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DESARROLLO DE UNA PLATAFORMA CIENTÍFICA TECNOLÓGICA PARA LA IDENTIFICACIÓN DE COMPUESTOS BIOACTIVOS DESDE ARRAYÁN (*LUMA APICULATA*) Y EVALUACIÓN DEL EFECTO DE LA NANOENCAPSULACIÓN SOBRE SU BIOACCESIBILIDAD

Tesis presentada a la Facultad de Farmacia de la Universidad de Concepción

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RESUMEN

Las Enfermedades Crónicas No Transmisibles (ECNT) son la principal causa de morbimortalidad del país y representan un problema nacional e internacional de salud pública. Como resultado, muchas especies nativas/autóctonas han sido estudiadas por su potencial fuente de compuestos fenólicos, los cuales pueden contribuir a la prevención y/o tratamiento de dichas enfermedades. Dentro de las especies nativas reconocidas por sus propiedades medicinales se encuentra el arrayán *(Luma apiculata)*, cuyos compuestos bioactivos presentes en el fruto y la hoja podrían ser un aporte relevante para disminuir el riesgo de padecer ECNT. Sin embargo, los estudios reportados de esta especie son muy escasos. En consecuencia, el presente trabajo de investigación tuvo como objetivo principal desarrollar metodologías químico-analíticas para obtener datos científicos que sustenten la característica funcional del fruto y hoja del arrayán y preservar los compuestos bioactivos identificados mediante el empleo de la nanotecnología.

Como paso previo al trabajo con arrayán, se desarrolló y optimizó un proceso de extracción asistida de ultrasonido de compuestos fenólicos desde semilla de quinoa. Basándose en este procedimiento, fue establecido y optimizado el proceso de extracción de dichos compuestos desde frutos y hojas de arrayán mediante extracción asistida por ultrasonido en baño ultrasónico. Esta

optimización contempló un diseño factorial completo y un diseño central compuesto.

Con las condiciones de extracción optimizadas, se evaluó la capacidad antioxidante de los extractos crudos y extractos ricos en compuestos fenólicos obtenidos por adsorción selectiva sobre la resina polimérica Amberlita XAD-7. La capacidad antioxidante se evaluó mediante dos metodologías: 2,2- Difenil-1-picrilhidrazilo (DPPH) y capacidad antioxidante reductor del hierro (FRAP). Además, se determinó el contenido de fenoles totales mediante la metodología de Folin-Ciocalteu (TPC). De acuerdo con los ensayos realizados, quedó en evidencia que los extractos de fruto y hoja de arrayán presentan una elevada concentración de compuestos fenólicos y una alta capacidad antioxidante, en especial, los extractos ricos en compuestos fenólicos. Para establecer el perfil fenólico de los extractos crudos, se implementó un método por cromatografía líquida de ultra-alta eficiencia (UHPLC) acoplada a espectrometría de masas (MS). El análisis cromatográfico, reveló la presencia de siete compuestos fenólicos en el extracto crudo de fruto de arrayán (quercetin 3-β-D glucósido, miricetina, quercetina, ácido p-cumárico, kaempferol glucósido, ácido gálico y kaempferol) y cinco compuestos fenólicos en el extracto crudo de hoja (quercetina 3-ß-D-glucósido, kaempferol 3-glucósido, miricetina, quercetina y ácido gálico).

Considerando el perfil fenólico y el efecto antimicrobiano reportado para algunos de los compuestos fenólicos identificados, se determinó la actividad

antiviral de extractos crudos (30% etanol) sobre el virus de la hepatitis A y norovirus murino, principales virus entéricos asociados a contaminación alimentaria. El extracto de hoja presentó un efecto antiviral significativo sobre ambos virus, mientras que el extracto de fruto tuvo un efecto moderado sobre el virus de la hepatitis A.

Luego de establecer el perfil fenólico, estándares de los compuestos fenólicos puros fueron sometidos a un proceso de digestión gastrointestinal *in vitro* para estudiar la bioaccesibilidad de dichos compuestos. Paralelamente, se evaluó la potencialidad de tres sistemas nanoparticulados (nanopartículas de zeina, zeína recubierta de alginato y zeína recubierta de complejo alginato/quitosano), los cuales fueron obtenidos mediante los métodos de nanoprecipitación y deposición electrostática. La fracción bioaccesible de los compuestos bioactivos fue determinada mediante cromatografía líquida de alta eficiencia asociada a detección UV (HPLC/UV). Los compuestos fenólicos presentaron una limitada bioaccesibilidad, que varió entre un 3.4 y 16.1%, mientras que los tres sistemas nanoparticulados incrementaron significativamente la bioaccesibilidad de todos los compuestos fenólicos, en especial, las nanoparticulas de zeina recubiertas con complejo de alginato/quitosano, la cual varió entre un 65.8 y 84.0%.

Finalmente, se implementó una plataforma científica-tecnológica por HPTLC/(bio)autografía/MS para detectar e identificar en los extractos crudos de fruto y hoja de arrayán compuestos con actividad antioxidante e inhibitoria sobre las enzimas α-glucosidasa y acetilcolinesterasa, consideradas como blancos

terapéuticos para tratar la diabetes mellitus tipo 2 y enfermedades neurodegenerativas, respectivamente. El extracto de hoja sólo presentó bandas con actividad antioxidante, mientras que el extracto de fruto presentó un compuesto con marcada actividad antioxidante e inhibitoria sobre ambas enzimas, el cual fue identificado tentativamente mediante la interfaz TLC-MS como fraxetina (hidroxicumarina).

Estos resultados permiten sustentar la característica funcional del fruto y hoja de arrayán, además de demostrar el potencial de la nanotecnología (sistemas nanoparticulados) en la preservación de los compuestos responsables de dicha funcionalidad.

ABSTRACT

Chronic non-communicable diseases (CNCD) are the leading cause of morbidity and mortality in Chile and constitute a national and international public health problem. Since CNCD incidence has continuously increased, the scientific community has studied many native species with important phenolic compounds content, which have shown positive effects over CNCD. Among the native species recognized for their medicinal properties, the arrayán (*Luma apiculata*) stands out due to its content of bioactive compounds in fruit and leaves, that could reduce the risk of suffering CNCD. However, the studies are very scarce. The objective of this work was to develop chemical-analytical methodologies to obtain scientific data for supporting the functional characteristics of arrayán fruit and leaf and to protect the identified bioactive compounds through nanotechnology. As a previous step, an ultrasound-assisted extraction process of phenolic compounds from quinoa seed was developed and optimized. Based on this procedure, the extraction process of phenolic compounds from arrayán fruits and leaves was established and optimized by means of ultrasound-assisted extraction. A full factorial design and a central composite design were used to optimize the extraction process.

Subsequently, the antioxidant capacity of raw extract and phenolic-rich extracts obtained by selective adsorption on the Amberlite XAD-7 polymeric resin were evaluated with the optimized extraction conditions. The antioxidant capacity was

evaluated using two methodologies: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). In addition, the total phenolic content (TPC) using Folin-Ciocalteu methodology was determined. These assays showed that arrayán fruit and leaf extracts have a high phenolic compound concentration and a high antioxidant capacity, especially the phenolic-rich extracts. Further, an ultra-high-performance liquid chromatography (UHPLC) coupled to mass spectrometry (MS) method was implemented to establish the phenolic profile of raw extracts. The chromatographic analysis showed the presence of seven phenolic compounds in the raw fruit extract (quercetin 3-ß-D-glucoside, myricetin, quercetin, p-coumaric acid, kaempferol 3-glucoside, gallic acid and kaempferol) and five phenolic compounds in the raw leaf extract (quercetin 3-ß-D-glucoside, myricetin, quercetin, kaempferol 3-glucoside and gallic acid).

According to the phenolic profile and the antimicrobial effect reported for some identified phenolic compounds, the antiviral activity of raw extracts (30% ethanol) was determined against hepatitis A virus and murine norovirus, both associated with foodborne outbreaks. The leaves extract had a significant antiviral effect on both viruses, while the fruit extract had a moderate effect on the hepatitis A virus.

After establishing the phenolic profile, standard solutions were subjected to *in vitro* gastrointestinal digestion process to study their bioaccessibility. Simultaneously, the potentiality of three nanoparticulate systems (zein particles,

alginate-coated zein particles, and alginate/chitosan complex-coated zein particles) were evaluated, which were prepared by nanoprecipitation and electrostatic deposition methods. The bioaccessible fraction of the bioactive compounds was determined by high-performance liquid chromatography with UV detection (HPLC/UV). Phenolic compounds presented limited bioaccessibility, which varied between 3.4 and 16.1%, while the three nanoparticulate systems significantly increased the bioaccessibility of all phenolic compounds, especially the alginate/chitosan complex-coated zein particles, which ranged between 65.8 and 84.0%.

Finally, a scientific-technological platform was implemented by HPTLC/(bio) autography/MS to detect and identify in the raw fruit and leaves extracts compounds with antioxidant and inhibitory activity against α-glucosidase and acetylcholinesterase enzymes, considered as therapeutic targets to treat type 2 diabetes mellitus and neurodegenerative diseases, respectively.

The leaves extract only presented bands with antioxidant activity, while the fruit extract presented a compound with significant antioxidant and inhibitory activity on both enzymes, which was tentatively identified by MS as fraxetin.

These results support the functional characteristic of arrayán fruit and leaves and demonstrate the nanotechnology potential (nanoparticulate systems) to protect the compounds responsible for this functionality.

CAPÍTULO I: Introducción y objetivos

1. INTRODUCCIÓN

1.1 Aspectos generales

Actualmente vivimos una transición epidemiológica, ya que las causas de enfermedades y mortalidad comienzan a ser diferentes de aquellas que nos aquejaban hace cincuenta años atrás. Las enfermedades infecciosas, la desnutrición, y las enfermedades neonatales están en franco retroceso, para dar paso a las enfermedades crónicas no transmisibles (ECNT), que son enfermedades caracterizadas por presentar una larga duración con una lenta progresión. Las principales son las enfermedades del aparato circulatorio, la diabetes, el cáncer y las enfermedades respiratorias crónicas (Valenzuela et al., 2014).

Las ECNT, son la principal causa de muerte a nivel mundial y son responsables de poco más del 70% de las muertes en todo el mundo (41 millones de personas). Estas enfermedades comparten factores de riesgo conductuales modificables, como el consumo de tabaco, una dieta poco saludable, la falta de actividad física y el uso nocivo del alcohol, que a su vez conducen al sobrepeso y la obesidad, el aumento de la presión arterial y el colesterol, y en última instancia, a la enfermedad. Siguen siendo un importante desafío de salud pública en todos los países, incluidos los países de bajos y medianos ingresos

donde ocurren más de las tres cuartas partes de las muertes por ECNT (WHO, 2020). En Chile se observa la misma tendencia. Las muertes causadas por estas patologías superan el 85% del total de muertes, significando un gran costo económico para el país y para las personas que las contraen (Mendoza-Torres et al., 2019). La preocupación actual sobre la morbimortalidad de las ECNT, ha aumentado el interés de la comunidad científica en buscar compuestos bioactivos (funcionales) en alimentos y plantas que permitan hacer frente y disminuir el riesgo relativo de padecer las enfermedades anteriormente mencionadas (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b). Como resultado, muchas especies nativas/autóctonas han sido estudiadas por su potencial fuente de compuestos bioactivos. Dentro de estas especies reconocidas por sus propiedades nutritivas y medicinales se encuentra la quinua y el arrayán.

1.2 Quinua (*Chenopodium quinoa* Will)

La quinua es un pseudocereal cultivado desde la antigüedad en la zona andina de América del Sur, que posee la capacidad de adaptación para crecer en diferentes condiciones climáticas, incluidos los factores abióticos más perjudiciales como la sequía, la gran altitud, el calor y la salinidad. Las semillas de quinua son la principal parte comestible de este grano, que también es la porción más conocida y valorada (Angeli et al., 2020). En el último tiempo, esta

semilla ha mostrado un aumento significativo en su consumo debido a su alto valor nutricional y posibles beneficios para la salud relacionados con el contenido de compuestos fenólicos que posee (Fischer et al., 2013; Fischer et al., 2017; Noratto et al., 2019; van den Driessche et al., 2018). Estos compuestos han sido ampliamente estudiados debido a que su ingesta está asociada con algunos efectos beneficiosos para la salud como la reducción de padecer ciertos cánceres, alergias, enfermedades neurodegenerativas, inflamatorias y cardiovasculares (Gómez-Caravaca et al., 2014; Repo-Carrasco-Valencia et al., 2010). Por tal motivo, la comunidad científica ha desarrollado una gran variedad de métodos químico analíticos destinados a la extracción, detección y cuantificación de compuestos fenólicos en semillas de quinua (Alvarez-Jubete et al., 2010; Gómez-Caravaca et al., 2011; Hirose et al., 2010; Repo-Carrasco-Valencia et al., 2010), los cuales luego han servido de base para el estudio de compuestos bioactivos en especies nativas menos investigadas como el arrayán (capitulo II sección 2 y 3).

1.3 Arrayán (*Luma apiculata*)

El arrayán, también llamado arrayán chileno, quetri o temu es una especie arbórea siempre verde de la familia de las mirtáceas, que ha sido utilizada en el ámbito culinario y en la medicina tradicional como antiasmático, antidiarreico y antiséptico (Araya-Contreras et al., 2019). Es un árbol de hoja perenne que se

encuentra en el sur de Chile y Argentina, mide aproximadamente 10 metros de altura con un tronco de color rojo anaranjado (Puntieri et al., 2018). Sus hojas son simples, de forma redonda u ovalada; brillante por el haz y terminada en una espícula o mucrón (Figura 1) (Aguilera et al., 2006). Produce flores hermafroditas en grupos de 3 a 5 unidades, blancas y olorosas, de hasta dos centímetros de diámetro; la floración tiene lugar en el verano y es relevante para la producción de miel de bosque (Fuentes et al., 2016). Su fruto es una baya negra comestible de 1,0-1,5 cm de diámetro, que madura a principios de otoño (Figura 1). Debido a su intenso sabor y aroma, es utilizado para preparar "chicha", una bebida fermentada nativa de América del Sur (Brito et al., 2014). Tanto el fruto como la hoja presentan una importante capacidad antioxidante que podría relacionarse con la gran cantidad de compuestos fenólicos que poseen, principalmente un alto contenido flavonoides, antocianinas y ácidos fenólicos (Simirgiotis et al., 2013). Estudios *in vitro* han demostrado que esta clase de compuestos son muy efectivos en neutralizar especies reactivas del oxígeno, como en su capacidad de inhibir la oxidación de las lipoproteínas LDL y la agregación plaquetaria. Por tanto, muchos de los compuestos fenólicos presentes en el fruto y hoja de arrayán, presentarían una importante actividad sobre las ECNT (Ghiselli et al., 1998).

Figura 1. Fruto y hoja de Arrayán

1.3 Compuestos fenólicos

Algunos de los efectos farmacológicos del fruto y hoja de arrayán son atribuidos a la presencia de compuestos fenólicos, a los cuales se les atribuye la capacidad de disminuir el riesgo de padecer ECNT. Estos compuestos son metabolitos secundarios de las plantas, necesarios para su normal crecimiento, desarrollo y defensa. Protegen a la especie contra factores adversos que amenazan su supervivencia en un entorno desfavorable, como la sequía, radiación UV, infecciones o daños físicos (Asami et al., 2003). Estos compuestos pueden tener potenciales beneficios terapéuticos debido a su actividad antioxidante y efecto depurador de radicales libres. Aunque la mayor parte de la evidencia de la actividad antioxidante de los compuestos fenólicos se basa en estudios *in vitro*, numerosas publicaciones indican que pueden

actuar más allá de las funciones antioxidantes *in vivo* (Mozaffarian y Wu, 2018; Parkinson y Cicerale, 2016; Tsao, 2010).

1.3.1 Estructura y clasificación

Los compuestos fenólicos son uno de los grupos con anillos aromáticos más numerosos y ampliamente distribuidos en el reino vegetal, con más de 8000 estructuras fenólicas actualmente conocidos, de los cuales más de 6000 corresponden a flavonoides (Garcia-Salas et al., 2010).

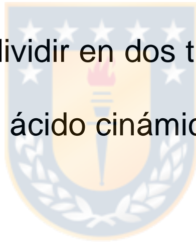
Existen varias clases y subclases de compuestos fenólicos que se definen en función del número de anillos fenólicos que poseen y de los elementos estructurales que presentan dichos anillos. De acuerdo con la función biológica, los compuestos fenólicos se pueden clasificar en diferentes clases. Sin embargo, se pueden identificar dos grupos principales: flavonoides y no flavonoides (Peña-Neira et al., 2007).

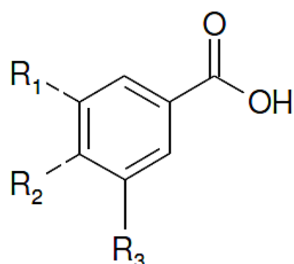
1.3.1.1 Compuestos fenólicos no flavonoides

Los no flavonoides se caracterizan por presentar un solo anillo fenólico. Pueden ser clasificados de acuerdo con su estructura química en los siguientes grupos:

ácidos fenólicos (derivados de los ácidos hidroxibenzoico e hidroxicinámico), estilbenos, lignanos y ligninas poliméricas (Zheng y Wang, 2001).

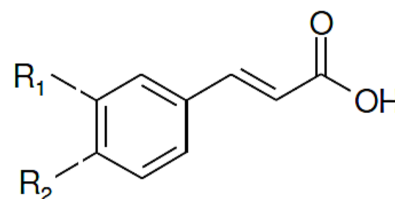
Ácidos fenólicos. Desde un punto de vista químico, los ácidos fenólicos contienen un grupo carboxilo con uno o más grupos hidroxilo unidos a un núcleo de benceno. Los ácidos fenólicos son los compuestos fenólicos más abundantes en la dieta humana. Se encuentran en los alimentos o plantas a la forma de aglicona (ácidos fenólicos libres), ésteres, glucósidos o unidos a complejos (Garcia-Salas et al., 2010). Según la posición del grupo hidroxilo, los ácidos fenólicos se pueden dividir en dos tipos, el ácido benzoico (C1-C6) y sus derivados y los derivados del ácido cinámico (C3-C6) (figura 2)(Tsao, 2010).





Ácido p-hidroxibenzoico (R_2 - OH)
Ácido gálico (R_1, R_2, R_3 - OH)
Ácido vanílico (R_2 - OH, R_3 - OCH_3)
Ácido siríngico (R_1, R_3 - OCH_3, R_2 - OH)

Ácidos hidroxibenzoicos



Ácido ferúlico (R_1 - OCH_3, R_2 - OH)
Ácido cafeico (R_1, R_2 - OH)

Ácidos hidroxicinámicos

Figura 2. Ejemplos representativos de ácidos fenólicos

Los ácidos hidroxibenzoicos más comunes en los alimentos son los ácidos vanílico, siríngico y gálico, mientras que los ácidos hidroxicinámicos más abundantes son los ácidos cafeico y ferúlico (Ndhlala et al., 2010).

Estilbenos. Los estilbenos son otra clase de compuestos que forman parte de los no flavonoides, teniendo como estructura básica el 1,2-difeniletileno. El resveratrol (Figura 3) es el principal representante de este grupo de compuestos, el cual existe en dos estereoisoformas, con configuración cis- o trans-, siendo esta última la más estudiada (Shrestha et al., 2019). El alto interés de este compuesto radica no sólo con su uso en el tratamiento de enfermedades cardiovasculares, sino también en la lucha contra deficiencias

motoras que conducen a problemas de movilidad en el adulto mayor (Kelsey et al., 2010)

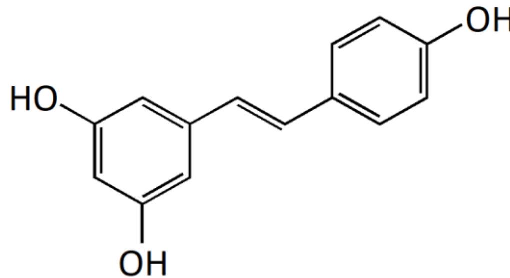


Figura 3. Estructura química de estilbeno (resveratrol)

Lignanos. Los lignanos son un grupo de compuestos fenólicos naturales formados por dos unidades de fenilpropano, enlazados por el átomo central de sus cadenas laterales (Figura 4). En la naturaleza, los lignanos están presentes en la forma de aglicona, mientras que sus glucósidos están presente solo en pequeñas cantidades (Den et al., 2018). Algunas actividades biológicas asociadas a estos compuestos incluyen actividad antihipertensiva, antioxidante, anticancerígena, antimicrobiana, antiinflamatoria, inmunosupresora y hepatoprotectora (Durazzo et al., 2018).

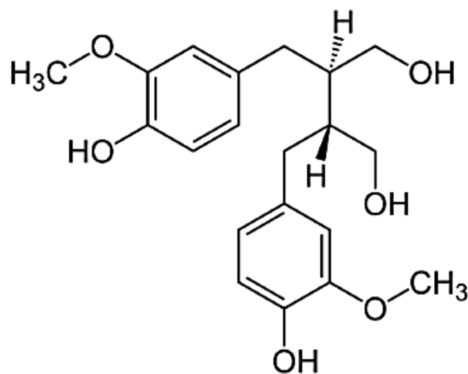


Figura 4. Estructura química de lignanos (Secoisolariciresinol)

Ligninas. Las ligninas son polímeros vegetales que comprenden entre el 16 y 33% de la biomasa de la madera y representan el segundo compuesto orgánico más grande después de la celulosa. La molécula de lignina tiene un alto peso molecular, que resulta de la combinación de varios ácidos fenilpropílicos y alcoholes (cumarílico, coniferílico y sinapílico) (Kawaguchi et al., 2016). Las ligninas no solo son una preciada materia prima para la biorefinería, sino también para la salud humana, debido a que posee múltiples propiedades, tales como antioxidante, antifúngica, antibiótica, anticancerígena, anti-VIH, entre otras (Popa et al., 2008).

1.3.1.2 Compuestos fenólicos flavonoides

Los flavonoides constituyen la subclase de polifenoles más abundante en el reino vegetal. Son compuestos de bajo peso molecular que comparten un esqueleto común difenilpirano (C6-C3-C6), compuesto por dos anillos fenilo (A y B) ligados a través de un anillo "C" de pirano heterocíclico (Figura 5). Todos los flavonoides son estructuras hidroxiladas en sus anillos aromáticos y por tanto, son estructuras polifenólicas (Quiñones et al., 2012).

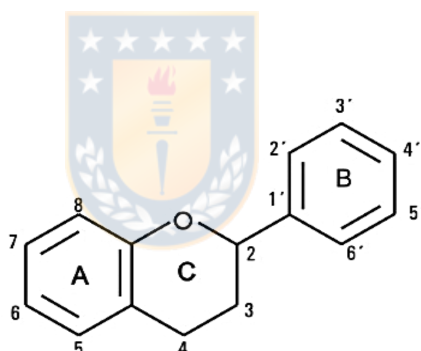


Figura 5. Estructura básica de los flavonoides (2-fenil-1-benzopirano)

Los flavonoides se encuentran principalmente como glucósidos, pero también pueden aparecer en la forma libre (aglicona). Además, se pueden presentar como sulfatos, dímeros o polímeros. Los glucósidos se pueden encontrar como C-glucósidos con los carbohidratos ligados a través de enlaces carbono-

carbono o como O-glucósidos con los carbohidratos ligados a través de átomos de oxígeno (enlace hemiacetal), siendo este último el mayoritario (Tapas et al., 2008).

Existen varios subgrupos de flavonoides. La clasificación de estos se hace en base al grado de oxidación del anillo heterocíclico "C" y la posición del anillo "B". La gran variedad de compuestos existentes en cada familia se diferencian por el número de grupos hidroxilos, su posición y los distintos grupos funcionales (azúcares, metilos, ácidos orgánicos). Según el estado de oxidación del anillo C, los flavonoides se dividen en seis subgrupos: flavonoles, flavanoles, isoflavonas, flavonas, flavanonas y antocianidinas (Figura 6) (Dai y Mumper, 2010).

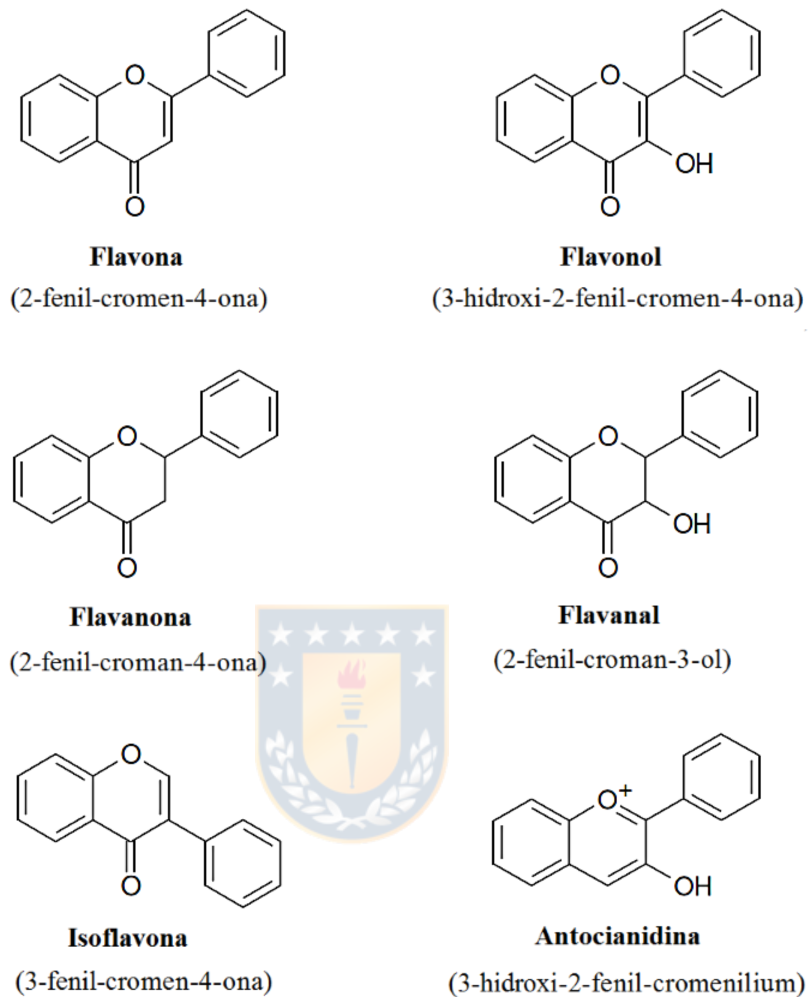


Figura 6. Estructura química de algunas subclases representativas de flavonoides

Flavonas y flavonoles. Las flavonas se caracterizan por la presencia de un doble enlace entre C2 y C3 en el heterociclo del esqueleto flavánico. Las

flavonas más estudiadas son la apigenina, la luteolina y sus derivados (Jiang et al., 2016). Los flavonoles tienen como columna vertebral la 3-hidroxiflavona, caracterizada precisamente por la presencia de un grupo hidroxilo en posición C3. Se encuentran en muchas frutas y verduras, incluyendo cebollas, manzanas, brócoli y bayas. Los flavonoles más importantes y estudiados son tres: kaempferol, quercetina y miricetina (Aherne y O'Brien, 2002).

Flavanonas y flavanoles. En comparación con los flavonoles y flavonas, estos dos grupos se caracterizan por la ausencia del doble enlace entre C2 y C3 y tener como precursor la 2-fenil-benzopirona. La principal fuente de flavanonas y flavanoles son los frutos y jugos cítricos. Entre las principales flavanonas podemos mencionar: hesperetina, naringina, pinocembrina y eriodictiol (Peterson et al., 2006). Los flavanoles también llamados flavan-3-oles existen como monómeros simples u oligómeros. Respecto a los primeros, sus representantes son la (+)-catequina y (-)-epicatequina, mientras que los segundos son llamados proantocianidinas, debido a que liberan antocianidinas cuando se calientan en soluciones ácidas (De Pascual-Teresa et al., 2010).

