homogalacturonan being the major domain (Yapo et al., 2007). Kaya et al. (2014) found low amounts of rhamnose and GalA as the major compound in orange peel pectin.

Since GalA was present in a considerable amount in FOR, DOR and AF (**Table 4**), the pectin extraction was done in these samples and the yields were 239.5, 119.3 and 165.6 g kg^{-1} DM, respectively. Yields of 259.0–299.0 g kg^{-1} DM have been previously reported for fresh orange peel pectin (Pandharipande & Makode, 2012; Farinas et al., 2010). The lower yields in the processed samples (DOR and AF) were probably due to the tissue hardening, which may influence the cellulase activity (Lopes da Silva et al., 2006).

Table 4. Monosaccharides derived from polysaccharides present in orange by-products (g kg^{-1} DM) analyzed by GC-FID after TFA hydrolysis and subsequent TMS-oxime formation.

Monosaccharide	FOR	DOR	PL	AF
Xylose	38.7±0.16b	43.3±0.41c	5.2±0.05a	43.5±0.42c
Arabinose	48.9±0.43c	51.5±0.22d	6.8±0.06b	1.7±0.02a
Rhamnose	23.3±0.17b	27.2±0.13c	5.9±0.03a	24.2±0.19b
Galactose	79.7±0.41b	62.5±0.27a	280.8±1.35b	83.4±0.74c
Mannose	51.7±0.41b	59.0±0.24c	33.7±0.18a	52.2±0.48b
Glucose	194.3±0.59c	68.7±0.29a	173.4±0.93d	161.3±0.91b
Galacturonic acid	160.7±0.90d	85.8±0.17b	7.9±0.07a	101.8±0.75c

Results are expressed in dry matter (DM). FOR: fresh orange residue. DOR: dry orange residue. PL: pressing liqueur. AF: orange animal feed. Means with different letters a-d denote significant difference (p <0.05) in the same file.

As shown in **Table 5**, the most striking feature is the high content of GalA in the three by-products, with values matching the requirement to be considered as a food

ingredient E-440 (>65%) (Food and Agriculture Organization of the United Nations, 2009).

Table 5. Monosaccharides present in orange by-products pectin (%) analyzed by GC-FID after enzymatic hydrolysis and subsequent TMS-oxime formation.

Monosaccharide	FORP	DORP	AFP
Xylose	5.8±0.10b	5.9±0.11b	5.2±0.10a
Arabinose	5.2±0.08a	5.2±0.07a	4.7±0.08a
Rhamnose	9.7±0.28a	12.2±0.18b	11.7±0.08b
Galactose	1.3±0.02a	4.0±0.02c	1.8±0.01b
Mannose	2.2±0.01c	1.5±0.01b	0.8±0.01a
Glucose	1.5±0.02b	0.8±0.01a	1.6±0.02b
Galacturonic acid	74.4±0.80b	70.4±1.05a	74.1±0.45b

Results are expressed in dry matter (DM). FORP: fresh orange residue pectin. DORP: dry orange residue pectin. AFP: animal feed pectin. Means with different letters a-d denote significant difference (p <0.05) in the same file.

Conclusions

According to the obtained results it is possible to conclude that furosine is a suitable indicator to retrospectively control the processing of orange juice industrial by-products. It is advisable to apply low temperatures, to avoid as much as possible, the loss of nutritive value due to the participation of lysine in the Maillard reaction. The by-products analysed constitute a source of polyphenols and, although their content can decrease during processing, the antioxidant activity is hardly affected, since the formation of the Maillard reaction compounds counteracts the effect. Their main components are carbohydrates, with fibre being the most abundant in the solid residues. In this case, the high content of GalA in all assessed samples indicates that the three by-products (fresh and processed) could be a suitable source of pectin derivatives with potential application as a food ingredient. In addition, the pressed liquor with its high fructose content could constitute an alternative to molasses with less caloric value and higher sweetening power. These results underline the importance of an adequate utilization of by-products by the food industry. Further in

vivo studies are being carried out to elucidate the possible beneficial effect of the consumption of these products.

4.2.3. Artículo VI. Efecto antiinflamatorio intestinal del consumo de subproductos industriales de naranja, en ratones tratados con DSS

Anti-inflammatory bowel effect of industrial orange by-products in DSS-treated mice

Pacheco, M. T., Vezza, T., Diez-Echave, P., Utrilla, P., Villamiel, M., and Moreno, F. J. *Food & Function*, 9(9), 4888–4896 (2018). DOI: 10.1039/ C8FO01060A

Abstract

This work addresses the role of different by-products derived from industrial extraction of orange juice on a possible anti-inflammatory effect in mice with colitis induced by dextran sulfate sodium (DSS). The fresh orange residue (FOR), the dry orange residue (DOR), orange liqueur (OL) and animal feed (AF), as well as commercial citrus pectin (CP) were supplied to C57BL/6J mice for 15 days before starting the DSS treatment. Macroscopic parameters such as Disease Activity Index (DAI) and colonic weight/length ratio revealed an anti-inflammatory effect following intake of FOR, AF or CP. Moreover, q-PCR of RNA from colonic tissue indicated measurable changes in the expression of TNF-α, IL-1β, iNOS, and intercellular adhesion molecules ICAM I, as well as in intestinal barrier proteins as MUC-3, Occludin, and ZO-1. Pectin, phenolic compounds and/or Maillard reaction products formed at initial steps were identified as relevant components exerting the ascribed beneficial effects. Our findings could open up the further application of a variety of orange by-products as food supplements in the potential amelioration of inflammatory bowel diseases.

Introduction

Inflammatory bowel disease (IBD) is commonly divided into ulcerative colitis (UC) and Crohn's disease (CD) (Szilagyi & Xue, 2018). UC is usually confined to the colon, while CD usually affects any part of the gastrointestinal tract. A small segment of patients with IBD is classified as undetermined IBD, showing symptoms of UC and CD (Moran, 2017). The IBD prevalence is between 37.5-248.6 per 100.000 in North America and 4.9-505 per 100.000 in Europe (Ananthakrishnan, 2015).

Among various potential causes implicated in the IBD pathology, genetic susceptibility coupled with environmental risk factors attributed to lifestyle changes such as dietary habits, smoking, stress and lack of exercise, as well as other changes associated with medications, surgery or those leading to alteration of the bacterial flora of the gut are the most frequently described (De Mattos et al., 2015; Lee et al., 2016).

Current strategies for the treatment of IBD involve first induction of remission, followed by maintaining remission. Patients are usually treated with corticosteroids, immunomodulators, and anti-TNFα agents; although immunosuppressive therapies and anti-TNFα agents are associated with a higher risk of infections (Frei et al., 2013) and they eventually require surgical intervention, indicating that current therapeutic options are insufficient (Andersson & Soderholm, 2009). Moreover, the high cost of biological therapies contributes to the increasing financial burden of health care. The disadvantages of pharmacological therapies on IBD emphasize the need for non-pharmacological options (Derikx et al., 2016). Exclusion diets are generally not recommended and there is little evidence to support any particular food when nutritional regimens are recommended (Forbes et al., 2017). In this sense, low dietary fiber intake has been associated with the incidence of IBD (Cazarin et al., 2016), since the prebiotic activity of fiber can act on IBD by stimulating the selective growth of the intestinal lactobacilli and bifidobacteria, which produce short-chain fatty acids (SCFA). SCFA could improve mucosal barrier functions and modulate the immune system (Gálvez et al., 2010). However, there is scarce evidence regarding prebiotics use in this type of pathologies (Lichtenstein et al., 2016).

Fiber can be found in a plethora of products and derivatives of vegetal origin. Industrial processing of citrus generates huge amounts of wastes (24.3 million of tons per year worldwide, and 1.3 million of tons in Spain) (FAO, 2016) that, without further treatment, cause environmental problems, since their fermentation implies high chemical and biological oxygen demand (Mamma & Christakopoulos, 2014; Lin et al., 2013). In most of the cases, these by-products are processed to obtain animal feed, and depending on the composition, and the thermal treatment applied, Maillard reaction products (MRPs) may be formed. Some of these MRPs may have anti-oxidant, anti-

mutagenic, carcinogenic and anti-bacterial activities (Hwang et al., 2011), but their anti-inflammatory effect is not yet well studied.

In past decades, dozens of animal models have been developed as indispensable tools for investigating the pathogenesis of IBD and evaluating therapeutic options (Perše & Cerar, 2012). These approaches are mainly based on spontaneous colitis models, inducible colitis models, genetically modified models, and adoptive transfer models (Neurath et al., 2000; Wirtz & Neurath, 2007; Wirtz et al., 2007). Chemically induced murine models of intestinal inflammation are one of the most commonly used models because they are simple to induce and the onset, duration, and severity of inflammation are immediate and controllable. The dextran sulfate sodium (DSS)-induced mouse colitis model is characterized by bloody faeces, diarrhea, weight loss and tissue inflammation (Okayasu et al., 1990), as well as an increase of proinflammatory cytokines (IL-1*β*, TNF-*α* and IL-6) release in the intestine (Shih & Targan, 2008), which can impair permeability of intestine and mucosal barrier function and correlate with the severity of intestine inflammation (Zhu et al., 2017). These changes are similar to those found in humans by using molecular techniques to demonstrate changes in the composition of the mucosa-associated and fecal microbiota in patients with Crohn's disease (Sartor, 2008). In addition, DSS-induced colitis model has some advantages when compared to other animal models of colitis. For example, the severity of the disease can be produced easily by changing the concentration of administration of DSS, and the dysplasia that resembles the clinical course of human UC occurs frequently in the chronic phase of DSS-induced colitis (De Robertis et al., 2011; Kanneganti et al., 2011).

In this context, a recent study allowed the determination of phenolic compounds, pectin and the Amadori compound *N*-*ε*-fructosyl-lysine (furosine) in by-products from the industrial extraction of orange juice (Pacheco et al., 2018), and, in order to explore their potential functionality, the aim of this work was to investigate the anti-inflammatory effect of a variety of orange by-products consumption in a DSS model in mice, which is the most commonly approach used to assess the *in vivo* therapeutic activity, since it exhibits certain characteristics similar to those present in human IBD (Sun et al., 2018).

Material and methods

Analytical standards

Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). DSS (36-50 kDa) was purchased from MP Biomedicals (Santa Ana, CA, USA). RNA later® was obtained from Sigma Aldrich (St. Louis, MO, USA), and Tri-Reagent® was acquired from Thermo Fisher Scientific (Invitrogen, USA). The oligo (dT) primers (Promega, Southampton, UK) and KAPA SYBRsFAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) were used to perform the qPCR analyses.

Orange by-products

Citrus pectin (CP) was provided by CEAMSA (O'Porriño, Spain). By-products from the orange juice extraction industry: fresh orange residue (FOR), the dry orange residue (DOR), orange liqueur (OL) and animal feed (AF) were provided by the company García-Carrión (Daimiel, Spain). After the industrial extraction of orange juice, the FOR was pressed with calcium oxide to facilitate the obtainment of the OL. Then, OL was concentrated from 10 to 50 °Brix by heating at 80 °C, and the pressed orange residues were dried from 10 to 30% dry matter at 70 °C, obtaining the DOR. In the final stage, OL and DOR were mixed, dried at 100 °C and grounded to get the AF in the form of pellets[27]. These samples were lyophilized, grounded and characterized as follows: dry matter, protein, fat, and fiber content were determined according to the AOAC methods. Total and reducing carbohydrates were measured using phenol-sulfuric acid and 3,5-dinitrosalicylic acid methods, respectively. Total phenolic content (TPC) was determined following the Folin-Ciocalteu method described by Soria et al. (2010). Antioxidant capacity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method (Brand-Williams et al., 1995). 2-Furoylmethyl-Amino Acids (2-FM-AA) were analyzed by ion-pair RP-HPLC-UV and monosaccharide composition was determined through the hydrolysis with 2 M trifluoroacetic acid and subsequent formation of trimethylsilyl-oximes derivatives by GC-FID (**Tables 1 y 2**) (Pacheco et al., 2018).

Table 1. Chemical and physicochemical characterization of orange by-products and citrus pectin.

Parameter	FOR*	DOR*	OL*	AF*	CP
Dry matter (DM) (g/100g)	88.53±0.05c	87.01±0.01b	31.02±0.09a	88.10±0.06c	99.49±0.04d
Fat (g/100g DM)	0.90±0.00b	1.24±0.00c	0.11±0.00a	1.73±0.00d	n.d.
Protein (g/100g DM)	5.89±0.08b	6.31±0.01b	6.00±0.05b	6.13±0.04b	0.97±0.01a
Total carbohydrates (g/100g DM)	79.84±0.07c	74.22±0.02b	20.67±0.03a	75.34±0.06b	98.35±0.03d
Reducing carbohydrates (g/100g DM)	3.91±0.03b	4.86±0.01c	7.03±0.05d	8.21±0.04e	1.79±0.01a
TDF (g/100g DM)	46.07±0.02b	67.42±0.00d	2.00±0.03a	57.69±0.09c	96.58±0.12e
IDF (g/100g DM)	22.60±0.02b	62.48±0.00d	0.25±0.00a	45.22±0.01c	n.d.
SDF (g/100g DM)	23.47±0.04d	4.94±0.00b	1.75±0.02a	12.46±0.10c	96.58±0.12e
Main minerals (g/100g DM)	1.72b	4.66d	4.08c	4.44cd	0.15a
Total phenols (mg GAE / 100 g)	211.9±0.36e	145.5±0.47d	115.6±0.52c	90.2±0.73b	0.1±0.01a
AC DPPH (mM of trolox /100 g)	23.3±0.50c	22.4±0.40b	21.6±0.30a	21.0±0.40a	n.d.
2-FM-Lys Furosine (mg / 100 g of protein)	9.0±0.14a	182.1±2.35c	19.5±0.35b	455.1±3.07d	827.4±8.60e

Results are expressed in dry matter (DM). FOR: fresh orange residue, DOR: dry orange residue, OL: orange liqueur, AF: animal feed, CP: citrus pectin. TDF: Total dietary fiber, IDF: Insoluble dietary fiber, SDF: soluble dietary fiber, AC: antioxidant capacity. n.d.: not detected. Means with different letters a-d, denote significant difference (p <0.05) in the same file.
*Data according to Pacheco et al. (2018).

Table 2. Monosaccharides derived from polysaccharides present in orange by-products (g kg^{-1} DM) and citrus pectin analyzed by GC-FID after TFA hydrolysis and subsequent TMS-oxime formation.

Monosaccharide	FOR*	DOR*	OL*	AF*	CP
Xylose	38.7±0.16c	43.3±0.41	5.2±0.05a	43.5±0.42d	25.14±0.18b
Arabinose	48.9±0.43d	51.5±0.22d	6.8±0.06b	1.7±0.02a	31.82±0.14c
Rhamnose	23.3±0.17b	27.2±0.13b	5.9±0.03a	24.2±0.19b	45.17±0.50c
Galactose	79.7±0.41b	62.5±0.27a	280.8±1.35d	83.4±0.74b	110.96±0.95c
Mannose	51.7±0.41c	59.0±0.24d	33.7±0.18b	52.2±0.48c	2.95±0.02a
Glucose	194.3±0.59d	68.7±0.29b	173.4±0.93c	161.3±0.91c	9.82±0.09a
Galacturonic acid	160.7±0.90d	85.8±0.17b	7.9±0.07a	101.8±0.75c	755.14±5.41e

Results are expressed in dry matter (DM). FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin. Means with different letters a-d denote significant difference (p <0.05) in the same file.
*Data according to Pacheco et al. (2018).

Animals and diets

This study was carried out in accordance to the Guide for the Care and Use of Laboratory Animals as promulgated by the National Institutes of Health. The experimental protocol was approved by the Commission of Ethics in Animal Experimentation (Protocol CEEA 2010-286) of the University of Granada (Spain). C57BL/6 male mice of 7-9 weeks old were obtained from Janvier (St Berthevin Cedex, France). They were housed in Makrolon cages, maintained in an air-conditioned atmosphere with a 12-h light–dark cycle, and provided with free access to tap water and standard rodent diet (Panlab A04 diet, Panlab S.A., Barcelona, Spain).

Experimental design

Mice (23±2 g) were maintained in specific pathogen-free conditions in the facilities of Licinio de la Fuente Center and were randomly assigned to seven groups (n=10): healthy, DSS control, FOR, DOR, OL, AF and CP. Solid by-products samples were added to standard food at 10%. CP and OL were diluted in water at concentrations of 2.5 and 1.25% (w/v), respectively, and supplied at the rate of 100 µL/mouse per day. Induction of colitis was

performed 15 days after the beginning of the experiment by adding 2.7% (w/v) DSS in the drinking water for seven days (**Table 3**).

Table 3. Experimental conditions applied to the study of the consumption effect of orange by-products.

Group	Animal weight (kg)	Daily dose of treatment	Average food intake (g/mice*day)	SDF content of treatment (g/kg animal*day)	Treatment supply (Days)	DSS (2.7%) supply (Days)
Healthy	0.025	0.0	14.6	0.0	-	-
DSS Control	0.025	0.0	10.5	0.0	-	7
FOR	0.025	10% in standard food	10.6	10.0	14	7
DOR	0.025	10% in standard food	10.9	2.1	14	7
OL	0.025	50 mg/kg of animal	11.2	0.9	14	7
AF	0.025	10% in the standard food	11.1	5.5	14	7
CP	0.025	10 mg/kg of animal	11.9	9.9	14	7

SDF: soluble dietary fiber. DSS: Dextran sulfate sodium. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin.

Macroscopic indicators

Weight variation, daily food intake, Disease Activity Index (DAI) and the ratio between colon weight and length were considered as macroscopic indicators. Mice were monitored recording the animal and food weight, the presence of gross blood in the faeces and the stool consistency. These parameters were each assigned a score according to the criteria proposed by Cooper, Murthy, Shah, and Sedergran (1993), and used to calculate the DAI (**Table 4**). Once the animals were sacrificed, the colon (from the ileocaecal junction to the anal verge) was quickly excised and carefully washed with a cold saline solution, weighed and its length was measured.

Table 4. Clinical parameters considered to determine the Disease Activity Index (DAI).

Bleeding	Stool consistency	WL (%)	Value assigned according to WL
0: normal	0: normal	0	0
1: presence of blood	1: moderate soft stools	1-4	1
2: moderate bleeding	2: soft stools	5-9	2
3: moderately high bleeding	3: soft stools and diarrhoea	10-19	3
4: abundant bleeding	4: diarrhoea	>20	4

WL: Weight loss.

Biochemical markers

The expression of pro-inflammatory cytokines as tumour necrosis factor (TNF)-α, interleukin (IL)-6, (IL)-1β and inducible nitric oxide synthase (iNOS), as well as barrier intestinal proteins as intercellular adhesion molecule (ICAM)-1, Mucin (MUC)-3, Occludin and Zonula occludens-1 (ZO)-1, were evaluated.

The colon tissue was longitudinally divided into different fragments and stored at -80 °C in RNAlater®. Total RNA from colonic samples was isolated using Tri-Reagent® following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2 µg of RNA was reverse transcribed using oligo (dT) primers (Promega). Real-time quantitative PCR was carried out on optical grade 48-well plates in an Eco™ Real-Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, the KAPA SYBR®FAST qPCR Master Mix (Kapa Biosystems), and specific primers at their annealing temperature (**Table 5**) (Cazarin et al., 2016). In order to normalize mRNA, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was measured. The mRNA relative quantitation was calculated using the $\Delta\Delta$Ct method.

Table 5. Primer sequences used in real-time qPCR assays in colonic tissue.

Gene	Sequence 5′–3′	Annealing T (°C)
GAPDH	FW: CCATCACCATCTTCCAGGAG	60
	RV: CCTGCTTCACCACCTTCTTG	
IL-1*β*	FW: TGATGAGAATGACCTCTTCT	55
	RV: CTTCTTCAAAGATGAAGGAAA	
IL-6	FW: TAGTCCTTCCTACCCCAATTTCC	60
	RV: TTGGTCCTTAGCCACTCCTTC	
TNF-*α*	FW: AACTAGTGGTGCCAGCCGAT	56
	RV: CTTCACAGAGCAATGACTCC	
ICAM-1	FW: AGGAGGTGAATGTATAAGTTATG	60
	RV: GGATGTGGAGGAGCAGAG	
iNOS	F: GGCAGAATGAGAAGCTGAGG	55
	R: GAAGGCGTAGCTGAACAAGG	
MUC-3	FW: CGTGGTCAACTGCGAGAATGG	62
	RV: CGGCTCTATCTCTACGCTCT	
ZO-1	FW: GGGGCCTACACTGATCAAGA	56
	RV: TGGAGATGAGGCTTCTGCT	
Occludin	FW: ACGGACCCTGACCACTATGA	56
	RV: TCAGCAGCAGCCATGTACTC	

Statistical analysis

Biochemical analyses were performed at least in duplicate. Indicators of each group were expressed as mean ± standard deviation. Means were compared using the Tukey test (significance limit was set at p <0.05). Statistical analysis was carried out using SPSS software 22.0.

Results

Physicochemical characterization of test materials

The main physicochemical characteristics determined in the orange by-products and citrus pectin are summarized in **Table 1**. Overall, all studied orange by-products had a similar composition in protein or fat content, whereas substantial differences were found in carbohydrate, fiber or total phenolic content. By comparing the general chemical

composition among the fresh orange residue (FOR), dry orange residue (DOR), orange liqueur (OL), animal feed (AF) and citrus pectin (CP), FOR presented the greatest total phenolic content (211.9 mg GAE/100 g) and the highest antioxidant capacity (23.3 mM of Trolox /100 g), whereas CP showed the greatest content of soluble dietary fiber (SDF) (96.58 g/100 g) as expected, followed by FOR (23.47 g/100 g) and animal feed (AF) (12.46 g/100 g). CP also showed the highest content in furosine, an indirect marker of the Amadori compound *N*-fructosyl-lysine (Erbersdobler & Somoza, 2007), (827.4 mg/100 g of protein), followed by AF (455.1 mg/100 g of protein).

All samples had galacturonic acid (GalA), xylose (Xyl), arabinose (Ara), rhamnose (Rha), galactose (Gal) and mannose (Man) (**Table 2**), which are core monomers of the main domains of pectin (homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II) (Holck et al., 2014).

Assessment of macroscopic indicators of Inflammatory bowel disease in DSS-treated mice

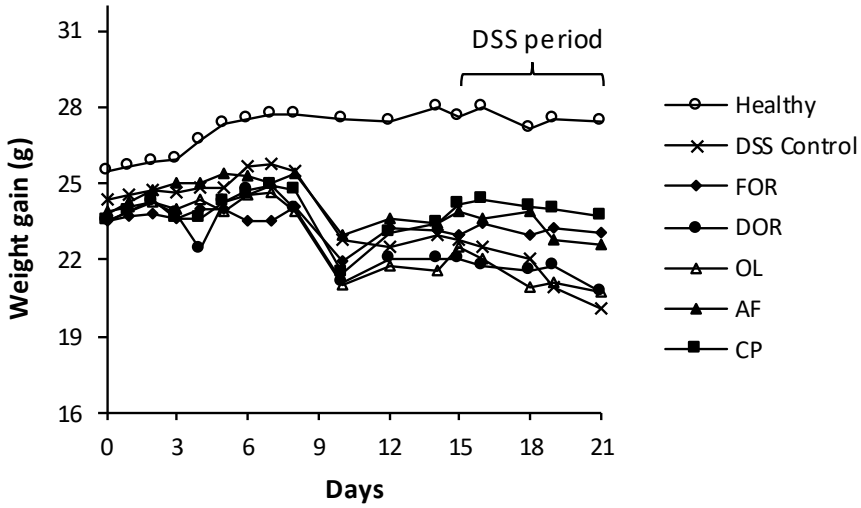
Among symptoms of colitis induced by the oral administration of DSS are haematochezia, body weight loss, shortening of the intestine, mucosal ulcers and infiltration of neutrophils.

Except for the healthy control group, the tested groups experimented a loss of weight (**Figure 1A**) which was higher in DSS control group, probably due to the inflammation located in the intestine, as well as to a systemic status of illness occasioned by DSS as a general toxic (Perše & Cerar, 2012). Remarkably, the weight reduction was significantly less in those animals that consumed CP, FOR and AF groups.