Antocianinas. Las antocianinas son la principal clase de flavonoides responsables del color rojo, azul o morado de la mayoría de las frutas, hojas y flores (Cheynier, 2012). Químicamente son glucósidos de las antocianidinas (aglicona) a la que se le une un azúcar por medio de un enlace β-glucosídico.

Los azúcares más comunes son la glucosa y la ramnosa, seguidos de la galactosa, xilosa y arabinosa (Yousuf et al., 2016). La estructura básica de estas agliconas es el ion flavilio (Figura 7), conformado por dos grupos aromáticos: un benzopirilo “A” y un anillo fenólico “B” (Ignat et al., 2011). Las diferencias entre estos compuestos se encuentra en el número de grupos hidroxilos y el grado de metilación de los anillos unidos al ion flavilio, el número, naturaleza y posición de los azúcares unidos a la molécula, y en la naturaleza y número de ácidos alifáticos o aromáticos unidos al azúcar (Yousuf et al., 2016). Estos compuestos presentan una estructura química adecuada para actuar como antioxidantes al donar hidrógenos o electrones a los radicales libres (Castañeda-Ovando et al., 2009; Zhao et al., 2010). El interés de estos pigmentos se ha intensificado recientemente por sus propiedades farmacológicas y terapéuticas. Los efectos terapéuticos de las antocianinas están relacionados principalmente con su capacidad antioxidante. Estos compuestos bioactivos permanecen intactos (en mamíferos) al pasar del tracto digestivo al torrente sanguíneo, ejerciendo efectos terapéuticos conocidos, que incluyen la reducción de la enfermedad coronaria, efectos anticancerígenos, antitumorales antidiabéticos y antiinflamatorios; además del mejoramiento del comportamiento cognitivo y agudeza visual (Castañeda-Ovando et al., 2009; Miyazawa et al., 1999; Yousuf et al., 2016).

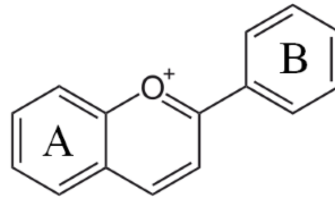


Figura 7. Estructura química del ion flavilio

Isoflavonas. Presentes abundantemente en vegetales, las isoflavonas son un grupo de compuestos derivado de las flavanonas. Tienen en común la presencia de una estructura derivada de la 3-fenil-benzopiran-4-ona (Zaheer y Humayoun Akhtar, 2017). Las isoflavonas también son llamadas fitoestrógenos, debido a que su estructura es análoga al estrógeno (Ignat et al., 2011). Las principales isoflavonas vegetales son la genisteína, la daidzeína, gliciteína y sus derivados glucosilados y metoxilados. Entre estos derivados, se han identificado la genistina y la biochanina A (precursores de la genisteína) y la daidzina y la formononetina (precursores de la daidzeína) (Zaheer y Humayoun Akhtar, 2017).

1.4 Actividad biológica de los compuestos fenólicos

Los compuestos fenólicos son conocidos por su rol en la regulación del sistema inmune, su efecto antiinflamatorio, quimioprevención, neuroprotección, cardioprotección y en el tratamiento de enfermedades como la diabetes, la enfermedad de Parkinson y el cáncer (Proestos et al., 2005; Tanase et al., 2019). Los flavonoides en particular, muestran una amplia gama de efectos biológicos, que incluyen actividad antibacteriana, antiviral, antialérgica, antiinflamatoria, antitrombótica, vasodilatadora y antioxidante (Siddhuraju y Becker, 2003; Toscano-Garibay et al., 2017). La actividad antioxidante de los compuestos fenólicos es el origen de las diversas funciones biológicas mencionadas anteriormente, por tanto, un aumento en el consumo de antioxidantes fenólicos naturales, se ha asociado con una mayor esperanza de vida (Granato et al., 2018).

1.5 Actividad antioxidante de los compuestos fenólicos

Los compuestos fenólicos, son sustancias con uno o más grupos hidroxilo unidos al anillo bencénico. Esta característica estructural proporciona al fenol un carácter ácido más fuerte que la mayoría de los alcoholes. Esta reactividad química es responsable del carácter antioxidante de los compuestos en cuestión (Kim et al., 2019; Subramanian et al., 2015). Los antioxidantes se

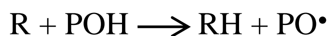
definen como compuestos que pueden retrasar, inhibir o prevenir la oxidación de materiales susceptibles a la oxidación, eliminando radicales libres y disminuyendo el estrés oxidativo. Este último, es un estado de desequilibrio, en el cual las cantidades de especies reactivas de oxígeno (ERO) y/o nitrógeno (ERN) (anión superóxido, peróxido de hidrógeno, radical hidroxilo y peroxinitrilo) superan la cantidad de moléculas antioxidantes endógenas, conduciendo a la oxidación de biomacromoléculas como enzimas, proteínas, ADN y lípidos. El estrés oxidativo es importante puesto que se ha relacionado con el desarrollo de enfermedades crónicas degenerativas, incluyendo las ECNT (Ames et al., 1993).

Recientemente, los compuestos fenólicos han sido considerados poderosos antioxidantes in vitro, demostrando ser incluso más potentes que las vitaminas C, E y los carotenoides (Farhoosh et al., 2016; Rice-Evans et al., 1995). La relación inversa entre ingesta de frutas y verduras y el riesgo de enfermedades asociadas al estrés oxidativo, como las enfermedades cardiovasculares, cáncer u osteoporosis se han atribuido parcialmente a los compuestos fenólicos (Scalbert et al., 2005; Williamson et al., 2018). Las propiedades antioxidantes de los compuestos fenólicos pueden estar mediadas por los siguientes mecanismos: (1) eliminación de especies radicales como ERO y/o ERN; (2) suprimir la formación de ERO y/o ERN, inhibiendo algunas enzimas o quelando metales traza implicados en la producción de radicales libres; (3) regulación

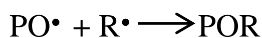
positiva (relación entre compuestos antioxidantes y compuestos oxidantes) (Suen et al., 2016).

1.5.1 Compuestos fenólicos como secuestradores de radicales libres y quelante de metales

Los compuestos fenólicos (POH) actúan como aceptores de radicales libres, interrumpiendo la reacción en cadena. Interfieren con la oxidación de lípidos y otras moléculas por donación de un átomo de hidrógeno a radicales (R):



Los radicales intermediarios fenoxi (PO•) son estabilizados por resonancia, por tanto, disminuye la probabilidad de iniciar una nueva reacción en cadena. Además, los radicales intermedios fenoxi también actúan como terminadores de la ruta de propagación al reaccionar con otros radicales libres:



La actividad de eliminación de radicales libres de los compuestos fenólicos se sustenta en su estructura química ya que presentan: (1) grupos hidroxilo fenólicos que son propensos a donar un átomo de hidrógeno o un electrón a un radical libre; (2) sistema aromático conjugado extendido para deslocalizar un electrón desapareado (Bors y Michel, 2002). Como propiedad antioxidante alternativa, algunos compuestos fenólicos con grupos di-hidroxi pueden conjugar metales de transición, evitando la formación de radicales libres

inducidos por metales. Los iones metálicos con propiedades redox como el Cu^{+} o Fe^{2+}, pueden interactuar con el peróxido de hidrógeno (H_2O_2), formando radicales hidroxilo ($OH^{\bullet}$) capaces de iniciar reacciones en cadena de radicales libres al extraer un hidrógeno de casi cualquier molécula. En este contexto, los compuestos fenólicos pueden inhibir la formación de radicales de oxígeno inducidos por metales, ya sea por coordinación con el ion Fe^{2+} (y mejorando la autooxidación de Fe^{2+}) o por la formación de complejos inactivos con Cu^{2+}, Fe^{2+} o Cu^{+} (Perron y Brumaghim, 2009). Teóricamente, estas dos acciones antioxidantes pueden causar una reducción en la cantidad de radicales libres y especies oxidantes, obteniendo como resultado una disminución en la oxidación de moléculas diana como lípidos, proteínas y ácidos nucleicos (Santos-Sánchez et al., 2019).

1.5.2 Métodos para evaluar la actividad antioxidante de compuestos fenólicos

1.5.2.1 Actividad secuestrante de radicales libres mediante el uso del radical DPPH

El método DPPH es el ensayo más utilizado para evaluar la capacidad de captura de radicales libres mediante los extractos de plantas. El mecanismo de

reacción implica la transferencia de un hidrógeno o electrón desde un compuesto fenólico al radical 2,2- difenil-1- picrilhidrazilo ($DPPH^{\bullet}$). El radical tiene una coloración púrpura que disminuye progresivamente cuando interacciona con compuestos fenólicos, efecto que puede ser monitoreado y cuantificado espectrofotométricamente a 515 nm (Mishra et al., 2012). La actividad antioxidante de los compuestos fenólicos es generalmente cuantificada por sus valores de EC_{50} (concentración necesaria para reducir el 50% de los radicales DPPH), estequiometría (moléculas de $DPPH^{\bullet}$ reducidas por una molécula de antioxidante)(Goupy et al., 2009; Vučić et al., 2013), o como micromoles equivalentes de estándar Trolox (Faria et al., 2005).

1.5.2.2 Método Folin-Ciocalteu

El método de Folin-Ciocalteu (FC) se basa en la capacidad de los compuestos fenólicos para reaccionar con agentes oxidantes mediante un mecanismo redox. Es utilizado para cuantificar el contenido de compuestos fenólicos totales en extractos de material vegetal, usando generalmente ácido gálico como estándar para todos los compuestos fenólicos presentes. Dado que su mecanismo es una reacción de óxido-reducción, el método de FC puede considerarse también un método para cuantificar la capacidad antioxidante (Craft et al., 2012). El reactivo de FC está formado por una mezcla de ácido fosfotúngstico y ácido fosfomolíbdico, el cual es reducido por los compuestos

fenólicos a óxidos azules de tungsteno y molibdeno, respectivamente. Estos compuestos coloreados tienen un máximo de absorbancia a 760 nm, la cual es proporcional a la cantidad total de compuestos fenólicos presentes en la muestra (López-Froilán et al., 2018).

1.5.2.3 Otros métodos

Otros métodos populares para evaluar la actividad antioxidante de los compuestos fenólicos son la capacidad de absorción de radicales de oxígeno (ORAC), capacidad antioxidante reductor de ion cúprico (CUPRAC), capacidad antioxidante reductor del hierro (FRAP) y capacidad antioxidante equivalente de Trolox (TEAC) (Pisoschi y Negulescu, 2011). El método ORAC es un ensayo que mide la capacidad de un compuesto para atrapar el radical peroxilo ($ROO^{\bullet}$) mediante un mecanismo de transferencia de un átomo de hidrógeno. En el protocolo básico del ensayo ORAC, los radicales peroxilo generados por iniciadores de radicales libres, reaccionan con una sonda fluorescente para formar un producto no fluorescente; es decir, a medida que avanza la reacción, la sonda fluorescente se consume y disminuye la fluorescencia. El antioxidante existente en el medio compite con la sonda fluorescente, manteniendo la fluorescencia. La mantención o disminución de este parámetro, es lo que se registra para determinar la capacidad antioxidante del medio (Prior et al., 2005). Las ventajas de este método incluyen no solo la elevada sensibilidad de los

métodos fluorescentes frente a los métodos espectrofotométricos, sino también el hecho de que permite medir el grado de inhibición de la fluorescencia ejercido por el antioxidante, así como el tiempo necesario para conseguirlo (Huang et al., 2002; Pisoschi y Negulescu, 2011).

Los métodos CUPRAC, FRAP Y TEAC son ensayos basados en transferencia de electrones y monitoreados por un cambio de color cuando los compuestos Cu^{2+}-neocuprina, Fe^{3+}- 2,4,6-tri(2-piridil)-s-triazina y radical $ABTS^{\bullet +}$ (ácido 2,2-azinobis-3etil benzotioazolín-6-sulfónico) son reducidos, respectivamente (Dai y Mumper, 2010).

1.6 Extracción de compuestos fenólicos

La gran importancia de los compuestos fenólicos ha dado lugar a un gran número de estudios enfocados a su extracción desde alimentos de origen vegetal, en especial desde frutas y vegetales presentes en la dieta (Aguilera et al., 2016). Sin embrago, debido a la variedad de compuestos fenólicos existentes y su capacidad para unirse a otros compuestos orgánicos, resulta difícil desarrollar un proceso capaz de recuperar todos los compuestos fenólicos presentes en una matriz. El proceso extractivo está influenciado principalmente por la naturaleza química de los compuestos diana, el método de extracción y las condiciones de extracción. La naturaleza y composición de los compuestos fenólicos a ser extraídos determinan la selección de las condiciones de

extracción (Haminiuk et al., 2012). Entre los factores más importantes que afectan la extracción de los compuestos fenólicos se tienen el disolvente, la agitación, la relación disolvente/sólido, el tamaño de partícula, pH del medio, temperatura, energía externa aplicada y tiempo de extracción (Haminiuk et al., 2012).

1.6.1 Variables de extracción

Agitación. La eficiencia del proceso extractivo es función del equilibrio de saturación del disolvente. La agitación provoca que las nuevas cantidades de disolvente, pobre en sustancias extraíbles, entren en contacto con el sólido y un nuevo punto de equilibrio de saturación sea alcanzado. El movimiento del líquido desplaza el equilibrio en el sentido de la saturación del disolvente, aumentando la eficiencia del proceso (Azmir et al., 2013)

Disolvente de extracción. La extracción con disolventes es una operación de separación que consiste en aplicar un disolvente para extraer o separar un componente deseado (soluto) de un alimento o material vegetal. En principio, un disolvente ideal debe presentar una alta capacidad de extracción de soluto, ser selectivo, disolver en gran medida al componente de interés y tener una capacidad mínima para disolver a los demás componentes, debe ser químicamente estable, presentar una baja viscosidad para facilitar la

transferencia de masa del analito desde la fase solida hacia el disolvente, y presentar una polaridad que le permita extraer compuestos afines (Mustafa y Turner, 2011). En relación a los compuestos fenólicos, se ha encontrado que el metanol es uno de los solventes más eficientes en la extracción de compuestos de bajo peso molecular, mientras que los compuestos de mayor peso molecular se extraen mejor con acetona acuosa o etanol, siendo este último considerado un solvente GRAS (*Generally Recognized As Safe*) (Dai y Mumper, 2010), representando además sus residuos una menor carga ambiental.

Temperatura y tiempo de extracción. La extracción también puede mejorarse aumentando la temperatura del disolvente. De la misma manera que la agitación, la temperatura contribuye al desplazamiento de la constante de equilibrio de saturación aumentando la eficiencia del proceso. Además, un aumento de temperatura suele producir una disminución de la viscosidad y tensión superficial del disolvente, facilitando su penetración en la matriz y generando una mejor tasa de extracción. Respecto al tiempo de extracción, este debe ser suficiente para permitir la separación de los compuestos de interés. Sin embargo, muchos compuestos fenólicos se hidrolizan o son oxidados fácilmente al utilizar altas temperaturas y largos tiempos de extracción, lo que conllevaría a una menor presencia de compuestos fenólicos en los extractos. Por tanto, es de suma importancia analizar y optimizar estas

variables para mantener la estabilidad de los compuestos de interés (Biesaga y Pyrzyńska, 2013; Davidov-Pardo et al., 2011)

Relación disolvente/sólido. La relación disolvente/solido es uno de los parámetros más relevantes y estudiados en un método de extracción sólido-líquido, ya que repercute directamente en la eficiencia de extracción del analito de interés. La forma más sencilla de llevar a cabo una extracción sólido-líquido es poner en contacto el disolvente con el sólido a tratar y separar luego el sobrenadante (disolución formada) del sólido residual insoluble. Esta operación recibe el nombre de "contacto simple". Si la cantidad total de disolvente que se va a utilizar en el proceso extractivo se subdivide en varias fracciones y el sólido es extraído sucesivamente con cada una de ellas se habla de "contacto múltiple", lo que mejora la recuperación del soluto, pero en disoluciones relativamente diluidas (Cardenas-Toro et al., 2015; Patel et al., 2019; Vorobiev y Lebovka, 2020). Según los principios de transferencia de masa, una mayor relación disolvente/sólido producirá en la mayoría de los casos un aumento en la extracción de los compuestos de interés. No obstante, tomando en consideración que el costo de utilización de disolvente repercute de manera directa en el costo del proceso extractivo y que un mayor volumen de disolvente significará probablemente un proceso de pre-concentración del analito, se hace imperioso investigar y optimizar la relación disolvente/sólido a utilizar (Bucić-Kojić et al., 2007).

Tamaño de partícula. El rendimiento de extracción de compuestos fenólicos puede verse afectado por las variaciones en el tamaño de partícula. La transferencia de masa en una extracción se puede mejorar si se utiliza un tamaño de partícula pequeño, debido a que facilita la penetración del disolvente en la matriz sólida. No obstante, se ha informado que un tamaño de partícula muy pequeño produce coeficientes de variación altos en el resultado por un posible efecto de aglomeración de partículas (Bucić-Kojić et al., 2007).

pH del medio. En la extracción de compuestos fenólicos, el pH puede tener un rol preponderante en su desempeño. Por lo general, el pH bajo (ácido) afecta favorablemente el proceso extractivo, ya que se aumenta la estabilidad de los compuestos fenólicos (principalmente de antocianinas), su solubilidad y la desintegración de las paredes celulares, facilitando la difusión de los compuestos del sólido al disolvente (Karvela et al., 2009). Sin embargo, la concentración del ácido en el disolvente de extracción debe ser baja, ya que la adición en exceso, puede escindir los grupos acilados y de azúcar de los compuestos fenólicos durante las etapas de extracción, lo que resulta en la formación de componentes indeseables (Putnik et al., 2016). Generalmente, el disolvente es acidificado a una concentración del 1%, utilizando ácido clorhídrico, ácido acético, ácido fórmico u otros ácidos (Galvan d'Alessandro et al., 2012).

1.6.2 Principales técnicas de extracción

Tradicionalmente, la extracción de compuestos fenólicos se fundamentaba únicamente en la extracción con diferentes solventes. Estos solventes en la mayoría de los casos eran éter, hexano, cloroformo, benceno, acetonitrilo, metanol y etanol. Todos ellos, a excepción del etanol, son tóxicos para el humano y peligrosos para el medioambiente. Actualmente se sigue utilizando la extracción con solventes pero se intenta, cada vez más, utilizar otros sistemas de extracción, promoviendo la utilización de solventes de grado alimentario reconocidos como GRAS (Gil-Chávez et al., 2013).

A continuación, se describe los diversos métodos y tecnologías de extracción utilizadas para la extracción de compuestos fenólicos.

1.6.2.1 Métodos clásicos de extracción

Los métodos clásicos de extracción con disolventes son los procedimientos más utilizados para preparar extractos de material vegetal debido a su facilidad de uso y amplia aplicabilidad. Los solventes más utilizados para llevar a cabo dicho proceso son el metanol, etanol, acetona, acetato de etilo, agua y combinaciones de estos. Entre los métodos convencionales de extracción más utilizados se tiene la maceración y la extracción Soxhlet (Xu y Chang, 2007).

Maceración. El proceso de maceración consiste en poner en contacto el material vegetal a extraer con el solvente de extracción durante varias horas o días. Se trata de un proceso que da como resultado un equilibrio de concentración entre los compuestos extraídos y el solvente, y depende de factores intrínsecos del material como su naturaleza, humedad, tamaño de partículas y factores relacionados con el disolvente, como su selectividad, viscosidad y cantidad (Azwanida, 2015). Este proceso conocido como maceración simple se caracteriza por ser demasiado lento. Para abreviar el tiempo de extracción, el material vegetal y el solvente pueden mantenerse en movimiento mediante agitación, procedimiento conocido como maceración dinámica. Las grandes desventajas del proceso de maceración son la lentitud del proceso y el hecho de no ser posible la extracción completa de los compuestos de interés (Azmir et al., 2013).

Extracción Soxhlet. La extracción Soxhlet ha sido utilizada ampliamente para la extracción de compuestos bioactivos desde diversas fuentes naturales. Se utiliza como modelo para la comparación de nuevas alternativas de extracción. Generalmente, consiste en colocar una pequeña cantidad de muestra seca en un dedal. El dedal se coloca luego en un matraz de destilación que contiene el disolvente seleccionado para llevar a cabo la extracción. Después de alcanzar un nivel de desbordamiento (producto de la destilación continua del disolvente), la solución del dedal es aspirada por un sifón, el cual descarga la solución que

contiene los compuestos de interés al matraz de destilación. Los analitos extraídos quedan en el matraz de destilación y el disolvente vuelve al dedal que contiene la muestra. El proceso se ejecuta repetidamente hasta que se completa la extracción (Azmir et al., 2013). Por lo general, este método requiere una menor cantidad de solvente en comparación con la maceración. Sin embargo, la extracción Soxhlet conlleva desventajas, como la exposición a sustancias peligrosas y disolventes orgánicos inflamables, con posibles emisiones tóxicas durante el proceso extractivo. Los disolventes utilizados en el sistema de extracción deben ser de alta pureza, lo que se traduce en un mayor costo. Este método, al igual que la maceración, no es considerado amigable con el medio ambiente (Azwanida, 2015).

Considerando las principales desventajas de los métodos convencionales de extracción, en el último tiempo han sido desarrollados métodos modernos de extracción o métodos no convencionales.

1.6.2.2 Métodos modernos de extracción

Las grandes desventajas de los métodos clásicos de extracción, ha impulsado la implementación de tecnologías novedosas de extracción asistidas por diversos mecanismos como el ultrasonido, la energía de microondas, fluidos supercríticos, campos eléctricos pulsados, alta presión hidrostática y acelerada por disolventes(Bendicho et al., 2012; Li et al., 2017; Wen et al., 2020). El

principal propósito de estas tecnologías es reducir el consumo de energía y tiempo, que es reflejado en la reducción del costo, por lo que han sido denominadas como sustentables, ya que protegen tanto al medio ambiente como a la salud de los consumidores, potenciando la competitividad económica e innovadora en el ámbito industrial (Armenta et al., 2015).

Extracción asistida por microondas (EAM). La extracción asistida por microondas se basa en el uso de energía de microondas para conseguir que los compuestos de interés pasen de la muestra (generalmente sólida) a un disolvente. Esta técnica presenta una reducción significativa del tiempo de extracción, obteniendo recuperaciones similares a las obtenidas con técnicas tradicionales (Kala et al., 2016; Mirzadeh et al., 2020). Cuando la extracción se lleva a cabo en sistemas cerrados, la temperatura alcanzada es mayor que la correspondiente de los puntos de ebullición de los disolventes a presión atmosférica, lo que da lugar a altas presiones (~200 psi). Ambos factores, la temperatura y la presión, inciden en la eficacia y velocidad de extracción, acortando el tiempo de extracción y aumenta la eficiencia de la misma. La alta temperatura alcanzada por el disolvente aumenta la solubilidad del analito y su desorción desde la matriz. Además, la difusión de metabolitos por aplicación de microondas resulta en un aumento de la permeabilidad y reblandecimiento del tejido material. Esto conlleva un aumento de la transferencia de masa debido a la alta penetración del disolvente en las estructuras celulares reblandecidas y

dañadas (Routray y Orsat, 2012). Se ha vuelto muy popular en la última década debido a la reducción del tiempo de extracción y el disolvente utilizado (Azmir et al., 2013; Kala et al., 2016; Li et al., 2017). La extracción asistida por microondas ofrece ventajas sobre las tecnologías utilizadas corrientemente, como un menor consumo de energía, menores volúmenes de disolventes utilizados durante la extracción y menor cantidad de residuos generados (Azwanida, 2015).

Extracción asistida por ultrasonido (EAU). La extracción asistida por ultrasonido es una de las técnicas más utilizadas en la industria para mejorar los fenómenos de transferencia de masa. Se basa en el fenómeno de la cavitación dado por la formación, crecimiento y colapso de burbujas de vapor a causa de la acción del campo ultrasónico dentro de un líquido. El tiempo de vida de las burbujas es del orden de los microsegundos, cuya implosión violenta genera, de manera localizada y transitoria, elevadas temperaturas (5000 °C) y presiones (100 MPa), contribuyendo a la erosión de la superficie del material vegetal y a la ruptura de las partículas en la superficie erosionada. Estos efectos físicos incrementan la permeabilidad del tejido vegetal, facilitando la liberación de las sustancias intracelulares en el disolvente de extracción (Arora et al., 2004; Gallo et al., 2018; Oroian y Escriche, 2015). La irradiación ultrasónica puede ser aplicada de dos maneras: por contacto directo con la muestra (sondas de ultrasonido), o a través de las paredes del recipiente que

contiene la muestra (indirecta), como los sistemas de baño ultrasónico. La principal desventaja del baño de ultrasonido, es que generalmente funcionan a una sola frecuencia (20 o 40 kHz), mientras que la sonda ultrasónica, al estar en contacto directo con la muestra, puede desarrollar una potencia de hasta 100 veces mayor que la proporcionada por el baño ultrasónico (Picó, 2013). Sin embrago, con ambas modalidades, se acorta el tiempo de extracción debido a que se produce un incremento en la presión, que favorece la penetración y el transporte de los analitos, mientras que el incremento de la temperatura aumenta la solubilidad de los compuestos y favorece la difusividad (Azmir et al., 2013; Wen et al., 2020).

Extracción asistida por campos eléctricos pulsados (ECEP). Esta técnica consiste en colocar la materia prima entre un set de electrodos que envuelven una cámara de tratamiento, suministrando a este último, pulsos eléctricos de elevado voltaje. El tratamiento puede ser realizado a temperatura ambiente o de refrigeración y los tiempos de aplicación de las descargas se encuentran en el orden de los microsegundos. El mecanismo de acción se basa en la destrucción de la pared celular cuando se aplica una intensidad de campo eléctrico debido a la diferencia de potencial entre ambos lados de la membrana. Cuando esta diferencia de potencial alcanza un valor crítico ocurre la electroporación, que corresponde a la formación de poros en la pared celular que trae como consecuencias pérdida de su integridad, incremento de la permeabilidad y

destrucción celular, mejorando a través de estos mecanismos las tasas de transferencia de masa del analito (Oroian y Escriche, 2015). Los cambios texturales al aplicar campos eléctricos pulsados producen una mejora de la suavidad de las estructuras celulares, permitiendo una mejor penetración del disolvente de extracción y que junto a la electroporación mejora la liberación de sustancias intracelulares en el disolvente (Soliva-Fortuny et al., 2009).

Extracción a alta presión hidrostática (EAPH). Es una técnica de proceso en frío consistente en someter la materia prima a altos niveles de presión hidrostática (transmitida por el agua) durante unos segundos a minutos, ocupando además poco solvente. En esta técnica, después de que el líquido llena los huecos de aire presente en el tejido vegetal, el aire ocluido en los poros es liberado por la alta presión hidrostática, causando daño en la membrana celular. Este fenómeno genera un aumento de la transferencia de masa debido al aumento de la permeabilidad celular (Oroian y Escriche, 2015).

Extracción con líquidos presurizados (EFP). Es una técnica que combina la extracción con disolventes a temperatura (50-200°C) y presiones (15000-2000 psi) elevadas para extraer rápida y eficazmente analitos de matrices sólidas. Esto resulta en tiempos de extracción más cortos y en una reducción significativa del uso (volumen) de disolvente. Las tecnologías emergentes de líquidos presurizados utilizan solventes ecológicos y respetuosos con el medio

ambiente, como etanol acuoso presurizado y agua caliente presurizada. La extracción con agua caliente a presión se ha convertido en un método de extracción verde popular para diferentes clases de compuestos presentes en numerosos tipos de matrices, como muestras ambientales, alimentarias y botánicas. El principio de esta tecnología se basa en el hecho de que la tensión superficial, la viscosidad y la constante dieléctrica del agua se acercan a las de los disolventes orgánicos a medida que la temperatura sube por encima de su punto de ebullición. Por lo tanto, se puede extraer eficientemente y a bajo costo una amplia gama de analitos de polaridad media y baja, evitando el uso de solventes orgánicos (Chen et al., 2007; Teo et al., 2010). Por lo general, es una técnica más rápida que la extracción asistida por ultrasonidos y otros métodos modernos de extracción (Sun et al., 2012). Se consigue una mayor eficiencia en la extracción debida a dos mecanismos (Azwanida, 2015; Sun et al., 2012):

a) Solubilidad y transferencia de masa: el trabajo a temperatura elevada provoca un aumento en la solubilidad del analito en el disolvente, una rápida difusión y una mejor transferencia de masa.

b) Interrupción del equilibrio en superficie: las temperaturas elevadas anulan las interacciones soluto-matriz causadas por fuerzas de Van der Waals, puentes de hidrógeno y atracción dipolar. La disminución de la viscosidad y tensión superficial del solvente, provocan una mayor penetración de este en la matriz.