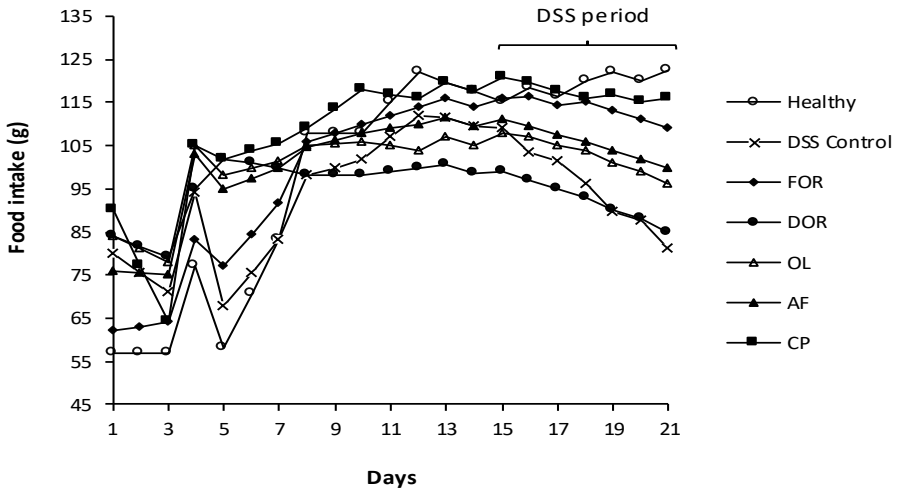
Reduction of food intake is also a symptom of illness (**Figure 1B**) possibly due to the discomfort caused by the intestinal inflammation (Yu & Rodriguez, 2017). During the DSS period the intake decreased significantly in the DSS control group, while animals treated with CP and FOR showed food consumption levels similar to that observed in the healthy group, and significantly different to DSS group (p <0.05). Similarly, the AF group showed a higher intake compared to DSS group, but non-significant differences were found.

The DAI, registered during the 7 days of DSS supply, allowed to infer that FOR, CP and AF groups *vs.* DSS control colitic group presented the lowest DAI (p <0.05) (**Figure 1C**). Weight/length ratio of the colon was significantly less in those groups that consumed FOR, AF and CP vs. the DSS group (**Figure 1D**), indicating a lower severity of inflammation and minor colonic cells infiltration (p <0.05).

A)



B)



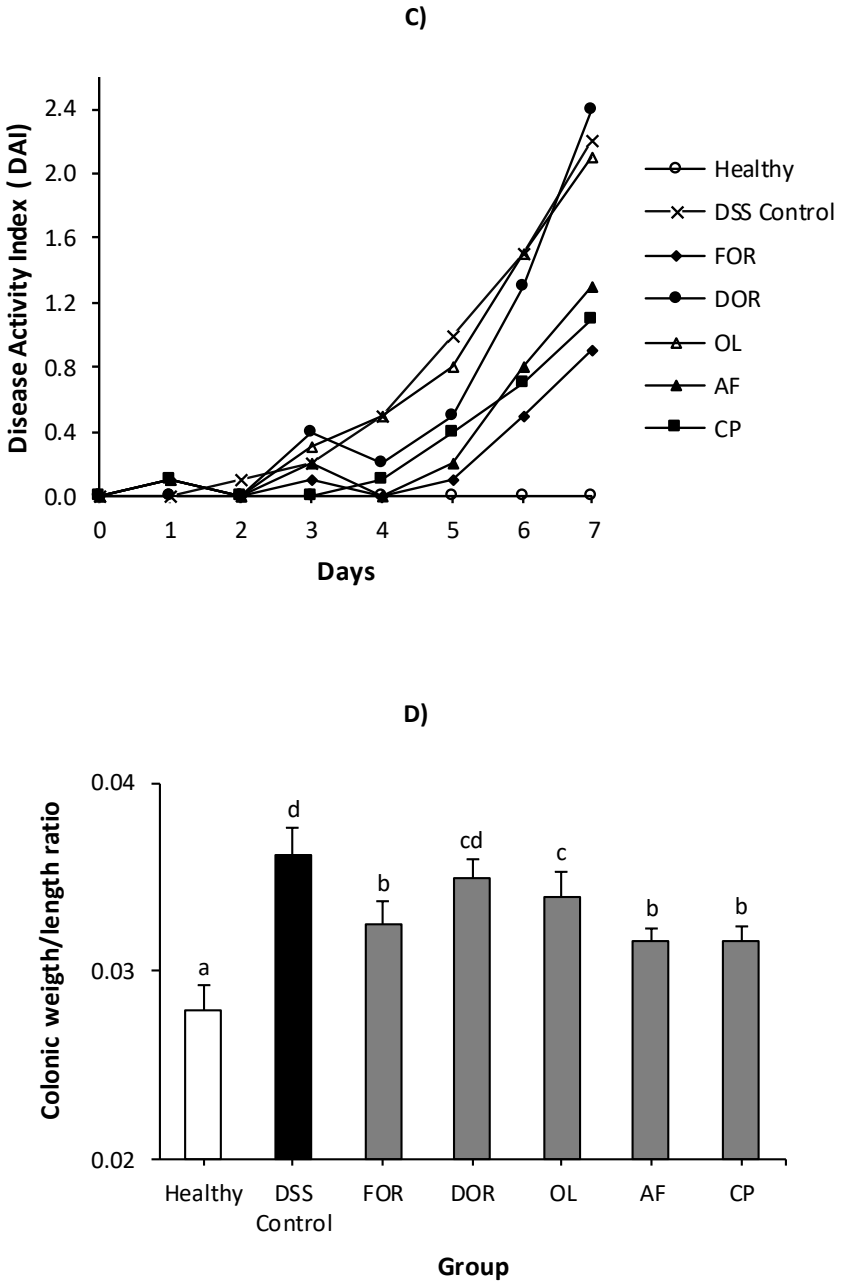


Figure 1. Macroscopic indicators. (A) Weight gain. (B) Food intake. (C) Disease Activity Index (DAI). (D) Colonic weight/length ratio. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin.

Overall, these macroscopic parameters showed a consistent anti-inflammatory effect for CP, FOR and AF, with a significant reduction of symptoms and improvement of the animals' general status. As mentioned in previous lines, the most important compositional differences found in the tested orange by-products were in fiber, total phenolic, and Amadori compounds (measured as furosine) content.

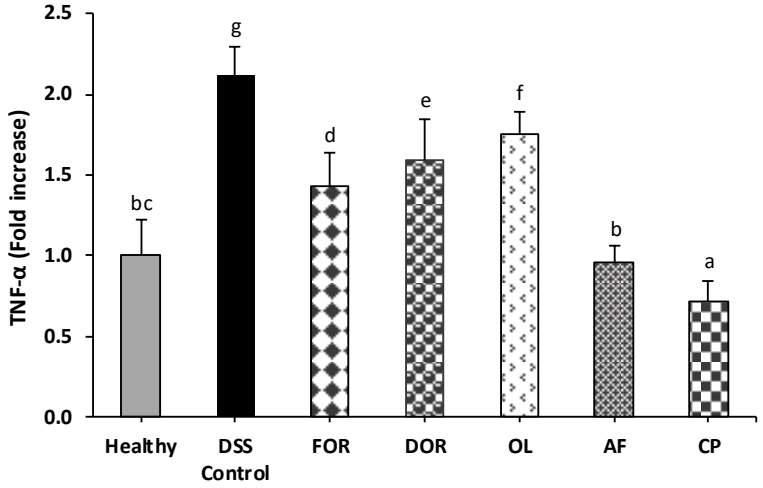
Assessment of biochemical markers of Inflammatory bowel disease in DSS-treated mice

Cytokine profile of DSS acute colitis is consistent with acute inflammatory response characterized by a macrophage-derived cytokine profile, strong chemotactic pattern and a polarized Th1 panel with high participation of TNF-α and IL-1β among others, similar to human IBD disease. This acute situation is also accompanied by a high expression of iNOS, and an increase of adhesion molecule ICAM I that facilitates the leukocyte endothelial transmigration, contributing to the tissue damage and the exacerbation of the gut inflammation (Abdelbaqi et al., 2006).

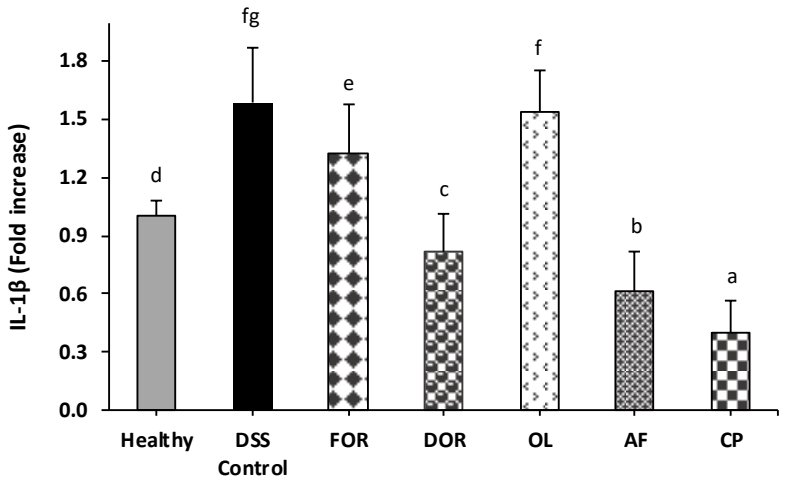
The expression of inflammatory cytokine panel (TNF-α, IL-1β, IL-6) was consistent and significantly reduced in the groups fed with FOR, AF and CP vs DSS control group (**Figures 2A-2C**); and additionally, a reduction in the expression of ICAM I was observed in those groups that consumed the orange by-products, in comparison with DSS control group (**Figure 2D**) (p <0.05). The expression of ICAM I was statistically similar between the healthy group and the CP and FOR groups, followed by AF group.

MUC 3, occludin and ZO-1 expression was significantly greater in FOR, AF and CP treated groups as compared with the DSS control group (p <0.05) (**Figure 2E-2H**). Increased expression of these proteins indicated a potential protective effect for those products which can not only reduce the symptoms and seriousness in acute conditions but also have a preventative effect against future IBD crisis (Nie et al., 2017).

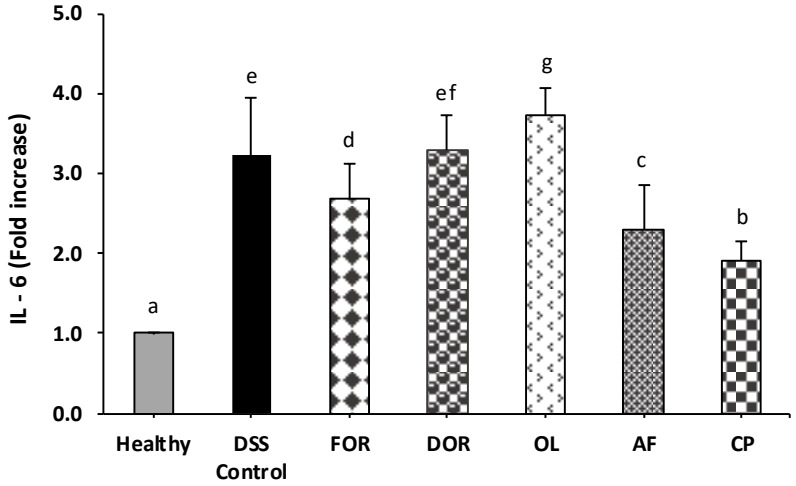
A)


TNF-α (Fold increase)
2.5
2.0
1.5
1.0
0.5
0.0
bc
g
d
e
f
b
a
Healthy
DSS Control
FOR
DOR
OL
AF
CP

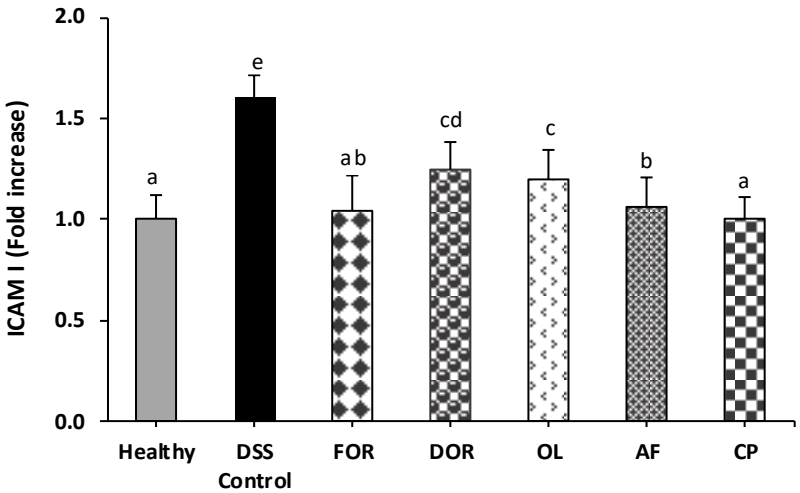
B)


IL-1β (Fold increase)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
d
fg
e
c
f
b
a
Healthy
DSS Control
FOR
DOR
OL
AF
CP

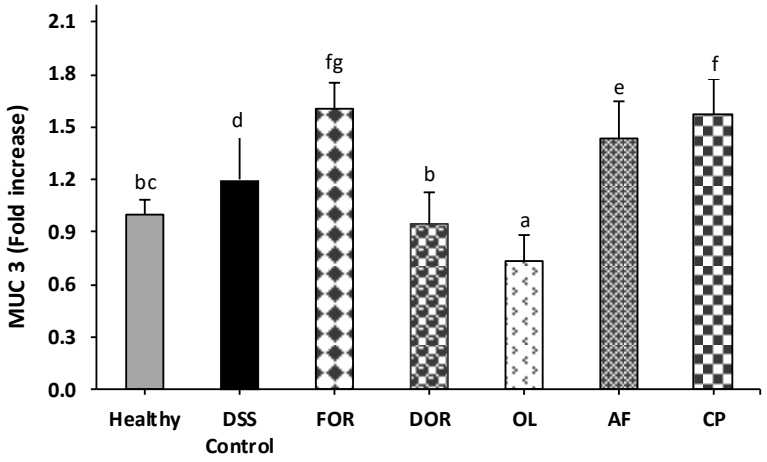
C)


IL - 6 (Fold increase)
5.0
4.0
3.0
2.0
1.0
0.0
a
e
d
ef
g
c
b
Healthy
DSS Control
FOR
DOR
OL
AF
CP

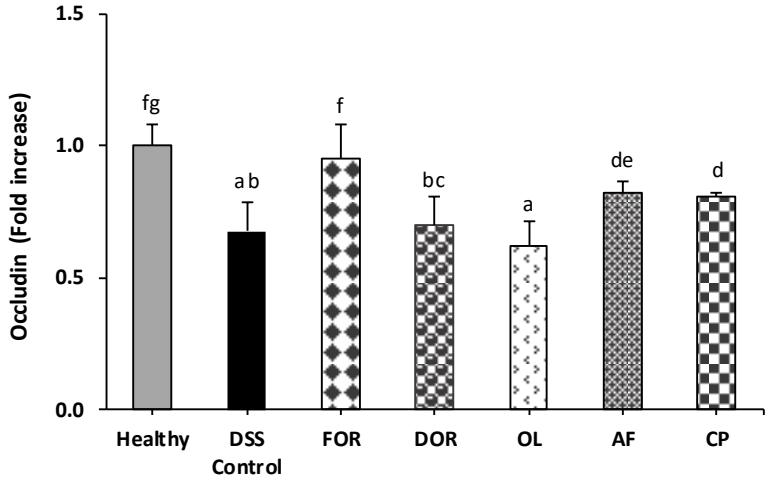
D)


ICAM I (Fold increase)
2.0
1.5
1.0
0.5
0.0
a
e
ab
cd
c
b
a
Healthy
DSS Control
FOR
DOR
OL
AF
CP

E)


MUC 3 (Fold increase)
2.1
1.8
1.5
1.2
0.9
0.6
0.3
0.0
bc
d
fg
b
a
e
f
Healthy
DSS Control
FOR
DOR
OL
AF
CP

F)


Occludin (Fold increase)
1.5
1.0
0.5
0.0
fg
ab
f
bc
a
de
d
Healthy
DSS Control
FOR
DOR
OL
AF
CP

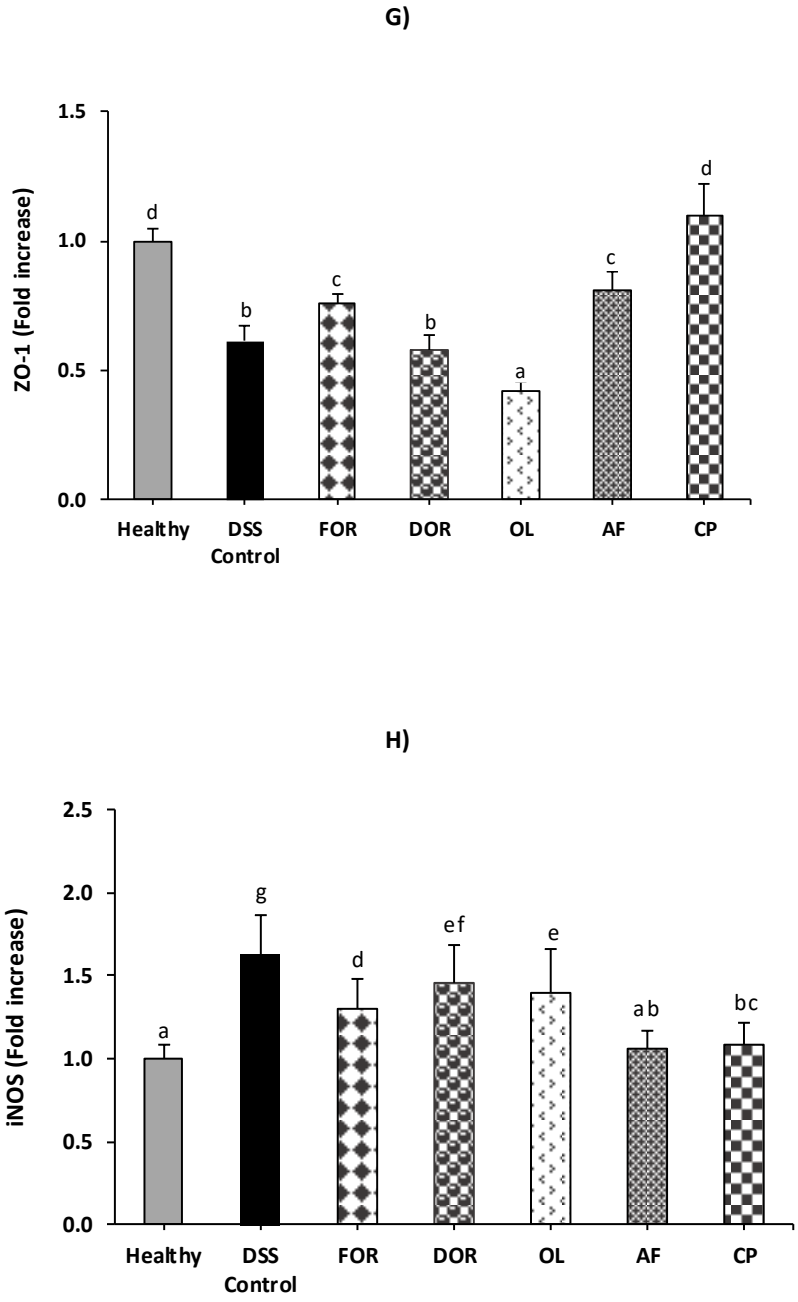


Figure 2. Effects of orange by-products intake on the expression of pro-inflammatory cytokines and chemokines in colonic tissue of the DSS mice colitis. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin. Data are expressed as mean ± S. Different letters on the bars, indicate statistical differences (p <0.05) amongst groups.

Nitric oxide synthase (iNOS) is an enzyme dominantly expressed during inflammatory reactions. The synthesis of high amounts of nitric oxide (NO) by iNOS has been demonstrated in pathophysiological processes, such as acute or chronic inflammation and tumorigenesis; however, the role of iNOS activity in these diseases is still not well understood (Gochman et al., 2012). Presence of nitric oxide (NO) is responsible for the generation of substantial reactive oxygen species (ROS) as peroxynitrites or anion superoxide that may affect the microbial agent and produce apoptosis of host cells (Soufli et al., 2016). Results indicated that all tested orange by-products produced a significant reduction in iNOS expression as compared to the DSS group (**Figure 2H**). There was no significant difference between the AF, CP and the healthy group; and the groups that consumed DOR and OL showed a higher expression of iNOS, slightly lower than that observed in the DSS control group, which could be related with their low GalA content (**Table 2**).

Discussion

By correlating the biochemical effects of orange by-products and citrus pectin ingestion to their physicochemical characteristics (**Table 1**), the beneficial effect observed following FOR consumption might be mainly due to its phenolic content and, consequently, antioxidant capacity. FOR had the highest content in total polyphenols, followed by DOR, OL, AF, and CP, respectively (**Table 1**). The reduction of phenols observed among the tested orange by-product samples seems to be mainly due to the heat treatment applied by the industry for the obtainment of animal feed from orange residues (Marić et al., 2018), since it is well known that phenolic compounds are vulnerable to heat (Brownmiller et al., 2008). Thus, the application of pressing and subsequent drying of FOR at 70 °C to obtain DOR generated a reduction of ~ 30% of initial phenolic content (**Table 1**). The process of evaporation and concentration of OL at 80 °C resulted in approximately a two-fold reduction in the phenolic content as compared to the initial sample (FOR). Finally, in the case of AF, despite being a product obtained by mixing the DOR and OL (145.5 and 115.6 mg GAE/100 g DM), its phenolic content was reduced to 90.2 mg GAE/100 g DM, which represents 42.6% of the phenolic content of the initial sample (211.9 mg GAE/100 g DM),

which may be due to the previous concentration step carried out at 100 °C. In consequence, the effect of phenolic compounds of FOR could be attributed to the fact that DSS acts mainly by breaching the intestinal barrier function, thereby exposing sub-epithelial immune cells to commensal bacteria (Dieleman et al., 1994; Axelsson et al., 1996) focusing on ROS and reactive nitrogen species (RNS) as the etiologic factors for IBD, and, thereby, phenols act as antioxidants counteracting the effect of ROS (Fang et al., 2013; Rezaie et al., 2007; Roessner et al., 2008). Recent studies have suggested that the administration of antioxidants from different sources, with the additional anti-inflammatory action may be beneficial in IBD's treatment (Ng et al., 2013; Rahimi et al., 2010). The beneficial effect observed after FOR consumption is in accordance to that described by Chen et al. (2012), who detected hesperidin, hesperetin, nobiletin and tangeretin in orange peel and reported a significant cytoprotective effect against oxidative stress in HepG2 cells. Consequently, FOR could be potentially considered as a useful and inexpensive source of compounds to prevent or minimize the effect of intestinal inflammation.

Prebiotic fiber (e.g. pectin, oligofructose inulin), on the other hand, helps to recover and/or maintain the normal state of the intestinal microbiota, which in turn produces short-chain fatty acids (SCFA) that decrease the synthesis or expression of inflammatory citoquines, and maintain the balance of the T regulatory cells (Treg) and T helper cells (Th) 17, counteracting and/or avoiding inflammation (Maslowski et al., 2009; Ivanov et al., 2008). Hartog et al. (2015) observed that oral intake of a multi-fibre mix (MF) counteracts IBD-like intestinal inflammation and weight loss in DSS treated mice, likely due to the fact that MF may induce a decrease in inflammatory cytokines levels and an increase in Treg cells in the mesenteric lymph nodes. These authors concluded that the optimization of enteral nutritional concepts dealing with the tested fibre mix could lead to the potential modulation of the gut microbiota composition and SCFA production to, subsequently, improve the inflammatory state and/or even induce remission.

In line with our results, Popov et al. (2014) demonstrated the *in vitro* anti-inflammatory and antioxidant activities of pectic polysaccharides from fresh plums. Lastly, the dissimilar behavior observed for other assayed products as DOR and OL, which did not show any positive effect, especially when analyzing macroscopic parameters, might be

explained by their low SDF and GalA content (**Tables 1 and 2**). Furthermore, Xiao et al. (2015) studied the preventive effects of cranberry (*Vaccinium macrocarpon*) products on experimental colitis induced by DSS in mice and indicated that dried cranberries were more effective in preventing colitis than cranberry extract. The same phenolic content and different blueberry fiber content in those products suggested that the fiber content could also contribute to the preventive effects on the development of colitis by its prebiotic action. A similar beneficial effect derived from the consumption of FOR or CP was observed by Cazarin et al. (2016) when supplying *Passiflora edulis* peel flour before colitis induction by DSS in drinking water to female C57BL/6J mice. These authors reported that *P. edulis* peel flour exerted an intestinal anti-inflammatory effect and attenuated the colonic damage due to its content of dietary fiber and polyphenols.

Patients with UC have presented deficiencies of antioxidant nutrients at the time of diagnosis (Razack & Seidner, 2007; Seidner et al., 2005), and patients with CD have shown a reduction of antioxidants in plasma and a decrease of total intestinal antioxidant capacity (Genser et al., 1999), suggesting an increase in ROS. Moura et al. (2015) indicated that this scenario could be balanced with the consumption of bioavailable functional foods, isolated nutrients, pro- and prebiotics, natural active compounds from vegetal sources, among other substances, all of them reported as effective antioxidants in IBD.

On the other hand, the anti-inflammatory effect caused by the AF ingestion could be associated to the *N*-*ε*-fructosyl-lysine content, formed as MRP during the concentration process and industrial drying at high temperature (100 °C) and determined through the quantification of furosine. Some MRPs have been associated with different beneficial properties, such as antioxidant activities (Echavarria et al., 2013) and anti-inflammatory effects (Kitts et al., 2012). Our results are in good agreement to those reported by Hong et al. (2017), who indicated that glucose-lysine MRPs ameliorated DSS-induced colitis as determined by a decrease in DAI, colon weight/length ratio, nitric oxide levels in serum, recovery of body weight loss, and serum lysozyme levels, as well as suppression of mRNA level of the inflammatory cytokines in colon tissues, highlighting the potential of these MRPs in preventing or treating IBDs. Oh et al. (2017) have recently reported that MRPs derived from lysine and galactose decreased the production of TNF-*α* in macrophages and

the expression of mRNA of interleukin IL-8 and IL-1b in Caco-2 cells in the model of DSS-induced colitis.

Concerning the effects derived from the intake of AF *vs.* CP, the variation of biochemical markers suggested a better anti-inflammatory response after the ingestion of CP, which could be due to its higher content of soluble dietary fibre (SDF) (96.58 *vs.* 12.46 g/100 g DM) and *N*-*ε*-fructosyl-lysine (827.4 *vs.* 455.1 mg of furosine/100 g of protein) as compared to AF (**Table 1**).

To summarize, polyphenol and/or fiber are the key components previously reported to exert intestinal anti-inflammatory activity based on therapies with natural products. In this sense, the anti-inflammatory effects related to the consumption of FOR or CP found in the current work seem to confirm previous findings regarding the functional activity of polyphenols and fiber on IBD. Additionally, further attention should be also paid to the potentially preventive role of certain Amadori compounds against IBD.

Conclusions

Samples rich in pectin, Amadori compounds and phenolic compounds, such as citrus pectin, animal feed and fresh orange residue, gave rise to a lower expression of pro-inflammatory cytokines, intercellular adhesion molecules ICAM I, iNOS enzyme, and a higher expression of protective chemokines MUC 3, occludin and ZO-1. These indicators underlined the potential role of pectin and Maillard reaction compounds in ameliorating some IBD symptoms. These promising results warrant the performance of further studies using superior doses of the selected orange by-products, as well as to broaden the assessment of biochemical indicators in order to increase certainty about the effect derived from the consumption of these singular by-products.

5. Discusión General

5. DISCUSIÓN GENERAL

La búsqueda de alimentos naturales, así como de nuevas alternativas que ayuden a prevenir problemas de salud presenta un gran interés en la actualidad. Al mismo tiempo, los efectos del cambio climático deterioran la calidad de los suelos y la disponibilidad de productos agrícolas en las diferentes épocas del año. Para poder enfrentar estos desafíos, el presente trabajo abordó la posibilidad de obtención de compuestos bioactivos a partir de tubérculos andinos escasamente estudiados y de fácil adaptación a condiciones adversas de cultivo y, por otro lado, a partir de residuos de la remolacha azucarera y de la extracción industrial de zumo de naranja, reconocidos por su gran volumen de producción e impacto ambiental.

Se estudiaron 5 variedades de tubérculos andinos ancestrales cultivadas en Ecuador: yacón (*Smallantus sonchifolius,* var. INIAP-ECU-1247), mashua (*Tropaeolum tuberosum,* var. INIAP-ECU-Izaño), melloco (*Ullucus tuberosus,* var. INIAP-ECU-amarillo-rosa), camote (*Ipomea batatas,* var. INIAP-ECU-morado) y zanahoria blanca (*Arracacia xanthorrhiza,* var. INIAP-ECU-blanca), cuyo estudio sobre composición y potencial industrial era limitado (**Artículo I**). Se observó que estos tubérculos presentan igual o mayor contenido de compuestos bioactivos que otros tubérculos de la misma especie, pero de distinta variedad y lugar de cultivo. El yacón mostró un elevedo contenido en FOS de tipo inulina (43,82 g FOS/100 g DM), en cantidad similar a la presente en la alcachofa de Jerusalén (30-50 g FOS/100 g DM (Long et al., 2016) y la achicoria (35,7-40,6 g FOS/100 g DM) (Moshfegh et al., 1999), consideradas como las principales fuentes de estos importantes oligosacáridos. Por lo tanto, el yacón analizado es una excelente fuente de FOS, siendo interesante estudiar en el futuro su extracción y posterior aplicación en el desarrollo de alimentos prebióticos con bajo índice glucémico. Estudios recientes han señalado que el consumo de FOS no sólo ejerce un efecto beneficioso en la microbiota intestinal, sino que también está relacionado con la reducción del índice de masa corporal, nivel de glucosa en ayunas e índice aterogénico (Fabersani et al., 2018). El tubérculo mashua mostró un alto contenido de carotenoides y vitamina C, compuestos relacionados con su alta actividad antioxidante,

mientras que el melloco presentó un alto contenido en proteína y fibra soluble. La cantidad de fenoles totales observada en el yacón y mashua analizados en este estudio fue tres veces superior que la descrita para estas mismas especies de variedades cultivadas en el Perú (Chirinos et al., 2013). El camote morado sobresalió por la abundante presencia de antocianinas y almidón, y la zanahoria blanca por su alto contenido en almidón y calcio. El almidón en el camote morado y en la zanahoria blanca estuvo presente en cantidades superiores al 90 %, observando rendimientos de extracción cercanos al 70%, que son similares al rendimiento de extracción de almidón de patata (*S. tuberosum*) (66-80%) (Bertoft & Blennow, 2016). Además, el almidón de camote morado destacó al presentar un mayor nivel de amilosa en comparación con el almidón rico en amilosa obtenido a partir de patata modificada genéticamente (72,26% *vs.* 40,8-57,1%; Zhao et al., 2018). Estos resultados ponen de manifiesto la idoneidad de estos tubérculos como fuentes de ingredientes con propiedades tecnológicas y funcionales con gran futuro dentro de la industria de biopolímeros y alimentaria.

Dada la importancia de los compuestos fenólicos y considerando su contenido total descrito en el **Artículo I** para los 5 tubérculos andinos, se planteó un siguiente trabajo enfocado en la identificación y cuantificación de compuestos fenólicos individuales mediante HPLC-DAD-ESI/MS^n (**Artículo II**). El yacón mostró el contenido más alto en compuestos fenólicos totales (˜2.2 mg/g DM) pero la variedad estructural de compuestos fue mínima, ya que todos correspondieron al grupo de derivados del ácido hidroxicinámico; siendo los más abundantes, los identificados como derivados del ácido cafeico. No obstante, el camote morado mostró un contenido en compuestos fenoles totales 6,5 veces menor, pero distribuido en tres grupos diferentes de fenoles: antocianinos (47,5%), flavonoles (26,5%) y derivados del ácido hidroxicinámico (26,0%). Los antocianos fueron exclusivos del camote morado de entre los 4 tubérculos estudiados. Mashua y melloco presentaron el menor contenido en compuestos fenólicos totales (27,5 y 53,0 veces menos que en yacón) y ambos tubérculos estuvieron dominados por flavonoles (69% y 85% para mashua y melloco, respectivamente). En mashua, el 31% de fenoles observados correspondieron a flavan-3-oles, grupo de compuestos que sólo se encontró en dicho tubérculo; mientras que el 15% de fenoles restantes en el melloco,

correspondieron a derivados del ácido hidróxicinámico. De entre los flavonoles observados en el melloco, se halló principalmente kaempferol - ramnosido - rutinósido (29.45 µg/g DM); siendo este el primer estudio, sobre determinación del perfil fenólico para esta especie. Finalmente, en la zanahoria blanca no se determinaron picos cuantificables.

Apenas se detectaron diferencias cualitativas en los perfiles de fenoles observados en el yacón, la mashua y el camote morado en comparación con otras variedades cultivadas en otros países y/o regiones, posiblemente debidas al factor genético, origen y condiciones de cultivo (Cotado et al., 2018). También es importante señalar que el camote cultivado en Ecuador (2.800-3.600 m.a.s.l.), presentó un contenido en antocianinas totales (TAC) mucho mayor que el camote morado var. *Covington* cultivado en Carolina del Norte (EE.UU) (449 mg/100 g de MS) (186 m.a.s.l.) (Grace et al., 2014); lo cual puede deberse a caracteres genéticos y a la diferencia de altitud de la zona de cultivo, relacionada a su vez con una mayor exposición UV, UVB y mayor producción de pigmentos fotoprotectores (Cotado et al., 2018).

Se conoce que los compuestos fenólicos en general, presentan actividad antioxidante asociada a propiedades anticancerígenas, antidiabéticas, antiinflamatorias y antimicrobianas, pudiendo también prolongar la vida útil de los alimentos (Santos-Sánchez et al., 2017). Además, Duque et al. (2017) observaron una relación directa entre el contenido de fenoles totales de extractos de yacón y su actividad protectora frente a radiaciones ultravioleta en el caso de fibroblastos dérmicos humanos. Por lo tanto, podría decirse que, todos los tubérculos analizados mostraron presencia de compuestos fenólicos de actividad terapéutica importante; no obstante, debido al contenido total encontrado, y según estudios previos documentados, principalmente el yacón, seguido del camote, mashua y melloco, poseen gran potencial como fuentes de antioxidantes alimentarios y de posible uso farmacológico; siendo importante estudiar a futuro, con más profundidad, el efecto de su uso sobre enfermedades y/o problemas oxidativos específicos.

Las propiedades morfológicas y estructurales del almidón de camote *(I. batatas)* y zanahoria blanca *(A. xhanthorriza)* han sido ampliamente descritas en la bibliografía

(Zhang et al., 2018; Castanha et al. 2018), sin embargo, en el caso del almidón de mashua y melloco se encontró muy poca información. Por ello, a continuación, se planteó un estudio enfocado en su caracterización morfológica, tecnológica y nutricional (**Artículo III**). El tamaño de partícula promedio del almidón extraído a partir de melloco y mashua fue 8,0 y 7,7 veces mayor que el tamaño de partícula promedio del almidón de patata (<90%) (p <0.05), lo que fue, en parte, atribuido a la presencia de otros componentes (fibra, proteína) difíciles de separar durante el proceso de extracción y que podrían formar aglomeración de gránulos de almidón. De acuerdo a los valores de potencial zeta se puede inferir que los almidones de mashua y melloco fueron más estables en dispersión acuosa que el almidón de patata a valores de pH entre 3 y 6,5, a la vez que presentaron una menor temperatura de gelificación. El almidón de melloco mostró poseer mayor viscosidad y elevada termorresistencia en comparación con el almidón de mashua y patata, efecto asociado a una mayor electronegatividad, mayor tamaño promedio de partícula y mayor contenido de amilosa (Genovese & Lozano, 2001; Alcázar-Alay & Meireles, 2015; Xie et al., 2009). Mediante ensayos *in vitro* se observó que el almidón cocido de mashua y melloco es altamente biodisponible (78,6 y 85,8%, respectivamente) y digerible (78,0 y 75,0%, respectivamente). La digestibilidad de la harina cocida de mashua, en cambio, fue menor que la digestibilidad de la harina de cocida de melloco (8,2 *vs.* 30,1 %), efecto relacionado con su mayor contenido en compuestos fenólicos, que han mostrado actuar como inhibidores de α-amilasas (Hernández-Uribe et al., 2007). La elevada tasa de digestión de la harina de melloco pone de manifiesto su potencial como alimento nutritivo, y la alta viscosidad y resistencia térmica de los almidones de mashua y melloco sugiere que estos tubérculos podrían ser fuentes no convencionales de almidón de gran potencial tecnológico.

Una vez conocida la composición y las propiedades de los tubérculos andinos, se abordó, en la segunda fase de la Tesis, el estudio del aprovechamiento de residuos procedentes de industrias agroalimentarias. Inicialmente, se procedió a la determinación de propiedades reológicas, estructurales y funcionales de pectina de subproductos de la remolacha azucarera, extraída por método ácido y enzimático

(**Artículo IV**). El análisis proximal permitió conocer el efecto del proceso de ensilado y secado sobre la pulpa de remolacha. El residuo ensilado presentó un incremento en la cantidad de grasa y SDF, en comparación al residuo prensado (p <0.05), al parecer, como resultado de una mayor liberación celular, generada por la disminución del pH, causada a su vez por la transformación de azúcares solubles en ácido acético y ácido láctico, como parte del metabolismo de bacterias anaerobias (Kung et al., 2018). También se detectó un mayor contenido de Na y Fe en este subproducto (p <0.05), lo cual podría deberse a un aumento en la solubilidad y biodisponibilidad de los minerales, ante la reducción del pH durante el ensilaje (Hansen & Spears, 2009). El proceso industrial de secado, en cambio, incrementó la cantidad de fibra insoluble y redujo el contenido de carbohidratos solubles, compuestos fenólicos y actividad antioxidante, debido probablemente a la aplicación de altas temperaturas (140°C/2-3 h) (Sharma et al., 2015).

Empleando celulasas o HNO_3 (0.4% v/v) (pH 1.2) sobre la pulpa prensada, ensilada o seca, se logró obtener compuestos pécticos de diferentes características. La pulpa de remolacha azucarera ha sido ampliamente empleada para extraer pectina (Lv et al., 2013; Guo et al., 2017; Huang et al., 2018), conociendo que ésta se diferencia de la pectina cítrica y de manzana por su menor grado de metoxilación, pero a la vez, por su mayor actividad emulsionante (Levigne et al., 2002; Siew & Williams, 2008). En el presente trabajo se logró extraer, mediante el método enzimático, pectina con alto contenido en ácido galacturónico (GalA) (>65%) a partir de la pulpa ensilada, lo cual permite considerarla como un potencial aditivo alimentario según la FAO (FAO & WHO, 2009), y aprovechar, de este modo, este importante residuo cuya valorización había sido ignorada hasta el momento. El método ácido permitió obtener pectina con mayor estabilidad en dispersión acuosa y mayor viscosidad, propiedades relacionadas con una mayor electronegatividad, como resultado del bajo valor de pH en el proceso de extracción y mayor tensión superficial (Genovese & Lozano, 2001). También se encontró una alta actividad emulsionante en la pectina extraída por el método ácido (58-75%), lo que pude deberse a su mayor contenido de proteína (Jankovská et al, 2001), en comparación con la pectina extraída por método enzimático.

Diversos estudios han demostrado que la pectina puede promover el crecimiento de bacterias beneficiosas y disminuir los niveles de colesterol en la sangre (Brouns et al., 2012), de modo que, en base a la información proporcionada en este estudio, se podría seleccionar el tipo de extracción y la materia prima (residuo de remolacha azucarera) más conveniente a emplear, según las características deseadas en la pectina final, ya sea para su uso como estabilizante, emulsionante y/o componente de alimentos funcionales.

Como se ha comentado con anterioridad, otro de los subproductos agroindustriales que se encuentran infrautilizados son aquéllos derivados de la elaboración del zumo de naranja. Aunque la piel y otros residuos se emplean para la extracción de algunos ingredientes, incluyendo la pectina, el destino principal es su utilización como pienso para animales. Además, no existía hasta el momento ningún estudio pormenorizado sobre la calidad de dichos subproductos tras su procesado, que suele realizarse bajo condiciones muy enérgicas en cuanto a tiempo y temperatura. Dichas condiciones pueden empeorar su calidad y comprometer el proceso de extracción de la pectina. Por ello, nos planteamos abordar un estudio empleando muestras procedentes de una importante industria elaboradora de zumo de naranja (**Artículo V**). El residuo fresco mostró el mayor contenido fenólico y de fibra soluble y los residuos tratados térmicamente, es decir, el residuo seco, licor de naranja y pienso para animales, presentaron compuestos de Amadori (*N*-*ε*-fructosil-lisina) (Erbersdobler & Hupe, 1991) intermediarios de la reacción de Maillard (MRPs), que podrían indicar pérdida de valor nutricional, y que a la vez, son precursores de otros MRPs que pueden presentar actividad antioxidante (Wang et al., 2011).

El licor de naranja mostró ser una alternativa para la obtención de fructosa, carbohidrato de menor poder calórico y mayor poder edulcorante que la sacarosa. Los residuos sólidos permitieron la extracción de pectina con contenidos de ácido galacturónico de 70,4 a 74,4 g/100 g DM; logrando el mayor rendimiento, al partir del residuo fresco (23,95 g/100 g DM).

La furosina resultó ser un indicador adecuado para controlar retrospectivamente el procesamiento de los subproductos industriales de la extracción de zumo de naranja,

y pese al tratamiento térmico aplicado en la industria, la reducción de actividad antioxidante se vio contrarrestada por la formación de los compuestos de reacción de Maillard.

Dado el potencial de este tipo de subproductos industriales de naranja, se planteó un estudio empleando un modelo DSS en ratones, el cual permite evaluar indicadores de inflamación muy similares a los observados en pacientes humanos con enfermedad de Crohn (Zhu et al., 2017; Sartor, 2008) (**Artículo VI**).

Tras la ingesta de los subproductos de naranja durante 14 días, y posterior suministro de DSS durante 7 días, se observó una menor reducción de peso corporal en los grupos que consumieron la pectina cítrica, la cáscara fresca y el pienso de naranja, y una mayor ingesta de alimento; así como también, mejores indicadores bioquímicos y un menor índice de actividad de la enfermedad (DAI), en comparación al grupo control-enfermo o -DSS (pienso estándar + DSS) ($p < 0.05$), señalando un menor avance de la inflamación ($p < 0.05$).

En base a lo observado, se puede concluir que principalmente la pectina cítrica, por su contenido de ácido galacturónico y *N*-*ε*-fructosil-lisina, podría ayudar a mitigar la sintomatología de la enfermedad inflamatoria intestinal, siendo necesario evaluar a futuro, el efecto del consumo de mayores dosis, así como el efecto de pectina procedente de diferentes fuentes vegetales.

6. Conclusiones

6. CONCLUSIONES

- De acuerdo a los resultados obtenidos sobre la caracterización de tubérculos se puede concluir que el yacón es una excelente fuente de FOS y compuestos fenólicos; la mashua destaca por su alta actividad antioxidante; el melloco, por su alto contenido de proteína y por poseer almidón de muy buenas propiedades nutricionales, reológicas y térmicas; el camote morado por su elevada cantidad de antocianinas y gran contenido en almidón rico en amilosa; y la zanahoria blanca por su contenido en calcio y almidón muy similar al del camote.

- Los almidones de camote morado, zanahoria blanca y melloco mostraron un alto contenido de amilosa relacionado con su alta viscosidad y resistencia a la digestión.

- Exceptuando la zanahoria blanca, todos los tubérculos andinos analizados en este estudio poseen carotenos, vitamina C y compuestos fenólicos relevantes, siendo destacable la alta cantidad de derivados del ácido clorogénico observada en el yacón.

- Se ha obtenido pectina con alto contenido en ácido galacturónico a partir de subproductos de la industria azucarera sometidos a un proceso de ensilado. La aplicación del método enzimático fue más eficaz respecto a este parámetro debido a la aplicación de condiciones más suaves, en comparación con el método ácido.

- Los resultados correspondientes al aprovechamiento de subproductos de la extracción de zumo de naranja nos permiten concluir que dichos subproductos (procesados o en fresco) constituyen una fuente importante de compuestos bioactivos, pudiendo ser empleados no sólo como piensos para la alimentación animal, sino también como nuevos recursos para ser considerados en la extracción de carbohidratos funcionales.

- La determinación de concentraciones variables de 2-furoilmetil-lisina en los subproductos de naranja muestran que dicho indicador de las etapas iniciales de la reacción de Maillard es un excelente parámetro para conocer retrospectivamente las condiciones del proceso, con el fin de lograr una optimización que minimice los cambios nutricionales en dichos subproductos.

- Se ha analizado el efecto beneficioso sobre la enfermendad inflamatoria intestinal, asociado a la ingesta de pectina de cítricos y subproductos de la industria elaboradora de zumo de naranja, empleando un modelo DSS en ratones; encontrándose el efecto beneficioso más marcado en el caso de la pectina cítrica, seguida del pienso, de acuerdo a los indicadores macroscópicos y bioquímicos estudiados.

Conclusión general

La caracterización y obtención de compuestos bioactivos a partir de tubérculos y residuos agroindustriales subutilizados constituye un aporte novedoso y valioso para promover su utilización, generando beneficios tanto para el sector agrícola como el industrial, incluyendo el desarrollo sostenible. La información sobre aspectos fisicoquímicos, nutricionales y tecnológicos y los procesos de obtención de sus compuestos bioactivos, puede promover la diversificación de materias primas de bajo coste, útiles para el desarrollo de nuevos alimentos funcionales, biopolímeros, y/o productos nutracéuticos.

7. Bibliografía

7. BIBLIOGRAFÍA

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8. Anexos

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Article

Structural and Rheological Properties of Pectins Extracted from Industrial Sugar Beet By-Products

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Abstract: In this work, the efficient extraction of pectin from sugar beet by-products (pressed, ensiled and dried pulp), by using an acid method or a commercial cellulose, is accomplished. The extraction method had an impact on the pectin monomeric composition, mainly in xylose, arabinose, and galacturonic acid content, as determined by GC-FID. FTIR and SEC analyses allowed the determination of similar degrees of methoxylation and molecular weights, respectively, in the extracted pectins. The acid extraction of pectin in the ensiled by-product led to the highest yield (19%) with a galacturonic acid content of 46%, whereas the application of the enzymatic extraction method resulted in a lower yield (13%) but higher galacturonic acid content (72%). Moreover, the stability in aqueous solution as well as the emulsifying activity index was higher for pectin extracted by the acid method, whereas the viscosity was higher in pectin extracted by the enzymatic method. To the best of our knowledge, this is the first study analyzing the physicochemical properties and exploring the potential reuse of ensiled and dried by-products from sugar beet industry for the extraction of pectin to be further used in the food and pharmaceutical areas.

Keywords: sugar beet; by-products; silage; pectin; viscosity; emulsifying activity

1. Introduction

In 2016, the largest area of root crops (1.7 million hectares) in the European Union (EU) was occupied by potatoes closely followed by sugar beet (*Beta vulgaris L.* subsp. *vulgaris* var. altissima Döll) (1.5 million hectares) [1]. These values point out the EU as the leading producer of sugar beet, providing approximately 50% of the global production, whose process generates a volume waste of 111.6 million tons per year. In addition, in Spain, sugar beet is the only source of sugar, producing 3000 tons of residues per year.

Only 30% of the world's sugar production comes from sugar beet, whereas the rest is derived from cane [1]; however, the obtainment of sugar from beet generates a significant volume of wastes each year, which is considered of great importance in terms of underexploited opportunities and generated levels [2]. When the sugar beet residues are exploited, habitually they are used as lignocellulosic material for the ethanol obtaining and pectin extraction [3].