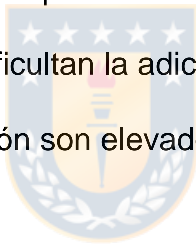
Extracción con fluidos supercríticos (EFS). Un fluido supercrítico es cualquier sustancia que se encuentra a una temperatura y presión por encima de su punto crítico termodinámico. Tiene la propiedad de difundir como un gas, y de disolver como un líquido. Además, puede cambiar rápidamente su densidad con pequeños cambios en presión o temperatura. Estas características lo convierten en un excelente sustituto de los solventes orgánicos utilizados en los procesos de extracción (Brunner, 2005). El CO_2 es el fluido supercrítico más utilizado debido a que no es tóxico, ni corrosivo, ni inflamable, incoloro, no es costoso, se elimina fácilmente, no deja residuos, sus condiciones críticas son relativamente fáciles de alcanzar, se puede trabajar a baja temperatura y por tanto, se pueden extraer compuestos termolábiles (Rosa y Meireles, 2005). Las principales ventajas de los fluidos supercríticos son las siguientes (Brunner, 2005; Khaw et al., 2017):

- Poseen un alto coeficiente de difusión y una viscosidad más baja que los líquidos.
- No presentan tensión superficial, lo cual mejora el proceso extractivo dada la rápida penetración de estos fluidos al interior de la matriz.
- La selectividad del proceso extractivo puede ser manipulada variando las condiciones de operación (temperatura y presión), afectando la solubilidad de los componentes en el fluido supercrítico.
- La extracción con fluidos supercríticos no deja residuos químicos

- La extracción con CO_2 supercrítico permite su fácil recuperación mediante procesos de reciclaje.

A pesar de las numerosas ventajas, esta técnica también presenta algunos inconvenientes, tales como (Lang y Wai, 2001):

- El equilibrio de fases entre el soluto y el solvente puede ser muy complejo
- Disuelve pocos compuestos de naturaleza no polar
- Los co-solventes utilizados para alterar la polaridad del CO_2, pueden quedar en el extracto, requiriendo una operación de separación posterior
- Las altas presiones dificultan la adición continua de sólidos al extracto
- Los costos de operación son elevados.



1.7 Aislamiento y purificación de compuestos fenólicos

Actualmente, existen numerosas técnicas a disposición para llevar a cabo el aislamiento y purificación de compuestos fenólicos. Una alternativa adecuada para el aislamiento y purificación de compuestos fenólicos es la cromatografía en contracorriente de alta velocidad (HSCCC), que corresponde a una cromatografía de reparto líquido-líquido compuesto por dos fases inmiscibles sin matriz de soporte. Presenta un bajo riesgo de desnaturalización de muestra, sin adsorción irreversible, gran capacidad de carga, recuperación total de la

muestra y bajo costo (Dai et al., 2013). Otros métodos utilizados para este propósito son la cromatografía líquida de alta eficiencia (HPLC) a escala semi- y preparativa, la ultrafiltración, la cromatografía de filtración en gel y la cromatografía de intercambio iónico (Lapsongphon y Yongsawatdigul, 2013).

Una técnica sencilla de utilizar es la cromatografía en columna abierta utilizando resinas poliméricas macroporosas de adsorción de distinta polaridad, las cuales han sido ampliamente utilizadas en el aislamiento y purificación de compuestos bioactivos a partir de extractos naturales debido a sus propiedades y ventajas de adsorción únicas que incluyen una estructura de poro ideal y varios grupos funcionales de superficie disponibles, bajo costo de operación, bajo consumo de solvente y fácil regeneración (Dahui et al., 2011; Jia y Lu, 2008; Lin et al., 2012). Son muy útiles para eliminar de extractos crudos azúcares y ácidos orgánicos, generalmente presentes en frutos mediante lavado con soluciones acuosas, recuperando luego los compuestos retenidos con un solvente apropiado como etanol.

1.8 Métodos para la determinación de compuestos fenólicos

A pesar de la gran cantidad de investigaciones publicadas, la determinación de varios grupos estructurales de compuestos fenólicos sigue siendo una tarea compleja (Ignat et al., 2011; Tsao y Yang, 2003). La cromatografía líquida asociada a espectrometría de masas (LC-MS), con frecuencia precedida de

detección UV-vis mediante detector de fila de fotodiodos (DAD), es hoy la técnica comúnmente aplicada para la determinación de compuestos fenólicos en diversas matrices vegetales. Otras técnicas relevantes para dicho fin son los ensayos espectrofotométricos (Xu et al., 2017).

1.8.1 Ensayos espectrofotométricos

La espectrofotometría es una de las técnicas más simples para la cuantificación de compuestos fenólicos en vegetales. Los métodos Folin-Denis y Folin-Ciocalteu fueron durante muchos años los dos ensayos espectrofotométricos más utilizados para medir los compuestos fenólicos totales en material vegetal (Baiano y Del Nobile, 2016). Ambos métodos se basan en una reducción química de los reactivos empleados. Los productos de esta reducción en presencia de compuestos fenólicos generan un color azul con un amplio espectro de absorción de luz alrededor de los 760 nm. Los reactivos de ambos métodos no solo reaccionan con compuestos fenólicos, sino también con otras sustancias, como el ácido ascórbico, aminas aromáticas y azúcares reductores (López-Froilán et al., 2018). Esta técnica se caracteriza por ser simple y económica, pero solo proporciona una estimación de la concentración de compuestos fenólicos y no cuantifica dichos compuestos de manera individual; sin embargo, puede ser muy útil para la detección rápida y relativamente económica de numerosas muestras (Sánchez-Rangel et al., 2013).

1.8.2 Métodos cromatográficos

Cromatografía en capa fina. La cromatografía en capa fina (TLC) es una técnica ampliamente utilizada para la purificación y separación de compuestos fenólicos utilizando diferentes sistemas de solventes (Naczk y Shahidi, 2006). La separación de los compuestos fenólicos entre sí y de otros componentes presentes en el extracto puede llevarse a cabo mediante un alto número de métodos desarrolladas por TLC (Cimpoiu, 2006). TLC es una técnica de separación rentable y ampliamente utilizada en el campo de los productos naturales y el análisis fitoquímico. Tiene la ventaja frente a la HPLC, que el lecho de separación se utiliza sólo una vez, la hace especialmente versátil para el análisis de extractos crudos. En las últimas décadas, la técnica ha avanzado mediante la introducción de la cromatografía de capa fina de alta eficiencia (HPTLC) (Bräm y Wolfram, 2017). Las sorprendentes mejoras en los equipos automatizados para la aplicación, la separación y la detección, así como las fases estacionarias estandarizadas disponibles comercialmente, con un tamaño de partícula menor, permiten una separación potente en poco tiempo y con una gran reproducibilidad. Además, TLC pertenece a los métodos de separación más populares para el análisis efecto-dirigido (Weller, 2012). Los ensayos bioautográficos representan una herramienta del análisis efecto-dirigido que se definen como métodos TLC combinados con detección biológica. Este procedimiento proporciona la separación cromatográfica simultánea de una

matriz compleja de componentes y la localización de los componentes con actividad biológica directamente en la placa de HPTLC, en un corto periodo de tiempo (Marston, 2011). En este contexto, varios ensayos farmacológicos han sido modificados para llevarse a cabo en placas de HPTLC. Actividad antimicrobiana, actividad antifúngica, actividad antioxidante e inhibición de enzimas involucradas en ECNT como la alfa glucosidasa y acetilcolinesterasa pueden ser detectadas rápidamente en placas de HPTLC, entregando información rápida de la actividad biológica del extracto y de las bandas (compuestos) responsables de dicha actividad (Cieśla et al., 2015; Choma y Jesionek, 2015; Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b; Marston, 2011). La popularidad de estos ensayos se sustenta sin lugar a dudas por las múltiples ventajas de TLC, tales como, gran flexibilidad, alto número de análisis de muestras, capacidad para detectar la actividad de compuestos individuales presentes en muestras complejas y equipamiento de fácil acceso (Naczk y Shahidi, 2006).

Cromatografía en capa fina de alta eficiencia-espectrometría de masa (HPTLC-MS). El acoplamiento directo de HPTLC con MS es de particular interés debido a la alta sensibilidad, el rápido análisis y la capacidad de este último para ayudar a la identificación y caracterización estructural de compuestos bioactivos (Gupta y Gupta, 2011). En este sentido, la interfaz TLC-MS es una herramienta importante para la identificación de compuestos

desconocidos, ya que permite la elución directa de la banda (compuesto) desde la placa de HPTLC, o la elución a un vial para un análisis complementario por otras técnicas analíticas como UHPLC-DAD-ESI-MS/MS o espectrometría de masas de alta resolución (HRMS). Estas estrategias son ampliamente utilizadas para una rápida identificación de nuevos compuestos bioactivos detectados mediante bioautografía en placas de HPTLC (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b; Marston, 2011).

Cromatografía líquida de alta eficiencia (HPLC). Entre los diversos métodos disponibles, HPLC es la más utilizada para la separación y cuantificación de compuestos fenólicos en materiales vegetales. Las condiciones cromatográficas de los métodos de HPLC incluyen, casi exclusivamente el uso de columnas de fase inversa, preferentemente C_{18}; UV-vis con tecnología de diodos y un sistema de solvente binario que contiene generalmente agua acidificada (solvente A) y un solvente orgánico polar (solvente B). HPLC de fase inversa se ha convertido en una herramienta analítica dominante para la separación y determinación de compuestos fenólicos con diferentes sistemas de detección, como el detector de arreglo de diodos (DAD), espectrometría de masas (MS) o espectrometría de masa en tándem (MS/MS) (Ignat et al., 2011). Los métodos de HPLC están acoplados habitualmente a detectores espectrofotométricos como el DAD, lo que puede generar ciertas limitaciones de detección y sensibilidad analítica, especialmente en matrices complejas, como extractos

crudos de plantas y muestras ambientales. Por ello, una pre-concentración inicial y purificación de los compuestos fenólicos de una matriz compleja, tiende a ser una buena alternativa previo al análisis instrumental por HPLC. El objetivo de la preconcentración es simplificar los cromatogramas obtenidos para que los compuestos diana puedan identificarse y cuantificarse de manera confiable, mientras que la etapa de purificación tiene como objetivo principal la eliminación de potenciales compuestos interferentes (Liu et al., 2008).

Cromatografía líquida-espectrometría de masa (LC-MS). Las técnicas acopladas de cromatografía-espectrometría de masa (LC-MS), son en la actualidad el mejor enfoque analítico disponible para estudiar los compuestos fenólicos en matrices vegetales. LC-MS permite la caracterización de estructuras complejas y proporciona evidencia experimental de estructuras que anteriormente solo se identificaban tentativamente (Ongkowijoyo et al., 2018). Esta técnica analítica es capaz de suministrar información sobre la composición cualitativa y cuantitativa tanto de compuestos orgánicos como inorgánicos en muestras complejas, las estructuras de una amplia variedad de especies moleculares y las relaciones isotópicas de los átomos en las muestras (de Villiers et al., 2016). Su uso permite la identificación de estructuras que han sido separadas previamente por HPLC. El registro de los resultados (espectros de masa) se obtienen por conversión de los componentes de una muestra en iones gaseosos que se mueven rápidamente y se separan en función de su relación

masa/carga. Cada compuesto es único, y por tanto, cada analito se fragmentará de una determinada manera. Comprender estas vías de fragmentación permite a los investigadores interpretar adecuadamente los diversos iones en un espectro de masas, lo que permite la identificación de una estructura dada (Gu et al., 2003). En caso de que dos analitos presenten el mismo ion molecular y patrones de fragmentación muy similares, sólo es posible diferenciarlos si es que se separan cromatográficamente y se cuenta con los compuestos de referencia respectivos o éstos se aíslan y caracterizan por un método espectroscópico independiente, por ejemplo resonancia magnética nuclear (RMN).

1.9 Bioaccesibilidad

Además de identificar y cuantificar los compuestos bioactivos in vitro, es importante evaluar los principales efectos in vivo, para lo cual el paso lógico es determinar la biodisponibilidad de estos compuestos en el organismo. Para ello, es necesario considerar que los compuestos fenólicos presentes en los alimentos pueden diferir considerablemente de los metabolitos que llegan realmente a destino en el organismo como resultado del exhaustivo metabolismo durante la absorción de nutrientes en los procesos gástricos e intestinales (Carbonell-Capella et al., 2014). Estos cambios estructurales podrían implicar un aumento o disminución de la bioactividad o biodisponibilidad

de los compuestos. Desde este enfoque, se hace necesario evaluar la bioaccesibilidad de los compuestos bioactivos detectados (Schulz et al., 2017). La bioaccesibilidad se define como “la fracción de un compuesto que se libera de la matriz alimenticia en el tracto gastrointestinal, encontrándose disponible para la absorción intestinal y posterior entrada al torrente sanguíneo (Cilla et al., 2018). La bioaccesibilidad se evalúa generalmente mediante procedimientos de digestión in vitro (Figura 9), simulando las condiciones del proceso digestivo. Las enzimas α-amilasa, pepsina, tripsina, pancreatina, quimotripsina y lipasa son utilizadas en los modelos de digestión *in vitro*, además de otros componentes de la digestión como sales biliares y mucina (Hur et al., 2011). Gran parte de los modelos *in vitro* sólo estudian la digestión gástrica y del intestino delgado, debido a que por medio de las enzimas y diferentes condiciones de pH puede detectarse el compuesto de interés, y por lo tanto, determinar la bioaccesibilidad. En estos modelos, la muestra es sometida a la digestión en estómago a un pH cercano a 2 e incubada con pepsina en un baño a 37°C con agitación por 2 horas. Luego, el pH es neutralizado para adicionar pancreatina y sales biliares por otras 2 horas para simular las condiciones del intestino delgado (fase intestinal) (Sun et al., 2019). Generalmente, los modelos de digestión *in vitro* no consideran el intestino grueso, debido a que la absorción de los compuestos ocurre habitualmente en el intestino delgado (Brandon et al., 2006).

La identificación de compuestos fenólicos permite muchas veces justificar y sustentar la actividad funcional de la matriz estudiada. Sin embargo, los compuestos fenólicos son inestables y muy propensos a degradarse y/o reaccionar con diferentes factores (oxígeno, iones metálicos, temperatura, pH, luz, etc.) que cambian sus estructuras, y dan como resultado una disminución de las actividades biológicas de los compuestos fenólicos (Deng et al., 2018). Por lo tanto, para asegurar la bioaccesibilidad y funcionalidad de dichos compuestos, se hace necesario que estos sean estables y no sufran modificaciones en el tracto gastrointestinal, pudiendo ser la nanotecnología una solución efectiva para este propósito.

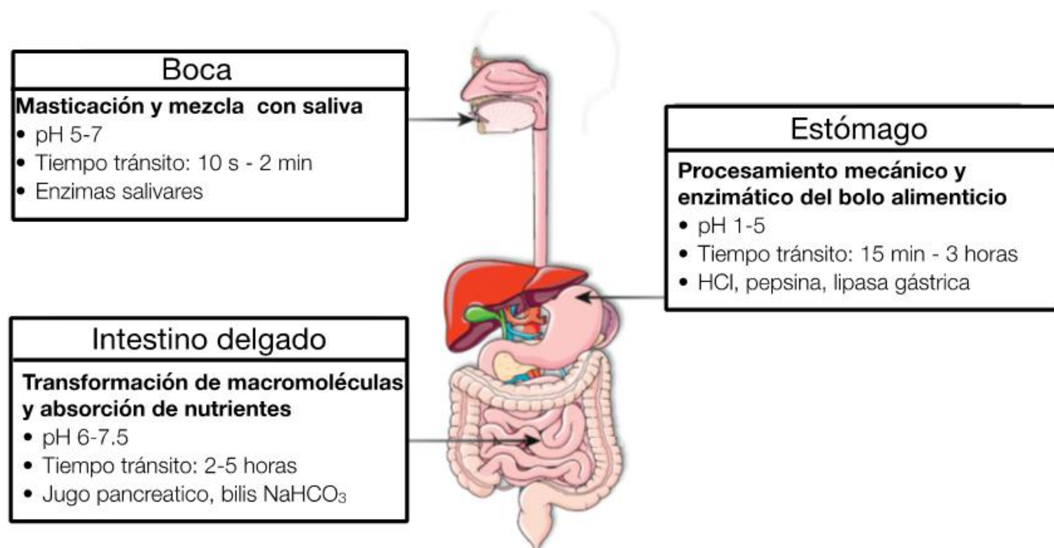


Figura 8. Simulación de las tres fases de la digestión: la masticación, la fase gástrica y la fase intestinal [adaptado de (Guerra et al., 2012)]

1.10 Nanotecnología

La nanotecnología se define como la actividad científica-tecnológica involucrada en el diseño, síntesis, caracterización y aplicación de materiales y dispositivos a escala nanométrica (generalmente 1 a 100 nm). Es una plataforma tecnológica que utiliza las propiedades únicas de la materia en la nanoescala, ya sea en forma compacta o en nanodispersiones, en las cuales los fenómenos físicos están dominados por diversas fuerzas, tales como Van der Walls, puentes de hidrógeno, carga electrónica, hidrofobicidad e hidrofilicidad (Gubin et al., 2005).

Aborda el estudio de sistemas complejos nanométricos (nanopartículas), formados al menos por dos componentes, un agente que actúa como vehículo y un compuesto con actividad biológica. En los últimos años ha tenido un incremento sin precedentes la investigación en dicha temática y se espera un impacto considerable en el campo de la medicina (Leso et al., 2019).

La nanotecnología trata de sistemas que permiten encapsular o adsorber en su exterior un compuesto con actividad biológica, por medio de unión no covalente (adsorción inespecífica o por afinidad) o mediante la formación de una unión covalente (Tian et al., 2020). Algunos de los propósitos de aplicar una técnica de encapsulación son (Champagne y Fustier, 2007; Onwulata, 2012):

- Proteger el compuesto activo de la degradación en el tracto gastrointestinal como también de las condiciones ambientales (calor, aire, luz, humedad, etc.)
- Modificar las características físicas del material original y hacer más fácil su manipulación. Por ejemplo, reducir la higroscopicidad, mejorar la fluidez, evitar la aglomeración (“caking”).
- Enmascarar sabores desagradables.
- Liberación controlada de un compuesto activo bajo condiciones específicas (pH, temperatura.)

Los materiales encapsulantes más utilizados para la protección de compuestos en la industria alimentaria y farmacéutica son las biomoléculas, debido a que son abundantes, no tóxicas y de baja alergenicidad (Tabla 1). La velocidad de

liberación de estos sistemas depende del grosor, el área y la permeabilidad del agente encapsulante como de la cantidad de compuesto activo encapsulado (Vinceković et al., 2017).

Tabla 1. Principales materiales encapsulantes utilizados para la protección de compuestos de ingredientes alimentarios y farmacéuticos [adaptado de (Desai y Jin Park, 2005)]

Materiales	**Ejemplos**
Proteínas	Albúmina, caseinatos, gelatina, gluten, péptidos, proteína de soja, proteínas de suero
Azúcares simples	Sacarosa, fructosa, galactosa, glucosa, maltosa, trehalosa
Polisacáridos y gomas	Alginatos, quitosano, almidones nativos y modificados (maíz, papa, etc.), ciclodextrina, maltodextrina, agar, carragenato, pectina, goma arábiga
Lípidos	Aceites y grasas comestibles, monoglicéridos, diglicéridos, liposomas, lecitina.
Celulosas	Carboximetilcelulosa, metilcelulosa, etilcelulosa, nitrocelulosa, acetilcelulosa, ftalato-acetato de celulosa, ftalato-butilato-acetato de celulosa

También se utilizan materiales poliméricos sintéticos como agentes encapsulantes. Estos deben ser biocompatibles y biodegradables, es decir, no deben causar ninguna respuesta adversa significativa del medio fisiológico que dañe el material y deben biodegradarse en componentes no tóxicos, tanto química como físicamente, o por combinación de ambas. Dentro de estos materiales podemos destacar: poli (ácido láctico) (PLA); poli (ácido glicólico) (PGA); poli (láctico-co-glicólico) (PLGA); poli (cianoacrilato de butilo) (PBCA), y poli (ε-caprolactona) (PCL) (Leyva-Gómez et al., 2015).

Se pueden obtener diversos tipos de sistemas, dependiendo de los materiales y del procedimiento de encapsulación. Entre los principales métodos de preparación podemos citar: nanoprecipitación, emulsión de difusión, doble emulsión, emulsión-coacervación, revestimiento de polímero, entre otros (Ephrem et al., 2018). La selección del proceso de encapsulación adecuado dependerá de múltiples factores, tales como el tamaño partícula requerido, las propiedades fisicoquímicas del material encapsulante y el compuesto activo, la finalidad del producto encapsulado, el mecanismo de liberación deseado y el costo (Munin y Edwards-Lévy, 2011).

Actualmente numerosos autores se encuentran estudiando la aplicación de nanotecnología sobre compuestos bioactivos para evitar la degradación que pudiesen sufrir ante variados mecanismos con el fin de mejorar la bioaccesibilidad de estos posterior a su digestión, permitiendo que estos compuestos puedan permanecer estables, protegidos de factores externos y ser

liberados en una ubicación específica en el tracto gastrointestinal para así ejercer su efecto benéfico (Jiang et al., 2020; Yao et al., 2015).

2. HIPÓTESIS Y OBJETIVOS

En base a los antecedentes planteados y discutidos en la sección previa, se plantean las siguientes hipótesis:

2.1 Hipótesis

Hipótesis científica-1

Los frutos y las hojas de arrayán poseen actividades funcionales alimentarias demostrables científicamente a través de (bio)ensayos químicos y analíticos.

Hipótesis científica-2

Los sistemas nanoparticulados protegen los compuestos bioactivos de la digestión gastrointestinal aumentando la bioaccesibilidad de dichos compuestos

2.2. Objetivo general

Desarrollar una plataforma científica tecnológica para la extracción e identificación de compuestos bioactivos desde arrayán (*Luma apiculata*) y evaluar el efecto de la nanoencapsulación sobre su bioaccesibilidad.

2.3 Objetivos específicos

2.3.1 Basándose en la metodología de extracción de compuestos fenólicos desde semillas de quinua, implementar y optimizar una metodología de extracción para el análisis de compuestos fenólicos en frutos y hojas de arrayán

2.3.2 Caracterizar químicamente el fruto y hoja de arrayán

2.3.3 Evaluar la bioaccesibilidad de los compuestos bioactivos aislados a través de un proceso de digestión *in-vitro*

2.3.4 Determinar el efecto de la nanoencapsulación sobre la bioaccesibilidad de los compuestos bioactivos seleccionados

2.3.5 Establecer metodologías químico-analíticas que permitan evaluar la actividad biológica del fruto y hoja de arrayán mediante la plataforma HPTLC/(bio)autografía/MS.

3. ESTRATEGIA ANALÍTICA

Las cuatro etapas de la figura 10, resumen la estrategia analítica del presente trabajo de investigación para dar cumplimiento a los objetivos propuestos y comprobar las hipótesis planteadas.

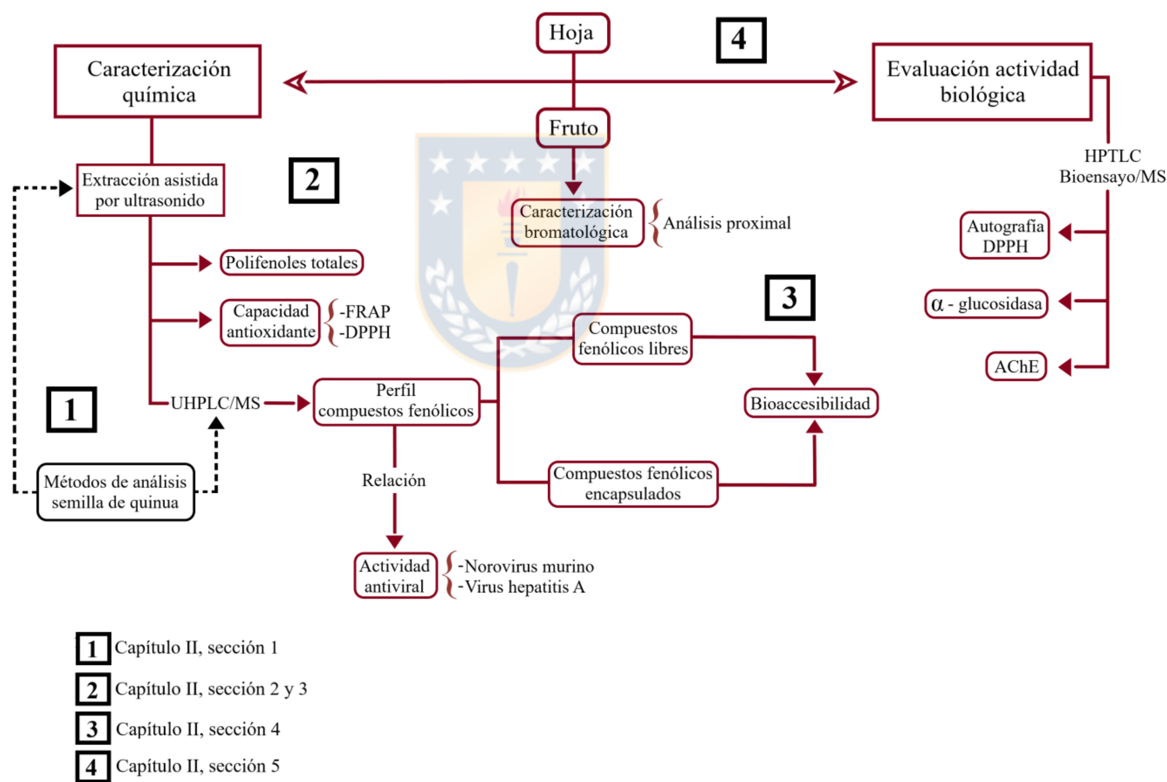


Figura 10. Estrategia analítica propuesta para el estudio de compuestos fenólicos

3.1 Desarrollo de una metodología de extracción de compuestos fenólicos

La extracción de compuestos bioactivos desde el fruto y hoja de arrayán se realizó en base al método desarrollado para la extracción de compuestos fenólicos desde semillas de quinua mediante extracción asistido por ultrasonido en baño ultrasónico (capitulo II, sección 1). Varios parámetros de extracción, incluyendo el porcentaje de etanol (en la mezcla hidroetánólica de extracción), temperatura de extracción, relación masa/solvente y tiempo de extracción fueron optimizadas mediante técnicas quimiométricas, con el objetivo de maximizar el rendimiento de extracción de los compuestos fenólicos. Luego de determinar y aplicar las condiciones óptimas de extracción, se evalúa en los extractos crudos generados la capacidad antioxidante mediante los ensayos FRAP y radical DPPH. También se evalúa el contenido de compuestos fenólicos y capacidad antioxidante de extractos ricos en compuestos fenólicos, obtenidos por adsorción selectiva sobre la resina polimérica Amberlita XAD-7 a partir de los extractos crudos. Los análisis realizados, las metodologías implementadas y los resultados obtenidos se presentan en el capítulo II, sección 2 y 3.