Pectin, an important anionic heteropolysaccharide, exists in the cell walls of dicotyledonous plants [4], and over the last years, pectin has gained increasing interest as thickening or gelling agent for the chemical and food industry [5]. Furthermore, pectin has been described as an emerging prebiotic with the ability to modulate the bacterial composition of the colon microbiota [6], being able to exert beneficial effects on health.

Sugar beet pectin (SBP), compared to the main sources of pectin that are citrus and apple, has poorer gelling properties due to its higher content of neutral sugars, low presence of acetyl groups (4–5%) [7], content of ferulic acid, higher protein content [8], and/or its relatively low molecular mass, but in contrast, these molecular characteristics give the SBP better emulsifying properties [9,10].

However, depending on the applied extraction method, the structure and technological properties of SBP can widely vary. A large number of studies have addressed the extraction and properties of pectin from sugar beet pulp pressed (SBP-P) in recent years, and most of the studies have been focused on the effect of extractants and extraction conditions on pectin yield, chemical composition, and technological behavior [11–13]. However, there is an excess of other underutilized industrial sugar beet by-products, such as ensiled sugar beet (SBP-E) and dried sugar beet pulp (SBP-D) whose potential as raw materials for obtaining similar compounds has not yet been addressed.

Therefore, the main objective of this work was to explore the potential use of different physico-chemically characterized sugar beet by-products (pressed, ensiled, and dried pulp) as efficient and alternative sources of pectin following its extraction by acid or enzymatic methods. Likewise, the potential of the extracted pectins as thickening or gelling agents is investigated through their rheological characterization.

2. Results and Discussion

2.1. Overall Characterization of Sugar Beet By-Products

The results of the physicochemical analysis of the sugar beet by-products, reported in Table 1, show a reduction of °Brix, pH, dry weight (DW), protein, total carbohydrates, reducing carbohydrates, total dietary fiber, insoluble dietary fiber, and Mg when comparing the chemical composition of the sugar beet pulp pressed (SBP-P), with the sugar beet pulp ensiled (SBP-E) ($p < 0.05$).

Table 1. Chemical composition of sugar beet pulp by-products (g/100 g DW).

Parameter	SBP-P	SBP-E	SBP-D
°Brix	5.00 ± 0.21^{b}	4.60 ± 0.07^{a}	4.40 ± 0.14^{a}
pH	4.62 ± 0.08^{c}	3.51 ± 0.03^{a}	$3.71 \pm 0.06^{a,b}$
Aw	0.88 ± 0.01^{b}	0.90 ± 0.02^{b}	0.73 ± 0.02^{a}
DW (%)	91.12 ± 0.16^{b}	83.41 ± 0.15^{a}	96.53 ± 0.22^{c}
Total fat (g/100 g DW)	0.84 ± 0.02^{a}	1.70 ± 0.04^{c}	1.33 ± 0.03^{b}
Protein (g/100 g DW)	10.42 ± 0.52^{c}	8.30 ± 0.30^{ab}	8.01 ± 0.31^{a}
Total carbohydrates (g/100 g DW)	82.64 ± 0.63^{c}	70.22 ± 0.32^{a}	78.14 ± 0.50^{b}
Reducing carbohydrates (g/100 g DW)	10.40 ± 0.11^{c}	6.73 ± 0.10^{b}	4.21 ± 0.08^{a}
TDF (g/100 g DW)	75.20 ± 0.24^{b}	64.52 ± 0.07^{a}	$76.84 \pm 0.32^{b,c}$
IDF (g/100 g DW)	47.58 ± 0.16^{b}	34.51 ± 0.09^{a}	$51.32 \pm 0.18^{b,c}$
SDF (g/100 g DW)	26.63 ± 0.47^{a}	$30.04 \pm 0.50^{b,c}$	29.78 ± 0.34^{b}
Ash (g/100 g DW)	$1.86^{a,b}$	$1.87^{a,b}$	1.81^{a}
Na^{+} (mg/100 g DW)	$17.66^{a,b}$	26.39^{c}	16.16^{a}
Mg^{+2} (mg/100 g DW)	244.97^{c}	$217.16^{a,b}$	214.30^{a}
P^{+3} (mg/100 g DW)	$27.51^{a,b}$	24.20^{a}	23.55^{a}
K^{+} (mg/100 g DW)	184.72^{b}	$189.84^{b,c}$	174.68^{a}
Ca^{+2} (mg/100 g DW)	$1327.90^{a,b}$	$1332.21^{b,c}$	1317.26^{a}
Fe^{+3} (mg/100 g DW)	54.63^{a}	84.95^{c}	$62.45^{a,b}$
Total phenols (mg GAE/100 g DW)	0.38 ± 0.05^{c}	0.29 ± 0.02^{b}	0.17 ± 0.01^{a}
Antioxidant capacity (mM de Trolox/100 g DW)	$2.34 \pm 0.14^{b,c}$	2.23 ± 0.09^{b}	1.16 ± 0.10^{a}

SBP-P: sugar beet pulp pressed, SBP-E: sugar beet pulp ensiled, SBP-D: sugar beet pulp dried. FM: fresh matter. DW: dry weight. TDF: total dietary fiber. IDF: insoluble dietary fiber. SDF: soluble dietary fiber. Means with different letters a–c denote significant difference ($p < 0.05$) in the same row.

This variation could be due to a fermentation process of DW (20–30%) during the storage time (seven–eight months) (Figure 1) carried out by saccharolytic and proteolytic bacteria; in a similar

way to that observed by Álvarez et al. [14] who studied the effect of fermentation during silage of banana by-products.

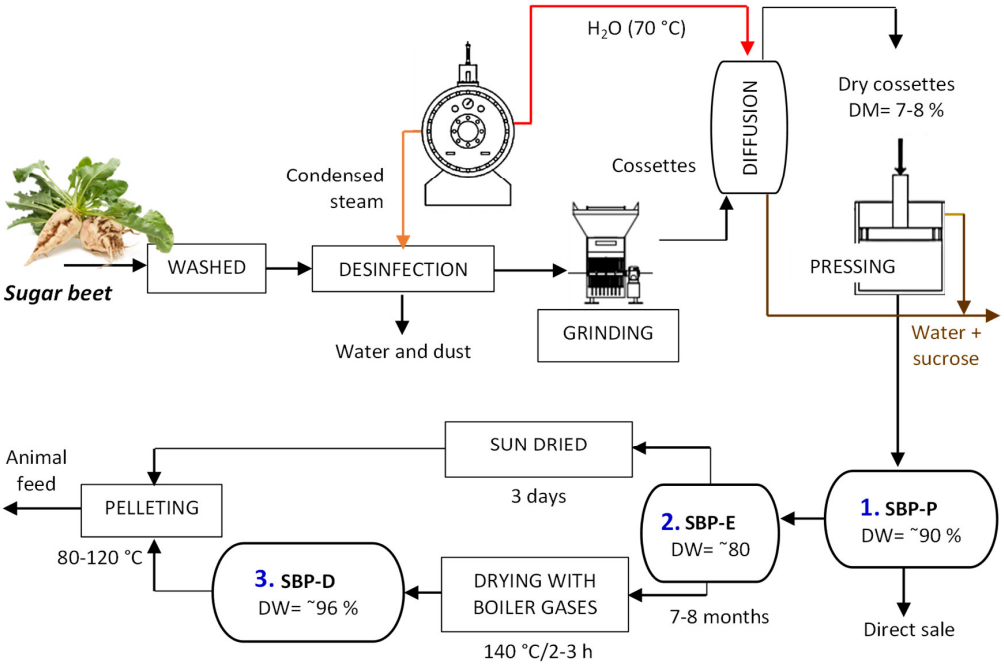

Figure 1. Industrial process of sugar extraction form sugar beet. By-products obtained: 1. SBP-P: sugar beet pulp pressed, 2. SBP-E: sugar beet pulp ensiled, 3. SBP-D: sugar beet pulp dried. DW: dry weight.

Moreover, the SBP-E showed an increase in the amount of fat and SDF ($p < 0.05$). The higher fat and SDF content may be due to a greater cellular release, generated by the decrease in pH caused, in turn, as a result of the transformation of soluble sugars in acetic and lactic acid, as part of the metabolism of anaerobic bacteria [15]. On the other hand, the increase of Na and Fe ($p < 0.05$) found in SBP-E could be due to an increase in the solubility and bioavailability of minerals, as an effect of the pH reduction during silage [16].

Conversely, in the case of the sugar beet pulp dried (SBP-D), an expected reduction of a_w, as well as a decrease in the content of reducing carbohydrates, soluble dietary fiber and antioxidant activity was observed, likely as a consequence of the heat treatment applied (Figure 1) (~100 °C/2–3 h) [17]. Lastly, a reduction of K was determined, probably due to a lower availability of this mineral for the analysis, as a consequence of the hardening of the sample by the drying effect ($p < 0.05$).

2.2. Pectin Extraction and Characterization

2.2.1. Yield, Monomeric Composition and Protein

The application of the acid method allowed for the achievement of higher yields in comparison with the enzymatic method, regardless the type of sugar beet waste used (Table 2). This result is in line to that reported by Lim et al. [18] who compared the acid and the enzymatic method to extract pectin from Yuza (*Citrus junos*) pomace. The maximum yield was observed in the case of the pectin extracted from SBP-E followed by SBP-D using acid conditions (18.9 and 16.7%, respectively) ($p < 0.05$), which seems to be related with the high amount of soluble dietary fiber (SDF) observed in the ensiled and dried residue (Table 1). Despite yields obtained by the enzymatic method being lower than those observed with the acid method, the sugar beet ensiled residue (P-SBP-E-EM) gave rise to a higher yield (13.4%) in comparison with that reported by Zykwinska et al. [19] (4.0%) using a similar method of extraction, but instead, starting from fresh sugar beet pulp as raw material.

Table 2. Yield extraction (g pectin/100 g DW), monomeric composition and protein of pectin from sugar beet by-products (g/100 g DW).

Pectin	Extraction Method	Yield	Xylose	Arabinose	Rhamnose	Galactose	Galacturonic Acid	Mannose	Glucose	Protein
P-SBP-P	AM	13.60	33.35 ± 1.11 ^{f}	3.60 ± 0.08 ^{e,f}	9.81 ± 0.31 ^{e}	14.50 ± 0.50 ^{d}	23.52 ± 0.11 ^{a}	6.14 ± 0.18 ^{e}	2.01 ± 0.02 ^{b}	4.3 ± 0.22 ^{e,f}
	EM	3.91	6.36 ± 0.19 ^{c}	0.10 ± 0.00 ^{a}	3.20 ± 0.06 ^{a}	8.26 ± 0.27 ^{a,b}	65.51 ± 0.30 ^{e}	4.04 ± 0.03 ^{c}	5.22 ± 0.09 ^{d}	1.6 ± 0.06 ^{a}
P-SBP-E	AM	18.94	25.48 ± 0.69 ^{d,e}	3.22 ± 0.06 ^{e}	4.94 ± 0.13 ^{c,d}	7.60 ± 0.28 ^{a}	42.74 ± 0.23 ^{b}	2.39 ± 0.04 ^{a}	3.30 ± 0.03 ^{c}	3.4 ± 0.14 ^{d}
	EM	13.40	4.53 ± 0.14 ^{a}	0.70 ± 0.01 ^{b}	3.50 ± 0.11 ^{a,b}	9.68 ± 0.33 ^{c}	66.98 ± 0.80 ^{ef}	5.05 ± 0.15 ^{d}	0.76 ± 0.01 ^{a}	2.0 ± 0.08 ^{b}
P-SBP-D	AM	16.72	21.53 ± 0.85 ^{d}	2.82 ± 0.06 ^{d}	4.74 ± 0.15 ^{c}	8.88 ± 0.24 ^{b}	48.92 ± 0.43 ^{d}	4.75 ± 0.17 ^{d}	0.62 ± 0.01 ^{a}	4.1 ± 0.20 ^{e}
	EM	7.50	5.22 ± 0.16 ^{a,b}	0.79 ± 0.02 ^{b,c}	16.79 ± 0.69 ^{f}	8.68 ± 0.26 ^{b}	44.80 ± 0.37 ^{b,c}	3.06 ± 0.09 ^{b}	12.57 ± 0.30 ^{e}	2.8 ± 0.1 ^{c}

DW: dry weight. P-SBP-P: pectin from sugar beet pulp pressed, P-SBP-E: pectin from sugar beet pulp ensiled, P-SBP-D: pectin from sugar beet pulp dried. AM: Acid method. EM: Enzymatic method. Means with different letters a–f denote significant difference ($p < 0.05$) in the same column.

The analysis by GC-FID of the extracted pectins revealed the presence of xylose, arabinose, rhamnose, galactose, and galacturonic acid, whereas glucose could be derived from the acid hydrolysis of cellulose [20], by disruption of β-1,4-glycosidic bonds [21], and mannose from mannans and galactomannans [22,23] (Table 2). The acid extraction led to high quantities of xylose and arabinose in all cases, whereas the content in galacturonic acid (GalA) was significantly less important, which could be attributed to its acid degradation [24]. The enzymatic method, instead allowed obtaining pectin with a higher amount of galacturonic acid (GalA), and a lower amount of xylose and arabinose ($p < 0.05$).

However, it is important to notice that GalA was present in all extracted pectins in the range from 23.5% to 67.0%, having the pectins of sugar beet pulp ensiled (P-SBP-E-EM) and pressed (P-SBP-P-EM), both extracted by the enzymatic method, the highest GalA content (67.0 and 65.5%, respectively). In fact, these values suggest that pectin extracted from these by-products could be considered as food additives, according to the recommendations given by a Joint FAO/WHO Expert Committee on Food Additives, which established that pectin should not contain less than 65% of GalA calculated on the ash-free and dried basis [25].

The protein content was higher in the case of the pectin extracted by the acid method compared to the pectin extracted by the enzymatic method, possibly due to the severity of the acid method, resulting in a greater amount of protein residues linked to the pectin obtained. The pectin sample with the higher amount of protein was the pectin of sugar beet pulp pressed (P-SBP-P-AM), which is consistent with the greater amount of protein observed in the pressed by-product (SBP-P) (Table 1).

2.2.2. Degree of Methoxylation (DM) and Molecular Weight (Mw)

FTIR spectra of pectins extracted from SBP-E by either acid or enzymatic methods are shown in Figure 2. The peak between 1052 and 1141 cm^{-1} is assigned to C=C double pectin bond. The absorption peaks at 1388 and 1633 cm^{-1} are related to the stretch bands of the pectin COO groups. These results indicate that the final products are true pectin compounds [26].

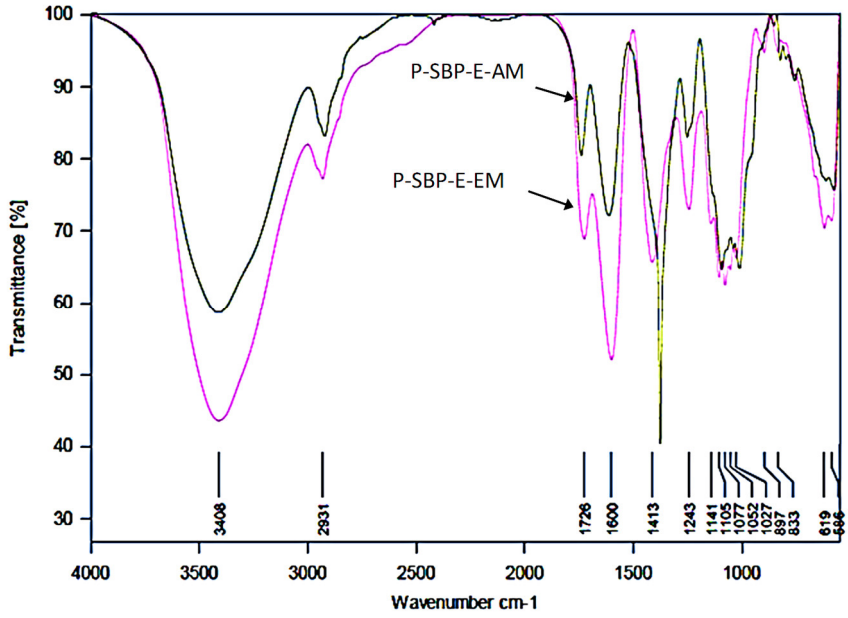

Figure 2. FTIR spectra of sugar beet pulp pectin. P-SBP-E-AM: pectin of sugar beet pulp ensiled, extracted by acid method. P-SBP-E-EM: pectin of sugar beet pulp ensiled, extracted by enzymatic method.

Pectins extracted by the enzymatic method regardless of the type of sugar beet pulp by-product (that is, pressed, silaged or dried) showed larger peaks at 1608 cm^{-1} and 1745 cm^{-1} than those observed at the same wavelengths for pectins extracted by the acid method. However, when the degree

of methoxylation (DM) was calculated by correlating the peak area of the esterified carboxyl groups to the peak area of total carboxyl groups, the DM values of pectin extracted by the acid method were statistically similar to the DM of pectins extracted by the enzymatic method ($p < 0.05$) (Table 3).

Table 3. Degree of methoxylation (DM) (%), and molecular weight (Mw)(kDa) of pectin extracted from sugar beet by-products.

Pectin	Extraction Method	DM	* Mw
P-SBP-P	AM	49.29 [a]	306 ± 7 [a]
	EM	47.08 [a]	311 ± 9 [a]
P-SBP-E	AM	50.14 [a]	303 ± 7 [a]
	EM	48.36 [a]	322 ± 10 [a]
P-SBP-D	AM	48.39 [a]	315 ± 9 [a]
	EM	45.21 [a]	319 ± 10 [a]

P-SBP-P: pectin of sugar beet pulp pressed, P-SBP-E: pectin of sugar beet pulp ensiled, P-SBP-D: pectin of sugar beet pulp dried. AM: Acid method. EM: Enzymatic method. Means with similar letter (a) in the same column do not present significant difference ($p < 0.05$). * Mw was calculated as the average at peak maximum observed in the analysis followed by triplicate.

DM values were in the range from 45.2 to 50.1%, which correspond to low-methoxyl pectins (DM < 50%). This type of pectins is known as "slow-gelling" and has the ability to form gels at slightly neutral or basic pH, with maximum consistency in the presence of calcium at concentrations ranging from 20 to 100 mg per gram of pectin, and/or with low amounts of sugar [27]. Therefore, low-methoxyl pectins are suitable as additives for the development of low-fat products, or foods by diabetic people [28].

Table 3 shows the molecular weight (Mw) of the extracted pectins estimated by SEC. This parameter was also statistically similar between the pectins extracted from sugar beet by-products pressed, silage, or dried by acid or enzymatic methods ($p < 0.05$). The determined Mw values were in the range from 303 to 322 kDa, in agreement to the maximum Mw values reported by Zykwinska et al. (2008) [19] for pectin extracted from fresh SBP (310 kDa).

2.2.3. Emulsifying Activity Index (EAI)

Figure 3 shows the emulsifying activity index (*EAI*) of the different pectins extracted by acid or enzymatic methods from sugar beet pulp pressed, ensiled, or dried, using the commercial citrus pectin as standard. In general, the *EAI* of sugar beet pectins extracted by the acid method were higher than those of the *EAI* of sugar beet pectins extracted by the enzymatic method. Furthermore, all pectin samples extracted from sugar beet residues, excluding P-SBP-P-EM, showed a significant EAI higher than that of citrus pectin ($p < 0.05$), according to the observed by Lerouxet al. [7].

Pectin from sugar beet pulp pressed extracted by the acid method (P-SBP-P-AM) and pectin of sugar beet pulp dried extracted by the same method (P-SBP-D-AM), presented the highest *EAI* (73.51, 75.53 m^2/g, respectively) among all the assayed pectins ($p < 0.05$). These values are in the range reported by Huang et al. [29] (75.3–104.9 m^2/g) for sugar beet pectin extracted from pulp dried. Remarkably, these authors pointed out that the studied pectin samples exhibited good emulsifying activity.

The difference in the emulsifying activity observed among the analysed pectins can be associated with the different content in protein (Table 2), which plays a predominant role in the emulsifying properties of sugar beet pectin [30]. Thus, it can be observed that pectins with higher *EAI* (P-SBP-P-AM and P-SBP-D-AM) had the highest protein content (4.3 and 4.1 g/100 g DW) (Table 2) and vice versa ($p < 0.05$). In fact, the emulsifying effect is related to the ability of protein molecules to open in aqueous-lipid media, allowing that their electrically charged outer groups to bind with water molecules, and the internal non-polar amino acids to be released and bound to the oily particles, linking both phases, until forming the stable mixture, called emulsion [31].

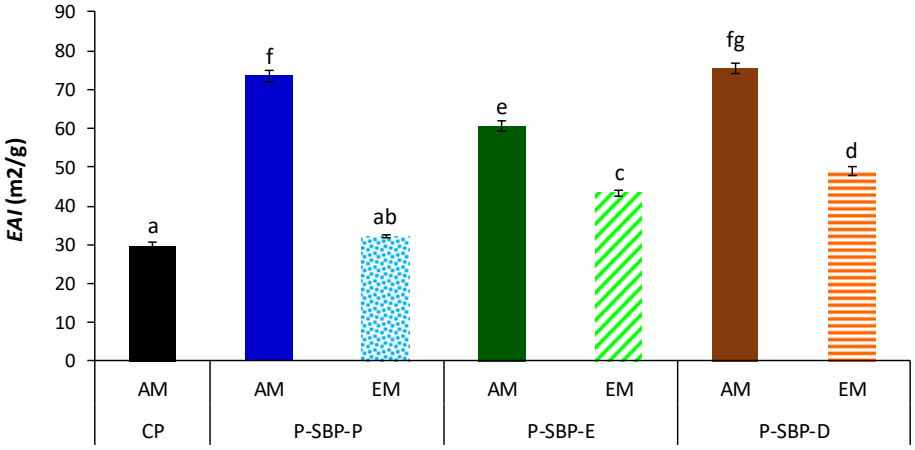

Figure 3. Emulsifying activity index *(EAI)* of pectin obtained from sugar beet by-products by acid or enzymatic methods. CP: citrus pectin. P-SBP-P: pectin of sugar beet pulp pressed, P-SBP-E: pectin of sugar beet pulp ensiled, P-SBP-D: pectin of sugar beet pulp dried. AM: Acid method. EM: Enzymatic method. Different letters (a–d) in the columns denote significant difference ($p < 0.05$).

2.2.4. Zeta Potential (ζ) and Apparent Viscosity (η)

The zeta potential (ζ) of sugar beet pectins extracted by acid or enzymatic methods from pressed, ensiled, and dried residues is presented in Figure 4. The isoelectric point was not reached in any of the samples, but it should occur at very acidic pHs, 1.5–2.0. Within the range of pH from 4.5 to 9, pectins extracted by the acid method had slightly higher absolute values of ζ (−25 to −34 mV) than pectins extracted by the enzymatic method (−20 to −28 mV) ($p < 0.05$). This indicates that pectin particles extracted by acid method showed higher stability in aqueous dispersion than those obtained by enzymatic method. This behavior is associated with the fact that acidic extraction increases the electronegativity of pectin, which causes the particles to move away from each other and remain suspended in the aqueous medium [32].

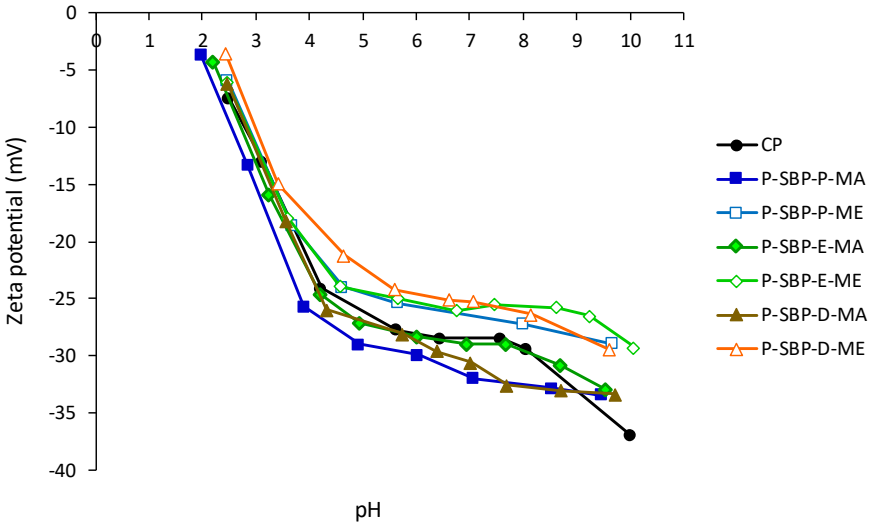

Figure 4. Zeta potential (ζ) curves of pectin. CP: citrus pectin. P-SBP-P-AM: pectin from sugar beet pulp pressed, acid method; P-SBP-P-EM: pectin from sugar beet pulp pressed, enzymatic method; P-SBP-E-AM: pectin from sugar beet pulp ensiled, acid method; P-SBP-E-EM: pectin from sugar beet pulp ensiled, enzymatic method; P-SBP-D-AM: pectin from sugar beet pulp dried, acid method; P-SBP-D-EM: pectin from sugar beet pulp dried, enzymatic method.