3.2 Estudio del perfil de compuestos fenólicos mediante UHPLC/MS

El método cromatográfico para la determinación de compuestos fenólicos en semillas de quinua (capítulo II, sección 1), fue ligeramente modificado y adaptado para establecer el perfil fenólico de los extractos crudos de fruto y hoja de arrayán. El perfil de ambos extractos fue establecido mediante UHPLC/MS empleando un espectrómetro de masa triple cuadrupolo. Además, se evaluó la actividad antiviral de extractos crudos frente al virus de la hepatitis A y norovirus murino debido a la reportada actividad que presentan los extractos ricos en compuestos fenólicos sobre los virus entéricos anteriormente mencionados. Las metodologías implementadas y los resultados obtenidos se presentan en el capítulo II, sección 2 y 3.

3.3 Evaluación de la bioaccesibilidad de los compuestos fenólicos aislados

Luego de establecer el perfil fenólico de los extractos, se determinó la bioaccesibilidad de los compuestos fenólicos aislados (estándares puros) después de ser sometidos a un proceso de digestión in vitro que simula la fase gástrica e intestinal. Paralelamente, se evaluó la potencialidad de tres sistemas nanoparticulados sobre la bioaccesibilidad de los compuestos estudiados. Para este fin, los compuestos fenólicos fueron cargados en nanopartículas de zeína,

zeína recubierta con alginato y zeína recubierta con complejo de alginato/quitosano, los cuales fueron obtenidos mediante los métodos de nanoprecipitación y deposición electrostática. La fracción bioaccesible de cada compuesto fenólico se determinó mediante el sistema HPLC/UV. Los análisis realizados, las metodologías implementadas y los resultados obtenidos se presentan en el capítulo II, sección 4

3.4 Evaluación de la actividad biológica mediante HPTLC/(bio)autografía

Por último, la actividad biológica de los extractos crudos de fruto y hoja de arrayán fue evaluada aplicando un análisis efecto-dirigido mediante HPTLC/(bio)autografía para la detección de compuestos con actividad antioxidante e inhibitoria de las bandas separadas en la placa de HPTLC sobre las enzimas alfa- glucosidasa y acetilcolinesterasa (consideradas como blancos terapéuticos para tratar ECNT como la diabetes mellitus tipo 2 y enfermedades neurodegenerativas, respectivamente). La identificación tentativa de compuestos con múltiples bioactividades se llevó a cabo mediante dos análisis complementarios: cribado fitoquímico sobre la placa de HPTLC y elución del compuesto (banda) desde la placa mediante la interfaz TLC-MS. Las metodologías implementadas y los resultados obtenidos son presentados en el capítulo II, sección 5.

4. REFERENCIAS

Aguilera, M.A., Casanueva, M.E., Hernández, C.E., 2006. Composición de la araneofauna en dos especies de árboles nativos Peumus boldus Mol. y Luma apiculata (D.C.) Burret en el parque botánico Pedro del río Zañartu (Hualpen), Concepción, VIII Región, Chile. Gayana (Concepción) 70, 176-185.

Aguilera, Y., Martin-Cabrejas, M.A., González de Mejia, E., 2016. Phenolic compounds in fruits and beverages consumed as part of the mediterranean diet: their role in prevention of chronic diseases. Phytochemistry Reviews 15, 405-423.

Aherne, S.A., O'Brien, N.M., 2002. Dietary flavonols: chemistry, food content, and metabolism. Nutrition 18, 75-81.

Alvarez-Jubete, L., Wijngaard, H., Arendt, E.K., Gallagher, E., 2010. Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking. Food Chemistry 119, 770-778.

Ames, B.N., Shigenaga, M.K., Hagen, T.M., 1993. Oxidants, antioxidants, and the degenerative diseases of aging. Proceedings of the National Academy of Sciences 90, 7915-7922.

Angeli, V., Miguel Silva, P., Crispim Massuela, D., Khan, M.W., Hamar, A., Khajehei, F., Graeff-Hönninger, S., Piatti, C., 2020. Quinoa (Chenopodium quinoa Willd.): An Overview of the Potentials of the "Golden Grain" and Socio-Economic and Environmental Aspects of Its Cultivation and Marketization. Foods 9, 216.

Araya-Contreras, T., Veas, R., Escobar, C.A., Machuca, P., Bittner, M., 2019. Antibacterial Effect of Luma apiculata (DC.) Burret Extracts in Clinically Important Bacteria. International Journal of Microbiology 2019, 7803726.

Armenta, S., Garrigues, S., de la Guardia, M., 2015. The role of green extraction techniques in Green Analytical Chemistry. TrAC Trends in Analytical Chemistry 71, 2-8.

Arora, M., Ohl, C.-D., Mørch, K.A., 2004. Cavitation Inception on Microparticles: A Self-Propelled Particle Accelerator. Physical Review Letters 92, 174501.

Asami, D.K., Hong, Y.-J., Barrett, D.M., Mitchell, A.E., 2003. Comparison of the total phenolic and ascorbic acid content of freeze-dried and air-dried marionberry, strawberry, and corn grown using conventional, organic, and sustainable agricultural practices. Journal of agricultural and food chemistry 51, 1237-1241.

Azmir, J., Zaidul, I.S.M., Rahman, M.M., Sharif, K.M., Mohamed, A., Sahena, F., Jahurul, M.H.A., Ghafoor, K., Norulaini, N.A.N., Omar, A.K.M., 2013. Techniques for extraction of bioactive compounds from plant materials: A review. Journal of Food Engineering 117, 426-436.

Azwanida, N., 2015. A review on the extraction methods use in medicinal plants, principle, strength and limitation. Med Aromat Plants 4, 2167-0412.1000196.

Baiano, A., Del Nobile, M.A., 2016. Antioxidant compounds from vegetable matrices: Biosynthesis, occurrence, and extraction systems. Critical reviews in food science and nutrition 56, 2053-2068.

Bendicho, C., De La Calle, I., Pena, F., Costas, M., Cabaleiro, N., Lavilla, I., 2012. Ultrasound-assisted pretreatment of solid samples in the context of green analytical chemistry. TrAC Trends in Analytical Chemistry 31, 50-60.

Biesaga, M., Pyrzyńska, K., 2013. Stability of bioactive polyphenols from honey during different extraction methods. Food Chemistry 136, 46-54.

Bors, W., Michel, C., 2002. Chemistry of the Antioxidant Effect of Polyphenols. Annals of the New York Academy of Sciences 957, 57-69.

Bräm, S., Wolfram, E., 2017. Recent Advances in Effect-directed Enzyme Assays based on Thin-layer Chromatography. Phytochemical Analysis 28, 74-86.

Brandon, E.F.A., Oomen, A.G., Rompelberg, C.J.M., Versantvoort, C.H.M., van Engelen, J.G.M., Sips, A.J.A.M., 2006. Consumer product in vitro digestion

model: Bioaccessibility of contaminants and its application in risk assessment. Regulatory Toxicology and Pharmacology 44, 161-171.

Brito, A., Areche, C., Sepúlveda, B., Kennelly, E.J., Simirgiotis, M.J., 2014. Anthocyanin characterization, total phenolic quantification and antioxidant features of some Chilean edible berry extracts. Molecules 19, 10936-10955.

Brunner, G., 2005. Supercritical fluids: technology and application to food processing. Journal of Food Engineering 67, 21-33.

Bucić-Kojić, A., Planinić, M., Tomas, S., Bilić, M., Velić, D., 2007. Study of solid–liquid extraction kinetics of total polyphenols from grape seeds. Journal of Food Engineering 81, 236-242.

Carbonell-Capella, J.M., Buniowska, M., Barba, F.J., Esteve, M.J., Frígola, A., 2014. Analytical Methods for Determining Bioavailability and Bioaccessibility of Bioactive Compounds from Fruits and Vegetables: A Review. Comprehensive Reviews in Food Science and Food Safety 13, 155-171.

Cardenas-Toro, F.P., Alcázar-Alay, S.C., Coutinho, J.P., Godoy, H.T., Forster-Carneiro, T., Meireles, M.A.A., 2015. Pressurized liquid extraction and low-pressure solvent extraction of carotenoids from pressed palm fiber: Experimental and economical evaluation. Food and Bioproducts Processing 94, 90-100.

Castañeda-Ovando, A., Pacheco-Hernández, M.d.L., Páez-Hernández, M.E., Rodríguez, J.A., Galán-Vidal, C.A., 2009. Chemical studies of anthocyanins: A review. Food Chemistry 113, 859-871.

Cieśla, Ł.M., Waksmundzka-Hajnos, M., Wojtunik, K.A., Hajnos, M., 2015. Thin-layer chromatography coupled with biological detection to screen natural mixtures for potential drug leads. Phytochemistry Letters 11, 445-454.

Cilla, A., Bosch, L., Barberá, R., Alegría, A., 2018. Effect of processing on the bioaccessibility of bioactive compounds – A review focusing on carotenoids, minerals, ascorbic acid, tocopherols and polyphenols. Journal of Food Composition and Analysis 68, 3-15.

Cimpoiu, C., 2006. Analysis of Some Natural Antioxidants by Thin-Layer Chromatography and High Performance Thin-Layer Chromatography. Journal of Liquid Chromatography & Related Technologies 29, 1125-1142.

Craft, B.D., Kerrihard, A.L., Amarowicz, R., Pegg, R.B., 2012. Phenol-Based Antioxidants and the In Vitro Methods Used for Their Assessment. Comprehensive Reviews in Food Science and Food Safety 11, 148-173.

Champagne, C.P., Fustier, P., 2007. Microencapsulation for the improved delivery of bioactive compounds into foods. Current Opinion in Biotechnology 18, 184-190.

Chen, C.-R., Lee, Y.-N., Chang, C.-M.J., Lee, M.-R., Wei, I.C., 2007. Hot-pressurized fluid extraction of flavonoids and phenolic acids from Brazilian propolis and their cytotoxic assay in vitro. Journal of the Chinese Institute of Chemical Engineers 38, 191-196.

Cheynier, V., 2012. Phenolic compounds: from plants to foods. Phytochemistry Reviews 11, 153-177.

Choma, I., Jesionek, W., 2015. Chapter 11 - Effects-Directed Biological Detection: Bioautography, in: Poole, C.F. (Ed.), Instrumental Thin-Layer Chromatography. Elsevier, Boston, pp. 279-312.

Dahui, L., Zaigui, W., Yunhua, Z., 2011. Antifungal activity of extracts by supercritical carbon dioxide extraction from roots of Echinacea angustifolia and analysis of their constituents using gas chromatography-mass spectrometry (GC-MS). Journal of Medicinal Plants Research 5, 5605-5610.

Dai, J., Mumper, R.J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules 15, 7313-7352.

Dai, X., Huang, Q., Zhou, B., Gong, Z., Liu, Z., Shi, S., 2013. Preparative isolation and purification of seven main antioxidants from Eucommia ulmoides Oliv. (Du-zhong) leaves using HSCCC guided by DPPH-HPLC experiment. Food Chemistry 139, 563-570.

Davidov-Pardo, G., Arozarena, I., Marín-Arroyo, M.R., 2011. Stability of polyphenolic extracts from grape seeds after thermal treatments. European Food Research and Technology 232, 211-220.

De Pascual-Teresa, D., Moreno, D.A., García-Viguera, C., 2010. Flavanols and anthocyanins in cardiovascular health: a review of current evidence. International journal of molecular sciences 11, 1679-1703.

de Villiers, A., Venter, P., Pasch, H., 2016. Recent advances and trends in the liquid-chromatography–mass spectrometry analysis of flavonoids. Journal of Chromatography A 1430, 16-78.

Den, W., Sharma, V.K., Lee, M., Nadadur, G., Varma, R.S., 2018. Lignocellulosic biomass transformations via greener oxidative pretreatment processes: access to energy and value-added chemicals. Frontiers in chemistry 6, 141.

Deng, J., Yang, H., Capanoglu, E., Cao, H., Xiao, J., 2018. 9 - Technological aspects and stability of polyphenols, in: Galanakis, C.M. (Ed.), Polyphenols: Properties, Recovery, and Applications. Woodhead Publishing, pp. 295-323.

Desai, K.G.H., Jin Park, H., 2005. Recent Developments in Microencapsulation of Food Ingredients. Drying Technology 23, 1361-1394.

Durazzo, A., Lucarini, M., Camilli, E., Marconi, S., Gabrielli, P., Lisciani, S., Gambelli, L., Aguzzi, A., Novellino, E., Santini, A., 2018. Dietary lignans: definition, description and research trends in databases development. Molecules 23, 3251.

Ephrem, E., Najjar, A., Charcosset, C., Greige-Gerges, H., 2018. Encapsulation of natural active compounds, enzymes, and probiotics for fruit juice fortification, preservation, and processing: An overview. Journal of Functional Foods 48, 65-84.

Farhoosh, R., Johnny, S., Asnaashari, M., Molaahmadibahraseman, N., Sharif, A., 2016. Structure–antioxidant activity relationships of o-hydroxyl, o-methoxy, and alkyl ester derivatives of p-hydroxybenzoic acid. Food Chemistry 194, 128-134.

Faria, A., Oliveira, J., Neves, P., Gameiro, P., Santos-Buelga, C., de Freitas, V., Mateus, N., 2005. Antioxidant properties of prepared blueberry (Vaccinium myrtillus) extracts. Journal of Agricultural and Food chemistry 53, 6896-6902.

Fischer, S., Wilckens, R., Jara, J., Aranda, M., 2013. Variation in antioxidant capacity of quinoa (chenopodium quinoa will) subjected to drought stress. Industrial Crops and Products 46, 341-349.

Fischer, S., Wilckens, R., Jara, J., Aranda, M., Valdivia, W., Bustamante, L., Graf, F., Obal, I., 2017. Protein and antioxidant composition of quinoa (Chenopodium quinoa Willd.) sprout from seeds submitted to water stress, salinity and light conditions. Industrial Crops and Products 107, 558-564.

Fuentes, L., Valdenegro, M., Gómez, M.-G., Ayala-Raso, A., Quiroga, E., Martínez, J.-P., Vinet, R., Caballero, E., Figueroa, C.R., 2016. Characterization of fruit development and potential health benefits of arrayan (Luma apiculata), a native berry of South America. Food Chemistry 196, 1239-1247.

Galarce-Bustos, O., Pavón-Pérez, J., Henríquez-Aedo, K., Aranda, M., 2019a. An improved method for a fast screening of α-glucosidase inhibitors in cherimoya fruit (Annona cherimola Mill.) applying effect-directed analysis via high-performance thin-layer chromatography-bioassay-mass spectrometry. Journal of Chromatography A 1608, 460415.

Galarce-Bustos, O., Pavón, J., Henríquez-Aedo, K., Aranda, M., 2019b. Detection and identification of acetylcholinesterase inhibitors in Annona cherimola Mill. by effect-directed analysis using thin-layer chromatography-bioassay-mass spectrometry. Phytochemical Analysis 30, 679-686.

Galvan d'Alessandro, L., Kriaa, K., Nikov, I., Dimitrov, K., 2012. Ultrasound assisted extraction of polyphenols from black chokeberry. Separation and Purification Technology 93, 42-47.

Gallo, M., Ferrara, L., Naviglio, D., 2018. Application of Ultrasound in Food Science and Technology: A Perspective. Foods 7, 164.

Garcia-Salas, P., Morales-Soto, A., Segura-Carretero, A., Fernández-Gutiérrez, A., 2010. Phenolic-compound-extraction systems for fruit and vegetable samples. Molecules 15, 8813-8826.

Ghiselli, A., Nardini, M., Baldi, A., Scaccini, C., 1998. Antioxidant Activity of Different Phenolic Fractions Separated from an Italian Red Wine. Journal of Agricultural and Food Chemistry 46, 361-367.

Gil-Chávez, G., Villa, J.A., Fernando Ayala-Zavala, J., Basilio Heredia, J., Sepulveda, D., Yahia, E.M., González-Aguilar, G.A., 2013. Technologies for extraction and production of bioactive compounds to be used as nutraceuticals and food ingredients: an overview. Comprehensive Reviews in Food Science and Food Safety 12, 5-23.

Gómez-Caravaca, A.M., Iafelice, G., Verardo, V., Marconi, E., Caboni, M.F., 2014. Influence of pearling process on phenolic and saponin content in quinoa (Chenopodium quinoa Willd). Food Chemistry 157, 174-178.

Gómez-Caravaca, A.M., Segura-Carretero, A., Fernández-Gutiérrez, A., Caboni, M.F., 2011. Simultaneous Determination of Phenolic Compounds and Saponins in Quinoa (Chenopodium quinoa Willd) by a Liquid Chromatography–Diode Array Detection–Electrospray Ionization–Time-of-Flight Mass Spectrometry Methodology. Journal of Agricultural and Food Chemistry 59, 10815-10825.

Goupy, P., Bautista-Ortin, A.-B., Fulcrand, H., Dangles, O., 2009. Antioxidant activity of wine pigments derived from anthocyanins: Hydrogen transfer reactions to the DPPH radical and inhibition of the heme-induced peroxidation of linoleic acid. Journal of agricultural and food chemistry 57, 5762-5770.

Granato, D., Shahidi, F., Wrolstad, R., Kilmartin, P., Melton, L.D., Hidalgo, F.J., Miyashita, K., Camp, J.v., Alasalvar, C., Ismail, A.B., Elmore, S., Birch, G.G., Charalampopoulos, D., Astley, S.B., Pegg, R., Zhou, P., Finglas, P., 2018. Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? Food Chemistry 264, 471-475.

Gu, L., Kelm, M.A., Hammerstone, J.F., Zhang, Z., Beecher, G., Holden, J., Haytowitz, D., Prior, R.L., 2003. Liquid chromatographic/electrospray ionization

mass spectrometric studies of proanthocyanidins in foods. Journal of Mass Spectrometry 38, 1272-1280.

Gubin, S.P., Koksharov, Y.A., Khomutov, G., Yurkov, G.Y., 2005. Magnetic nanoparticles: preparation, structure and properties. Russian Chemical Reviews 74, 489.

Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., Alric, M., 2012. Relevance and challenges in modeling human gastric and small intestinal digestion. Trends in Biotechnology 30, 591-600.

Gupta, A.P., Gupta, S., 2011. HPTLC–MS Coupling: New Dimension of HPTLC, in: Srivastava, M. (Ed.), High-Performance Thin-Layer Chromatography (HPTLC). Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 311-333.

Haminiuk, C.W.I., Maciel, G.M., Plata-Oviedo, M.S.V., Peralta, R.M., 2012. Phenolic compounds in fruits – an overview. International Journal of Food Science & Technology 47, 2023-2044.

Hirose, Y., Fujita, T., Ishii, T., Ueno, N., 2010. Antioxidative properties and flavonoid composition of Chenopodium quinoa seeds cultivated in Japan. Food Chemistry 119, 1300-1306.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J.A., Deemer, E.K., 2002. Development and Validation of Oxygen Radical Absorbance Capacity Assay for Lipophilic Antioxidants Using Randomly Methylated β-Cyclodextrin as the Solubility Enhancer. Journal of Agricultural and Food Chemistry 50, 1815-1821.

Hur, S.J., Lim, B.O., Decker, E.A., McClements, D.J., 2011. In vitro human digestion models for food applications. Food Chemistry 125, 1-12.

Ignat, I., Volf, I., Popa, V.I., 2011. A critical review of methods for characterisation of polyphenolic compounds in fruits and vegetables. Food Chemistry 126, 1821-1835.

Jia, G., Lu, X., 2008. Enrichment and purification of madecassoside and asiaticoside from Centella asiatica extracts with macroporous resins. Journal of Chromatography A 1193, 136-141.

Jiang, N., Doseff, A.I., Grotewold, E., 2016. Flavones: from biosynthesis to health benefits. Plants 5, 27.

Jiang, T., Liao, W., Charcosset, C., 2020. Recent advances in encapsulation of curcumin in nanoemulsions: A review of encapsulation technologies, bioaccessibility and applications. Food Research International 132, 109035.

Kala, H.K., Mehta, R., Sen, K.K., Tandey, R., Mandal, V., 2016. Critical analysis of research trends and issues in microwave assisted extraction of phenolics: Have we really done enough. TrAC Trends in Analytical Chemistry 85, 140-152.

Karvela, E., Makris, D.P., Kalogeropoulos, N., Karathanos, V.T., Kefalas, P., 2009. Factorial design optimisation of grape (Vitis vinifera) seed polyphenol extraction. European Food Research and Technology 229, 731-742.

Kawaguchi, H., Hasunuma, T., Ogino, C., Kondo, A., 2016. Bioprocessing of bio-based chemicals produced from lignocellulosic feedstocks. Current Opinion in Biotechnology 42, 30-39.

Kelsey, N.A., Wilkins, H.M., Linseman, D.A., 2010. Nutraceutical antioxidants as novel neuroprotective agents. Molecules 15, 7792-7814.

Khaw, K.-Y., Parat, M.-O., Shaw, P.N., Falconer, J.R., 2017. Solvent supercritical fluid technologies to extract bioactive compounds from natural sources: a review. Molecules 22, 1186.

Kim, D.-H., Kim, M.-J., Kim, D.-W., Kim, G.-Y., Kim, J.-K., Gebru, Y.A., Choi, H.-S., Kim, Y.-H., Kim, M.-K., 2019. Changes of phytochemical components (urushiols, polyphenols, gallotannins) and antioxidant capacity during Fomitella fraxinea–mediated fermentation of Toxicodendron vernicifluum bark. Molecules 24, 683.

Lang, Q., Wai, C.M., 2001. Supercritical fluid extraction in herbal and natural product studies — a practical review. Talanta 53, 771-782.

Lapsongphon, N., Yongsawatdigul, J., 2013. Production and purification of antioxidant peptides from a mungbean meal hydrolysate by Virgibacillus sp. SK37 proteinase. Food Chemistry 141, 992-999.

Leso, V., Fontana, L., Iavicoli, I., 2019. Biomedical nanotechnology: Occupational views. Nano Today 24, 10-14.

Leyva-Gómez, G., Cortés, H., Magaña, J.J., Leyva-García, N., Quintanar-Guerrero, D., Florán, B., 2015. Nanoparticle technology for treatment of Parkinson's disease: the role of surface phenomena in reaching the brain. Drug Discovery Today 20, 824-837.

Li, F., Chen, G., Zhang, B., Fu, X., 2017. Current applications and new opportunities for the thermal and non-thermal processing technologies to generate berry product or extracts with high nutraceutical contents. Food Research International 100, 19-30.

Lin, L., Zhao, H., Dong, Y., Yang, B., Zhao, M., 2012. Macroporous resin purification behavior of phenolics and rosmarinic acid from Rabdosia serra (MAXIM.) HARA leaf. Food Chemistry 130, 417-424.

Liu, Q., Cai, W., Shao, X., 2008. Determination of seven polyphenols in water by high performance liquid chromatography combined with preconcentration. Talanta 77, 679-683.

López-Froilán, R., Hernández-Ledesma, B., Cámara, M., Pérez-Rodríguez, M.L., 2018. Evaluation of the Antioxidant Potential of Mixed Fruit-Based Beverages: a New Insight on the Folin-Ciocalteu Method. Food Analytical Methods 11, 2897-2906.

Marston, A., 2011. Thin-layer chromatography with biological detection in phytochemistry. Journal of Chromatography A 1218, 2676-2683.

Mendoza-Torres, E., Bravo-Sagua, R., Villa, M., Flores, N., Olivares, M.J., Calle, X., Riquelme, J.A., Bambs, C., Castro, P., Lavandero, S., 2019. Enfermedades cardiovasculares y cáncer: ¿dos entidades mutuamente relacionadas? Revista chilena de cardiología 38, 54-63.

Mirzadeh, M., Arianejad, M.R., Khedmat, L., 2020. Antioxidant, antiradical, and antimicrobial activities of polysaccharides obtained by microwave-assisted extraction method: A review. Carbohydrate Polymers 229, 115421.

Mishra, K., Ojha, H., Chaudhury, N.K., 2012. Estimation of antiradical properties of antioxidants using DPPH assay: A critical review and results. Food Chemistry 130, 1036-1043.

Miyazawa, T., Nakagawa, K., Kudo, M., Muraishi, K., Someya, K., 1999. Direct intestinal absorption of red fruit anthocyanins, cyanidin-3-glucoside and cyanidin-3, 5-diglucoside, into rats and humans. Journal of agricultural and food chemistry 47, 1083-1091.

Mozaffarian, D., Wu, J.H.Y., 2018. Flavonoids, Dairy Foods, and Cardiovascular and Metabolic Health. Circulation Research 122, 369-384.

Munin, A., Edwards-Lévy, F., 2011. Encapsulation of natural polyphenolic compounds; a review. Pharmaceutics 3, 793-829.

Mustafa, A., Turner, C., 2011. Pressurized liquid extraction as a green approach in food and herbal plants extraction: A review. Analytica Chimica Acta 703, 8-18.

Naczk, M., Shahidi, F., 2006. Phenolics in cereals, fruits and vegetables: Occurrence, extraction and analysis. Journal of Pharmaceutical and Biomedical Analysis 41, 1523-1542.

Ndhlala, A.R., Moyo, M., Van Staden, J., 2010. Natural antioxidants: fascinating or mythical biomolecules? Molecules 15, 6905-6930.

Noratto, G.D., Murphy, K., Chew, B.P., 2019. Quinoa intake reduces plasma and liver cholesterol, lessens obesity-associated inflammation, and helps to prevent hepatic steatosis in obese db/db mouse. Food Chemistry 287, 107-114.

Ongkowijoyo, P., Luna-Vital, D.A., Gonzalez de Mejia, E., 2018. Extraction techniques and analysis of anthocyanins from food sources by mass spectrometry: An update. Food Chemistry 250, 113-126.

Onwulata, C.I., 2012. Encapsulation of New Active Ingredients. Annual Review of Food Science and Technology 3, 183-202.

Oroian, M., Escriche, I., 2015. Antioxidants: Characterization, natural sources, extraction and analysis. Food Research International 74, 10-36.

Parkinson, L., Cicerale, S., 2016. The Health Benefiting Mechanisms of Virgin Olive Oil Phenolic Compounds. Molecules 21, 1734.

Patel, K., Panchal, N., Ingle, D., 2019. Review of Extraction Techniques. Int. J. Adv. Res. Chem. Sci 6, 6-21.

Peña-Neira, A., Cáceres, A., Pastenes, C., 2007. Low molecular weight phenolic and anthocyanin composition of grape skins from cv. Syrah (Vitis vinifera L.) in the Maipo Valley (Chile): Effect of clusters thinning and vineyard yield. Food science and technology international 13, 153-158.

Perron, N.R., Brumaghim, J.L., 2009. A Review of the Antioxidant Mechanisms of Polyphenol Compounds Related to Iron Binding. Cell Biochemistry and Biophysics 53, 75-100.

Peterson, J.J., Beecher, G.R., Bhagwat, S.A., Dwyer, J.T., Gebhardt, S.E., Haytowitz, D.B., Holden, J.M., 2006. Flavanones in grapefruit, lemons, and limes: A compilation and review of the data from the analytical literature. Journal of Food Composition and Analysis 19, S74-S80.

Picó, Y., 2013. Ultrasound-assisted extraction for food and environmental samples. TrAC Trends in Analytical Chemistry 43, 84-99.

Pisoschi, A.M., Negulescu, G.P., 2011. Methods for total antioxidant activity determination: a review. Biochem Anal Biochem 1, 106.

Popa, V.I., Dumitru, M., Volf, I., Anghel, N., 2008. Lignin and polyphenols as allelochemicals. Industrial Crops and Products 27, 144-149.

Prior, R.L., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of agricultural and food chemistry 53, 4290-4302.

Proestos, C., Chorianopoulos, N., Nychas, G.-J., Komaitis, M., 2005. RP-HPLC analysis of the phenolic compounds of plant extracts. Investigation of their antioxidant capacity and antimicrobial activity. Journal of agricultural and food chemistry 53, 1190-1195.

Puntieri, J., Torres, C., Magnin, A., Stecconi, M., Grosfeld, J., 2018. Structural differentiation among annual shoots as related to growth dynamics in Luma apiculata trees (Myrtaceae). Flora 249, 86-96.

Putnik, P., Bursać Kovačević, D., Radojčin, M., Dragović-Uzelac, V., 2016. Influence of acidity and extraction time on the recovery of flavonoids from grape skin pomace optimized by response surface methodology. Chemical and biochemical engineering quarterly 30, 455-464.

Quiñones, M., Miguel, M., Aleixandre, A., 2012. Los polifenoles, compuestos de origen natural con efectos saludables sobre el sistema cardiovascular. Nutrición hospitalaria 27, 76-89.

Repo-Carrasco-Valencia, R., Hellström, J.K., Pihlava, J.-M., Mattila, P.H., 2010. Flavonoids and other phenolic compounds in Andean indigenous grains: Quinoa (Chenopodium quinoa), kañiwa (Chenopodium pallidicaule) and kiwicha (Amaranthus caudatus). Food Chemistry 120, 128-133.

Rice-Evans, C.A., Miller, N.J., Bolwell, P.G., Bramley, P.M., Pridham, J.B., 1995. The relative antioxidant activities of plant-derived polyphenolic flavonoids. Free radical research 22, 375-383.

Rosa, P.T.V., Meireles, M.A.A., 2005. Rapid estimation of the manufacturing cost of extracts obtained by supercritical fluid extraction. Journal of Food Engineering 67, 235-240.

Routray, W., Orsat, V., 2012. Microwave-Assisted Extraction of Flavonoids: A Review. Food and Bioprocess Technology 5, 409-424.

Sánchez-Rangel, J.C., Benavides, J., Heredia, J.B., Cisneros-Zevallos, L., Jacobo-Velázquez, D.A., 2013. The Folin–Ciocalteu assay revisited: improvement of its specificity for total phenolic content determination. Analytical Methods 5, 5990-5999.

Santos-Sánchez, N.F., Salas-Coronado, R., Villanueva-Cañongo, C., Hernández-Carlos, B., 2019. Antioxidant compounds and their antioxidant mechanism, Antioxidants. IntechOpen.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., Jiménez, L., 2005. Dietary Polyphenols and the Prevention of Diseases. Critical Reviews in Food Science and Nutrition 45, 287-306.

Schulz, M., Biluca, F.C., Gonzaga, L.V., Borges, G.d.S.C., Vitali, L., Micke, G.A., de Gois, J.S., de Almeida, T.S., Borges, D.L.G., Miller, P.R.M., Costa, A.C.O., Fett, R., 2017. Bioaccessibility of bioactive compounds and antioxidant potential of juçara fruits (Euterpe edulis Martius) subjected to in vitro gastrointestinal digestion. Food Chemistry 228, 447-454.

Shrestha, A., Pandey, R.P., Sohng, J.K., 2019. Biosynthesis of resveratrol and piceatannol in engineered microbial strains: achievements and perspectives. Applied Microbiology and Biotechnology 103, 2959-2972.

Siddhuraju, P., Becker, K., 2003. Antioxidant properties of various solvent extracts of total phenolic constituents from three different agroclimatic origins of drumstick tree (Moringa oleifera Lam.) leaves. Journal of agricultural and food chemistry 51, 2144-2155.

Simirgiotis, M.J., Bórquez, J., Schmeda-Hirschmann, G., 2013. Antioxidant capacity, polyphenolic content and tandem HPLC–DAD–ESI/MS profiling of phenolic compounds from the South American berries Luma apiculata and L. chequén. Food Chemistry 139, 289-299.

Soliva-Fortuny, R., Balasa, A., Knorr, D., Martín-Belloso, O., 2009. Effects of pulsed electric fields on bioactive compounds in foods: a review. Trends in Food Science & Technology 20, 544-556.

Subramanian, R., Raj, V., Manigandan, K., Elangovan, N., 2015. Antioxidant activity of hopeaphenol isolated from Shorea roxburghii stem bark extract. Journal of Taibah University for Science 9, 237-244.

Suen, J., Thomas, J., Kranz, A., Vun, S., Miller, M., 2016. Effect of flavonoids on oxidative stress and inflammation in adults at risk of cardiovascular disease: a systematic review, Healthcare. Multidisciplinary Digital Publishing Institute, p. 69.

Sun, H., Ge, X., Lv, Y., Wang, A., 2012. Application of accelerated solvent extraction in the analysis of organic contaminants, bioactive and nutritional compounds in food and feed. Journal of Chromatography A 1237, 1-23.

Sun, Y., Tao, W., Huang, H., Ye, X., Sun, P., 2019. Flavonoids, phenolic acids, carotenoids and antioxidant activity of fresh eating citrus fruits, using the coupled in vitro digestion and human intestinal HepG2 cells model. Food Chemistry 279, 321-327.

Tanase, C., Coșarcă, S., Muntean, D.-L., 2019. A critical review of phenolic compounds extracted from the bark of woody vascular plants and their potential biological activity. Molecules 24, 1182.

Tapas, A.R., Sakarkar, D., Kakde, R., 2008. Flavonoids as nutraceuticals: a review. Tropical journal of Pharmaceutical research 7, 1089-1099.

Teo, C.C., Tan, S.N., Yong, J.W.H., Hew, C.S., Ong, E.S., 2010. Pressurized hot water extraction (PHWE). Journal of Chromatography A 1217, 2484-2494.

Tian, B., Hua, S., Liu, J., 2020. Cyclodextrin-based delivery systems for chemotherapeutic anticancer drugs: A review. Carbohydrate Polymers 232, 115805.

Toscano-Garibay, J.D., Arriaga-Alba, M., Sánchez-Navarrete, J., Mendoza-García, M., Flores-Estrada, J.J., Moreno-Eutimio, M.A., Espinosa-Aguirre, J.J., González-Ávila, M., Ruiz-Pérez, N.J., 2017. Antimutagenic and antioxidant activity of the essential oils of Citrus sinensis and Citrus latifolia. Scientific Reports 7, 11479.

Tsao, R., 2010. Chemistry and biochemistry of dietary polyphenols. Nutrients 2, 1231-1246.

Tsao, R., Yang, R., 2003. Optimization of a new mobile phase to know the complex and real polyphenolic composition: towards a total phenolic index using high-performance liquid chromatography. Journal of Chromatography A 1018, 29-40.

Valenzuela, A., Valenzuela, R., Sanhueza, J., Morales I, G., 2014. Alimentos funcionales, nutraceúticos y foshu: ¿vamos hacia un nuevo concepto de alimentación? Revista chilena de nutrición 41, 198-204.

van den Driessche, J.J., Plat, J., Mensink, R.P., 2018. Effects of superfoods on risk factors of metabolic syndrome: a systematic review of human intervention trials. Food & Function 9, 1944-1966.

Vinceković, M., Viskić, M., Jurić, S., Giacometti, J., Bursać Kovačević, D., Putnik, P., Donsì, F., Barba, F.J., Režek Jambrak, A., 2017. Innovative technologies for encapsulation of Mediterranean plants extracts. Trends in Food Science & Technology 69, 1-12.

Vorobiev, E., Lebovka, N., 2020. Solid/Liquid Extraction and Expression, Processing of Foods and Biomass Feedstocks by Pulsed Electric Energy. Springer International Publishing, Cham, pp. 113-148.

Vučić, D.M., Petković, M.R., Rodić-Grabovac, B.B., Stefanović, O.D., Vasić, S.M., Čomić, L.R., 2013. Antibacterial and antioxidant activities of bilberry (Vaccinium myrtillus L.) in vitro. African Journal of Microbiology Research 7, 5130-5136.

Weller, M.G., 2012. A unifying review of bioassay-guided fractionation, effect-directed analysis and related techniques. Sensors 12, 9181-9209.

Wen, L., Zhang, Z., Sun, D.-W., Sivagnanam, S.P., Tiwari, B.K., 2020. Combination of emerging technologies for the extraction of bioactive compounds. Critical Reviews in Food Science and Nutrition 60, 1826-1841.

WHO, 2020. Noncommunicable diseases progress monitor 2020. https://www.who.int/publications/i/item/ncd-progress-monitor-2020.

Williamson, G., Kay, C.D., Crozier, A., 2018. The Bioavailability, Transport, and Bioactivity of Dietary Flavonoids: A Review from a Historical Perspective. Comprehensive Reviews in Food Science and Food Safety 17, 1054-1112.

Xu, B.J., Chang, S.K.C., 2007. A Comparative Study on Phenolic Profiles and Antioxidant Activities of Legumes as Affected by Extraction Solvents. Journal of Food Science 72, S159-S166.

Xu, C.-C., Wang, B., Pu, Y.-Q., Tao, J.-S., Zhang, T., 2017. Advances in extraction and analysis of phenolic compounds from plant materials. Chinese Journal of Natural Medicines 15, 721-731.

Yao, M., McClements, D.J., Xiao, H., 2015. Improving oral bioavailability of nutraceuticals by engineered nanoparticle-based delivery systems. Current Opinion in Food Science 2, 14-19.

Yousuf, B., Gul, K., Wani, A.A., Singh, P., 2016. Health benefits of anthocyanins and their encapsulation for potential use in food systems: a review. Critical reviews in food science and nutrition 56, 2223-2230.

Zaheer, K., Humayoun Akhtar, M., 2017. An updated review of dietary isoflavones: Nutrition, processing, bioavailability and impacts on human health. Critical reviews in food science and nutrition 57, 1280-1293.

Zhao, Q., Duan, C.-Q., Wang, J., 2010. Anthocyanins profile of grape berries of Vitis amurensis, its hybrids and their wines. International Journal of Molecular Sciences 11, 2212-2228.

Zheng, W., Wang, S.Y., 2001. Antioxidant Activity and Phenolic Compounds in Selected Herbs. Journal of Agricultural and Food Chemistry 49, 5165-5170.

CAPÍTULO II: Resultados y Discusión

CAPITULO II SECCIÓN 1

Title: A fast and selective method to determine phenolic compounds in quinoa (*Chenopodium quinoa* Will) seeds applying ultrasound-assisted extraction and high-performance liquid chromatography

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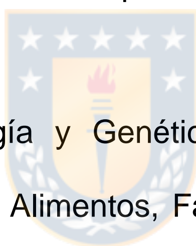

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Abstract

A simple and fast ultrasound-assisted extraction (UAE) method was optimized employing a chemometric technique to determine caffeic acid, vanillic acid, quercetin 3-ß-D-glucoside, *p*-coumaric acid, vanillin, *trans*-ferulic acid, benzoic acid, quercetin, kaempferol and myricetin in quinoa seeds. Applying a central composite design, UAE conditions were established at 39°C for 10 min, which is up to 12-fold lower than reported extraction times. Chromatography was performed on Phenomenex Kinetex XB C_{18} (150 mm x 4.6 mm, S-5 µm) column using a binary mobile phase composed of ammonium formate (0.01 mol L^{-1}, pH 3.0) and acetonitrile (0.1% formic acid) at a flow rate of 0.5 mL min^{-1}. Validation was performed following International Conference on Harmonization recommendations. Calibration data fitted a linear regression model with R^2 >0.999. Repeatability (*n*=6) and intermediate precision (*n*=3) in matrix showed relative standard deviation (RSD) values lower than 2.89 and 3.61%, respectively. Selectivity was evaluated by mass spectrometry (MS), without observing interference compounds. The proposed method was applied to determine the phenolic compound content in 10 quinoa seeds samples from different varieties, finding a concentration range from 1.1 to 2.0 mg kg^{-1}. The proposed method proved to be a fast and selective alternative to evaluate phenolic compounds in quinoa seeds.

Keywords: Ultrasound-assisted extraction, quinoa, polyphenols, chromatography, design of experiment.

1. Introduction

Quinoa (*Chenopodium quinoa* Willd) is a pseudocereal that belongs to Amaranthaceae family. This crop is cultivated from ancient times in the Andean region of South America and it is capable of adapting and growing in different climate conditions, including the most detrimental abiotic factors like drought, hailstone, high altitude, heat, and salinity. Quinoa seeds are the main edible part of this grain, which is also the most known and valued portion (Angeli et al., 2020). In the last time, this seed has shown a significant increase in its consumption due to its high nutritional value and possible health benefits (Noratto et al., 2019; van den Driessche et al., 2018). Further, another relevant feature of quinoa seeds is that most of the cultivars contain safe levels of gluten-forming proteins and therefore could an excellent option for gluten free-diet (Alvarez-Jubete et al., 2010; Stoven et al., 2013). From a nutritional point of view, edible seeds can be defined as a complete food because it possess a well-balanced essential amino acid content and it is a good source of protein, fiber, phosphorus, magnesium, iron and phenolic compounds (Ogungbenle, 2003). Recently, taking into account quinoa polyphenols content, some studies have been focused on its antioxidant properties and how this can be affected/modulated by different cultivation conditions (Fischer et al., 2013; Fischer et al., 2017). Polyphenols are secondary metabolites produced as defense against a variety of herbivores and pathogen microorganisms, as well

as in response of different kind of abiotic stresses (Harborne and Williams, 2000). Polyphenols can be found in free or bound form (glycosides) and they have been extensively studied because its intake is associated with some beneficial health effects like reduction of the risk of certain cancer, allergies, neurodegenerative, inflammatory and cardiovascular diseases (Gómez-Caravaca et al., 2014; Repo-Carrasco-Valencia et al., 2010). The main phenolic compounds present in quinoa are phenolic acids like ferulic and vanillic acids and flavonoids such as kaempferol, quercetin and their glucosides forms (Tang et al., 2016). Several analytical techniques have been applied to determine phenolic compounds content in different food and plant matrices, e.g., gas chromatography (GC), high performance thin-layer chromatography (HPTLC), capillary electrophoresis (CE) and high-performance liquid chromatography (HPLC). The latter, coupled to diode array detection (DAD), is the preferred technique to perform both, separation and quantification (Khoddami et al., 2013). Phenolic extraction from food and plant matrices have been carried out employing conventional techniques like maceration and Soxhlet extraction as well as advanced technologies such as pressurized liquid extraction, supercritical fluid extraction, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (Azmir et al., 2013a). Further, other less common approaches like QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) (Galarce-Bustos et al., 2019a), and disperse solid phase extraction (d-SPE) (Senes et al., 2020) have been also assayed. UAE technique has become an

excellent alternative for polyphenol extraction because it is possible to accomplish high extraction yields in a very short period of time (Vinatoru, 2001) using effortless procedures and low-cost equipment (Wang and Weller, 2006). The present work reports a chemometric optimization of UAE of phenolic compounds from quinoa seeds. A face-centered composite design was applied to optimize UAE conditions and a fast and validated HPLC/UV method was developed for separation and quantification.

2. Experimental

2.1 Reagents, chemicals and standard solutions

Caffeic acid (≥98%), vanillic acid (≥97%), quercetin 3-β-D-glucoside (≥90%), *p*-coumaric acid (≥98%), vanillin (≥99%), *trans*-ferulic acid (99%), benzoic acid (≥99.5), quercetin hydrate (95%), kaempferol (≥90%), myricetin (96%) and ammonium formate (≥99%) were purchased from Sigma (St. Louis, MO, USA). Acetonitrile and methanol, both HPLC grade, ethyl acetate, and formic acid were obtained from Merck (Darmstadt, Germany). Ultrapure water (18 MΩ cm) was produced using a Simplicity system from Millipore (Bedford, MA, USA). Phenolic compounds stock solution was prepared in methanol: water (7:3 v/v) for a given concentration of 0.15 mg mL^{-1}. Standard solutions were prepared by aliquot dilution from the stock solution.

2.2 Ultrasound-assisted extraction

Quinoa seed samples were homogenized employing an IKA (Staufen, Germany) A 11 basic analytical mill. A proper amount of powdered quinoa sample (0.8 g) was accurately weighed and transferred to 15 mL centrifuge tube. Thereafter, 8.0 mL of ethyl acetate (1:10 ratio) were added and vortex-mixed during 1 min. UAE was carried out for 10 min at 39 ºC out using a Getidy (Wuyi Tongqin, China) ultrasonic bath with a frequency of 40 kHz and a potency of 40 WL^{-1}. Then, the suspension was centrifuged for 10 min at 9050 x *g,* and 6.0 mL of clear supernatant was collected and evaporated to dryness under a nitrogen stream (to avoid phenolic compounds oxidation) at 40 °C and protected from light. Prior chromatography samples were reconstituted with 600 µL (10-fold concentrated solution) of methanol: water (7:3 v/v) and filtered through 13-mm polyvinylidene fluoride (PVDF) syringe filter (0.22 µm).

2.3 Experimental design

Optimization of phenolic compounds extraction was carried out applying a response surface methodology based on central composite design. The experimental design was established with two factors at three levels each. The effect of extraction time and extraction temperature on extraction yields of phenolic compounds was studied. Experiment plan (runs), independent and

dependent variables values are shown in (Table 1). Linear interaction and quadratic coefficients were evaluated using ANOVA ($p < 0.05$), while extractive process conditions were optimized using the response surface methodology

2.4 Chromatography

Chromatographic analysis was performed in a Shimadzu (Kyoto, Japan) Prominence HPLC system composed of LC-20AT pump, DGU-20AR5R degassing unit, CTO-20AC column oven, CBM-20A communication module, SIL-20A autosampler and SPD-20AV UV/VIS detector. Data were acquired, recorded and analyzed by means of Shimadzu LabSolutions 5.54 software. Separation was performed on Phenomenex (Torrance, CA, USA) Kinetex C_{18} (150 mm x 4.6 mm, S-5 μm) column connected to Kinetex guard column, both set at 35°C, using a binary mobile phase composed of ammonium formate (0.01 mol L^{-1}) adjusted to pH 3 with formic acid (A) and acetonitrile containing 0.1% of formic acid (B). The following gradient program was applied at a flow rate of 0.5 mL min^{-1}: 0-3 min 20%-%20 B (isocratic step), 3-4 min 20%-30% B, 4-6 min 30%-30% B (isocratic step), 6-8 min 30%-75% B, 8-10 min 75%-90% B, 10-14 min 90%-90% B (isocratic step), 14-15 min 90%-20% B, and 15-28 min 20%-20% B (column conditioning). Complete separation was accomplished in less than 18 min for a total run time of 28 min. Detection was performed by UV absorption at 280 nm. Mass spectrometry analysis was performed employing a

Shimadzu Nexera X2 UHPLC system composed of LC30AD bin pump, DGU-20AR 5R vacuum degasser, SIL-30AC autosampler, CTO-20AC column oven, and SPD-M20A photodiode array detector, coupled to a Shimadzu LCMS-8030 triple quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source. MS analysis was performed in Scan mode (*m/z* 100-2000) using the following settings: ESI (-) voltage of 4.5 kV, nebulizer gas (N_2) flow: 3.0 L min^{-1}, drying gas flow: 15 L min^{-1}, desolvation line temperature 250 °C and heat block temperature 400 °C.

2.5 Statistical analysis

Data were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. Calibrations were established applying a linear regression model. Calibrations with and without matrix were compared using F-test. All the above statistical analyses were carried out with a significance level (α) of 0.05 using GraphPad (San Diego, CA, USA) Prism 6.0 software. Central composite designs were prepared and analyzed using Statgraphics Centurion XV version 15.1.02 software (Rockville, MD, USA).

3. Results and discussion

3.1 Optimization of ultrasound-assisted extraction

Following early reports (Alvarez-Jubete et al., 2010; Hirose et al., 2010; Repo-Carrasco-Valencia et al., 2010), the first extraction solvents evaluated were methanol and aqueous methanol solution (7:3 v/v). Quinoa samples (0.8 g) were spiked with 2.5 mg kg^{-1} (2.5 mg L^{-1} due to 10-fold concentration) of each phenolic compound and then extracted with both solvents applying UAE general conditions (40ºC for 20 min) and a mass to solvent ratio of 1:10. Even when extraction yields were adequate, both solvents were discarded due to the high levels of interference peaks observed at 280 and 370 nm. Using the same conditions, ethyl acetate and acetone were assayed obtaining with both satisfactory yields and low interference levels at UV range. From both solvents, ethyl acetate was chosen due to its higher boiling point (77.1ºC vs 56.0ºC), with which it was possible to extend the temperature range under study in the experimental design. Optimization was carried out using a face-centered central composite design with two central points; this chemometric technique is one the most used due to its high efficiency regarding the number of experiments and the information obtained (Ferreira et al., 2007; Pineda et al., 2012). From the factors that may influence phenolic compound extraction applying UAE, the most relevant ones were defined according to preliminary assays and published

reports (Carciochi et al., 2015; Jovanović et al., 2017). Thus, the following factors and ranges were studied: temperature-X_1 (25 to 71°C) and extraction time-X_2 (from 10 to 45 min) keeping mass to solvent ratio (1:10) constant. The response/variable studied was extraction yield of each phenolic compound determined via chromatographic peaks area. An experimental plan composed of 10 runs was established (Table 1), which was assayed at least in duplicate (n=2) in randomized order to minimize the effects of uncontrolled factors using quinoa samples spiked with 2.5 mg kg^{-1} (2.5 mg L^{-1}) of each phenolic compound. An analysis of variance (ANOVA) with a significance level (α) of 0.05 was carried out to determine which experimental factors significantly affect the yield of each phenolic compound. Yield experimental data fitted second-degree model with a cubic domain. According to the results (table 2), extraction time showed a significant influence on extraction yield of two phenolic compounds, i.e. myricetin and quercetin 3-β-D-glucoside. All other phenolic compounds yields were not statistically affected by this factor. Regarding extraction temperature, only quercetin 3-β-D-glucoside yields were significantly affected, without observing statistically significant effect over the other phenolic compounds. No significant effect over phenolic compounds yields was observed due to factors interaction (p>0.05). By means of multiple response optimization, which considers all responses of all variables, an optimal extraction yield for all phenolic compounds was established at 71°C for 10 min. These optimal conditions were the same for most phenolic compounds, except for caffeic acid

(25°C, 15 min), vanillic acid (71°C, 30 min), quercetin 3-β-D-glucoside (39°C, 10 min) and quercetin (25°C, 10 min).

Table 1. Experimental runs for a central composite design with the corresponding yield of each phenolic compound (peak area).

Run	Temp	Time	Caffeic acid	Vanillic acid	Quercetin-3-β-D-gl.	*p*-coumaric acid	Vanillin	*t*-ferulic acid	Myricetin	Benzoic acid	Quercetin	Kaempferol
	(°C)	(min)						AU				
1-1	25	10	74832	56900	13894	120389	80926	45989	10222	25160	34741	30123
1-2	25	10	75046	56982	14168	120779	80528	45653	10694	25248	35129	29883
2-1	48	20	74640	58521	10904	129399	64568	56720	7549	26432	37321	31534
2-2	48	20	74762	58611	10498	129739	64828	57062	7905	25994	37643	31220
3-1	48	10	78540	60156	14899	137765	71199	55545	8320	26430	39999	34513
3-2	48	10	78600	60788	15145	137973	71799	55939	8680	26830	39167	34305
4-1	48	30	64196	52432	8001	115001	59940	48916	7009	20513	31970	26710
4-2	48	30	65390	51772	8477	115153	59982	48496	7197	19925	31692	26102
5-1	71	20	69876	60867	9101	122987	72897	53888	8752	22784	36892	29112
5-2	71	20	70656	59715	9309	123259	73567	53368	8734	23056	37120	28972
6-1	48	20	64843	51867	8105	114333	59999	48390	6890	20239	32151	26114
6-2	48	20	65103	52139	8487	114941	59599	47974	6948	20067	31957	26556
7-1	25	30	69144	50232	8543	120103	55317	54022	6159	18810	29479	26415
7-2	25	30	69544	51242	8841	120487	55917	53930	6345	18950	29135	26361
8-1	71	30	59898	72650	0	114629	85239	57001	10001	18146	37591	28235
8-2	71	30	61096	73104	0	115237	85619	56893	10189	18744	37965	27973
9-1	25	20	83452	58199	11643	130783	73261	58225	9131	28199	38337	32082
9-2	25	20	83810	58583	12051	131205	73703	58847	9077	28467	38209	31916
10-1	71	10	73310	60189	10413	133367	97412	54189	12179	25391	34148	31997
10-2	71	10	73418	60215	10615	132879	97896	54155	12197	24875	33894	31727

Table 2. ANOVA results showing the effect of independent variables on phenolic compounds extraction yields

Phenolic compounds	p-value Extraction time	p-value Extraction temperature
Caffeic acid	0.0784	0.1578
Vanillic acid	0.8872	0.0986
Quercetin-3β-D-gl.	0.0122	0.0465
p-coumaric acid	0.1071	0.9739
Vanillin	0.0547	0.0627
t-Ferulic acid	0.7961	0.6728
Myricetin	0.0445	0.1218
Benzoic acid	0.0578	0.4667
Quercetin	0.3314	0.5104
Kaempferol	0.0890	0.9327

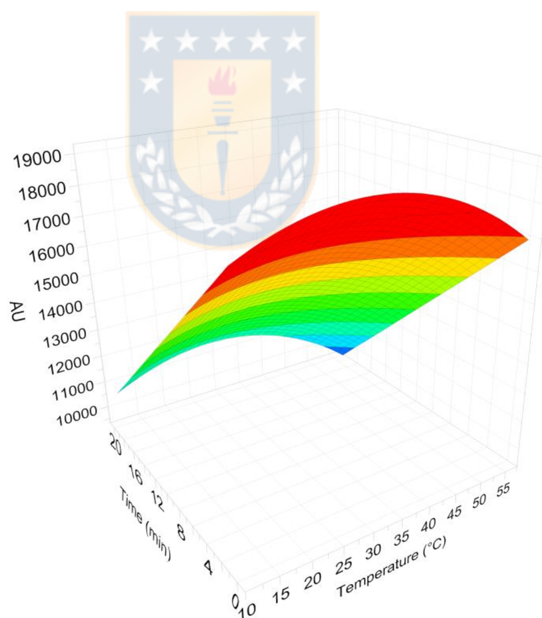

Fig. 1. Response surface plot for quercetin 3-β-D-glucoside obtained with UAE showing extraction yield changes relating to factors extraction time and temperature.

Since quercetin 3-β-D-glucoside detection level was the lowest one, it was preferred to use those optimal conditions in order to improve its extraction yield. Thus, the final extractions conditions using UAE were established at 39°C for 10 min (Fig. 1). Using these conditions, it was possible to obtain adequate extraction yields for all polyphenols (>81%), except for

quercetin 3-β-D-glucoside (*ca.* 45%), and low levels of interference peaks at UV range (Figure 2). Comparatively with other extraction methods, such as dynamic maceration (Alvarez-Jubete et al., 2010), ultrasound-assisted extraction (Gómez-Caravaca et al., 2011; Repo-Carrasco-Valencia et al., 2010) and dynamic maceration/ultrasound-assisted extraction (Carciochi et al., 2015; Tang et al., 2015), the proposed method significantly reduces extraction time (up to 12-fold).

3.2 Method validation

Optimized method was validated following the International Conference on Harmonization guidelines (International Conference on Harmonization (ICH), 2005). Calibration curves of each phenolic compound were established with and without quinoa matrix in order to evaluate a possible matrix effect. Since some slopes were statistically different (p<0.0001), all calibrations were established with matrix using six levels in triplicate from 0.02 – 5.0 mg L^{-1}. For spiking, a quinoa sample was used as a blank sample subtracting the initial content of

each phenolic compound. Calibration data fitted a linear regression model with determination coefficients (R^2) higher than 0.999 (Table 3).