Citrus pectin exhibited an intermediate stability between the samples extracted by the enzymatic and the acid method, and lastly, the P-SBP-P-MA showed the highest stability in aqueous solution among all analyzed samples ($p < 0.05$).

Figure 5 shows the apparent viscosity (η) of pectin solutions prepared in water (20 mg/mL). The solutions prepared with all types of sugar beet pectin showed lower viscosity than solutions prepared with commercial citrus pectin; in turn, solutions prepared with sugar beet pectin extracted by the enzymatic method presented higher apparent viscosity values than solutions prepared with sugar beet pectin extracted by the acid method, regardless of the type of by-product used ($p < 0.05$). The higher viscosity observed in pectin extracted by the enzymatic method, could be explained by the presence of polyelectrolytes, since they affect the conformation of the macromolecule and the nature of the counterions, which act as a brake on the flow of polymers [33]. In this sense, the pectin extracted from sugar beet pulp ensiled by the enzymatic method (P-SBP-E-EM) reached the highest final viscosity (40 m Pa.s), followed by pectin of sugar beet pulp dried extracted by the enzymatic method (P-SBP-D-EM) (18 m Pa.s), while the pectin of sugar beet pulp dried, extracted by the acid method (P-SBP-D-AM), exhibited the lowest apparent viscosity value (4 m Pa.s).

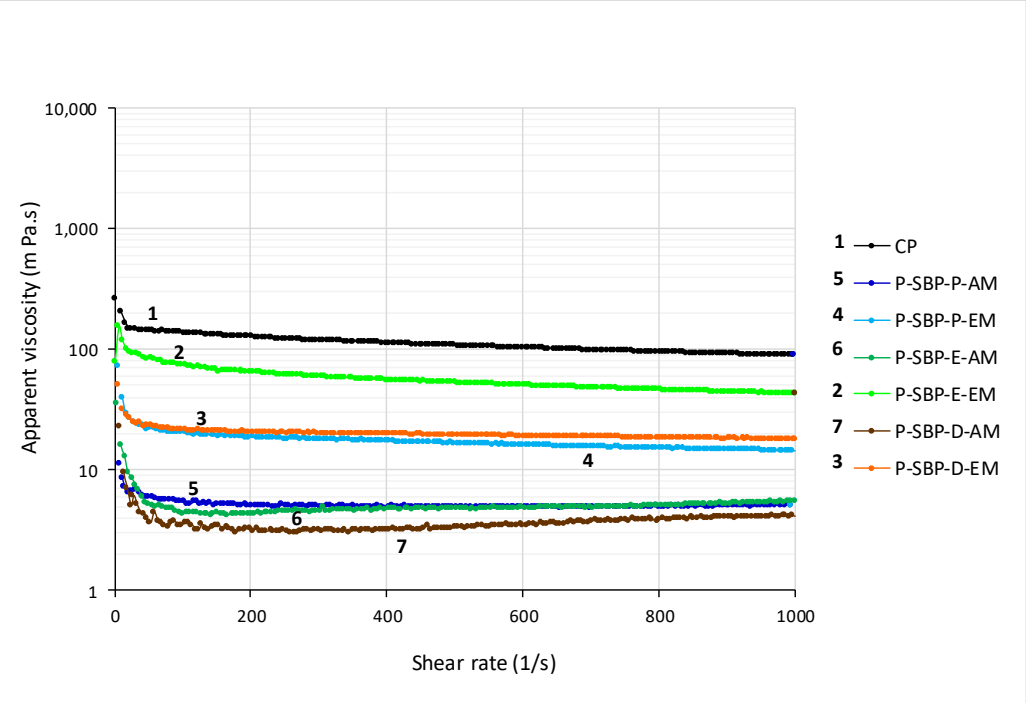

Figure 5. Apparent viscosity of pectin solutions (20 mg/mL). CP: citrus pectin. P-SBP-P-AM: pectin of sugar beet pulp pressed, acid method. P-SBP-P-EM: pectin of sugar beet pulp pressed, enzymatic method. P-SBP-E-AM: pectin of sugar beet pulp ensiled, acid method. P-SBP-E-EM: pectin of sugar beet pulp ensiled, enzymatic method. P-SBP-D-AM: pectin of sugar beet pulp dried, acid method. P-SBP-D-EM: pectin of sugar beet pulp dried, enzymatic method.

The lower viscosity observed in the solutions prepared with pectin obtained from the dried beet residue may be due to the drying processes that can negatively affect the properties of the rheological properties of gum [29]. However, the viscosity of the pectin obtained in the present study, by the enzymatic method from the dry sugar beet by-product (P-SBP-D-EM) (18 m Pa.s), obtained in the industry by application of 140 °C/2–3 h with boiler gases (Figure 1), was higher than that reported by Huang et al. [29] for a sugar beet pectin obtained by the acid method (12 M HCl), from dehydrated pulp at 40, 50, and 60 °C/8h, in a hot air oven (10 m Pa.s); reaffirming the advantage of using the enzymatic method in the extraction process of pectin, in order to obtain high viscosity, since in both cases the waste used presented a moisture content close to 4.6% (~96% DW) Table 1.

It should be noted that, the degree of methoxylation (DM) and the molecular weight (Mw) of the extracted pectin (Table 3), could not have influenced the viscosity observed in the present study, since both the DM and the Mw did not have a statistically significant difference in all extracted pectins ($p < 0.05$).

Overall, the apparent viscosity of the pectin solutions decreased when the shear rate increased, which is indicative of a pseudoplastic (shear-thinning) flow behavior due to a decrease of entanglements of their structure, as is the case of gums [34].

3. Materials and Methods

3.1. Samples

Commercial citrus pectin was purchased from Acofarma (Barcelona, Spain). Industrial sugar beet by-products were provided by Azucarera Ebro (Madrid, Spain). Figure 1 shows the industrial process of sugar extraction and derived by-products. Briefly, sugar beet is washed, disinfected with hot water and grinded to obtain small particles named cossettes. Then, sugar is extracted from cossettes by a diffusion process with water heated at 70 °C. Water and sucrose are concentrated and dried, and the cossettes with a 7%–8% of dry weight (DW) are pressed to extract more sugar, obtaining the Sugar Beet Pulp Pressed (SBP-P), with a DW of 28–29%. This residue is stored in silos during 7–8 months obtaining the Sugar Beet Pulp Ensiled (SBP-E), and then, it can be dried by sun (3 days), or with boiler gas caldera 2–3 h until 88-96 % DW obtaining the Sugar Beet Pulp Dried (SBP-D). Those residues are destined to the direct sell or used in the production of animal feed. SBP-P, SBP-E and SBP-D were selected performing a simple, non-stratified random sampling. The beet used in the extraction process came from different cultivars of *Beta vulgaris* var. altissima Döll, harvested in early-January, in Spain. All samples were lyophilized, ground, sieved thought 250 µm mesh, and maintained at −20 °C until analysis.

3.2. Physicochemical Characterization of Sugar Beet by-Products

°Brix, pH, water activity (a_w), dry weight (DW) and protein content were determined according to the AOAC methods described by Megías-Pérez, Gamboa-Santos, Soria, Villamiel, and Montilla, (2014) [35]. Fat content was determined by the soxhlet method using propanol during 2 h of heating. Minerals content was determined in the Interdepartmental Research Service (SIdI-UAM) (Madrid, Spain), by ICP-MS in an Elan 6000 Perkin-Elmer Sciex instrument (Concord, Canada).

Total carbohydrates were determined according to the phenol sulfuric method described by Masuko et al. [36]. Working inside a fume hood, 278 µL of aqueous dilutions of the samples (70 µg/mL) were disposed in Eppendorf tubes of 2 mL, and 167 µL of phenol sulfuric solution (5% *w/v*) were added on the dilutions. Tubes were stirred in a vortex, 1 mL of sulfuric acid were carefully added, and the mixture was shaken again, and then kept for 30 min without agitation. Afterwards, the absorbance was measurement at 480 nm in a Synergy HT Multi-Mode Microplate reader (BioTek® Instruments, Inc., Winooski, VT, 05404-0998 USA) (Gen 5 software) and using a calibration curve of galacturonic acid (0–0.2 mg/mL). Results were expressed as the total carbohydrates (g/100 g DW).

Reducing carbohydrates were measured using the method described by Sumner et al. [37], by adding 100 µL of 3,5-dinitrosalicylic acid (DNS) reactive to 100 µL of the diluted sample previously located in the Eppendorf tubes of 1.5 mL. The mixture was stirred and boiled during 5 min, and then cooled in an ice bath, and 750 µL of milli-Q water was added. After shaking again, 280 µL of mix were transferred to a multiwell plate and the absorbance was measured at 540 nm. The calibration curve was prepared with GalA (0–0.4 mg/mL), and data were expressed as reducing carbohydrates (g/100 g DW).

Fiber content was determined by the enzymatic-gravimetric method described by McCleary et al. [38]. Samples were milled and sieved through 250 µm mesh. A dilution of 1 g of sample in 50 mL of sodium phosphate buffer 0.08 M pH 6 was prepared, and 100 µL of α-amylase from hog pancreas

(Sigma-Aldrich Química SL, Madrid, Spain, ≥5000 U/mL) was added to remove the starch, heating at 95 °C for 15 min in a water bath with agitation. After cooling down, the pH was adjusted to 7.5 with 0.275M NaOH, and 5 mg of protease from *Streptomyces griseus* (Sigma Aldrich, ≥3500 U/g) was added and the mixture was heated (60 °C/ 30 min) to remove the protein.

The pH was adjusted to 4–4.6 with HCl 0.325 N, and 300 µL of amyloglucosidase from *Aspergillus niger* (Sigma Aldrich, 72,500 U/g) (1 mg/mL) (30 min, 60 °C) were added to remove the gelatinized starch. Afterwards, 280 mL of water at 60 °C were added and left to stand for 1 h to precipitate the insoluble fiber. The precipitate was filtered through a porous crucible of 0.8 µ, washed successively with 60 mL of ethanol 78%, 20 mL of ethanol 95%, and 20 mL of acetone. The solid detained was dehydrated for 24 h at 100 °C, and its final weight was corrected depending on the protein and ash value, to obtain the insoluble dietary fiber (IDF) content. Total dietary fiber (TDF) was determined by replacing the 280 mL of water by ethanol 95% and filtering all the precipitate; and soluble dietary fiber (SDT) was calculated by subtracting IDF from TDF values.

Total phenolic content was determined in methanolic extracts of samples by the Folin-Ciocalteu method described by Soria et al. [39]. To obtain the extracts, 0.2 g of powder sample were homogenized in 5 mL of methanol using an Ultra Turrax (IKA Labortechnik, Janke and Kunkel, Staufen, Germany) at 24,000 rpm for 1 min. The homogenates were placed in tubes of 15 mL and stirred at 750 rpm (50 °C/20 min) in an Eppendorf ThermoMixer® incubator (15 mL). Mixtures were centrifuged at 2000× *g* for 15 min and filtered through Acrodisc PVDF syringe filters (0.45 µm, Sigma-Aldrich).

The reaction was carried out, by adding 100 µL of MeOH and 100 µL of Folin-Ciocalteu 2N to 100 µL of the filtered extract, disposed in Eppendorf tubes of 1.5 mL. After 5 min, 700 µL of Na_2CO_3 (75 g/L) were added, and tubes were left in the dark for 20 min. Mixtures were centrifuged at 28,000× *g* for 3 min, and the absorbance was measured in the supernatant at 735 nm using a Synergy HT Multi-Mode Microplate reader (BioTek® Instruments, Inc., Winooski, VT 05404-0998, USA) (Gen 5 software). The calibration curve was prepared with gallic acid (0–60 mg/L) and the results were expressed as mg of gallic acid equivalent (GAE)/g DW.

Antioxidant capacity was determined according to the method proposed by Brand-Williams et al. [40], by the addition of 193 µL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) 2 mM diluted in methanol (1:15) to 7 µL of methanolic extract of powder sample, in the Eppendorf tubes of 1.5 mL. Mixture was stirred and transferred to a multiwell cell (280 µL). After 30 min without agitation under dark conditions, the absorbance was measured at 517 nm. The calibration curve was prepared with Trolox (Sigma 648471, 500 mg; ≥98%) (0.25–2.5 mM in methanol). Results were expressed as mM Trolox/100 g DW.

3.3. Pectin Extraction

3.3.1. Acid Method

Pectin was extracted by the traditional acidifying method optimized by Neha Babbar et al. [41] with slight modifications. The sample was mixed with deionized water (5%, *w/v*) and the pH was adjusted to 1.2 with HNO_3 12 M. The suspended samples were heated at 90 °C with continuous stirring at 200 rpm for 3 h. After the reaction was completed, the resulting slurries were cooled down to 40 °C, and the pH was adjusted to 4.5 with $NH_3.H_2O$ 25% and centrifuged at 2600× *g* at 4 °C for 10 min, to separate insoluble fiber, protein, and other non-pectin compounds. The supernatant was collected and stored in a refrigerator at 4 °C for subsequent purification. One volume of supernatant was precipitated using two volumes of ethanol 95% for 1 h at room temperature. The centrifugation was repeated and the precipitate was washed three times with ethanol at 70%. After purification, the pectin was dried by lyophilization, and stored until its analysis.

3.3.2. Enzymatic Method

According to the method described by Liew et al. [42], pectin was extracted from sugar beet by-products by dilution of powder samples in buffer sodium citrate 0.05 M at pH 4.5 (1:20 *w/v*) and heating with continuous stirring (125 rpm) with the commercial cellulase Celluclast®, derived from *Trichoderma reesei* (Novozymes Corp., Bagsvaerd, Denmark. 700 U/g) (1.17 U/g powder sample) at 61 °C during 102 min. Mixtures were left without stirring at room temperature during 24 h, to degrade the cellulose; and then, they were centrifuged at 2600× *g* at 4 °C for 10 min, to separate insoluble fiber, protein, and other non-pectin compounds. Ethanol 95% was added to the supernatants (2:1 *v/v*), and ethanolic mixtures were kept under dark conditions at 4 °C for 24 h to allow the flotation of pectin. Pectin solutions were centrifuged at 3400× *g* by 15 min and the precipitate was washed twice with ethanol 70%, mixed and centrifuged before each addition. Finally, pectin was de-colorated by adding acetone drop-by-drop and dried through lyophilization. The pectin yield was calculated by means of Equation (1):

$$\text{Pectin yield } (\%) = \frac{\text{Weight of product obtained (g)}}{\text{Weight of powder sample (g)}} \times 100 \quad (1)$$

3.4. Pectin Characterization

3.4.1. Monomeric Composition

Sample was hydrolyzed with trifluoroacetic acid (TFA) 2 M (30 mg/1.5 mL) at 110 °C during 4 h [43]. Then, 500 µL of hydrolysate were placed in a flask and evaporated under vacuum at 43 °C. 400 µL of phenyl-β-D-glucoside (0.5 mg/mL) (internal standard, I.S.) were added, and the flask was evaporated again. For the oximes formation, 250 µL of hydroxylamine chloride in pyridine (2.5%) were added and the mixture was vortexed and heated at 70 °C during 30 min, stirring the sample at the beginning, at the middle, and at the final of the 30 min. Samples were persilylated with 250 µL of hexamethyldisylazane (HMDS) and 25 µL of TFA at 50 °C for 30 min, agited, and centrifuged at 10,000× *g* for 2 min.

The released monomers were analyzed by GC-FID (Agilent Technologies 7890A gas chromatograph, Agilent Technologies, Wilmington, DE, USA) using a DB-5HT capillary column (15 m × 0.32 mm × 0.10 µm) (J&W Scientific, Folsom, CA, USA). Injector and detector temperatures were 280 and 350 °C, respectively; oven temperature program was increasing from 150 °C to 165 °C at 1 °C/min and up to 300 °C at a heating rate of 10 °C/min. Nitrogen was used as the carrier gas, at a flow of 1 mL/min, and injections were made in split mode 1:20. Data acquisition was done using a HPChem Station software (Hewlett-Packard, Palo Alto, CA, USA). The response factors were calculated after the analysis of standard solutions (xylose, arabinose, rhamnose, galactose, mannose, glucose, and galacturonic acid), in concentrations of 0.01–2 mg, and 0.2 mg of I.S.

3.4.2. Protein Content

Protein content was determined in all the pectin samples following the Bradford assay [44] using the Bio-Rad protein assay kit, which includes Coomasie Blue and bovine serum albumin (BSA) (0–2 mg/mL) for the calibration curve. The absorbances were measured at 595 nm and protein content was expressed as g/100 g DW.

3.4.3. Degree of Methylesterification (DM)

The degree of methylesterification (DM) of extracted pectin was determined by Fourier transform infrared spectroscopy (FTIR) analysis. KBr discs were prepared mixing the pectin with KBr (1:100) and pressed. FTIR spectra Bruker IFS66v (Bruker Optics, Ettlingen, 76275 Germany) were collected at absorbance mode in the frequency range of 400–4000 cm^{-1}, at a resolution of 4 cm^{-1} (mid infrared region) with 250 coadded scans. The DM was expressed as the ratio between the peak area of methylesterified carboxyl groups: $COOCH_3$, measured at 1745 cm^{-1}; and the sum of the peak areas of

esterified carboxyl groups: $COOCH_3$ at 1745 and free carboxyl groups COO^- measured at 1608 cm^{-1}; according to the Equation described by Singthong et al. [45], Equation (2):

$$DM = \frac{\text{Methylesterified carboxyl groups}}{\text{Total carboxyl groups}} \times 100 \quad (2)$$

3.4.4. Molecular Weight (Mw)

The distribution of Mw of pectin samples was determined by Size Exclusion Chromatography (SEC) according to the method described by Muñoz-Almagro et al. [46] with slight modifications. Dilutions of sample in milli-Q water (1 mg/mL) were eluted with ammonium acetate 0.01 M at a flow rate of 0.5 mL/min for 50 min at 30 °C. The eluent was monitored using a refractive index detector (Boeblingen, Germain) at 30 °C, disposed in a LC Agilent Technologies 1220 Infinity LC System 1260 (Agilent Technologies, Boeblingen, Germain), equipped with two consecutive TSK-GEL columns (G5000 PWXL, 7.8 × 300 mm, particle size 10 µm, and G2500 PWXL, 7.8 × 300 mm, particle size 6 µm; Tosoh Bioscience, Stuttgart, Germany). Calibration curves were prepared using pullulans of Mw 788, 473, 212, 100, 1.3, and 0.34 kDa; and, Mw values were the average weight at peak maximum obtained in the analysis by triplicate.

3.4.5. Emulsifying Activity

Emulsifying activity was calculated by turbidity according to the method described by Wang et al. [47] with slight modifications.

A volume of 100 mL of pectin solution in water (20%, *w/v*), were mixed with 5 g of corn oil using an Ultraturrax at 24,000 rpm for 1 min to obtain an emulsion. The emulsion was diluted 30, 500 and 900-folds with sodium dodecyl sulphate (SDS) (1 g/L). Turbidity of emulsions was measured in a UV spectrophotometer SPECORD®210 and the WinASPECT® PLUS software (Analitik Jena AG, Jena, Germany), at 500 nm, using the SDS solution as the blank sample. The turbidity was calculated by Equation (3):

$$T = \frac{2.303 \times A \times F}{I} \quad (3)$$

where T is turbidity of emulsions (m^{-1}), A is the absorbance at 500 nm, F is the dilution factor, and I is path length, which is 0.01 m.

The emulsion activity index (*EAI*) was calculated using Equation (4):

$$EAI = \frac{2 \times T}{\varnothing \times c} \quad (4)$$

where Ø is the oil volume fraction of the dispersed phase, and c is the concentration of pectin in the emulsion.

3.4.6. Zeta potential (ζ)

Zeta potential (ζ) of pectin in aqueous dilution was determined according to the method described by Falk et al. [48], using a Malvern Zeta sizer Nano ZS instrument (Malvern Instruments Ltd., Worcestershire, UK). A volume of 250 mL of pectin solution was prepared by dissolving the extracted pectin in KCl 0.1 M (1 mg/mL). The solution was agitated, sonicated during 1 min, and its pH was measured (mixture 1). Then, 10 mL of mixture 1 and 90 mL of KCl 0.1 M were agitated, sonicated during 1 min, and its pH was recorded (mixture 2). Briefly, mixture 2 was injected into the clear disposable zeta cell and the ζ was measured. The procedure for the preparation of mixtures was repeated, in order to obtain dilutions of mixture 1, at different pH values (2 to 10) by adding HCl 0.1 M or KOH 0.1 M drops, and their respective ζ values were measured. The measuring cell was carefully washed after each reading, using deionized water and the next dilution, avoiding bubbles inside to evade measurement errors.

3.4.7. Apparent Viscosity

Following the method described by Huang et al. [29], extracted pectin was dissolved in deionized water (20 mg/mL) using a magnetic stirrer at ambient temperature during 1 h. The apparent viscosity of the sample was determined using a Modular Advanced Rheometer System (MARS) (Thermo Fisher Scientific Inc., Waltham, MA, USA). Flow curves over the shear rate (1–100 s^{-1}) were measured at 25 °C. The measuring geometry used was a double-cone and plate system with a truncated cone with an angle of 2° and a diameter pf 60 mm. The apparent viscosity and steady shear rate measurement were fitted to the Herschel-Bulkley model, Equation (5):

$$\sigma = \sigma_0 + k \times \dot{\Upsilon}^n \quad (5)$$

where σ is the shear stress (Pa), σ_0 is the yield stress (Pa), k is the consistency index ($Pa.s^n$), $\dot{\Upsilon}$ is shear rate (s^{-1}), and n is the flow behavior index.

3.5. Statistical Analysis

Extractions and analysis were carried out at least in triplicate and means were compared by Tukey's test ($p < 0.05$), using SPSS Statistics 22.0 (IBM Corp., Armonk, NY, USA). Differences were expressed as mean ± standard deviation.

4. Conclusions

The present study compared the compositional and rheological properties of sugar beet pectin, which was efficiently extracted from pressed, ensiled, and dried residues by acid or enzymatic methods. The silage process caused a reduction in the protein and insoluble carbohydrates content of sugar beet pulp, as well as an increase in the fat and soluble dietary fiber amount, likely due to a lactic fermentation process. The drying process, instead, caused a reduction in the reducing carbohydrates, soluble fiber and antioxidant capacity. Either the type of sugar beet by-product or the extraction method had no impact on the degree of methoxylation and molecular weight of extracted pectin. Nevertheless, the enzymatic method allowed the extraction of pectin with a significantly higher content of galacturonic acid as compared to the acid method, due to the milder conditions of the former. The rheological analysis showed that all pectins obtained presented a pseudoplastic flow behavior. Furthermore, the zeta potential and *EAI* values indicated that pectins extracted by the acid method showed good stabilizing behavior in aqueous dispersion and good emulsifying activity, whereas pectins enzymatically extracted had a higher apparent viscosity that was linked to the presence of polyelectrolytes that impede the polymer flow.

To conclude, the information provided in the present work could be very useful for the potential reuse of ensiled and dried by-products from sugar beet industry in the cost-effective production of pectin with different technological properties depending on the applied extraction method. Pectins were conveniently characterized and with suitable rheological properties are known to find immediate applications in the pharmaceutical and/or food fields.

Author Contributions: M.V. and F.J.M. designed the study; M.T.P. performed and analyzed the experiments; F.J.M., M.V., and R.M. supervised the progress; M.T.P. prepared the original draft, which was later reviewed by all authors.

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Sample Availability: Samples of the pectin are available from the authors.

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Research Article

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Chemical and physicochemical characterization of orange by-products derived from industry

M Teresa Pacheco, F Javier Moreno and Mar Villamiel*

Abstract

BACKGROUND: Industrial extraction of orange juice produces a large amount of waste that affects the environment and gives rise to important economic losses; at the same time, information about the composition of the waste is still limited. The present study carried out an exhaustive chemical and physicochemical characterization of the residues in the waste, aiming to increase their potential application for the extraction of functional ingredients.

RESULTS: Four different products (three solids and one liqueur) were provided by the industry. The overall characterization indicated that carbohydrates comprised the main components. During processing, carbohydrate derivatives were formed such as those corresponding to the initial steps of the Maillard reaction. In this sense, furosine was demonstrated to be a suitable indicator with respect to the control of the process. Although the phenolic content substantially decreased (by up to 57%) as the processing proceeded, the antioxidant capacity was affected to a much lesser extent (~10%). Dehydrated products were rich in galacturonic acid and hardly any change was detected during their elaboration. The liqueur by-product was found to have a much higher level of fructose than glucose and sucrose.

CONCLUSION: Orange juice waste obtained industrially under the conditions described in the present study could be used as a source of pectic derivatives or fructose in the case of solid or liquid by-products, respectively. The results reported here could diversify the present application of these products as a source of food ingredients, contributing to an improvement in their utilization.
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Keywords: orange by-products; antioxidant activity; feed; Maillard reaction; furosine; carbohydrates; galacturonic acid

INTRODUCTION

Citrus (*Citrus* spp.) is one of the most important fruit crops worldwide.[1] Between 2013 and 2014, global citrus production was 121.3 million tons (58% of this corresponds to oranges), reaching 6.5 million tons in Spain; this country is the largest producer in the European Union and the fifth in the world. Around 40% of the fruit is used by the industry to extract juice, leaving a half of its weight as waste (mainly peel, seeds and pulp). Citrus wastes reach 24.3 million tons per year from which, 1.3 million tons correspond to Spain.[2]

Without further treatment, orange wastes can contribute to environmental problems, as a result of their fermentation having a high chemical and biological oxygen demand.[2] Citrus pulp amounts to 50–70% of the fresh weight of the original fruit and it is contained in the peel (60–65%), internal tissues (30–35%) and seeds (0–10%).[1] Citrus peel is rich in fibre and polysaccharides with water and oil-holding and cation-exchange capacities, as well as swelling properties much higher than those of cellulose. Thus, this dietary fibre constitutes a low-calorie bulk ingredient in food applications requiring oil or moisture retention.[3] Over the last decade, orange peel wastes have been reported as an excellent source of essential oils, natural antioxidants, ethanol, organic acids, pectic oligosaccharides and pectin.[2,4] In this sense, a huge interest in the latter has emerged not only because of their well-known technological properties, but also because of the benefits for gut microbiota, amongst others.[5]

Devatkal *et al.*[6] replaced synthetic antioxidants in meat products with powder extract of Kinnow mandarin bark and found that this extract, which is rich in phenolic compounds with free radical scavenging activity, has the potential to be used as a safer alternative to synthetic forms. Additionally, Sharma *et al.*[7] showed that citrus wastes can act as potent antioxidants.

Thus, orange waste processing may provide an efficient, inexpensive and environment-friendly platform for obtaining new nutraceutical products or for improving existing ones.[8]

Among the different citrus by-products applications, their use as animal feed constitutes the cheapest and most realistic option for the food industry. Particularly, fresh citrus pulp is often used locally to feed animals; it has a natural acidity, although it is still a perishable product as a result of its high content of water and soluble sugars. Dried citrus pulp is used as a cereal substitute because of its high energy content in ruminant species. It can be used to support growth and lactation with good digestibility and fewer negative effects on rumen fermentation than starch rich feeds.[1]

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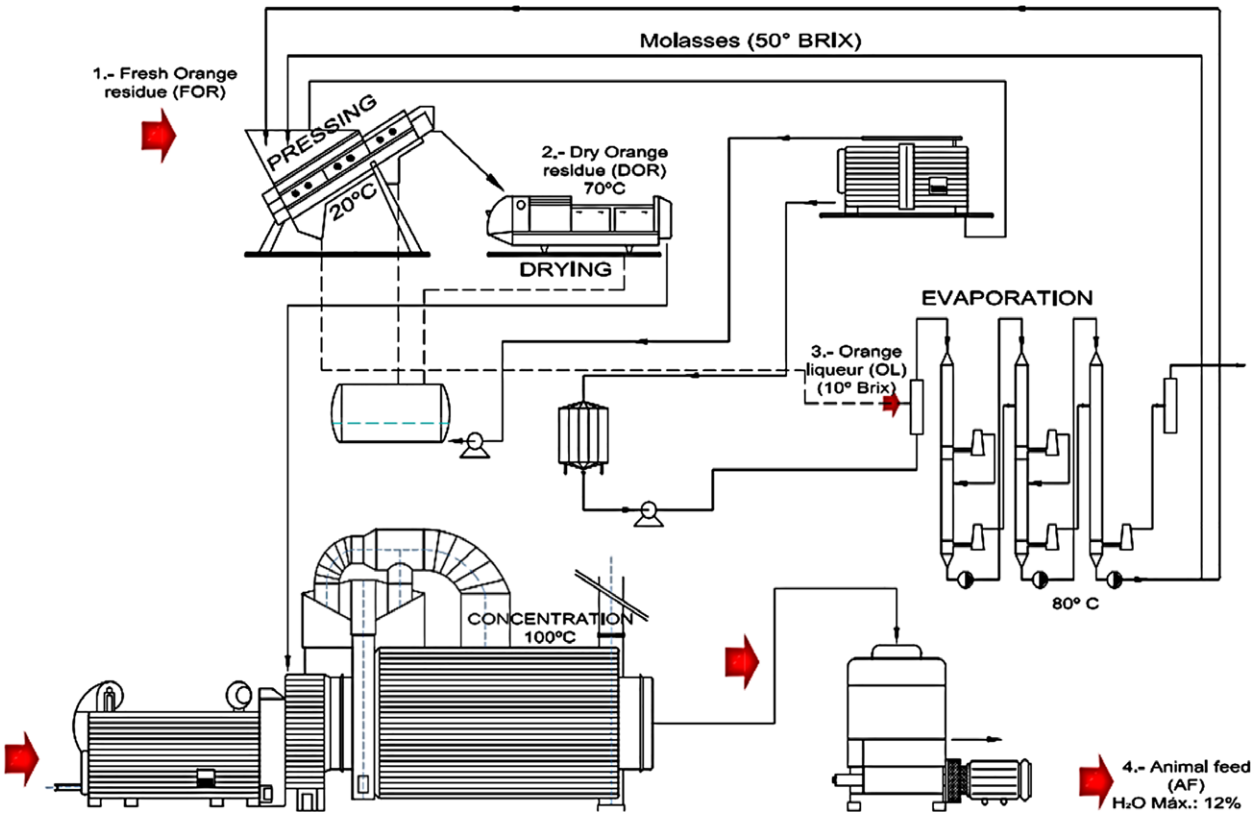

Figure 1. General scheme of the obtainment of by-products from the industrial extraction of orange juice.

The transformation of raw materials to animal feed can involve treatments at high temperatures to achieve gelatinization of the starch and destruction of microorganisms, which can also give rise to chemical modifications, with the Maillard reaction (MR) being one of the most important.[9] This reaction mainly takes place between amino acids, peptides or proteins and reducing sugars; the advanced stages may favour an undesirable colour development together with nutritive losses as a result of the participation of lysine.[10] The MR is often used by food manufacturers to develop an appealing aroma, colour or texture in food products (cereal-based food, coffee, meat, etc.). However, despite some positive aspects, the MR could generate, in advanced stages of the reaction, potentially harmful compounds (e.g. acrylamide, furans, heterocyclic amines).[11] To date, the Amadori and Heyns compounds represent indicators of the initial steps of the MR, and they are formed before subsequent major changes in composition and functionality. In this regard, furosine (2-furoylmethyl-lysine) and other 2-furoylmethyl amino acids, which are formed after the acid hydrolysis of the Amadori and Heyns compounds, have been observed as one of the best and most sensitive indicators of the initial steps of the MR in products of various sources.[12]

Another reaction that can also take place during processing of by-products is the degradation of polyphenols, affecting antioxidant activity[13] as a result of the action of enzymes and oxygen and the thermal processes used. However, during certain conditions of processing and storage, an increase of phenols can also be observed, which is attributed to the formation of compounds by hydrolysis.

Despite such evidence, to the best of our knowledge, no information is available on either the control of the processes involved or on the quality of orange-juice by-products, making it difficult to guarantee their nutritive value and health benefits.

Accordingly, the present study carried out an exhaustive overall characterization of industrial by-products derived from orange juice extraction, which can afford useful information to retrospectively determine the changes taking place during their manufacture, including carbohydrate and antioxidant activity modifications. A complete compositional study of these by-products could diversify their use not only as animal feed, but also as potential sources of functional ingredients for human applications.

MATERIALS AND METHODS

Samples

Samples derived from the processing of Valencia late oranges were provided by García Carrión (Daimiel, Spain). Figure 1 shows the industrial process of orange juice extraction and transformation of by-products. The fresh orange residue (FOR) was pressed with $CaOH_2$ to facilitate the obtainment of orange liqueur (OL). OL was concentrated into molasses, from 10 to 50 °Brix (approximately), in a triple-effect evaporator with recirculation, and the pressed orange solid residue was dried. Then, the dry orange residue (DOR) was triturated, mixed with the molasses, and the whole mass was concentrated at 100 °C to obtain the animal feed (AF) in the form of pellets up to a maximum moisture content of 12%. FOR, DOR, OL and AF were selected performing a simple, non-stratified random sampling. The waste used in the extraction process came from oranges of different cultivars, harvested in early May. All samples were lyophilized, ground, sieved and maintained at −20 °C until analysis.

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Physicochemical characterization

°Brix, pH, water activity (a_w), dry matter (DM), fat and protein content were measured in accordance with Association of Official Agricultural Chemists (AOAC) methods, as described by Megías-Pérez *et al.*[14] The mineral composition of samples was determined using an ICP-MS Elan 6000 Perkin-Elmer Sciex instrument (Perkin Elmer, Waltham, MA, USA) from the Service Interdepartmental Research (SIdI-UAM) (Madrid). Either a semiquantitative or a quantitative analysis of the elements of interest using the external calibration method, and internal standards to correct instrumental drift, were carried out.[15]

Total and reducing carbohydrates

Total carbohydrates were determined by the phenol–sulphuric acid method described by Masuko *et al.*,[16] with small variations. In total, 278 µL of diluted samples were placed in Eppendorf tubes of 2 mL. Tubes were subjected to an extraction sorbonne, and 278 µL of 5% aqueous phenol solution were added. After slight vortex agitation, 1000 µL of sulphuric acid were added carefully to each tube, following all chemical safety regulations using the appropriate protective protocols. Then, after 30 min, absorbance was measured at 480 nm using a Power Wave XS 201595 spectrophotometer (BioTek Inc., Winooski, VT, USA), equipped with a plate reader (Biotek KcJunior). Galacturonic acid (GalA) (G2125 ≥ 98%; Sigma-Aldrich, St Louis, MO, USA) was used for the calibration curve (20–100 µg mL^{-1}). The results were expressed as g kg^{-1} DM.

Reducing sugar determination was carried out using the 3,5-dinitrosalicylic acid method described by Sumner *et al.*[17] The calibration curve was constructed using GalA (0–5 mg mL^{-1} in methanol). Absorbance was measured at 540 nm.

Dietary fibre

Samples of orange by-products were analyzed for total and insoluble fibre by the modified enzymatic–gravimetric method described by McCleary *et al.*[18] (AOAC 985.29 and 991.42) (AOAC, 1995). The AOAC method uses a heat-stable, α-amylase, amyloglucosidase and protease treatment. Insoluble dietary fibre (IDF) was determined by weighing the dried residue that remained after the enzymatic treatment. Total dietary fibre (TDF) was determined on a different aliquot by weighing the dry precipitate resulting from the addition of ethyl alcohol to the enzymatically treated samples. Soluble dietary fibre (SDF) values were obtained by the difference between the total and insoluble dietary fibre fractions.

2-Furoylmethyl-amino acid content

The determination of these compounds was carried out by ion-pair reversed-phase high-performance liquid chromatography-UV analysis (model 1260 Infinity LC System; Agilent Technologies, Böblingen, Germany)[19] using a C8 column (250 cm × 4.6 mm inside diameter) (furosine-dedicated; Alltech, Nicolasville, KY, USA) at 35 °C, a linear binary gradient at a flow rate of 1.2 mL min^{-1} with two mobile phases: (A) 0.4% acetic acid and (B) 0.34% KCl in phase A, and a UV detector at 280 nm.

Samples (0.25 g) were hydrolyzed with 4 mL of 8 mol L^{-1} HCl at 110 °C for 24 h, filtered (Whatman No. 40; Whatman, Maidstone, UK) and 0.5 mL of the filtrate was applied to a Sep-Pack C18 cartridge (Millipore, Milford, MA, USA). Furosine was eluted with 3 mL of 3 mol L^{-1} HCl and injected in the system. For quantification, a commercial standard (Neosystem Laboratoire, Strasbourg, France) was used. The content was expressed as mg 100 g^{-1} protein.[19]

Total phenolic content

Following the Folin–Ciocalteu method described by Soria *et al.*[20] methanolic extracts were obtained by dilution of 0.2 g of powder sample in 5 mL of methanol, trituration in Ultraturrax (24 000 rpm for 1 min), heating with continuous stirring (50 °C for 20 min), centrifugation (2000 × *g* for 15 min) and filtration of supernatant (1 mL using Sep-Pak Cartridges of 0.45 µL).

Once the methanolic extracts were obtained, 100 µL of methanol, 100 µL of 2 N Folin, and 700 µL of Na_2CO_3 were added to 100 µL of each methanolic extract, and absorbance was read at 735 nm. The calibration curve was constructed with gallic acid (GAE) (0–60 mg L^{-1}). The results were expressed as mg GAE 100 g^{-1} DM.

Antioxidant capacity

According to the method proposed by Brand-Williams *et al.*,[21] 7 µL of methanolic extract obtained as described above and 193 µL of 2 mmol L^{-1} 2,2-diphenyl-1-picrylhydrazyl diluted in ethanol (1:15) were transferred to a multiwell cell and kept in the dark for 30 min. The absorbance was read at 517 nm (UV–visible Lambda 40 spectrophotometer; Perkin Elmer). The calibration curve was obtained with Trolox (0.25–2.5 mmol L^{-1} in methanol). The results were expressed as Trolox equivalent antioxidant capacity (mmol L^{-1} Trolox 100 g^{-1} DM).

Colour development

The colour of the samples was measured using a CM-508i colorimeter (Minolta Co. Ltd, Tokyo, Japan) and the CIE Lab colour system, where L^* is lightness, a^* is redness and b^* is yellowness.[22] The chroma (C^*), the huge angle ($h°$) and the total colour differences (ΔE) were calculated by the equations[23]:

$$C^* = (a^{*2} + b^{*2})0.5$$
$$h° = \arctan(b^*/a^*)$$
$$\Delta E = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{0.5}$$

Monomeric composition

For this, a previous hydrolysis of 30 mg of sample was carried out with 1500 µL of 2 mol L^{-1} trifluoroacetic acid (TFA) at 110 °C for 4 h. The monomers released were analyzed by gas chromatography-flame ionization detection (GC-FID) (7890A gas chromatograph; Agilent Technologies, Wilmington, DE, USA). Then, 500 µL of the hydrolyzed samples were evaporated under vacuum and mixed with 400 µL of phenyl-β-D-glucoside (0.5 mg mL^{-1}) internal standard. After the formation of oximes,[24] samples were persilylated with hexamethyldisylazane (250 µL) and TFA (25 µL) at 50 °C for 30 min and centrifuged at 11 200 × *g* for 2 min. Analyses were carried out using a DB-5HT capillary column (15 m × 0.32 mm × 0.10 µm) (J&W Scientific, Folsom, CA, USA). Chromatographic conditions were selected according to Muñoz-Almagro *et al.*[25]

The carbohydrates with a degree of polymerization < 6 were analyzed by GC-FID as trimethylsilyl derivatives of their oximes. Samples (5 mg) and 400 µL of β-phenyl glucoside (0.5 mg mL^{-1}) were evaporated, derivatized and analyzed in a similar way as that described above.

Pectin extraction and characterization

Pectin was extracted from by-products with the highest content of GalA, in accordance with the enzymatic method of Wikiera *et al.*,[26] with some modifications. Samples diluted in 0.05 mol L^{-1}

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Table 1. Chemical and physicochemical composition of orange by-products obtained from the orange juice extraction (g kg^{-1} DM)

Parameter	FOR	DOR	OL	AF
°Brix	29.7 ± 0.00 a	29.7 ± 0.00 a	39.0 ± 0.15 b	29.7 ± 0.00 a
pH	3.9 ± 0.01 a	6.4 ± 0.01 b	5.1 ± 0.01 a	5.1 ± 0.01 a
Aw	0.3 ± 0.00 a	0.5 ± 0.00 b	0.9 ± 0.00 c	0.5 ± 0.00 b
Dry matter (DM)	885.3 ± 0.50 c	870.1 ± 0.10 b	310.2 ± 0.90 a	881.0 ± 0.60 b
Total fat	9.0 ± 0.00 b	12.4 ± 0.00 c	1.1 ± 0.00 a	17.3 ± 0.00 d
Protein	58.9 ± 0.79 a	63.1 ± 0.10 c	60.0 ± 0.51 b	61.3 ± 0.44 b
Na	0.21 a	0.25 b	0.39 d	0.33 c
Mg	1.14 a	2.60 c	2.13 b	2.67 d
P	0.29 a	0.41 b	0.51 d	0.47 c
K	10.87 a	12.85 b	21.33 d	16.09 c
Ca	6.09 a	33.50 d	19.07 b	27.95 c
Fe	0.02 a	0.43 d	0.10 b	0.26 c
Ca/P ratio	21.0:1	81.7:1	37.4:1	59.5:1
Total carbohydrates	798.4 ± 0.70 c	742.2 ± 0.20 b	206.7 ± 0.30 a	753.4 ± 0.60 b
Reducing carbohydrates	39.1 ± 0.34 a	48.6 ± 0.06 b	70.3 ± 0.45 c	82.1 ± 0.37 d
TDF	460.7 ± 0.19 b	674.2 ± 0.02 d	20.0 ± 0.25 a	576.9 ± 0.93 c
IDF	226.0 ± 0.16 b	624.8 ± 0.02 d	2.5 ± 0.01 a	452.2 ± 0.09 c
SDF	234.7 ± 0.35 c	49.4 ± 0.03 b	17.5 ± 0.24 a	124.6 ± 1.02 c
Furosine (mg furosine 100 g^{-1} protein)	9.0 ± 0.14 a	182.1 ± 2.35 c	19.5 ± 0.35 b	455.1 ± 3.07 d
Total phenols (mg GAE 100 g^{-1} DM)	211.9 ± 0.36 d	145.5 ± 0.47 c	115.6 ± 0.52 b	90.2 ± 0.73 a
AC (mmol L^{-1} de Trolox 100 g^{-1} DM)	23.3 ± 0.15 d	22.4 ± 0.07 c	21.6 ± 0.12 b	21.0 ± 0.06 a

AC, antioxidant capacity; AF, animal feed; DOR, dry orange residue; FOR, fresh orange residue; IDF, Insoluble dietary fiber; OL, orange liqueur; SDF, soluble dietary fiber; TDF, total dietary fibre. Means with different lowercase letters indicate a statistically significant difference ($P < 0.05$) in the same row.

buffer sodium citrate pH 4.5 were treated with Celluclast 1.5 L (Novozymes Corp., Bagsvaerd, Denmark) (50 µL g^{-1}). After stirring at 50 °C for 18 h, samples were maintained at room temperature, and centrifuged. The supernatant was filtered and a double volume of 95% ethanol was added to allow the pectin flotation, which was subsequently filtered, and washed using 70% ethanol and acetone to remove the colour.[27,28] The resulting pectin was lyophilized and stored at −20 °C. The yield was determined by the equation:

$$\text{Pectin yield} = \frac{\text{Pectin (g)}}{\text{Powder sample (g)}} \times 100\%$$

The monomeric composition of pectin was determined after acid and enzymatic hydrolysis with 1500 µL of 2 mol L^{-1} TFA at 110 °C for 4 h and 25 µL mL^{-1} of Viscozyme (Novozymes Corp.) at 50 °C for 24 h as described by Zhang *et al.* [29] The monomers released were analyzed as described previously.[24,25]

Statistical analysis

Data were analyzed for comparison of means using SPSS, version 22.0 (IBM Corp., Armonk, NY, USA). Differences between means were obtained using Tukey's test at $\alpha = 0.05$ and the values were expressed as the mean ± SD. Analyses were performed at least in duplicate.

RESULTS AND DISCUSSION

Overall characterization

As shown in Table 1, °Brix data are similar in all the samples, except in the OL sample that displayed the highest value (39.0 °Brix), probably as a result of the extractability of soluble sugars during the pressing of FOR (Fig. 1). To the best of our knowledge, the value of °Brix of similar by-products obtained from citrus fruit has not been reported. Cane[30] and beet[31] molasses were found to have 50.0 and 83.6 °Brix, respectively.

Regarding pH, FOR showed the lowest value, probably because of the presence of organic acids that could have been lastly removed or transformed during processing. The pH value of DOR was higher probably a result of the addition of $CaOH_2$ during the pressing (Fig. 1).

The a_w of the solid samples (FOR, DOR and AF) had low values, being considered as low moisture products with high stability over time.[32] The highest a_w corresponded to OL. As expected, DM content was opposite to a_w; the highest value was observed in FOR (885.3 g kg^{-1}) and the lowest was found in OL (310.2 g kg^{-1}) samples.

Fat content was found to be between 9 and 17.3 g kg^{-1} in all solid samples, whereas, in the liqueur, the value was lower (1.1 g kg^{-1}). Cerón and Cardona[33] reported a fat content of 15.5 g kg^{-1} in orange peel wastes. The differences can be the result of a different proportion of essential oils depending on the variety and method of extraction. Protein content was similar in all samples analyzed, which varied from 58.9 to 63.1 g kg^{-1}.

Regarding mineral analysis, the most abundant elements were K and Ca, ranging from 10.87 to 16.09 g kg^{-1} and 6.09 to 33.50 g kg^{-1}, respectively. Bampidis and Robinson,[1] and the National Research Council (NRC)[34] reported 11.00 and 7.10 g kg^{-1} of K and Ca, respectively, for dried citrus pulp.

The DOR sample presented the highest calcium content (33.50 g kg^{-1}) followed by AF (27.95 g kg^{-1}) and OL (19.07 g kg^{-1}) as a result of the addition of $CaOH_2$ during the pressing and concentration (Fig. 1). NRC,[35] reported a Ca content of 17.2 g kg^{-1} for citrus molasses, which is similar to the value shown in the present study for the OL sample.

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Table 2. Colour parameters of orange by-products

Colour parameter	FOR	DOR	OL	AF
L^*	52.85 ± 0.57 d	32.76 ± 0.47 b	30.25 ± 0.46 a	34.77 ± 0.19 c
a^*	6.89 ± 0.10 c	3.67 ± 0.03 a	9.46 ± 0.09 d	3.90 ± 0.03 b
b^*	31.35 ± 0.11 c	17.16 ± 0.34 a	32.83 ± 0.42 d	19.96 ± 0.27 b
C^*	32.10 ± 0.13 b	17.55 ± 0.33 a	34.16 ± 0.39 c	20.33 ± 0.27 b
$h°$	1.35 ± 0.00 b	1.36 ± 0.01 b	1.29 ± 0.00 a	1.38 ± 0.00 c
		FOR and DOR	**FOR and OL**	**FOR and AF**
ΔE		24.81 ± 0.90c	22.79 ± 0.33 b	21.58 ± 0.50 a

AF, animal feed; DOR, dry orange residue; FOR, fresh orange residue; OL, orange liqueur. Means with different lowercase letters indicate a statistically significant difference ($P < 0.05$) in the same row.