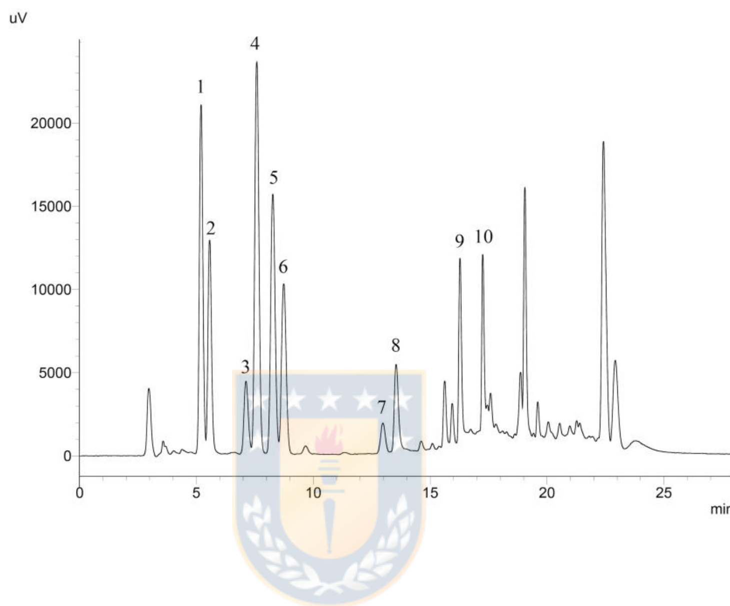

Fig. 2. HPLC/UV chromatogram at 280 nm of a quinoa seed sample spiked with 5 mg L^{-1} of each phenolic compound. Caffeic acid (1), vanillic acid (2), quercetin 3-β-D-glucoside (3), *p*-coumaric acid (4), vanillin (5), *trans*-ferulic acid (6), myricetin (7), benzoic acid (8), quercetin (9) and kaempferol (10).

Method precision was evaluated through repeatability and intermediate precision. Repeatability was measured injecting in sextuplicate (*n*=6) a quinoa sample spiked with a mixture of phenolic compounds (1.0 mg L^{-1}), showing RSD values from 0.14% to 2.89%. Intermediate precision was determined analyzing

in duplicates the same spiked sample (1.0 mg L^{-1}), showing RSD from 0.38% a 3.61% for all studied phenolic compounds. Method accuracy was determined through recovery evaluation at three levels (0.2, 0.4 and 0.6 mg kg^{-1}). Each level was prepared daily and measured in duplicate during three days. All polyphenols showed recovery values higher than 81%, except quercetin 3-β-D-glucoside, that showed *ca.* 45% with mean RSD values lower than 2.1% (Table 3)

Detection and quantification limits were calculated using signal-to-noise ratios (S/N) of 3 and 10, respectively. Considering an injection volume of 5 µL, detection and quantification limits ranged from 0.01 to 0.04 mg kg^{-1} and from 0.02 to 0.15 mg kg^{-1}, respectively. Selectivity was evaluated by peak identification through retention time (t_R), and UV-absorption spectra comparison with pure standard, and then, it was evaluated by mass spectrometry analysis in full scan mode (*m/z* 100 to 2000), showing the unique and corresponding *m/z* value of each phenolic compounds at their respective t_R (Table 3). Comparatively with other methods for determination of main phenolic compounds present in quinoa (Gómez-Caravaca et al., 2011), this method showed similar validation results in terms of recovery (accuracy), and dispersion levels, but with lower detection and quantification limits. Considering flow rates and running times (Alvarez-Jubete et al., 2010; Repo-Carrasco-Valencia et al., 2010; Tang et al., 2015), the proposed method reduced at least two times the

mobile phase consumption and double the number of samples analyzed per day.

Table 3. Summary of method validation results

Analyte	Regression equation[a]	R^2	Confidence interval		Repeat.[b]	Inter. precision[c]	LOD[d]	LOQ[e]	Selectivity	Recovery[h]
	y=ax±SD+b±SD		a	b	(%RSD)	(%RSD)	(mg kg^{-1})	(mg kg^{-1})	*m/z*[g]	(%)
Caffeic acid	36992±309+978±772	0.9997	36134 to 37850	-1168 to 3123	0.30	1.76	0.01	0.03	179	81.1-84.3
Vanillic acid	21271±198+1109±495	0.9997	20721 to 21821	-266 to 2484	0.67	1.73	0.01	0.04	167	92.8-95.5
Quercetin-3β	8718±72+464±192	0.9997	8518 to 8916	-68 to 997	0.87	2.16	0.04	0.14	463	44.6-45.6
p-coumaric acid	49833±364+1115±971	0.9998	48823 to 50843	-1580 to 3810	0.14	1.65	0.01	0.02	163	88.6-90.3
Vanillin	33342±294+1105±784	0.9997	32526 to 34157	-1072 to 3281	0.49	0.38	0.01	0.03	151	88.1-90.6
t-Ferulic acid	21575±221-99±590	0.9996	20961 to 22188	-1736 to 1538	0.97	1.77	0.02	0.06	193	86.3-88.6
Myricetin	4664±46+816±114	0.9996	4537 to 4791	499 to 1134	2.89	2.51	0.04	0.15	317	84.1-86.3
Benzoic acid	12146±154-1830±385	0.9994	11718 to 12574	-2900 to -760	2.06	3.57	0.04	0.14	121	82.0-85.6
Quercetin	15689±104+5601±243	0.9998	15399 to 15978	4925 to 6277	1.65	1.26	0.01	0.02	301	89.2-93.1
Kaempferol	16945±231-2321±424	0.9993	16304 to 17586	-3498 to -1144	1.43	3.61	0.02	0.07	285	83.0-85.8

[a] Range: 0.02–5.0 mg L^{-1}; [b] *n*=6; [c] *n*=3; [d] S/N=3; [e] S/N=10; [f] *n*=3 (each level)

3.3 Method application

Optimized and validated method was applied to analyze ten quinoa seed samples cultivated applying different agronomic conditions, mainly water stress. Samples showed a total phenolic compound content from 1.10 to 1.99 mg kg^{-1} (Table 4). Comparatively, these results are similar to those reported elsewhere for quinoa samples cultivated in other countries (Gómez-Caravaca et al., 2011). Analyzing the phenolic compound profile, only caffeic acid, vanillic acid, vanillin and *trans*-ferulic acid were found in quantifiable concentrations. This profile is similar to those reported by other studies (Repo-Carrasco-Valencia et al., 2010; Tang et al., 2015).

Table 4. Phenolic compounds content (mg kg^{-1})[a] in quinoa seeds samples

Sample code	Caffeic acid	Vanillic acid	Vanillin	*t*-ferulic acid	Total
R4T0	0.19 ± 0.00	0.82 ± 0.01	0.08 ± 0.00	0.66 ± 0.01	1.75
R1T70	0.33 ± 0.02	0.56 ± 0.06	0.08 ± 0.00	1.02 ± 0.07	1.99
R3T0	0.20 ± 0.00	0.60 ± 0.01	0.04 ± 0.01	0.65 ± 0.01	1.49
R1T100	0.15 ± 0.00	0.70 ± 0.00	ND	0.48 ± 0.01	1.33
R1T0	0.17 ± 0.00	0.77 ± 0.00	ND	0.21 ± 0.00	1.15
R3T40	0.24 ± 0.01	0.54 ± 0.01	ND	1.03 ± 0.00	1.81
R2T20	0.15 ± 0.02	0.34 ± 0.01	0.05 ± 0.01	0.56 ± 0.01	1.10
R3T70	0.20 ± 0.01	0.41 ± 0.02	ND	0.64 ± 0.01	1.25
R4T100	0.14 ± 0.00	0.59 ± 0.01	0.05 ± 0.00	0.48 ± 0.01	1.26
R3T100	0.18 ± 0.00	0.95 ± 0.01	0.09 ± 0.00	0.67 ± 0.02	1.89

[a]Results are expressed as mean ± standard deviation (*n*=3); ND: not detected (<LOD)

4. Conclusions

A method based on UAE and HPLC has been established for the determination of ten phenolic compounds in quinoa seeds. Chemometric optimization of ultrasound-assisted extraction conditions reduced up to 12-fold the extraction time. A selective, fast and MS compatible chromatographic method was developed for a suitable determination of phenolic compounds without major interferences. Separation conditions reduced at least two times the mobile phase consumption and double the number of samples analyzed per day. The proposed method was successfully applied to determine phenolic compounds content in 10 quinoa seed samples. Considering validation results and sample analysis, the method proved to be reliable for phenolic compound evaluation in different varieties of quinoa seeds.

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Conflict of interest statement

On behalf of all authors, the corresponding author states that there is no conflict of interest.

References

Alvarez-Jubete L, Wijngaard H, Arendt EK, Gallagher E (2010) Polyphenol composition and in vitro antioxidant activity of amaranth, quinoa buckwheat and wheat as affected by sprouting and baking Food Chem 119:770-778 doi:10.1016/j.foodchem.2009.07.032

Angeli V et al. (2020) Quinoa (Chenopodium quinoa Willd.): An Overview of the Potentials of the "Golden Grain" and Socio-Economic and Environmental Aspects of Its Cultivation and Marketization Foods 9:216 doi:10.3390/foods9020216

Azmir J et al. (2013) Techniques for extraction of bioactive compounds from plant materials: A review J Food Eng 117:426-436 doi:10.1016/j.jfoodeng.2013.01.014

Carciochi RA, Manrique GD, Dimitrov K (2015) Optimization of antioxidant phenolic compounds extraction from quinoa (Chenopodium quinoa) seeds J Food Sci Technol 52:4396-4404 doi:10.1007/s13197-014-1514-4

Ferreira SLC et al. (2007) Statistical designs and response surface techniques for the optimization of chromatographic systems J Chromatogr A 1158:2-14 doi:10.1016/j.chroma.2007.03.051

Fischer S, Wilckens R, Jara J, Aranda M (2013) Variation in antioxidant capacity of quinoa (chenopodium quinoa will) subjected to drought stress Ind Crop Prod 46:341-349 doi:10.1016/j.indcrop.2013.01.037

Fischer S et al. (2017) Protein and antioxidant composition of quinoa (Chenopodium quinoa Willd.) sprout from seeds submitted to water stress, salinity and light conditions Ind Crop Prod 107:558-564 doi:10.1016/j.indcrop.2017.04.035

Galarce-Bustos O, Novoa L, Pavon-Perez J, Henriquez-Aedo K, Aranda M (2019) Chemometric Optimization of QuEChERS Extraction Method for Polyphenol Determination in Beers by Liquid Chromatography with Ultraviolet Detection Food Anal Methods 12:448-457 doi:10.1007/s12161-018-1376-x

Gómez-Caravaca AM, Iafelice G, Verardo V, Marconi E, Caboni MF (2014) Influence of pearling process on phenolic and saponin content in quinoa (Chenopodium quinoa Willd) Food Chem 157:174-178 doi:10.1016/j.foodchem.2014.02.023

Gómez-Caravaca AM, Segura-Carretero A, Fernández-Gutiérrez A, Caboni MF (2011) Simultaneous Determination of Phenolic Compounds and Saponins in Quinoa (Chenopodium quinoa Willd) by a Liquid Chromatography–Diode Array Detection–Electrospray Ionization–Time-of-Flight Mass Spectrometry Methodology J Agric Food Chem 59:10815-10825 doi:10.1021/jf202224j

Harborne JB, Williams CA (2000) Advances in flavonoid research since 1992 Phytochemistry 55:481-504 doi:10.1016/S0031-9422(00)00235-1

Hirose Y, Fujita T, Ishii T, Ueno N (2010) Antioxidative properties and flavonoid composition of Chenopodium quinoa seeds cultivated in Japan Food Chem 119:1300-1306 doi:10.1016/j.foodchem.2009.09.008

International Conference on Harmonization (ICH) (2005) Validation of analytical procedures: text and methodology Q2 (R1) 1

Jovanović AA, Đorđević VB, Zdunić GM, Pljevljakušić DS, Šavikin KP, Gođevac DM, Bugarski BM (2017) Optimization of the extraction process of polyphenols from Thymus serpyllum L. herb using maceration, heat- and ultrasound-assisted techniques Sep Purif Technol 179:369-380 doi:10.1016/j.seppur.2017.01.055

Júnior DS, Krug FJ, Pereira MdG, Korn M (2006) Currents on ultrasound-assisted extraction for sample preparation and spectroscopic analytes determination Appl Spectrosc Rev 41:305-321 doi:10.1080/05704920600620436

Khan MK, Abert-Vian M, Fabiano-Tixier A-S, Dangles O, Chemat F (2010) Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (Citrus sinensis L.) peel Food Chem 119:851-858 doi:10.1016/j.foodchem.2009.08.046

Khoddami A, Wilkes MA, Roberts TH (2013) Techniques for analysis of plant phenolic compounds Molecules 18:2328-2375 doi:10.3390/molecules18022328

Noratto GD, Murphy K, Chew BP (2019) Quinoa intake reduces plasma and liver cholesterol, lessens obesity-associated inflammation, and helps to prevent hepatic steatosis in obese db/db mouse Food Chem 287:107-114 doi:10.1016/j.foodchem.2019.02.061

Ogungbenle HN (2003) Nutritional evaluation and functional properties of quinoa (Chenopodium quinoa) flour Int J Food Sci Nutr 54:153-158 doi:10.1080/0963748031000084106

Pineda A, Carrasco J, Pena-Farfal C, Henriquez-Aedo K, Aranda M (2012) Preliminary evaluation of biogenic amines content in Chilean young varietal wines by HPLC Food Control 23:251-257 doi:10.1016/j.foodcont.2011.07.025

Repo-Carrasco-Valencia R, Hellström JK, Pihlava J-M, Mattila PH (2010) Flavonoids and other phenolic compounds in Andean indigenous grains: Quinoa (Chenopodium quinoa), kañiwa (Chenopodium pallidicaule) and kiwicha (Amaranthus caudatus) Food Chem 120:128-133 doi:10.1016/j.foodchem.2009.09.087

Senes CER, Nicácio AE, Rodrigues CA, Manin LP, Maldaner L, Visentainer JV (2020) Evaluation of Dispersive Solid-Phase Extraction (d-SPE) as a Clean-up Step for Phenolic Compound Determination of Myrciaria cauliflora Peel Food Anal Methods 13:155-165 doi:10.1007/s12161-019-01566-9

Stoven S, Murray JA, Marietta EV (2013) Latest in vitro and in vivo models of celiac disease Expert Opin Drug Discov 8:445-457 doi:10.1517/17460441.2013.761203

Tang Y, Li X, Zhang B, Chen PX, Liu R, Tsao R (2015) Characterisation of phenolics, betanins and antioxidant activities in seeds of three Chenopodium quinoa Willd. genotypes Food Chem 166:380-388 doi:10.1016/j.foodchem.2014.06.018

Tang Y, Zhang B, Li X, Chen PX, Zhang H, Liu R, Tsao R (2016) Bound Phenolics of Quinoa Seeds Released by Acid, Alkaline, and Enzymatic Treatments and Their Antioxidant and α-Glucosidase and Pancreatic Lipase Inhibitory Effects J Agric Food Chem 64:1712-1719 doi:10.1021/acs.jafc.5b05761

van den Driessche JJ, Plat J, Mensink RP (2018) Effects of superfoods on risk factors of metabolic syndrome: a systematic review of human intervention trials Food Funct 9:1944-1966 doi:10.1039/C7FO01792H

Vinatoru M (2001) An overview of the ultrasonically assisted extraction of bioactive principles from herbs Ultrason Sonochem 8:303-313 doi:10.1016/S1350-4177(01)00071-2

Wang L, Weller CL (2006) Recent advances in extraction of nutraceuticals from plants Trends Food Sci Technol 17:300-312 doi:10.1016/j.tifs.2005.12.004

CAPITULO II SECCIÓN 2

Title: Multivariable optimization of ultrasound-assisted extraction for the determination of phenolic compounds and antioxidants from Arrayán (*Luma apiculata*) leaves by microplate-based methods and mass spectrometry

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Abstract

Introduction. Arrayán (*Luma apiculata*) is a tree species native from Chile that has been extensively used in traditional medicine like astringent and anti-inflammatory.

Objective. To establish a multivariable optimization of ultrasonic-assisted extraction (UAE) of phenolic compounds from arrayán leaves to obtain a polyphenol-rich extract (PRE) applying microplate-based methods and mass spectrometry.

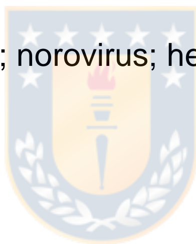
Material and Methods. Extraction parameters were optimized applying full factorial and central composite designs. PRE of arrayán leaves was obtained via purification on XAD-7 resin and phenolic profile was established by UHPLC/DAD/MS in Scan and SIM modes.

Results. The optimal conditions for phenolic compound extraction were: 42% ethanol in extraction solvent, extraction time 27 min, extraction temperature 50ºC and a mass to solvent ratio 1:33.4 g mL^{-1}. Under these conditions, a total phenolic content (TPC) of 128.16±1.18 and 593.64±6.49 mg of gallic acid equivalent per g of dry weight (DW) were obtained for raw extract (RE) and PRE. Antioxidant capacity determined via FRAP analysis showed values of 1349.53±28.99 and 6175.47±127.64 µmol Trolox equivalent per g of DW for RE and PRE, and DPPH IC_{50} values of 831.40±0.80 and 132.21±2.51 µg mL^{-1}. Polyphenols profile showed the presence of quercetin 3-ß-D-glucoside,

myricetin, quercetin, kaempferol 3-glucoside and gallic acid; the last two reported for the first time. A dose-dependent manner effect was observed against norovirus and hepatitis A.

Conclusions. Results showed that arrayán leaves, particularly PRE, could be a good source of bioactive compounds for functional foods, nutraceuticals and active pharmaceutical ingredients development, with special attention on its antioxidant and antiviral activities.

Keywords: central composite design; liquid chromatography; full factorial design; polyphenols; antiviral; norovirus; hepatitis A; nutraceuticals



1. Introduction

Chronic non-communicable diseases (CNCD) are pathologies characterized for showing long duration and slow progression. The most relevant ones are cardiovascular, cancer, diabetes, and chronic respiratory diseases. It is estimated that CNCD are responsible for 71% of all deaths worldwide. Amongst the risk factors that cause higher mortality, unhealthy behaviors like tobacco use, unhealthy diets, sedentary lifestyle and overweight-obesity are the main contributors[1]. The current concern about CNCD morbimortality has increased the interest of searching bioactive compounds (functional) in plants and foods, finding several types of molecules with different healthy properties[2, 3]. Arrayán (*Luma apiculata*) is a tree species native from Chile that has been extensively used in traditional medicine. Its leaves are commonly used for medicinal purposes like astringent, balsamic, and anti-inflammatory[4]. Additionally, its leaves and stems have also a high antioxidant capacity that could be related to the presence of a large amount of phenolic compounds[4]. Some healthy leaf properties are attributed precisely to this type of bioactive compounds, which could have the ability to reduce the risk of developing CNCD[5]. Numerous studies have documented the biological properties of phenolic compounds, which are mainly related to their antioxidant capacity showing vasodilator, vasoprotective, anti-thrombotic, anti-atherosclerotic and anti-inflammatory effects[6]. Different techniques have been used for phenolic compounds

extraction, from traditional ones like maceration and Soxhlet extraction to advanced technologies such as pressurized liquid extraction, supercritical fluid extraction, microwave-assisted extraction and ultrasound-assisted extraction[7, 8]. This last extraction technology has received much attention for bioactive compounds extraction because it is possible to achieve high extraction yields in a short period of time[9] using simple procedures and low-cost equipment[10, 11]. To the best of our knowledge, the present work reports for the first time a multivariable optimization of ultrasonic-assisted extraction (UAE) of phenolic compounds from arrayán leaves to obtain a polyphenol-rich extract (PRE). A complementary study was performed by ultra-high-performance liquid chromatography-diode array-mass spectrometry detection (UHPLC/DAD/MS) to establish the polyphenols profile of arrayán leaves. The type of polyphenol present is relevant due to the different activities ascribed to these bioactive compounds. Particularly, antiviral effect, which is gaining attention especially for the food and pharmaceutical industry since the worldwide concern regarding foodborne health risks[12]. For that reason, the activity of arrayán leaves extract against human norovirus and hepatitis A virus was studied, both associated with foodborne outbreaks[13-16].

2. Material and Methods

2.1 Reagents, chemicals, and standard solutions

Caffeic acid (≥98%), *p*-coumaric acid (≥98%), gallic acid (≥99%), *trans*-ferulic acid (99%), vanillic acid (≥97%), quercetin 3-β-D-glucoside (≥90%), vanillin (≥99%), quercetin hydrate (95%), kaempferol (≥90%), kaempferol 3-glucoside (≥90%), myricetin (96%), 2,4,6-tri (2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and amberlite XAD–7 resin were purchased from Sigma (St. Louis, MO, USA). Folin-Ciocalteu phenol reagent (2N), sodium hydrogen carbonate (>99%) formic acid (98%) and analytical grade solvents (ethyl acetate, ethanol, methanol, acetone, and acetonitrile) were obtained from Merck (Darmstadt, Germany). Ultrapure water (18.2 MΩ cm at 25ºC) was produced using a simplicity system from Millipore (Bedford, MA, USA). Stock solution of phenolic compounds was prepared in methanol: water (7:3 v/v) for a given concentration of 0.30 μg mL^{-1}.

2.2 Sample preparation and extraction process

Arrayán leaves were collected in Concepcion, Biobio region, Chile (36º 50´ 0´´S and 73º 3´ 0´´W) in November 2018. Leaves samples without blemishes or

damage were cleaned, frozen (-24 ºC for 48h), and then ground and homogenized using an IKA (Staufen, Germany) A11 basic analytical mill. After a second freezing step (-24 ºC for 24h) samples were freeze-dried for 48 h at -55°C using a Martin Christ (Osterode am Harz, Germany) Alpha 1-2 LD plus freeze-dryer. Raw extract (RE) of arrayán leaves was prepared as follows: 0.1 g of freeze-dried leaves powder was vortex-mixed for 1 min with 3.34 mL of extraction solvent composed of ethanol: water: formic acid (42:57:1 v/v/v). Then, ultrasound-assisted extraction (UAE) was carried out for 27 min at 50 ºC using an ultrasonic bath (Getidy, China) with a frequency of 40 kHz and a potency of 200 W. Thereafter, samples were centrifuged for 30 min at 16162 x *g,* and 1.5 mL of clear supernatant was collected and filtered through 13 mm polyvinylidene fluoride (PVDF) syringe filter (0.22 µm). This filtrate was used for total phenolic content (TPC) and antioxidant capacity (AC) analysis (DPPH and FRAP assay). PRE of arrayán leaves was obtained from 334 mL of RE. First, ethanol content of RE was removed under vacuum in a Büchi (Flawil, Switzerland) R-100 rotavapor at 40 °C. Ethanol-free extract was loaded onto a column containing 100 g Amberlite XAD-7 resin, previously conditioned with a proper volume of ultrapure water. Column was washed with four portions of 50 mL of ultrapure water and phenolic compounds were eluted with four portions of 25 mL of ethanol. This solution was evaporated to dryness under vacuum giving 1115.8 mg of arrayán leaves powder rich in phenolic compounds. In order to compare with RE results, PRE solution was prepared dissolving 0.1 g of PRE powder with

3.34 mL of extraction solvent. This solution was filtered through 13 mm PVDF syringe filter (0.22 µm) and used for TPC and AC analysis (DPPH and FRAP assay).

2.3 Total phenolic content (TPC)

TPC was determined by Folin-Ciocalteu assay following previously reported the protocols[4, 17] with some modifications to adapt the assay to 96-well microplates. First, RE and PRE were diluted with extraction solvent and 15 µL of each solution were mixed with 100 µL of Folin–Ciocalteu reagent (previously diluted 10-fold) and 100 µL of sodium hydrogen carbonate (60 g L^{-1}). This solution was incubated into BioTek (Winooski, VT, USA) Epoch 2 microplate reader under continuous agitation for 90 minutes at room temperature (23±2 °C). Optical density (OD) was measured at 725 nm using gallic acid as reference standard, and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW).

2.4 DPPH Radical Scavenging Assay

FRAP and DPPH assay were used to estimate AC, the chemistry of both methods is described in detail elsewhere[18]. DPPH assay was performed

according to the procedure described previously[4, 19] with some modifications to adapt the protocol to 96-well microplate. Stock solution was prepared by dissolving 11.83 mg of DPPH with 3 mL of methanol, this solution was 10-fold diluted to prepare the working solution. After appropriate dilution with extraction solvent, 5 µL of sample solution were mixed with 95 µL of methanol and 100 µL of DPPH working solution. After gentle mixing, 96-well microplate was incubated inside Epoch 2 microplate reader under continuous agitation for 30 minutes at room temperature (23±2 °C). OD or absorbance (A) was determined at 517 nm. Trolox reagent was used as equivalent standard. DPPH scavenging capacity as percentage (Pi) was calculated as Pi= [(Acontrol - Asample)/Acontrol] *100. Calibration data of % DPPH bleaching activity versus concentration were used to calculate IC_{50} (value at which 50% of radical is scavenged).

2.5 Ferric reducing antioxidant (FRAP) assay

FRAP assay was done according to earlier reports[4, 20] with some modifications to adapt the protocol to 96-well microplate. Stock solution was composed of 0.3 mol L^{-1} of acetate buffer (pH 3.6), 0.01 mol L^{-1} of TPTZ solution in 0.04 mol L^{-1} of HCl, and 0.02 mol L^{-1} of $FeCl_3 \cdot 6H_2O$ solution. Fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3 \cdot 6H_2O$. Thirty microliters of appropriately diluted sample were mixed with 150 µL of working solution (previously diluted 5-fold with ultrapure water) and incubated

under continuous agitation for 30 minutes at 37ºC inside the microplate reader. Colored product (ferrous tripyridyltriazine complex) formation was determined by measuring the OD at 593 nm. Calibration was established using Trolox (from 0.2 to 2.5 µmol mL^{-1}), and results were expressed in µmol Trolox equivalent (TE) per g of DW.

2.6 Polyphenols profile

Samples were analyzed using a Shimadzu (Kyoto, Japan) Nexera X2 UHPLC system composed of LC30AD pump, DGU-20AR 5R vacuum degasser, SIL-30AC autosampler, CTO-20AC column oven, and SPD-M20A diode array detector coupled to a Shimadzu LCMS-8030 triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI). Separation of phenolic compounds was carried out on Phenomenex (Torrance, CA, USA) Kinetex C_{18} core-shell column (150 mm x 4.6 mm, S-5 µm) connected to Kinetex guard column, both set at 32°C, using a binary mobile phase composed of acidified water (0.1% v/v formic acid-A) and acetonitrile (B). The following gradient program was applied at a flow rate of 0.5 mL min^{-1}: 0-17 min 20%-45% B; 17-18 min 45%-45% B (isocratic step); 18-20 min 45%-20% B; and 20-30 min 20%-20% B (column conditioning). Complete separation was accomplished in less than 15 min for a total run time of 30 min. UV-detection was performed from 200-400 nm and MS analysis was carried out using the following settings: ESI (-

) voltage of 4.5 kV, nebulizer gas (N_2) flow: 3.0 L min^{-1}, drying gas flow: 15 L min^{-1}, desolvation line temperature 250 °C and heat block temperature 400 °C. Analytes were evaluated in Full Scan (*m/z* 100-2000) and Selected Ion Monitoring (SIM) modes using the following *m/z* values: caffeic acid ($[M-H]^-$, *m/z* 179); vanillic acid ($[M-H]^-$, *m/z* 167); quercetin 3-ß-D-glucoside ($[M-H]^-$, *m/z* 463); *p*-coumaric acid ($[M-H]^-$, *m/z* 163); vanillin ($[M-H]^-$, *m/z* 151), *trans*-ferulic acid ($[M-H]^-$, *m/z* 193); quercetin ($[M-H]^-$, *m/z* 301), kaempferol ($[M-H]^-$, *m/z* 285); gallic acid ($[M-H]^-$, *m/z* 169), kaempferol 3-glucoside ($[M-H]^-$, *m/z* 447); and myricetin ($[M-H]^-$, *m/z* 317).