In animal feed, the determination of the Ca/P ratio is important because a value of 2:1 is recommended to avoid modifications in the nutrient uptake for ruminants.[36] This ratio in AF was 59.5:1, which could be improved by the addition of P, or substitution of $CaOH_2$ by enzymes with a similar effect to alkaline hydrolysis, as employed to dissolve lignin during the degradation process of agro wastes.[37]

The DM of this type of sample consists mostly of total carbohydrates (Table 1). OL was the by-product with the lowest content because the solid residue retains a large part of insoluble carbohydrates. The highest total carbohydrate content was observed in FOR, compared to DOR, and AF, possibly because the fresh residue is more porous, which could facilitate hydrolysis during the application of the phenol–sulphuric method.

The content of reducing sugars observed in the fresh (39.1 g kg^{-1}) and dry (48.6 g kg^{-1}) residue was in the range reported for orange peel (23.2–59.9 g kg^{-1}).[38] The liquor showed a higher content (70.3 g kg^{-1}) because, during the pressing of the fresh residue, reducing sugars may have easily migrated to the soluble phase. In the final product (AF), the reducing carbohydrates were higher (82.1 g kg^{-1}) than expected, most likely because of the hydrolysis of other non-reducing carbohydrates, such as sucrose, during the heat treatment (Fig. 1).

DOR presented the highest content of TDF (674.2 g kg^{-1}) and IDF (624.8 g kg^{-1}). Its content of TDF was similar to that reported by Figuerola *et al.*[39] for orange peel (643.0 g kg^{-1}) and the SDF content of FOR (234.7 g kg^{-1}) was higher than that reported for peel of *Citrus sinensis* L. cv. Liucheng (94.1 g kg^{-1}).[3] The mixture of DOR and OL led to a lower fibre content in the final product (AF) (576.9 g kg^{-1}); however, this quantity is well suited to the needs of cattle. This product is classified as an effective fibre for maintaining the optimum ruminal pH (6.2–6.6).[40]

The SDF was high in FOR (234.7 g kg^{-1}) and remained mostly in this fraction during the pressing; thus, a very small amount appeared in OL (17.5 g kg^{-1}). In DOR, the small amount of SDF (49.4 g kg^{-1}) could be related to an increase in the degree of sample hardness during drying (Fig. 1), which apparently affects the hydrolysis of the sample with respect to the enzymatic method applied for SDF determination. Conversely, AF had a higher SDF content (124.6 g kg^{-1}), probably because it was made from the crushed dry residue, which facilitates the extraction of SDF.

Assessment of initial steps of MR

The initial steps of MR were assessed by the formation of furosine. The FOR sample presented 9 mg of furosine 100 g^{-1} protein, and higher values were observed for DOR, OL and AF (182.1, 19.5, 455.1 mg of furosine 100 g^{-1} protein, respectively), mainly as a result of the heat treatment applied during industrial extraction (Fig. 1) and the composition of each by-product (Table 1). The higher pH values of DOR, OL and AF, compared to FOR, could also have favoured the MR. The highest value of furosine in AF could also be a result of the intensity of the process that can increase the hydrolysis of non-reducing sugars, thus, increasing the amount of reducing sugars able to participate in the MR. No data on the formation of furosine in these types of products have been reported to date. Megías-Pérez *et al.*[14] found joint levels of furosine and 2-FM-Arg ranging from 38.4 to 3766.3 mg 100 g^{-1} protein in dehydrated fruits.

Total phenolic content and antioxidant capacity

The total phenolic content of FOR (211.9 mg GAE 100 g^{-1} DM) was in accordance with the results reported by Ghasemi *et al.*[41] for *Citrus sinensis* var. Washington Navel (peel 160.3 mg GAE 100 g^{-1} DM and tissues 232.5 mg GAE 100 g^{-1} DM). During processing, this parameter decreased with the intensity of the treatment (Table 1). Comparing the phenolic content between FOR and AF, a significant reduction was observed.

The antioxidant capacity of citrus by-products in the initial sample (23.3 mmol L^{-1} Trolox 100 g^{-1}) was lower than the value reported by Escobedo *et al.*[42] for flavedo of Valencia orange (46.4 mmol L^{-1} Trolox 100 g^{-1}), probably because of the presence of other non-antioxidant vegetal tissues (e.g. rag) in FOR[43] or a different cultivar, amongst other factors.

The antioxidant capacity decreased significantly from the initial to the final product, although to a much lower extent (10%) than the total polyphenol content (57%). This could be a result of the formation of antioxidant compounds during the progress of the MR because of the intensity of processing.[44] Ghasemi *et al.*[41] did not find any correlation between the total phenolic content and antioxidant activity in tissues or peels of 13 citrus species.

Colour changes

L^* decreased significantly ($P < 0.05$) during processing. The greatest decrease was between FOR and DOR (Table 2), which could be a result of the loss of water and MR progress, related to the browning (< L^*).

The tone $h°$ was low in all by-products and hardly any difference was detected. The redness a^* was also low in all the by-products analyzed, which might be a result of the low amount of anthocyanins in orange wastes. a^*, b^* and C^* parameters presented a similar trend, with the highest change occurring after the first step

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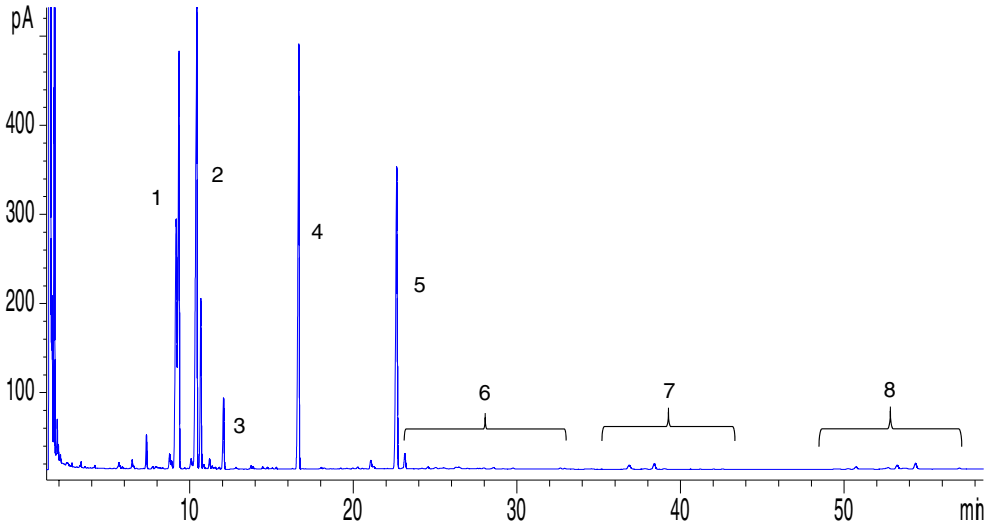

Figure 2. Chromatogram of low molecular weight carbohydrates (water-soluble) (analyzed as their trimethylsilylated oximes) present in the fresh orange residue (FOR), determined by GC-FID, without previous hydrolysis with TFA. 1: fructose; 2: galactose; 3: glucose; 4: β-phenyl-glucoside (internal standard); 5: sucrose; 6: disaccharides; 7: trisaccharides; 8: tetrasaccharides.

Table 3. Carbohydrates of low molecular weight (water-soluble) in orange by-products (g kg^{-1} DM) determined by GC-FID

Carbohydrates	FOR	DOR	OL	AF
Fructose	131.7 ± 3.13 b	91.4 ± 2.28 a	154.2 ± 4.12 c	92.9 ± 2.20 a
Galactose	2.1 ± 0.02 a	22.0 ± 0.55 b	86.1 ± 3.80 d	69.7 ± 0.97 c
Glucose	105.3 ± 2.48 d	5.2 ± 0.12 a	53.3 ± 1.13 c	17.9 ± 0.40 b
Sucrose	65.4 ± 1.37 b	8.1 ± 0.27 a	77.2 ± 2.30 d	68.7 ± 1.07 c
Total disaccharides	7.9 ± 0.11 b	8.1 ± 0.12 b	16.2 ± 0.38 c	6.1 ± 0.14 a
Total trisaccharides	5.0 ± 0.11 c	4.0 ± 0.07 b	4.9 ± 0.11 c	3.3 ± 0.08 a
Total tetrasaccharides	8.0 ± 0.04 d	1.2 ± 0.02 b	1.9 ± 0.04 c	0.9 ± 0.01 a

Results are expressed in dry matter (DM). AF, animal feed; DOR, dry orange residue; FOR, fresh orange residue; OL, orange liqueur. Means with different lowercase letters indicate a statistically significant difference ($P < 0.05$) in the same row.

of drying to obtain DOR. These parameters were higher in the liquor because of the presence of coloured soluble compounds. When OL was added to DOR to obtain AF, intermediate values of a^*, b^* and C^* were observed. Therefore, redness, yellowness and the chroma decreased with the reduction of humidity and intensity of heating. The parameter C^*, which correlates redness (a^*), yellowing (b^*) and luminosity (L^*), can be an indicator of non-enzymatic browning; the lower the value, the greater the non-enzymatic browning (Tables 1 and 2).

Soluble fraction of carbohydrates and monomeric composition

The analysis by GC-FID of the samples without previous hydrolysis allowed the investigation of the presence of free carbohydrates of a low degree of polymerization. Figure 2 shows the presence of fructose, galactose, glucose, sucrose, disaccharides, trisaccharides and tetrasaccharides in FOR. The most abundant water-soluble carbohydrate (Table 3) in FOR was fructose (131.7 g kg^{-1}), showing a value similar to that reported by Wilkins *et al.*[45] for Valencia orange peel waste (136.6 g kg^{-1}) hydrolyzed enzymatically, followed by glucose (105.3 g kg^{-1}) and sucrose (65.4 g kg^{-1}).

In DOR, sucrose could have been hydrolyzed, although fructose and glucose decreased with respect to FOR, probably as a result of their participation in the MR, in agreement with the high furosine formation indicated above. Total tetrasaccharides also decreased because of hydrolysis during processing. The most abundant sugar in the liquor was fructose, the content of which was much higher compared to the other by-products. This is probably a result, amongst other factors, of its higher solubility compared to glucose.[39] It can be inferred that the OL could be an enriched source of fructose in a similar way to the cane molasses that are used to extract sucrose. Thus, cane molasses contain 200–300 g kg^{-1} of sucrose, 140–250 g kg^{-1} of glucose and 150–250 g kg^{-1} of fructose,[30] whereas OL contains 77.2 g kg^{-1} of sucrose, 53.3 g kg^{-1} of glucose and 154.2 g kg^{-1} of fructose.

With respect to the final product, AF, the amount of carbohydrates could be explained by the combination of several factors: (i) the mixture of DOR and OL; (ii) the participation of monosaccharides in the MR; and (iii) hydrolysis of sucrose, tri- and tetrasaccharides during processing.

When evaluating the monomeric composition after acid hydrolysis (Fig. 3 and Table 4), xylose, arabinose, rhamnose, galactose, mannose, glucose and GalA were found in all samples. Apart from glucose and mannose, the other monosaccharides could originate mainly from pectic polysaccharides. Glucose could be derived from the acid hydrolysis of cellulose,[46] by disruption of β-1,4-glycosidic bonds,[47] and mannose from mannans and galactomannans.[48,49] This composition indicates the presence of

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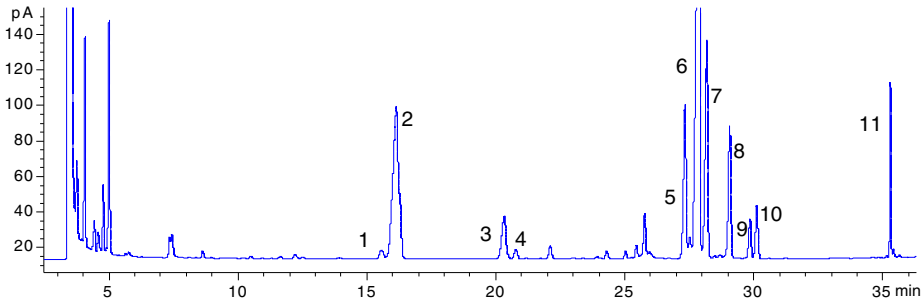

Figure 3. Chromatographic profile obtained by GC-FID of trimethylsilylated oximes of carbohydrates present in the FOR after its hydrolysis with 2 mol L^{-1} TFA for 4 h at 110 °C. 1: xylose (Xyl); 2: xylose (Xyl) + arabinose (Ara); 3: rhamnose1 (Rha); 4: rhamnose2 (Rha); 5: galactose (Gal); 6: mannose1 (Man); 7: glucose1 (Glc); 8: galactose2 (Gal) + mannose2 (Man) + glucose2 (Glc); 9: galacturonic acid1 (GalA); 10: galacturonic acid2 (GalA); 11: Internal standard. Sub-indices represent the elution order of compounds.

Table 4. Monosaccharides derived from polysaccharides present in orange by-products (g kg^{-1} DM) analyzed by GC-FID after TFA hydrolysis and subsequent *trimethylsilylated* oxime formation

Monosaccharide	FOR	DOR	PL	AF
Xylose	38.7 ± 0.16 b	43.3 ± 0.41 c	5.2 ± 0.05 a	43.5 ± 0.42 c
Arabinose	48.9 ± 0.43 c	51.5 ± 0.22 d	6.8 ± 0.06 b	1.7 ± 0.02 a
Rhamnose	23.3 ± 0.17 b	27.2 ± 0.13 c	5.9 ± 0.03 a	24.2 ± 0.19 b
Galactose	79.7 ± 0.41 b	62.5 ± 0.27 a	280.8 ± 1.35 b	83.4 ± 0.74 c
Mannose	51.7 ± 0.41 b	59.0 ± 0.24 c	33.7 ± 0.18 a	52.2 ± 0.48 b
Glucose	194.3 ± 0.59 c	68.7 ± 0.29 a	173.4 ± 0.93 d	161.3 ± 0.91 b
Galacturonic acid	160.7 ± 0.90 d	85.8 ± 0.17 b	7.9 ± 0.07 a	101.8 ± 0.75 c

Results are expressed in dry matter (DM). AF, animal feed; DOR, dry orange residue; FOR, fresh orange residue; PL, pressing liqueur. Means with different lowercase letters indicate a statistically significant difference ($P < 0.05$) in the same row.

Table 5. Monosaccharides present in orange by-products pectin (%) analyzed by GC-FID after enzymatic hydrolysis and subsequent trimethysilyl oxime formation

Monosaccharide	FORP	DORP	AFP
Xylose	5.8 ± 0.10 b	5.9 ± 0.11 b	5.2 ± 0.10 a
Arabinose	5.2 ± 0.08 a	5.2 ± 0.07 a	4.7 ± 0.08 a
Rhamnose	9.7 ± 0.28 a	12.2 ± 0.18 b	11.7 ± 0.08 b
Galactose	1.3 ± 0.02 a	4.0 ± 0.02 c	1.8 ± 0.01 b
Mannose	2.2 ± 0.01 c	1.5 ± 0.01 b	0.8 ± 0.01 a
Glucose	1.5 ± 0.02 b	0.8 ± 0.01 a	1.6 ± 0.02 b
Galacturonic acid	74.4 ± 0.80 b	70.4 ± 1.05 a	74.1 ± 0.45 b

Results are expressed in dry matter (DM). AFP, animal feed pectin; DORP, dry orange residue pectin; FORP, fresh orange residue pectin. Means with different lowercase letters indicate a statistically significant difference ($P < 0.05$) in the same row.

pectic derivatives in the by-products, with homogalacturonan being the major domain.[50] Kaya *et al.*[51] found low amounts of rhamnose and GalA as the major compound in orange peel pectin.

Because GalA was present in a considerable amount in FOR, DOR and AF (Table 4), the pectin extraction was performed in these samples and the yields were 239.5, 119.3 and 165.6 g kg^{-1} DM, respectively. Yields of 259.0–299.0 g kg^{-1} DM have been reported previously for fresh orange peel pectin.[52,53] The lower yields in the processed samples (DOR and AF) were probably a result of the tissue hardening, which may influence cellulase activity.[54] As shown in Table 5, the most striking feature is the high content of GalA in the three by-products, with values matching the requirement to be considered as a food ingredient E-440 (> 65%).[55]

CONCLUSIONS

According to the results obtained in the present study, it is possible to conclude that furosine is a suitable indicator for retrospectively controlling the processing of orange juice industrial by-products. The application of low temperatures is advised to avoid, as much as possible, a loss of nutritive value as a result of the participation of lysine in the MR. The by-products analyzed constitute a source of polyphenols and, although their content can decrease during processing, the antioxidant activity is scarcely affected because the formation of the MR compounds counteracts the effect. Their main components are carbohydrates, with fibre being the most abundant in the solid residues. In this case, the high content of GalA in all assessed samples indicates that the three by-products (fresh and processed) could be a suitable source of pectin derivatives with potential application as a food ingredient. In addition, the pressed liquor with its high fructose content could constitute an alternative to molasses with less caloric value and a higher sweetening power. These results highlight the importance of the adequate utilization of by-products by the food industry. Further *in vivo* studies are being carried out to clarify the possible beneficial effects of the consumption of these products.

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Anti-inflammatory bowel effect of industrial orange by-products in DSS-treated mice†

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This work addresses the role of different by-products derived from the industrial extraction of orange juice in a possible anti-inflammatory effect in mice with colitis induced by dextran sulfate sodium (DSS). Fresh orange residue (FOR), dry orange residue (DOR), orange liqueur (OL) and animal feed (AF), as well as commercial citrus pectin (CP), were administered to C57BL/6J mice for 15 days before starting the DSS treatment. Analysis of macroscopic parameters such as the Disease Activity Index (DAI) and the colonic weight/length ratio revealed an anti-inflammatory effect following intake of FOR, AF or CP. Moreover, q-PCR of RNA from colonic tissue indicated measurable changes in the expression of TNF-α, IL-1β, iNOS, and intercellular adhesion molecules ICAM I, as well as in intestinal barrier proteins such as MUC-3, occludin, and ZO-1. Pectin, phenolic compounds and/or Maillard reaction products formed at initial steps were identified as relevant components exerting the ascribed beneficial effects. Our findings could open up the further application of a variety of orange by-products as food supplements in the potential amelioration of inflammatory bowel diseases.

1. Introduction

Inflammatory bowel disease (IBD) is commonly divided into ulcerative colitis (UC) and Crohn's disease (CD).[1] UC is usually confined to the colon, while CD usually affects any part of the gastrointestinal tract. A small segment of patients with IBD is classified as undetermined IBD, showing symptoms of UC and CD.[2] The IBD prevalence is in the range of 37.5–248.6 per 100 000 in North America and 4.9–505 per 100 000 in Europe.[3]

Among the various potential causes implicated in the IBD pathology, genetic susceptibility coupled with environmental risk factors attributed to lifestyle changes such as dietary habits, smoking, stress and lack of exercise, as well as other changes associated with medications and surgery or those leading to alteration of the bacterial flora of the gut, are the most frequently described causes.[4,5]

Current strategies for the treatment of IBD involve first the induction of remission, followed by maintaining the remission. Patients are usually treated with corticosteroids, immunomodulators, and anti-TNFα agents, although immunosuppressive therapies and anti-TNFα agents are associated with a higher risk of infections[6] and they eventually require surgical intervention, indicating that current therapeutic options are insufficient.[7] Moreover, the high cost of biological therapies contributes to the increasing financial burden of health care. The disadvantages of pharmacological therapies on IBD emphasize the need for non-pharmacological options.[8] Exclusion diets are generally not recommended and there is little evidence to support any particular food when nutritional regimens are recommended.[9] In this sense, low dietary fiber intake has been associated with the incidence of IBD,[10] since the prebiotic activity of fiber can act on IBD by stimulating the selective growth of the intestinal lactobacilli and bifidobacteria, which produce short-chain fatty acids (SCFA). SCFA could improve mucosal barrier functions and modulate the immune system.[11] However, there is scarce evidence of prebiotics use in this type of pathologies.[12]

Fiber can be found in a plethora of products and derivatives of vegetal origin. Industrial processing of citrus generates huge amounts of wastes (24.3 million tons per year worldwide and 1.3 million tons in Spain)[13] that, without further treatment, may cause environmental problems, since their fermentation implies high chemical and biological oxygen demand.[14,15] In most of the cases, these by-products are processed to obtain animal feed, and depending on the composition and the thermal treatment applied, Maillard reaction products (MRPs) may be formed. Some of these MRPs may

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have anti-oxidant, anti-mutagenic, carcinogenic and anti-bacterial activities,[16] but their anti-inflammatory effect is not yet well studied.

In the past few decades, dozens of animal models have been developed as indispensable tools for investigating the pathogenesis of IBD and evaluating therapeutic options.[17] These approaches are mainly based on spontaneous colitis models, inducible colitis models, genetically modified models, and adoptive transfer models.[18–20] Chemically induced murine models of intestinal inflammation are among the most commonly used models because they are simple to induce and the onset, duration, and severity of inflammation are immediate and controllable. The dextran sulfate sodium (DSS)-induced mouse colitis model is characterized by bloody faeces, diarrhea, weight loss and tissue inflammation,[21] as well as an increase of proinflammatory cytokines (IL-1β, TNF-α and IL-6) released in the intestine,[22] which can impair intestine permeability and mucosal barrier function and correlate with the severity of intestine inflammation.[23] These changes are similar to those found in humans by using molecular techniques to demonstrate changes in the composition of the mucosa-associated and fecal microbiota in patients with Crohn's disease.[24] In addition, a DSS-induced colitis model has some advantages when compared to other animal models of colitis. For example, the severity of the disease can be regulated easily by changing the concentration of administration of DSS, and the dysplasia that resembles the clinical course of human UC occurs frequently in the chronic phase of DSS-induced colitis.[25,26]

In this context, a recent study allowed the determination of phenolic compounds, pectin and the Amadori compound *N*-fructosyl-lysine (furosine) in by-products from the industrial extraction of orange juice,[27] and, in order to explore their potential functionality, the aim of this work was to investigate the anti-inflammatory effect of consumption of a variety of orange by-products in a DSS model in mice, which is the most commonly used approach to assess the *in vivo* therapeutic activity, since it exhibits certain characteristics similar to those present in human IBD.[28]

2. Materials and methods

2.1. Analytical standards

Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). DSS (36–50 kDa) was purchased from MP Biomedicals (Santa Ana, CA, USA). RNAlater® was obtained from Sigma Aldrich (St Louis, MO, USA), and Tri-Reagent® was acquired from Thermo Fisher Scientific (Invitrogen, USA). The oligo (dT) primers (Promega, Southampton, UK) and KAPA SYBRsFAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) were used to perform the qPCR analyses.

2.2. Orange by-products

Citrus pectin (CP) was provided by CEAMSA (O'Porriño, Spain). By-products from the orange juice extraction industry: fresh orange residue (FOR), the dry orange residue (DOR), orange liqueur (OL) and animal feed (AF) were provided by the company García-Carrión (Daimiel, Spain). After the industrial extraction of orange juice, the FOR was pressed with calcium oxide to facilitate the obtainment of the OL. Then, OL was concentrated from 10 to 50° Brix by heating at 80 °C, and the pressed orange residues were dried from 10 to 30% dry matter at 70 °C to obtain the DOR. In the final stage, OL and DOR were mixed, dried at 100 °C and ground to obtain the AF in the form of pellets.[27] These samples were lyophilized, ground and characterized as follows: dry matter, protein, fat, and fiber content were determined according to the AOAC methods. Total and reducing carbohydrates were measured using phenol–sulfuric acid and 3,5-dinitrosalicylic acid methods, respectively. Total phenolic content (TPC) was determined following the Folin–Ciocalteu method described by Soria *et al*.[29] The antioxidant capacity was measured by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging method.[30] 2-Furoylmethyl-Amino Acids (2-FM-AA) were analyzed by ion-pair RP-HPLC-UV and monosaccharide composition was determined through hydrolysis with 2 M trifluoroacetic acid and subsequent formation of trimethylsilyl-oxime derivatives by GC-FID (ESI Tables S1 and S2†).[27]

2.3. Animals and diets

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals as promulgated by the National Institutes of Health. The experimental protocol was approved by the Commission of Ethics in Animal Experimentation (Protocol CEEA 2010-286) of the University of Granada (Spain). C57BL/6 male mice (7–9 weeks old) were obtained from Janvier (St Berthevin Cedex, France). They were housed in Makrolon cages, maintained under an air-conditioned atmosphere with a 12 h light–dark cycle, and provided with free access to tap water and a standard rodent diet (Panlab A04 diet, Panlab S.A., Barcelona, Spain).

2.4. Experimental design

Mice (23 ± 2 g) were maintained under specific pathogen-free conditions in the facilities of Licinio de la Fuente Center and were randomly assigned to seven groups (n = 10): healthy, DSS control, FOR, DOR, OL, AF and CP. Solid by-product samples were added to standard food at 10%. CP and OL were diluted with water at concentrations of 2.5 and 1.25% (w/v), respectively, and administered at the rate of 100 μL per mouse per day. Induction of colitis was performed 15 days after the start of the experiment by adding 2.7% (w/v) DSS to the drinking water for seven days (Table 1).

2.5. Macroscopic indicators

Weight variation, daily food intake, Disease Activity Index (DAI) and the ratio between colon weight and length were considered to be macroscopic indicators. Mice were monitored recording the animal and food weight, the presence of gross blood in the faeces and the stool consistency. These parameters were each assigned a score according to the criteria proposed by Cooper *et al*.[31] and used to calculate the DAI

Table 1 Experimental conditions applied to study the effect of consumption of orange by-products

Group	Animal weight (kg)	Daily dose of treatment	Average food intake (g per mice per day)	SDF content of treatment (g per kg animal per day)	Treatment supply (days)	DSS (2.7%) supply (days)
Healthy	0.025	0.0	14.6	0.0	—	—
DSS Control	0.025	0.0	10.5	0.0	—	7
FOR	0.025	10% in standard food	10.6	10.0	14	7
DOR	0.025	10% in standard food	10.9	2.1	14	7
OL	0.025	50 mg kg^{-1} of animal	11.2	0.9	14	7
AF	0.025	10% in the standard food	11.1	5.5	14	7
CP	0.025	10 mg kg^{-1} of animal	11.9	9.9	14	7

SDF: soluble dietary fiber. DSS: dextran sulfate sodium. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin.

Table 2 Clinical parameters considered to determine the Disease Activity Index (DAI)

Bleeding	Stool consistency	Weight loss (WL) (%)	Value assigned according to WL
0: normal	0: normal	0	0
1: presence of blood	1: moderate soft stools	1–4	1
2: moderate bleeding	2: soft stools	5–9	2
3: moderately high bleeding	3: soft stools and diarrhoea	10–19	3
4: abundant bleeding	4: diarrhoea	>20	4

(Table 2). Once the animals were sacrificed, the colon (from the ileocaecal junction to the anal verge) was quickly excised and carefully washed with a cold saline solution, weighed and its length was measured.

2.6. Biochemical markers

The expressions of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-6, (IL)-1β and inducible nitric oxide synthase (iNOS), as well as barrier intestinal proteins such as intercellular adhesion molecule (ICAM)-1, mucin (MUC)-3, occludin and zonula occludens-1 (ZO)-1, were evaluated.

The colon tissue was longitudinally divided into different fragments and stored at −80 °C in RNAlater®. Total RNA from colonic samples was isolated using Tri-Reagent® following the manufacturer's protocol. All RNA samples were quantified with the Thermo Scientific NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 2 μg of RNA was reverse transcribed using oligo (dT) primers (Promega). Real-time quantitative PCR was carried out on optical grade 48-well plates in an Eco™ Real-Time PCR System (Illumina, CA, USA) with 20 ng of cDNA, KAPA SYBR®FAST qPCR Master Mix (Kapa Biosystems), and specific primers at their annealing temperatures (Table 3).[10] In order to normalize mRNA, the expression of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was measured. The relative quantitation of mRNA was calculated using the $\Delta\Delta C_t$ method.

Table 3 Primer sequences used in real-time qPCR assays in colonic tissue

Gene	Sequence 5′–3′	Annealing T (°C)
GAPDH	FW: CCATCACCATCTTCCAGGAG RV: CCTGCTTCACCACCTTCTTG	60
IL-1β	FW: TGATGAGAATGACCTCTTCT RV: CTTCTTCAAAGATGAAGGAAA	55
IL-6	FW: TAGTCCTTCCTACCCCAATTTCC RV: TTGGTCCTTAGCCACTCCTTC	60
TNF-α	FW: AACTAGTGGTGCCAGCCGAT RV: CTTCACAGAGCAATGACTCC	56
ICAM-1	FW: AGGAGGTGAATGTATAAGTTATG RV: GGATGTGGAGGAGCAGAG	60
iNOS	F: GGCAGAATGAGAAGCTGAGG R: GAAGGCGTAGCTGAACAAGG	55
MUC-3	FW: CGTGGTCAACTGCGAGAATGG RV: CGGCTCTATCTCTACGCTCT	62
ZO-1	FW: GGGGCCTACACTGATCAAGA RV: TGGAGATGAGGCTTCTGCT	56
Occludin	FW: ACGGACCCTGACCACTATGA RV: TCAGCAGCAGCCATGTACTC	56

2.7. Statistical analysis

Biochemical analyses were performed at least in duplicate. Indicators of each group were expressed as the mean ± standard deviation. Means were compared using Tukey's test (the significance limit was set at $p < 0.05$). Statistical analysis was carried out using the SPSS 22.0 software.

3. Results

3.1. Physicochemical characterization of test materials

The main physicochemical characteristics determined in the orange by-products and citrus pectin are summarized in Table S1.† Overall, all the studied orange by-products had a similar composition in protein or fat content, whereas substantial differences were found in carbohydrate, fiber or total phenolic content. Comparing the general chemical compositions of fresh orange residue (FOR), dry orange residue (DOR), orange liqueur (OL), animal feed (AF) and citrus pectin (CP), FOR presented the highest total phenolic content (211.9 mg GAE per 100 g) and the highest antioxidant capacity (23.3 mM of Trolox per 100 g), whereas CP showed the highest

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content of soluble dietary fiber (SDF) (96.58 g per 100 g) as expected, followed by FOR (23.47 g per 100 g) and animal feed (AF) (12.46 g per 100 g). CP also showed the highest content of furosine, an indirect marker of the Amadori compound *N*-fructosyl-lysine,[32] (827.4 mg per 100 g of protein), followed by AF (455.1 mg per 100 g of protein).

All samples had galacturonic acid (GalA), xylose (Xyl), arabinose (Ara), rhamnose (Rha), galactose (Gal) and mannose (Man) (Table S2†), which are core monomers of the main domains of pectin (homogalacturonan, rhamnogalacturonan-I, and rhamnogalacturonan-II).[33]

3.2. Assessment of macroscopic indicators of inflammatory bowel disease in DSS-treated mice

Among the symptoms of colitis induced by the oral administration of DSS are haematochezia, body weight loss, shortening of the intestine, mucosal ulcers and infiltration of neutrophils.

Except for the healthy control group, the tested groups experimented a loss of weight (Fig. 1A), which was higher in the DSS control group, probably due to the inflammation located in the intestine, as well as to a systemic status of illness occasioned by DSS as a general toxic.[17] Remarkably, the weight reduction was significantly less in the animals that consumed CP, FOR and AF groups.

Reduction of food intake is also a symptom of illness (Fig. 1B) possibly due to the discomfort caused by the intestinal inflammation.[34] During the DSS period the intake decreased significantly in the DSS control group, while animals treated with CP and FOR showed food consumption levels similar to that observed in the healthy group, and significantly different from the DSS group ($p < 0.05$). Similarly, the AF group showed a higher intake compared to the DSS group, but non-significant differences were found.

The DAI registered during the 7 days of DSS supply allowed us to infer that FOR, CP and AF groups *vs.* DSS control colitic group presented the lowest DAI ($p < 0.05$) (Fig. 1C). The weight/length ratio of the colon was significantly less in the groups that consumed FOR, AF and CP *vs.* the DSS group (Fig. 1D), indicating a lower severity of inflammation and minor colonic cell infiltration ($p < 0.05$).

Overall, these macroscopic parameters showed a consistent anti-inflammatory effect for CP, FOR and AF, with a significant reduction of symptoms and improvement of the animals' general status. As mentioned in subsection 3.1, the most important compositional differences found in the tested orange by-products were in fiber, total phenolic, and Amadori compounds (measured as furosine) contents.

3.3. Assessment of biochemical markers of inflammatory bowel disease in DSS-treated mice

The cytokine profile of DSS acute colitis is consistent with the acute inflammatory response, characterized by a macrophage-derived cytokine profile, strong chemotactic patterns and a polarized Th1 panel with a high participation of TNF-α and IL-1β among others, similarly to human IBD disease. This acute situation is also accompanied by a high expression of

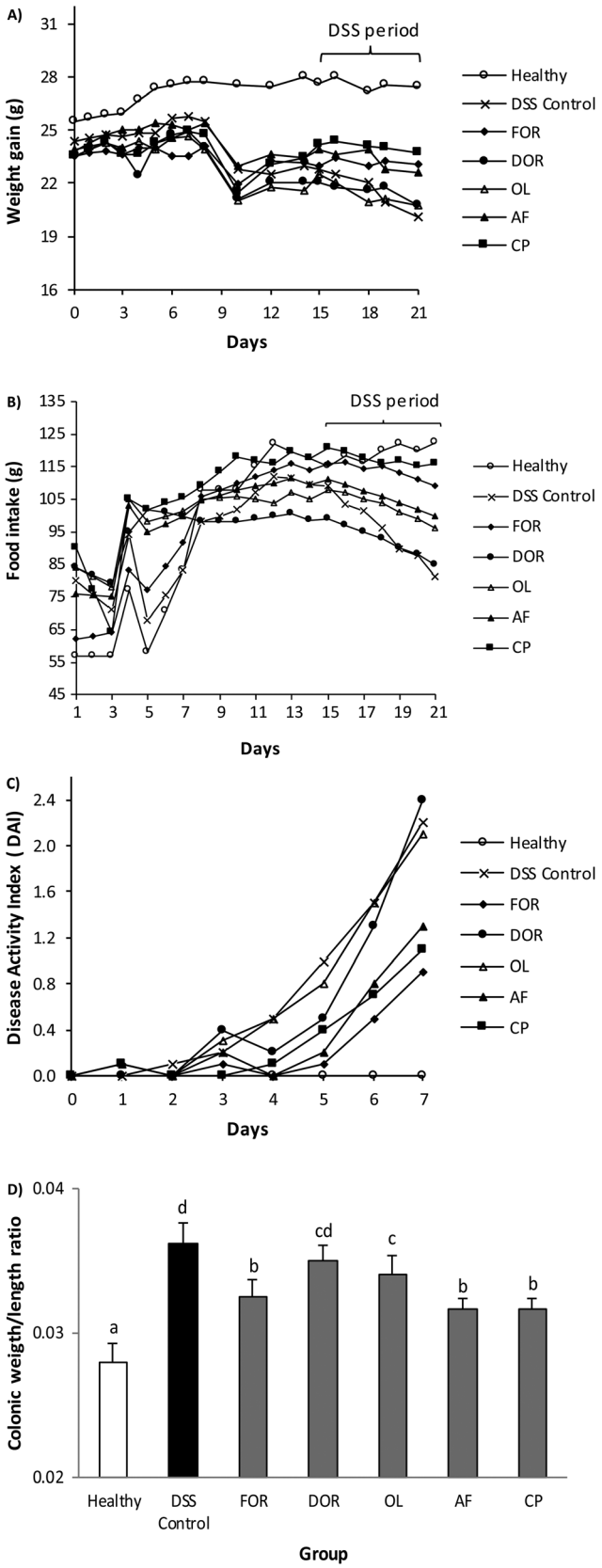

Fig. 1 Macroscopic indicators. (A) Weight gain. (B) Food intake. (C) Disease Activity Index (DAI). (D) Colonic weight/length ratio. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin.

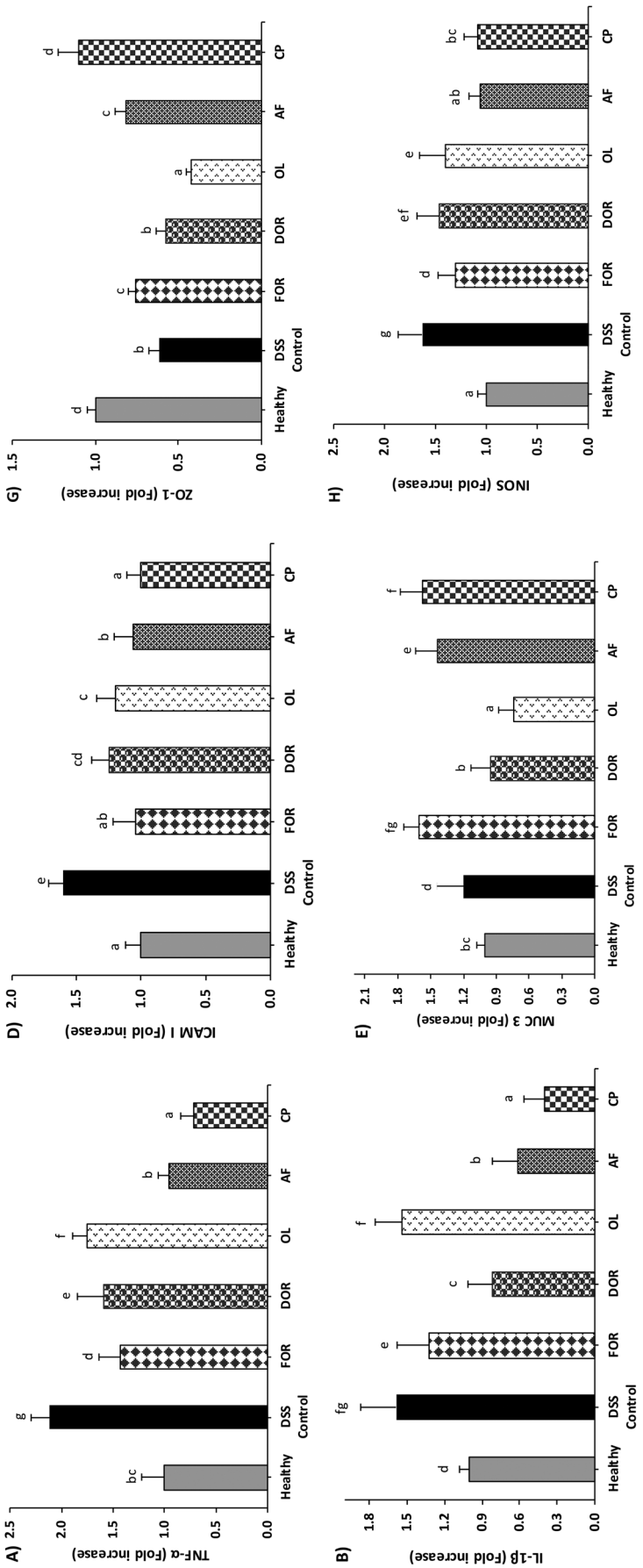

Fig. 2 Effects of orange by-product intake on the expression of pro-inflammatory cytokines and chemokines in colonic tissue of the DSS colitis mice. FOR: fresh orange residue. DOR: dry orange residue. OL: orange liqueur. AF: animal feed. CP: citrus pectin. Data are expressed as the mean ± S.D. Different letters on the bars indicate statistical differences ($P < 0.05$) amongst groups.

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iNOS, and an increase of the adhesion molecule ICAM I that facilitates the leukocyte endothelial transmigration, contributing to tissue damage and exacerbation of gut inflammation.[35]

The expression of the inflammatory cytokine panel (TNF-α, IL-1β, IL-6) was consistent and significantly reduced in the groups fed with FOR, AF and CP *vs.* the DSS control group (Fig. 2A–C); additionally, a reduction in the expression of ICAM I was observed in the groups that consumed the orange by-products, in comparison with the DSS control group (Fig. 2D) ($p < 0.05$). The expression of ICAM I was statistically similar comparing the healthy group and the CP and FOR groups, followed by AF group.

MUC 3, occludin and ZO-1 expression was significantly greater in FOR, AF and CP treated groups as compared with the DSS control group ($p < 0.05$) (Fig. 2E–H). The increased expression of these proteins indicated a potential protective effect by these products, which can not only reduce the symptoms and seriousness in acute conditions but also have a preventative effect against a future IBD crisis.[36]

Nitric oxide synthase (iNOS) is an enzyme dominantly expressed during inflammatory reactions. The synthesis of large amounts of nitric oxide (NO) by iNOS has been demonstrated in pathophysiological processes, such as acute or chronic inflammation and tumorigenesis; however, the role of iNOS activity in these diseases is still not well understood.[37] The presence of nitric oxide (NO) is responsible for the generation of substantial reactive oxygen species (ROS) as peroxynitrites or anion superoxide that may affect the microbial agent and produce apoptosis of host cells.[38] Results indicated that all the tested orange by-products showed a significant reduction in iNOS expression as compared to the DSS group (Fig. 2H). There was no significant difference between the AF, CP and healthy groups; the groups that consumed DOR and OL showed a higher expression of iNOS, slightly lower than that observed in the DSS control group, which could be related with their low GalA content (Table S2†).

4. Discussion

By correlating the biochemical effects of orange by-products and citrus pectin ingestion to their physicochemical characteristics (Table S1†), the beneficial effect observed following FOR consumption might be mainly due to its phenolic content and, consequently, antioxidant capacity. FOR had the highest content of total polyphenols, followed by DOR, OL, AF, and CP, respectively (Table S1†). The reduction of phenols observed among the tested orange by-product samples seems to be mainly due to the heat treatment applied by the industry for the obtainment of animal feed from orange residues,[39] since it is well known that phenolic compounds are vulnerable to heat.[40] Thus, the application of pressing and subsequent drying of FOR at 70 °C to obtain DOR caused a reduction of ~30% of initial phenolic content (Table S1†). The process of evaporation and concentration of OL at 80 °C resulted in an approximately two-fold reduction in the phenolic content as compared to the initial sample (FOR). Finally, in the case of AF, despite being a product obtained by mixing the DOR and OL (145.5 and 115.6 mg GAE per 100 g DM), its phenolic content was reduced to 90.2 mg GAE per 100 g DM, which represents 42.6% of the phenolic content of the initial sample (211.9 mg GAE per 100 g DM), which may be due to the previous concentration step carried out at 100 °C. DSS acts mainly by breaching the intestinal barrier function, thereby exposing sub-epithelial immune cells to commensal bacteria,[41,42] focusing on ROS and reactive nitrogen species (RNS) as the etiologic factors for IBD. Thereby, phenols present in FOR could act as antioxidants counteracting the effect of ROS.[43–45] Recent studies have suggested that the administration of antioxidants from different sources, with the additional anti-inflammatory action, may be beneficial in IBD treatment.[46,47] The beneficial effect observed after FOR consumption is in accordance with that described by Chen *et al.*,[48] who detected hesperidin, hesperetin, nobiletin and tangeretin in orange peel and reported a significant cytoprotective effect against oxidative stress in HepG2 cells. Consequently, FOR could be potentially considered as a useful and inexpensive source of compounds to prevent or minimize the effect of intestinal inflammation.

Prebiotic fiber (*e.g.* pectin, oligofructose, inulin), on the other hand, helps to recover and/or maintain the normal state of the intestinal microbiota, which in turn produces short-chain fatty acids (SCFA) that decrease the synthesis or expression of inflammatory citoquines, and maintain the balance between the T regulatory cells (Treg) and T helper cells (Th) 17, counteracting and/or avoiding inflammation.[49,50] Hartog *et al.*[51] observed that oral intake of a multi-fibre mix (MF) counteracts IBD-like intestinal inflammation and weight loss in DSS treated mice, likely due to the fact that MF may induce a decrease in inflammatory cytokine levels and an increase in Treg cells in the mesenteric lymph nodes. These authors concluded that the optimization of enteral nutritional concepts dealing with the tested fibre mix could lead to the potential modulation of gut microbiota composition and SCFA production to subsequently improve the inflammatory state and/or even induce remission.

In line with our results, Popov *et al.*[52] demonstrated the *in vitro* anti-inflammatory and antioxidant activities of pectic polysaccharides from fresh plums. Lastly, the dissimilar behavior observed for other assayed products such as DOR and OL, which did not show any positive effect, especially when analyzing macroscopic parameters, might be explained by their low SDF and GalA content (Tables S1 and S2†). Furthermore, Xiao *et al.*[53] studied the preventive effects of cranberry (*Vaccinium macrocarpon*) products on experimental colitis induced by DSS in mice and indicated that dried cranberries were more effective at preventing colitis than cranberry extract. The same phenolic content and different blueberry fiber contents in those products suggested that the fiber content could also contribute to the preventive effects on the development of colitis by its prebiotic action. A similar beneficial effect derived from the consumption of FOR or CP was observed by Cazarin *et al.*[10] when supplying *Passiflora edulis*

peel flour before colitis induction by DSS in drinking water to female C57BL/6J mice. These authors reported that *P. edulis* peel flour exerted an intestinal anti-inflammatory effect and attenuated the colonic damage due to its content of dietary fiber and polyphenols.

Patients suffering from UC have presented deficiencies of antioxidant nutrients at the time of diagnosis,[54,55] and patients suffering from CD have shown a reduction of antioxidants in plasma and a decrease of the total intestinal antioxidant capacity,[56] suggesting an increase in ROS. Moura *et al*.[57] indicated that this scenario could be balanced with the consumption of bioavailable functional foods, isolated nutrients, pro- and prebiotics, and natural active compounds from vegetal sources, among other substances, all of them reported as effective antioxidants in IBD.

On the other hand, the anti-inflammatory effect caused by AF ingestion could be associated with the *N*-fructosyl-lysine content, formed as MRP during the concentration process and industrial drying at high temperature (100 °C) and determined through the quantification of furosine. Some MRPs have been associated with different beneficial properties, such as antioxidant activities[58] and anti-inflammatory effects.[59] Our results are in good agreement with those reported by Hong *et al*.,[60] who indicated that glucose-lysine MRPs ameliorated DSS-induced colitis as determined by a decrease in DAI, colon weight/length ratio, nitric oxide levels in serum, recovery of body weight loss, and serum lysozyme levels, as well as suppression of the mRNA level of the inflammatory cytokines in colon tissues, highlighting the potential of these MRPs in preventing or treating IBDs. Oh *et al*.[61] have recently reported that MRPs derived from lysine and galactose decreased the production of TNF-α in macrophages and the expression of mRNA of interleukin IL-8 and IL-1b in Caco-2 cells in the model of DSS-induced colitis.

Concerning the effects derived from the intake of AF *vs.* CP, the variation of biochemical markers suggested a better anti-inflammatory response after the ingestion of CP, which could be due to its higher content of soluble dietary fibre (SDF) (96.58 *vs.* 12.46 g per 100 g DM) and *N*-fructosyl-lysine (827.4 *vs.* 455.1 mg of furosine per 100 g of protein) as compared to AF (Table S1†).

To summarize, polyphenol and/or fiber are the key components previously reported to exert intestinal anti-inflammatory activity based on therapies with natural products. In this sense, the anti-inflammatory effects related to the consumption of FOR or CP found in the current work seem to confirm previous findings regarding the functional activity of polyphenols and fiber on IBD. Additionally, further attention should also be paid to the potentially preventive role of certain Amadori compounds against IBD.

5. Conclusions

Samples rich in pectin, Amadori compounds and phenolic compounds, such as citrus pectin, animal feed and fresh orange residue, gave rise to a lower expression of pro-inflammatory cytokines, intercellular adhesion molecules ICAM I, iNOS enzyme, and a higher expression of protective chemokines MUC 3, occludin and ZO-1. These indicators underlined the potential role of pectin and Maillard reaction compounds in ameliorating some IBD symptoms. These promising results warrant the initiation of further studies using superior doses of the selected orange by-products, as well as broadening the assessment of biochemical indicators, in order to increase certainty about the effect of consumption of these singular by-products.

Conflicts of interest

The authors declare no conflict of interest.

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