2.7 Antiviral activity

Murine norovirus (MNV) and hepatitis A virus (HAV) strain HM-175/18f (ATCC VR-1402) were assayed and propagated in RAW 264.7 and FRhK-4 (ATCC CRL-1688) cell lines, respectively. Virus stocks were harvested by infecting cell lines for 2 days for MNV and 12 days for HAV, followed by three thaw cycles at 660 x *g* for 30 min. Fifty percent of tissue culture infectious dose ($TCID_{50}$) was determined to enumerate infectious viruses using the Spearmen-Karber method[21]. A proper amount of arrayán leaves powder was dissolved in 30% v/v ethanol solution for a given concentration of 0.5 and 5 mg mL^{-1}. Each solution was incubated in equal volume of MNV and HAV (*ca.* 5 log $TCID_{50}$ mL^{-1}) for 2 h at 37°C. Samples were then ten-fold diluted in Dulbecco's Modified Eagle's

Medium supplemented with 10% fetal calf serum and the residual infectivity was determined by $TCID_{50}$. Ten-fold dilutions of treated and untreated virus suspensions were inoculated into confluent cell monolayers in 96-well plates. Virus suspensions added to 30% v/v ethanol under the same experimental conditions were used as positive control. Each treatment was done in triplicate. Virus decay titer was calculated as $\log_{10}(N_x/N_0)$, where N_0 is the infectious virus titer for untreated samples and N_x is the infectious virus titer for arrayán leaves extract treated samples[15].

2.8 Statistical analysis

Experimental data were analyzed using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. Full factorial design and central composite design were prepared and analyzed using MODDE version 7.0.0.1 software (Umetrics. Umea, Sweden). All statistical tests were performed with a significance level (α) of 0.05.

3. Results and Discussion

3.1 Full factorial design 2k (FFD)

Combination of two-level full factorial design (FFD)[22] and central composite design (CCD)[23, 24] is commonly used for screening and optimization of different analytical parameters. From the factors that may influence TPC extraction by UAE, the most relevant ones were defined according to preliminary assays and published reports[25-27]. Thus, the following factors and ranges were studied: extraction time (20-30 minutes), extraction temperature (50-65°C), mass to solvent ratio (1:20-1:30 g mL^{-1}) and ethanol percentage in extraction solvent (30-50 % v/v). Four-factor two-level FFD was built with three central points resulting in 19 experiments (Table 1). Each run was performed in duplicate (n=2) in random order to minimize the effects of uncontrolled factors. The response studied was TPC determined by the Folin-Ciocalteu assay as described in section 2.3. According to ANOVA results (Table 1 Supporting Information), extraction temperature was the only factor without a significant effect on TPC (p=0.148). Ethanol concentration (p=0.001), mass to solvent ratio (p=0.005) and extraction time (p=0.008) showed a significant effect on TPC. Regarding factor interactions, only ethanol-ethanol percentage interaction showed a significant effect (p<0.001), all other interactions did not show a statistically effect on TPC [ethanol percentage-extraction time (p=0.898), ethanol percentage-extraction

temperature (p=0.372), ethanol percentage-mass to solvent ratio (p=0.075), extraction time-mass to solvent ratio (p=0.925), extraction temperature-mass to solvent ratio (p=0.346), extraction time-extraction temperature (p=0.185)]. All factors with a significant effect on TPC were studied via CCD, while extraction temperature was set at 50°C.

3.2 Central composite design (CCD)

A CCD with three factors and three central points was established (Table 2) and analyzed in duplicate (*n*=2) in random order to avoid systematic errors.

Table 1. Experimental runs (n=2) of full factorial design (FFD) showing independent variables and experimental data for total phenolic content (TPC) response

N° run	Etanol concentration (%v/v)	Extraction time (min)	Extraction temperature (°C)	Mass to solvent ratio (g mL^{-1})	TPC (mg GAE g^{-1} DW)*
1	30	20	50	1:20	99.98 ± 0.31
2	50	20	50	1:20	101.52 ± 0.41
3	30	30	50	1:20	102.23 ± 0.61
4	50	30	50	1:20	108.29 ± 0.15
5	30	20	65	1:20	100.37 ± 0.58
6	50	20	65	1:20	105.23 ± 0.46
7	30	30	65	1:20	103.78 ± 0.55
8	50	30	65	1:20	109.65 ± 0.46
9	30	20	50	1:30	104.03 ± 0.49
10	50	20	50	1:30	107.92 ± 0.58
11	30	30	50	1:30	105.88 ± 0.54
12	50	30	50	1:30	109.32 ± 0.25
13	30	20	65	1:30	106.00 ± 0.31
14	50	20	65	1:30	107.48 ± 0.44
15	30	30	65	1:30	109.17 ± 0.20
16	50	30	65	1:30	111.86 ± 0.30
17	40	25	57.5	1:25	109.23 ± 0.31
18	40	25	57.5	1:25	107.76 ± 0.29
19	40	25	57.5	1:25	108.95 ± 0.45

*Mean ± standard deviation (n=3)

Table 2. Experimental runs (*n*=2) for a central composite design (CCD) showing independent variables and experimental data for total phenolic content (TPC) response

N° run	Etanol Concentration (%v/v)	Extraction time (min)	Mass to solvent ratio (g mL^{-1})	TPC (mg GAE g^{-1} DW)*
1	30	20	1:20	99.98 ± 0.31
2	50	20	1:20	101.52 ± 0.41
3	30	30	1:20	102.23 ± 0.61
4	50	30	1:20	108.29 ± 0.15
5	30	20	1:30	104.03 ± 0.49
6	50	20	1:30	107.92 ± 0.58
7	30	30	1:30	105.88 ± 0.54
8	50	30	1:30	109.32 ± 0.25
9	23.2	25	1:25	92.00 ± 0.35
10	56.8	25	1:25	97.15 ± 0.48
11	40	16.6	1:25	103.65 ± 0.31
12	40	33.4	1:25	107.43 ± 0.52
13	40	25	1:16.6	105.45 ± 0.43
14	40	25	1:33.4	111.01 ± 0.61
15	40	25	1:25	109.47 ± 0.55
16	40	25	1:25	106.28 ± 0.36
17	40	25	1:25	108.42 ± 0.47

*Mean ± standard deviation (n=3)

As for FFD, the response evaluated was TPC determined by the Folin-Ciocalteu assay. ANOVA results (Table 2 Supporting Information) showed that the following factors and interactions had a significant effect on TPC: ethanol percentage ($p=0.016$), mass to solvent ratio ($p=0.013$), extraction time ($p=0.041$) and ethanol percentage-ethanol percentage interaction ($p<0.001$). All other interactions did not show a statistically effect on TPC [mass to solvent ratio-mass to solvent ratio ($p=0.350$), extraction time-extraction time ($p=0.579$), ethanol percentage-extraction time ($p=0.498$), ethanol percentage-mass to solvent ratio ($p=0.964$), extraction time-mass to solvent ratio ($p=0.344$)]. Through response optimization, the optimal UAE conditions were defined as follows: 42% v/v of ethanol in extraction solvent, extraction time of 27 min, and a mass to solvent ratio of 1:33.4 g mL^{-1} (Fig. 1). Under UAE optimized conditions, TPC showed a value of 116.50±1.18 mg GAE g^{-1} of DW, which was very similar to the predicted content (112.82 mg GAE g^{-1} of DW).

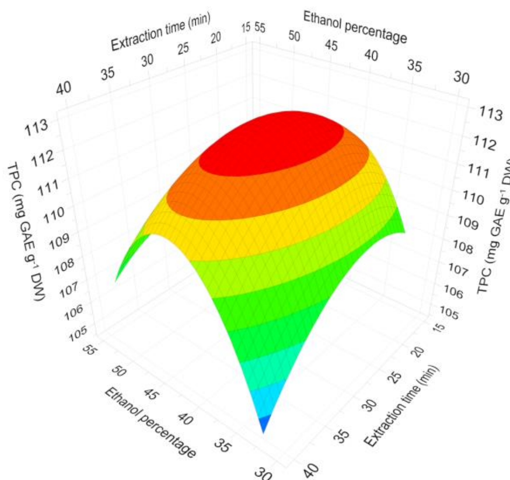


Fig. 1. Response surface plot showing the optimal extraction conditions for total phenolic content (TPC) and the influence of the variables in the extraction yield.

3.3 Total phenolic compounds content (TPC) and Antioxidant Capacity (AC) analysis

Using optimized UAE conditions, TPC values were 116.50±1.18 and 593.64±6.49 mg of GAE g^{-1} of DW for RE and PRE, respectively. AC of RE and PRE showed FRAP values of 1349.53±28.99 and 6175.47±127.64 μmol TE g^{-1} of DW; and DPPH IC_{50} of 831.40±0.80 μg mL^{-1} and 132.21±2.51 μg mL^{-1}, respectively. PRE showed TPC, FRAP and DPPH values at least 4.6-fold higher than RE. This value clearly demonstrated the importance of purification step with XAD-7 column. Additionally, DPPH IC_{50} value of PRE (132.21±2.51 μg mL^{-1})

was in the same magnitude but significantly lower ($p<0.001$) than the one observed with Trolox reagent (165.04±1.44 µg mL^{-1}). The only article that reported the TPC in arrayán aerial parts, applied an extraction process via maceration with methanol with a posterior purification by solid-phase extraction using C_{18} sorbent[4]. Comparing both purified extracts, PRE showed TPC and FRAP mean values 3.3 and 36 times higher than the ones described previously (179.83±0.38 mg of GAE g^{-1} of DW and 170.5±0.1 µmol TE g^{-1} of DW). DPPH IC_{50} values were not possible to compare because the reported value was much lower (2.44±0.03 µg mL^{-1}) even compared with Trolox standard. Although this unusual result, TPC and FRAP results showed the importance of optimizing the extraction conditions using a design of experiment. A key factor was the extraction technology which allowed the obtention of extracts rich in polyphenols. UAE has emerged as an excellent choice for polyphenol extraction, especially due to the high yields accomplished compared with traditional methods, and in some cases, superior to more advanced extraction technologies such as supercritical fluid extraction and microwave-assisted extraction[28, 29]. Another factor to consider is the extraction solvent; due to the polar nature of polyphenols, polar protic medium such as hydroalcoholic solutions are the most used binary mixture to carry out extractive processes from food matrices[30, 31].

3.4 Polyphenols profile

UHPLC/DAD/MS method was carried out to quantify the following phenolic compounds in arrayán leaves: gallic acid, caffeic acid, *trans*-ferulic acid, vanillic acid, *p*-coumaric acid, quercetin 3-ß-D-glucoside, vanillin, quercetin, kaempferol, kaempferol 3-glucoside, and myricetin. Using the chromatographic conditions described in section 2.6 an adequate separation of eleven phenolic compounds was achieved in less than 15 minutes (Fig. 2). Five phenolic compounds were detected and quantified in arrayán leaves samples (Fig. 3), i.e., quercetin 3-ß-D-glucoside, myricetin, quercetin, kaempferol 3-glucoside and gallic acid (Table 3). The last two, to the best of our knowledge, are reported for the first time. As can be observed in Fig. 3, mass chromatogram also shows an important signal at retention time (t_R) of 6.663 min with a *m/z* value of 447, which is the same *m/z* value as kaempferol 3-glucoside but appears at a slightly higher t_R.

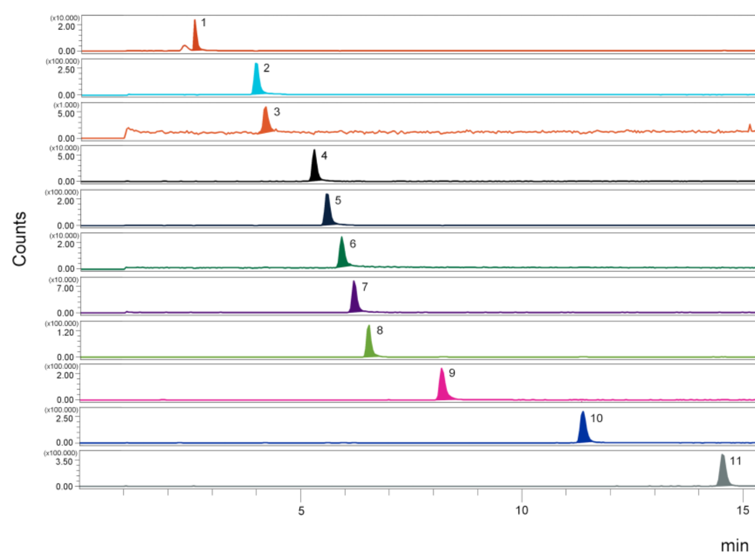


Fig. 2. Mass chromatogram of phenolic compounds analyzed. Gallic acid (1), caffeic acid (2), vanillic acid (3), quercetin 3-β-D-glucoside (4), *p*-coumaric acid (5), vanillin (6), *trans*-ferulic acid (7), kaempferol 3-glucoside (8), myricetin (9), quercetin (10) and kaempferol (11).

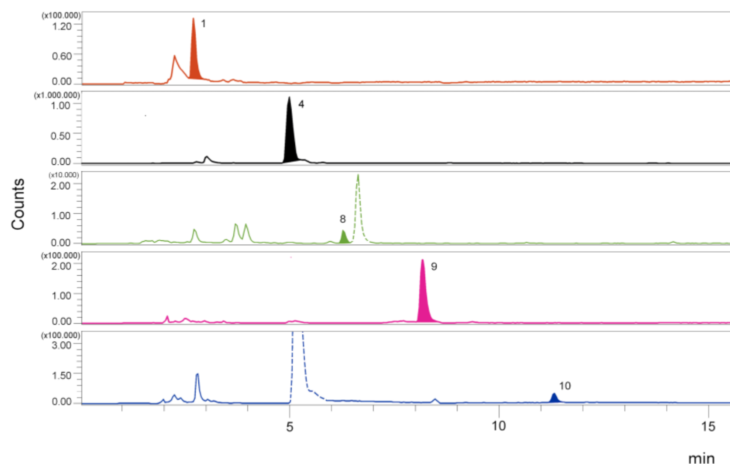


Fig. 3. Mass chromatogram of phenolic compounds found in arrayán leaf samples. Gallic acid (1), quercetin 3-β-D-glucoside (4), kaempferol 3-glucoside (8), myricetin (9), and quercetin (10). See content in Table 3.

Based on this chromatographic behavior (t_R and elution order) and *m/z,* it is possible to infer that this signal could correspond to quercitin-3-O-rhamnoside (Quercitrin)[32, 33], previously identified in arrayán leaves[4]. Additionally, it could also correspond to kaempferol derivatives such as kaempferol 7-glucoside, which has the same *m/z* value and present similar t_R than kaempferol 3-glucoside[34]. Regarding the intense signal observed at t_R 5.240 min with *m/z* 301, this could correspond to ellagic acid[35, 36].

Table 3. Phenolic compounds profile after optimized ultrasound-assisted extraction of arrayán leaf

Peak	Compounds	t_R [a] (min)	λ max (nm)	*m/z* [b]	Concentration (mg kg^{-1}) [c]
1	Gallic acid	2.504	275	169	31.48 ± 0.52
2	Caffeic acid	3.856	322	179	ND
3	Vanillic acid	4.087	263	167	ND
4	Quercetin 3-β-D-glucoside	5.183	353	463	2638.72 ± 42.69
5	*p*-coumaric acid	5.506	309	163	ND
6	Vanillin	5.823	284	151	ND
7	*trans*-ferulic acid	6.095	320	193	ND
8	Kaempferol 3-glucoside	6.427	265	447	0.86 ± 0.02
9	Myricetin	8.109	372	317	57.41 ±1.61
10	Quercetin	11.259	372	301	3.32 ± 0.07
11	Kaempferol	14.450	365	285	ND

[a]: Retention time; [b]: Mass to charge value; [c]: Mean ± standard deviation (*n*=3); ND: not detected (<0.01 mg kg^{-1})

3.5 Antiviral activity

Arrayán leaves extract (30% v/v ethanol) was tested against MNV, a well-studied human norovirus surrogate, and HAV at concentrations of 0.5 and 5 mg mL^{-1}. Results showed a clear antiviral activity reducing the viral titers in a dose-dependent manner. Incubation of MNV with arrayán extract at concentrations of 5 mg mL^{-1} for 2 h at 37°C reduced titers to undetectable levels, while for HAV the decrease was more than 2 log (Fig. 4). These results are in concordance with phenolic compounds profile and with early reports that showed antiviral activity of this type of bioactive compound against numerous foodborne viruses, such as MNV and HAV[13-16].

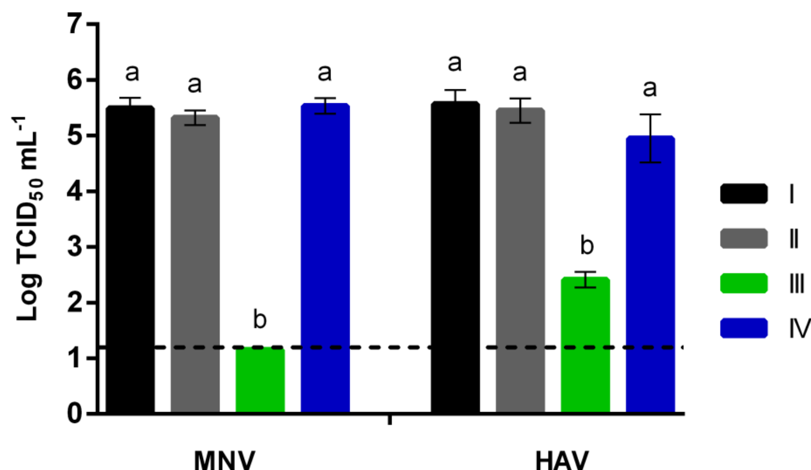


Fig. 4. Reduction of murine norovirus (MNV) and hepatitis A virus (HAV) titers (log $TCID_{50}$ mL^{-1}) treated with arrayán leaf extract at concentrations of 5 and 0.5 mg mL^{-1}. Virus control (I); ethanol control (II); 5 mg mL^{-1} of arrayán leaf extract treatment (III); 0.5 mg mL^{-1} arrayán leaf extract treatment (IV).

Each bar represents the mean of at least triplicate determination.

Different letters denote significant differences between treatments. Horizontal dashed-line depicts the detection limit.

Overall, to the best of our knowledge, a multivariable optimization of UAE of phenolic compounds from arrayán leaves was established for the first time. With this optimized UAE process, it was possible to obtain an arrayán leaves extract with TPC and FRAP values 3.3 and 36-fold higher than the ones described in previous reports. Extract purification via XAD-7 column increased TPC, FRAP,

and DPPH values at least 4.6-fold compared with the unpurified extract. Employing UHPLC/DAD/MS analysis it was possible to identify the presence of five phenolic compounds, reporting the novel presence of kaempferol 3-glucoside and gallic acid. To the best of our knowledge, for the first time, the antiviral effect of arrayán leaves extract against MNV and HAV was demonstrated. These results showed that arrayán leaves extract, particularly PRE, could be a good source of bioactive compounds for functional foods, nutraceutical and/or active pharmaceutical ingredients development, with special attention on its antioxidant and antiviral activities.

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Declarations of interest

None.

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References

1 World Health Organization. 2018. Non communicable diseases. URL [https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases]; accessed March 2020

2 Galarce-Bustos O, J Pavon, K Henriquez-Aedo and M Aranda. Detection and identification of acetylcholinesterase inhibitors in Annona cherimola Mill. by effect-directed analysis using thin-layer chromatography-bioassay-mass spectrometry. *Phytochem Anal* 2019; 30: 679-686. https://doi.org/10.1002/pca.2843

3 Galarce-Bustos O, J Pavon-Perez, K Henriquez-Aedo and M Aranda. An improved method for a fast screening of alpha-glucosidase inhibitors in cherimoya fruit (Annona cherimola Mill.) applying effect-directed analysis via high-performance thin-layer chromatography-bioassay-mass spectrometry. *J Chromatogr A* 2019; 1608. https://doi.org/10.1016/j.chroma.2019.460415

4 Simirgiotis MJ, J Bórquez and G Schmeda-Hirschmann. Antioxidant capacity, polyphenolic content and tandem HPLC–DAD–ESI/MS profiling of phenolic compounds from the South American berries Luma apiculata and L. chequén. *Food Chem* 2013; 139: 289-299. https://doi.org/10.1016/j.foodchem.2013.01.089

5 Fuentes L, M Valdenegro, M-G Gómez, A Ayala-Raso, E Quiroga, J-P Martínez, R Vinet, E Caballero and CR Figueroa. Characterization of fruit development and potential health benefits of arrayan (Luma apiculata), a native berry of South America. *Food Chem* 2016; 196: 1239-1247. https://doi.org/10.1016/j.foodchem.2015.10.003

6 Li A-N, S Li, Y-J Zhang, X-R Xu, Y-M Chen and H-B Li. Resources and Biological Activities of Natural Polyphenols. *Nutrients* 2014; 6: 6020-6047. https://doi.org/10.3390/nu6126020

7 Azmir J, I Zaidul, M Rahman, K Sharif, A Mohamed, F Sahena, M Jahurul, K Ghafoor, N Norulaini and A Omar. Techniques for extraction of bioactive compounds from plant materials: A review. *J Food Eng* 2013; 117: 426-436. https://doi.org/10.1016/j.jfoodeng.2013.01.014

8 Ordoñez-Torres A, C Torres-León, A Hernández-Almanza, T Flores-Guía, D Luque-Contreras, CN Aguilar and J Ascacio-Valdés. Ultrasound-microwave-assisted extraction of polyphenolic compounds from Mexican “Ataulfo” mango peels: Antioxidant potential and identification by HPLC/ESI/MS. *Phytochem Anal* 2020. https://doi.org/10.1002/pca.2997

9 Vinatoru M. An overview of the ultrasonically assisted extraction of bioactive principles from herbs. *Ultrason Sonochem* 2001; 8: 303-313. https://doi.org/10.1016/S1350-4177(01)00071-2

10 Wang L and CL Weller. Recent advances in extraction of nutraceuticals from plants. *Trends Food Sci Technol* 2006; 17: 300-312. https://doi.org/10.1016/j.tifs.2005.12.004

11 Tomaz I, L Maslov, D Stupić, D Preiner, D Ašperger and J Karoglan Kontić. Multi-response optimisation of ultrasound-assisted extraction for recovery of flavonoids from red grape skins using response surface methodology. *Phytochem Anal* 2016; 27: 13-22. https://doi.org/10.1002/pca.2582

12 World Health Organization. 2013. Advancing food safety initiatives: strategic plan for food safety including foodborne zoonoses 2013-2022.

13 Li D, L Baert and M Uyttendaele. Inactivation of food-borne viruses using natural biochemical substances. *Food Microbiol* 2013; 35: 1-9. https://doi.org/10.1016/j.fm.2013.02.009

14 Seo DJ, SB Jeon, H Oh, B-H Lee, S-Y Lee, SH Oh, JY Jung and C Choi. Comparison of the antiviral activity of flavonoids against murine norovirus and feline calicivirus. *Food control* 2016; 60: 25-30. https://doi.org/10.1016/j.foodcont.2015.07.023

15 Falcó I, W Randazzo, LG Gómez-Mascaraque, R Aznar, A López-Rubio and G Sánchez. Fostering the antiviral activity of green tea extract for sanitizing purposes through controlled storage conditions. *Food control* 2018; 84: 485-492. https://doi.org/10.1016/j.foodcont.2017.08.037

16 Fabra MJ, I Falcó, W Randazzo, G Sánchez and A López-Rubio. Antiviral and antioxidant properties of active alginate edible films containing phenolic

extracts. *Food Hydrocoll* 2018; 81: 96-103. https://doi.org/10.1016/j.foodhyd.2018.02.026

17 Quispe-Fuentes I, A Vega-Galvez and M Aranda. Evaluation of phenolic profiles and antioxidant capacity of maqui (Aristotelia chilensis) berries and their relationships to drying methods. *J Sci Food Agric* 2018; 98: 4168-4176. https://doi.org/10.1002/jsfa.8938

18 Huang D, OU Boxin and RL Prior. The chemistry behind antioxidant capacity assays. *J Agric Food Chem* 2005; 53: 1841-1856. https://doi.org/10.1021/jf030723c

19 Fischer S, R Wilckens, J Jara and M Aranda. Variation in antioxidant capacity of quinoa (chenopodium quinoa will) subjected to drought stress. *Ind Crops Prod* 2013; 46: 341-349. http://doi.org/10.1016/j.indcrop.2013.01.037

20 Chavez F, M Aranda, A Garcia and E Pastene. Antioxidant polyphenols extracted from Avocado epicarp (Persea americana var. Hass) inhibit Helicobacter pylori urease. *B Latinoam Caribe Pl* 2011; 10: 265-280.

21 Pintó RM, JM Diez and A Bosch. Use of the colonic carcinoma cell line CaCo-2 for in vivo amplification and detection of enteric viruses. *J Med Virol* 1994; 44: 310-315. https://doi.org/10.1002/jmv.1890440317

22 Ćujić N, K Šavikin, T Janković, D Pljevljakušić, G Zdunić and S Ibrić. Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. *Food Chem* 2016; 194: 135-142. https://doi.org/10.1016/j.foodchem.2015.08.008

23 Angulo MF, M Flores, M Aranda and K Henriquez-Aedo. Fast and selective method for biogenic amines determination in wines and beers by ultra high-performance liquid chromatography. *Food Chem* 2020; 309. https://doi.org/10.1016/j.foodchem.2019.125689

24 Galarce-Bustos O, L Novoa, J Pavon-Perez, K Henriquez-Aedo and M Aranda. Chemometric Optimization of QuEChERS Extraction Method for Polyphenol Determination in Beers by Liquid Chromatography with

Ultraviolet Detection. *Food Anal Methods* 2019; 12: 448-457. https://doi.org/10.1007/s12161-018-1376-x

25 He B, L-L Zhang, X-Y Yue, J Liang, J Jiang, X-L Gao and P-X Yue. Optimization of Ultrasound-Assisted Extraction of phenolic compounds and anthocyanins from blueberry (Vaccinium ashei) wine pomace. *Food Chem* 2016; 204: 70-76. https://doi.org/10.1016/j.foodchem.2016.02.094

26 Carrasco-Sandoval J, P Rebolledo, D Peterssen-Fonseca, S Fischer, R Wilckens, M Aranda and K Henríquez-Aedo. A fast and selective method to determine phenolic compounds in quinoa (Chenopodium quinoa Will) seeds applying ultrasound-assisted extraction and high-performance liquid chromatography. *Chem Pap* 2020. https://doi.org/10.1007/s11696-020-01313-z

27 Jovanović AA, VB Đorđević, GM Zdunić, DS Pljevljakušić, KP Šavikin, DM Gođevac and BM Bugarski. Optimization of the extraction process of polyphenols from Thymus serpyllum L. herb using maceration, heat- and ultrasound-assisted techniques. *Sep Purif Technol* 2017; 179: 369-380. https://doi.org/10.1016/j.seppur.2017.01.055

28 Khan MK, M Abert-Vian, A-S Fabiano-Tixier, O Dangles and F Chemat. Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (Citrus sinensis L.) peel. *Food Chem* 2010; 119: 851-858. https://doi.org/10.1016/j.foodchem.2009.08.046

29 Júnior DS, FJ Krug, MdG Pereira and M Korn. Currents on ultrasound-assisted extraction for sample preparation and spectroscopic analytes determination. *Appl Spectrosc Rev* 2006; 41: 305-321. https://doi.org/10.1080/05704920600620436

30 Thoo YY, SK Ho, JY Liang, CW Ho and CP Tan. Effects of binary solvent extraction system, extraction time and extraction temperature on phenolic antioxidants and antioxidant capacity from mengkudu (Morinda citrifolia). *Food Chem* 2010; 120: 290-295. https://doi.org/10.1016/j.foodchem.2009.09.064

31 Fontana AR, A Antoniolli and Rn Bottini. Grape pomace as a sustainable source of bioactive compounds: extraction, characterization, and biotechnological applications of phenolics. *J Agric Food Chem* 2013; 61: 8987-9003. https://doi.org/10.1021/jf402586f

32 He L, H Xu, X Liu, W He, F Yuan, Z Hou and Y Gao. Identification of phenolic compounds from pomegranate (Punica granatum L.) seed residues and investigation into their antioxidant capacities by HPLC–ABTS+ assay. *Food Res Int* 2011; 44: 1161-1167. https://doi.org/10.1016/j.foodres.2010.05.023

33 Vallverdú-Queralt A, N Boix, E Piqué, J Gómez-Catalan, A Medina-Remon, G Sasot, M Mercader-Martí, JM Llobet and RM Lamuela-Raventos. Identification of phenolic compounds in red wine extract samples and zebrafish embryos by HPLC-ESI-LTQ-Orbitrap-MS. *Food Chem* 2015; 181: 146-151. https://doi.org/10.1016/j.foodchem.2015.02.098

34 Nouman W, F Anwar, T Gull, A Newton, E Rosa and R Domínguez-Perles. Profiling of polyphenolics, nutrients and antioxidant potential of germplasm's leaves from seven cultivars of Moringa oleifera Lam. *Ind Crops Prod* 2016; 83: 166-176. https://doi.org/10.1016/j.indcrop.2015.12.032

35 Kajdžanoska M, V Gjamovski and M Stefova. HPLC-DAD-ESI-MSn identification of phenolic compounds in cultivated strawberries from Macedonia. *Maced J Chem Chem Eng* 2010; 29: 181-194. https://doi.org/10.20450/mjcce.2010.165

36 Chernonosov AA, EA Karpova and EM Lyakh. Identification of phenolic compounds in Myricaria bracteata leaves by high-performance liquid chromatography with a diode array detector and liquid chromatography with tandem mass spectrometry. *Rev Bras Farmacogn* 2017; 27: 576-579. https://doi.org/10.1016/j.bjp.2017.07.001

Capítulo II Sección 2 Supporting information

Table SI-1. Analysis of variance (ANOVA) for full factorial design

	SS[a]	d.f[b]	MS[c]	F-value	p-value
TPC (mg GAE g^{-1})					
Constant	214471	1	214471		6.617×10^{-14}
Factors					
Ethanol concentration [%v/v] (1)	55.676	1	55.676	15.170	0.0008
Extraction time [min] (2)	47.743	1	47.743	13.010	0.0084
Extraction temperature [°C] (3)	12.875	1	12.875	3.510	0.1480
Mass to solvent ratio [g mL^{-1}] (4)	58.432	1	58.432	15.920	0.0047
Interaction of two factors					
1 by 2	2.460	1	2.460	0.670	0.898
1 by 3	8.281×10^{-5}	1	8.281×10^{-5}	0.000	0.372
1 by 4	2.911	1	2.911	0.790	0.075
2 by 3	0.600	1	0.600	0.160	0.185
2 by 4	2.305	1	2.305	0.630	0.925
3 by 4	0.008	1	0.008	0.000	0.346
Model	183.001	10	18.300	9.903	0.005
Residual	29.359	8	3.670		
Corrected total	212.359	18			

[a] Sum of squares. [b] Degrees of freedom. [c] Mean square.

Table SI-2. Analysis of variance (ANOVA) for central composite design

	SS[a]	d.f[b]	MS[c]	F-value	p-value
TPC (mg GAE g^{-1})					
Constant	186385	1	186385		4.393×10^{-12}
Factors					
Ethanol concentration [%v/v] (1)	40.758	1	40.758	10.040	0.016
Extraction time [min] (2)	25.368	1	25.368	6.250	0.041
Mass to solvent ratio [g mL^{-1}] (3)	43.813	1	43.813	10.790	0.013
Interaction of two factors					
1 by 1	201.086	1	201.086	49.530	2.042×10^{-4}
2 by 2	1.367	1	1.367	0.340	0.579
3 by 3	4.085	1	4.085	1.010	0.350
1 by 2	2.073	1	2.073	0.510	0.498
1 by 3	0.009	1	0.009	0.000	0.964
2 by 3	4.175	1	4.175	1.030	0.344
Model	362.502	9	40.278	9.926	0.003
Residual	28.404	7	4.058		
Lack of fit	23.112	5	4.623	1.747	0.403
Pure error	5.292	2	2.646		
Corrected total	390.906	16	24.432		
	$R^2 = 0.927$	$R^2_{Adj} = 0.834$	CV = 2.014		

[a] Sum of squares. [b] Degrees of freedom. [c] Mean square.

CAPITULO II SECCIÓN 3

Title: Optimization of ultrasonic-assisted extraction of phenolic compounds from arrayán fruit (*Luma apiculata*): UHPLC-MS profile and *in vitro* biological activities

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Abstract

In this study, phenolic compounds from arrayán fruit were extracted using an ultrasonic-assisted extraction (UAE) method. The extraction parameters were optimized using a full factorial design (FFD) and a central composite design (CCD) in order to maximize the total

phenolic content (TPC). The optimal extraction conditions were as follows: 39% ethanol, extraction temperature of 62°C, extraction time of 10 min, with a mass to solvent ratio of 1:33.4 g mL^{-1}. Under the selected conditions, TPC of raw extract (RE) and polyphenols-rich extract (PRE) were 57.51±0.84 and 578.10±8.26 mg of GAE g^{-1} of dry weight (DW), respectively. Using the optimized extraction conditions, the antioxidant capacity (AC) showed FRAP values of 493.81±19.97 and 4497.33±81.69 μmol TE g^{-1} of DW for RE and PRE; and IC_{50} of DPPH assay was 2390.70±1.60 μg mL^{-1} and 186.07±1.28 μg mL^{-1} for RE and PRE. Phenolics profile showed the presence of quercetin 3-ß-D-glucoside, myricetin, quercetin, p-coumaric acid, kaempferol 3-glucoside, gallic acid, and kaempferol. The last four are reported for the first time in arrayán fruits. Due to the presence of phenolic compounds, antiviral activity was assayed against murine norovirus and hepatitis A virus, showing a significant effect in a dose-dependent manner against hepatitis A virus.

Keywords: central composite design; full factorial design; total phenolic compounds; antioxidant; antiviral; norovirus; hepatitis A.

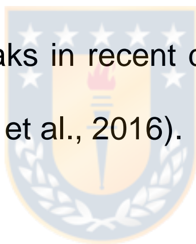
1. Introduction

Chronic non-communicable diseases (CNCD) are long-term diseases whose evolution is generally slow. Together they are responsible for more than 71% of deaths worldwide. The main CNCD are diabetes, cardiovascular diseases, cancer and chronic respiratory diseases and are characterized by sharing the same risk factors, such as smoking, poor diet, lack of physical activity and consumption of excessive alcohol (WHO, 2018). The complex scenario of CNCD has caused an increase in the search for bioactive compounds present in plants and foods with different healthy properties (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b). The arrayan (*Luma Apiculata*) is a Chilean myrtle that has been used in traditional medicine (Massardo and Rozzi, 1996; Rozzi and Massardo, 1994). Its fruit is an edible fleshy purplish-black berry with an intense flavor and aroma (Fuentes et al., 2016). This fruit has a high antioxidant capacity that could be related to the large number of phenolic compounds that it presents (Simirgiotis et al., 2013). Healthy fruit properties are attributed precisely to these types of bioactive compounds related to the ability to reduce the risk of developing CNCD (Fuentes et al., 2016). Phenolic compounds have attracted great interest for their antioxidant properties and health-promoting beneficial effects, specifically aimed at being incorporated within functional foods and nutraceuticals as natural antioxidants (Frevel et al., 2012). Currently, there are different techniques for the extraction of the phenolic compounds, from

traditional techniques such as Soxhlet extraction and static maceration to more modern techniques such as supercritical fluid extraction, pressurized liquid extraction, ultrasonic-assisted extraction, and microwave-assisted extraction (Azmir et al., 2013). In recent years, ultrasonic-assisted extraction has received much attention for extracting bioactive compounds from various food sources (Cuoco et al., 2009; Chemat et al., 2004). This extraction technology is attractive due to its simplicity, low energy consumption and low cost of equipment compared to other extraction technologies such as microwave-assisted extraction and supercritical fluid extraction (Wang and Weller, 2006). The enhancement in extraction obtained by using ultrasound is mainly attributed to the effect of acoustic cavitations produced in the solvent by the passage of an ultrasound wave (Wang et al., 2008). Ultrasonic-assisted extraction (UAE) of bioactive compounds is usually performed in an ultrasonic bath because of its more widely available and the greater number of samples that can be processed at the same time, in contrast to only one at a time with the ultrasonic probe (Rostagno et al., 2003).

Recent reports highlighted some extraction methods and potential applications of the bioactive components of arrayán fruit. However, there is no available information on the optimization of UAE procedure to obtain extracts rich in phenolic compounds from this matrix using indirect sonication in an ultrasonic bath. Therefore, the present study was mainly aimed at the optimization of UAE process parameters using full factorial design and central composite design to

maximize the content of the extracted phenolics. Besides, a complementary study was performed by ultra-high-performance liquid chromatography-diode array-mass spectrometry detection (UHPLC/DAD/MS) to study the phenolics profile of arrayán fruit. The type of phenolic compounds present is significant due to the different activities ascribed to these bioactive compounds. Particularly, antiviral effect, which is gaining increasing interest in the food and pharmaceutical industry since the worldwide concern regarding foodborne health risks (WHO, 2013). For that reason, the activity of arrayán fruit extract against human norovirus and hepatitis A virus was studied, both associated with numerous foodborne outbreaks in recent decades (Fabra et al., 2018; Falcó et al., 2018; Li et al., 2013; Seo et al., 2016).



2. Material and Methods

2.1 Reagents, chemicals, and standard solutions

2,4,6-tri(2-pyridyl)-s-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Amberlite XAD–7 resin, caffeic acid (≥98%), gallic acid (≥99%), vanillic acid (≥97%), quercetin 3-β-D-glucoside (≥90%), p-coumaric acid (≥98%), vanillin (≥99%), *trans*-ferulic acid (99%), quercetin hydrate (95%), kaempferol (≥90%), kaempferol 3-glucoside (≥90%) myricetin (96%) and ammonium formate (≥99%) were purchased from Sigma (St. Louis, MO, USA). Ethanol, acetone, ethyl acetate and formic acid used were analytical reagent grade and purchased from Merck (Darmstadt, Germany). Acetonitrile and methanol were HPLC grade and purchased from Merck (Darmstadt, Germany). Folin-Ciocalteu phenol reagent (2N), and sodium hydrogen carbonate (>99%) were obtained from Merck (Darmstadt, Germany). Ultrapure water was produced (18 MΩ cm) using a Simplicity system from Millipore (Bedford, MA, USA). Phenolic compounds stock solution was prepared in methanol/water (7:3 v/v) for a given concentration of 0.30 μg mL^{-1}.

2.2 Collection and proximal chemical analysis of the arrayán fruits

The fresh fruits of arrayán (*Luma apiculata*), were collected on Concepcion, Biobio region, Chile (36º 50´ 0´´ S and 73º 3´ 0´´ W) in May 2018. Fruits were washed with deionized water, frozen and deposited at the Laboratory of Advanced Research on Foods and Drugs, University of Concepcion, Concepción, Chile.

Proximate chemical analysis was done according to the procedures described in the AOAC standard methods (AOAC, 1990). Moisture, crude fat, crude protein, crude fiber and ash contents were all determined in triplicate. Carbohydrate content was estimated by the difference of mean value: 100 – (sum of percentages of moisture, ash, fiber, protein and crude fat) (Watts, 1964).

2.3 Sample preparation and extraction process

Raw extract (RE) of arrayán fruit was prepared from fresh fruit samples which were ground and homogenized using an IKA (Staufen, Germany). After frozen again for 24 h, samples were freeze-dried for 48 hours at -55°C using an Alpha 1-2 LDplus freeze-dryer from Martin Christ (Osterode am Harz, Germany). Freeze-dried fruit powdered (0.1 g) was extracted with 3.34 mL of extraction solvent (1:33.4 g mL^{-1}) composed of ethanol/water/formic acid (39:60:1 v/v/v). The mixture was vortex-mixed for 1 min and incubated in ultrasonic-assisted

extraction (UAE) for 10 min at 62 ºC using an ultrasonic bath (Getidy, China) with a potency of 200 W and frequency of 40 kHz. The samples were centrifuged for 15 min at 16162 x *g,* and 1 mL of clear supernatant was collected and filtered through 13 mm polyvinylidene fluoride (PVDF) syringe filters (0.22 µm) for total phenolic compounds (TPC) and antioxidant capacity (AC) analysis (DPPH and FRAP assay). Polyphenols-rich extract (PRE) of arrayán fruit was obtained from 334 mL of RE. First, the organic solvent of the RE was removed under vacuum in a Büchi (Flawil, Switzerland) R-100 rotavapor at 40 °C. The ethanol-free extract was loaded on a column containing 100 g Amberlite XAD-7 resin, previously conditioned with proper volume of ultrapure water. Then, column was washed with four portions of 50 mL of ultrapure water and the phenolic compounds were eluted with four portions of 50 mL of ethanol. This solvent was evaporated under vacuum to give 815.8 mg of arrayán fruit powder rich in phenolic compounds.

In order to compare with RE results, PRE solution was prepared dissolving 0.01 g of polyphenols-rich powder with 3.34 mL of extraction solvent. This solution was filtered through 13 mm polyvinylidene fluoride (PVDF) syringe filters (0.22 µm) for TPC and AC analysis (FRAP and DPPH assay).

2.4 Total phenolic content (TPC)

TPC was determined by the Folin-Ciocalteu assay following the protocol reported by Simirgiotis et al. (2013) with some modifications to adapt the protocol to 96-well microplate. First, RE and PRE were appropriately diluted with extraction solvent and 15 µL of this solution was mixed with 100 µL of 10 fold-diluted Folin–Ciocalteu reagent and 100 µL of sodium hydrogen carbonate (60 g L^{-1}). After mixing, the solution was incubated into BioTek (Winooski, VT, USA) Epoch 2 microplate reader at room temperature (23±2 °C) and under continuous agitation for 90 minutes. Absorbance was measured at 725 nm. Gallic acid has been used as a reference standard, and the results were expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW).

2.5 Antioxidant Capacity (AC)

AC was evaluated on RE and PRE through two assays: 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) and the ferric reducing antioxidant power assay (FRAP). The DPPH method acts on phenolic compounds that donate hydrogen atoms or electrons to radical oxidants, stabilizing them and thus decreasing their oxidizing power, while the FRAP method acts on phenolic compounds able to donate electrons by reducing oxidized intermediates. Therefore, using these two methods, it is possible to determine the total antioxidant capacity of the fruit by

evaluating the neutralizing effect of free radicals as their reduction capacity (Brand-Williams et al., 1995).

2.5.1 DPPH Radical Scavenging Assay

The free radical scavenging activity of the fruit extracts was analyzed using DPPH assay according to the protocol reported by Simirgiotis et al. (2013) with some modifications to adapt the protocol to 96-well microplate. The DPPH radical solution was prepared by dissolving 11.83 mg of radical with 3 mL of methanol. Then, 100 µL of this solution was mixed with 4.9 mL of methanol (working solution). First, samples were appropriately diluted with the extraction solvent and 5 µL of this solution was mixed with 95 µL of methanol and 100 µL of DPPH working solution. After mixing, 96-well microplate was incubated inside Epoch 2 microplate reader under continuous agitation for 30 minutes at room temperature (23±2 °C). Absorbance was measured at 517 nm. Trolox reagent was used as the standard. The DPPH scavenging ability as percentage (Pi) was calculated as $Pi= [(A_{control} – A_{sample})/A_{control}] *100$. Afterward, a curve of % DPPH bleaching activity versus concentration was plotted, and IC_{50} values were calculated (value at which 50% of radical is scavenged).

2.5.2 The ferric reducing antioxidant (FRAP) assay

The FRAP determination was done according to the protocol reported by Simirgiotis et al. (2013) with some modifications to adapt the protocol to 96-well microplate. The stock solutions included 0.3 mol L^{-1} of acetate buffer pH 3.6, 0.01 mol L^{-1} of TPTZ solution in 0.04 mol L^{-1} HCl, and 0.02 mol L^{-1} of $FeCl_3 \cdot 6H_2O$ solution. The working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ and 2.5 mL $FeCl_3 \cdot 6H_2O$. Thirty microliters of appropriately diluted sample were mixed with 150 µL of 5-fold-diluted working solution and incubated under continuous agitation for 30 minutes at 37ºC inside the microplate reader. The readings of the colored product (ferrous tripyridyltriazine complex) were measured at 593 nm. Quantification was performed using a standard calibration curve of antioxidant Trolox (from 0.2 to 2.5 µmol mL^{-1}), and the results were expressed in µmol Trolox equivalent (TE) per g of DW.

2.6 UHPLC/DAD-MS analysis

Chromatographic analysis was performed using a Shimadzu Nexera X2 UPLC system (LC30AD bin pump, DGU-20AR 5R vacuum degasser, SIL-30AC autosampler, CTO-20AC column oven, and SPD-M20A photodiode array detector), coupled to a Shimadzu LCMS-8030 triple quadrupole mass

spectrometer (Kioto, Japan) equipped with an electrospray ionization source (ESI). Separation was performed on a Phenomenex (Torrance, CA, USA) Kinetex C_{18} (150 mm x 4.6 mm, S-5 µm) column preceded by a Kinetex guard column, both set at 32°C, using a binary mobile phase composed of water containing 1 mL L^{-1} formic acid (A) and acetonitrile (B) applying the following gradient program at a flow rate of 0.7 mL min^{-1}: 0-17 min, 20%-%45 B; 17-18 min, 45%-45% B (isocratic step); 18-20 min, 45%-20% B; and 20-30 min, 20%-20% B (column conditioning). The complete separation was accomplished in less than 15 min for a total run time of 30 min. UV-detection was performed from 200-400 nm and MS analysis was performed using ESI in negative mode under the following conditions; ESI voltage 4.5 kV, nebulizer gas (N_2) flow: 3.0 L min^{-1}, drying gas flow: 15 L min^{-1}, desolvation line temperature 250 °C and heat block temperature 400 °C. Analytes were evaluated in Scan (*m/z* 100-2000) and Selected Ion Monitoring (SIM) mode using the following *m/z* values: gallic acid ($[M{-}H]^-$, *m/z* 169); caffeic acid ($[M{-}H]^-$, *m/z* 179); vanillic acid ($[M{-}H]^-$, *m/z* 167); quercetin 3-ß-D-glucoside ($[M{-}H]^-$, *m/z* 463); *p*-coumaric acid ($[M{-}H]^-$, *m/z* 163); vanillin ($[M{-}H]^-$, *m/z* 151); *trans*-ferulic acid ($[M{-}H]^-$, *m/z* 193); kaempferol 3-glucoside ($[M{-}H]^-$, *m/z* 447); myricetin ($[M{-}H]^-$, *m/z* 317); quercetin ($[M{-}H]^-$, *m/z* 301) and kaempferol ($[M{-}H]^-$, *m/z* 285).

2.7 Antiviral activity

Hepatitis A virus (HAV) strain HM-175/18f (ATCC VR-1402) and murine norovirus (MNV-1) were assayed and propagated in FRhK-4 (ATCC CRL-1688) and RAW 264.7 cell lines, respectively. MNV and RAW 264.7 cells were kindly provided by Prof. H. W. Virgin, Washington University School of Medicine, USA. Virus stocks were harvested by infecting cell lines during 2 days for MNV and 12 days for HAV, followed by three freeze-thaw cycles at 660 x g for 30 min to remove cell debris. Fifty percent of tissue culture infectious dose ($TCID_{50}$) was determined to enumerate infectious viruses using Spearmen-Karber method (Pintó et al., 1994). A proper amount of arrayán fruit powder was dissolved in 30% v/v ethanol solution for given concentration of 0.5 to 5 mg mL^{-1}. Each solution was incubated in equal volume of HAV and MNV (*ca.* 5 log $TCID_{50}$ mL^{-1}) for 2 h at 37°C. Samples were then ten-fold diluted in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS) and the residual infectivity was determined by $TCID_{50}$. Ten-fold dilutions of treated and untreated virus suspensions were inoculated into confluent cell monolayers in 96-well plates. Virus suspensions added to 30% v/v ethanol under the same experimental conditions were used as positive control. Each treatment was done in triplicate. The decay of MNV and HAV titers was calculated as $\log_{10} (N_x/N_0)$, where N_0 is the infectious virus titer for untreated samples and N_x is the infectious virus titer for arrayán fruit extract treated samples (Falcó et al., 2018).

2.8 Statistical analysis

Data were evaluated using descriptive statistics [mean, standard deviation (SD) and relative standard deviation (RSD)]. FFD and CCD were prepared and analyzed using MODDE version 7.0.0.1 software (Umetrics. Umea, Sweden). All statistical tests were performed with a significance level (α) of 0.05.

3. Results and discussion

3.1 Proximal chemical analysis

The proximate chemical evaluation of moisture, crude protein, ash, carbohydrate, crude fiber, and crude fat was analyzed for the first time in the arrayán fruit (Table 1). This fruit has a moisture content lower than various berries, such as strawberry (89.9%), cranberry (86.5%) y blackberry (88.15%) (Chen, 1993; Konja and Lovric, 1993), while its ash content is higher than the berries mentioned (cranberry 0.2%, strawberry 0.5%, and blackberry 0.5%) (Ne et al., 2016). Concerning the protein content, the arrayán fruit has a higher content than most berries (strawberry 0.8%, cranberry 0.4%, blueberry 0.74%) and other fruits such as banana (1-1.3%), orange (0.9%) or apple (0.3-0.4%) (Ne et al., 2016; Wall, 2006). However, the crude fiber content is lower than the berries reported (blackberries 3.2%, raspberries 4.7%, strawberries 1.6%) (Ne et

al., 2016). The crude fat content is similar or higher than the values reported for most fruits (pineapple 0.1%, orange 0.1%, banana 0.4%) except avocado (17-20%) (Ne et al., 2016). Regarding the carbohydrate content, arrayán fruit presented a similar content to most berries (blackberry 13.8-18.0%, blueberry 14.5%, cherry 11.9%) (de Souza et al., 2014; Imran et al., 2010; Mazza, 2005).

Table 1. Proximate chemical analysis composition of arrayán fruit

Component[a,b]	Content (%)
Moisture	80.88 ± 0.32
Crude fat	0.40 ± 0.01
Crude protein	1.62 ± 0.09
Crude fiber	1.20 ± 0.08
Ash	0.77 ± 0.01
Total carbohydrate	15.10 ± 0.04

[a]: Measurements are expressed as mean ± SD of three determinations (*n=3*); [b]:Values except moisture expressed on a dry weight basis.

3.2 Optimization of the ultrasonic-assisted extraction process

A full factorial design (FFD) following the methodology used by Ćujić et al. (2016) and a central composite design (CCD) (Pineda et al., 2012) were used for the screening and optimization of extraction process variables. The effects of several variables on TPC extraction were defined according to preliminary assays and published reports (Pompeu et al., 2009; Prasad et al., 2011).

Ethanol percentage in extraction solvent (varying between 30-50 % v/v), mass to solvent ratio (varying between 1:20-1:30 g mL^{-1}), extraction temperature (varying between 50-65°C) and extraction time (varying between 10-20 minutes) were investigated, as independent variables in FFD. The purpose of this step was to identify which variable had a significant effect on TPC extraction. Based on the results obtained in FFD, response surface methodology (RSM) through CCD was used to investigate the effect between variables and choose the optimum values of extraction parameters.

3.2.1 Full factorial design 2^k (FFD)

Full factorial design (2^4) was built, composed of an experimental plan with 19 runs, with three central points (Table 2). Each run was carried out in duplicate (*n*=2) in randomized order to minimize the effects of uncontrolled factors. The response/variable evaluated was TPC determined by the Folin-Ciocalteu assay, as described in section 2.4. Analysis of variance (ANOVA) (supporting information, Table 1) was carried out to determine which experimental factors significantly affected the extractive capacity of phenolic compounds. According to the observed results, extraction time (p=0.343) and extraction temperature (p=0.958) did not have a significant effect on the extraction of the phenolic compounds. However, the extraction temperature-mass to solvent ratio interaction was statistically significant (p=0.033); therefore, extraction

temperature was a factor to consider in the optimization process. The ethanol percentage (*p*=0.0449) and mass to solvent ratio (p=1.559x10^{-5}) were also statistically significant in the extractive process. All these factors with a significant effect on TPC were studied via CCD, while the extraction time was not considered due to little statistical importance being set at the lowest level (10 minutes).

3.2.2 Central composite design (CCD)

After identifying the variables that had a significant effect on TPC, RSM through CDD was employed to investigate the effect and interactions of the variables and determine the optimal extraction conditions. The design was composed of 17 runs with three central points (Table 3), each one analyzed in duplicate (*n*=2) in randomized order to minimize the effects of uncontrolled factors. As for FFD, the response evaluated was TPC determined by the Folin-Ciocalteu assay. ANOVA (supporting information, Table 2) was carried out to determine which experimental factors significantly affected the extractive capacity of phenolic compounds. According to the observed results, ethanol percentage (*p*=0.048), mass to solvent ratio (p=3.837x10^{-7}), ethanol percentage-ethanol percentage interaction (*p*=0.013), extraction temperature-extraction temperature interaction (p=6.248x10^{-4}), and extraction temperature-mass to solvent ratio interaction (*p*=0.008) were statistically significant in the extractive process, while extraction

temperature (*p*=0.725), mass to solvent ratio-mass to solvent ratio interaction (p=0.625), ethanol percentage-extraction temperature interaction (*p*=0.411) and ethanol percentage-mass to solvent ratio interaction (*p*=0.297) did not have a significant effect on the extraction of the phenolic compounds. By means of a response optimization, the optimal extraction conditions using UAE were 39% ethanol, extraction temperature of 62°C, with a mass to solvent ratio of 1:33.4 g mL^{-1} (Fig. 1). Under optimized conditions, the experimental TPC of RE was 57.51 mg GAE g^{-1} DW, which is very similar to the predicted value (55.84 mg GAE g^{-1} of DW).

Table 2. Experimental runs (n=2) of full factorial design (FFD) showing independent variables and experimental data for total phenolic content (TPC) response

N° run	Etanol concentration (%v/v)	Extraction time (min)	Extraction temperature (°C)	Mass to solvent ratio (g mL^{-1})	TPC (mg GAE g^{-1} DW)*
1	30	10	50	1:20	33.29 ± 0.17
2	50	10	50	1:20	38.37 ± 0.40
3	30	20	50	1:20	36.07 ± 0.28
4	50	20	50	1:20	40.77 ± 1.04
5	30	10	65	1:20	32.34 ± 0.57
6	50	10	65	1:20	35.15 ± 0.29
7	30	20	65	1:20	33.13 ± 0.57
8	50	20	65	1:20	35.84 ± 0.23
9	30	10	50	1:30	42.84 ± 0.11
10	50	10	50	1:30	45.37 ± 0.25
11	30	20	50	1:30	44.55 ± 0.27
12	50	20	50	1:30	46.12 ± 0.42
13	30	10	65	1:30	47.00 ± 0.04
14	50	10	65	1:30	48.16 ± 0.08
15	30	20	65	1:30	47.04 ± 0.26
16	50	20	65	1:30	48.21 ± 0.58
17	40	15	57.5	1:25	45.20 ± 0.44
18	40	15	57.5	1:25	43.55 ± 0.38
19	40	15	57.5	1:25	45.67 ± 0.28

*Mean ± standard deviation (n=3)

Table 3. Experimental runs (*n*=2) for a central composite design (CCD) showing independent variables and experimental data for total phenolic content (TPC) response

N° run	Etanol Concentration (%v/v)	Extraction temperature (°C)	Mass to solvent ratio (g mL^{-1})	TPC (mg GAE g^{-1} DW)*
1	30	50	1:20	34.25 ± 0.29
2	50	50	1:20	38.17 ± 0.39
3	30	65	1:20	29.34 ± 0.40
4	50	65	1:20	31.02 ± 0.23
5	30	50	1:30	44.17 ± 0.17
6	50	50	1:30	45.18 ± 0.10
7	30	65	1:30	49.02 ± 0.17
8	50	65	1:30	47.65 ± 0.17
9	23.2	58	1:25	34.76 ± 0.37
10	56.8	58	1:25	41.46 ± 0.45
11	40	45	1:25	33.48 ± 0.07
12	40	70	1:25	34.79 ± 0.14
13	40	57.5	1:16.6	31.83 ± 0.05
14	40	57.5	1:33.4	53.28 ± 0.32
15	40	57.5	1:25	44.90 ± 0.29
16	40	57.5	1:25	43.29 ± 0.21
17	40	57.5	1:25	45.59 ± 0.18

*Mean ± standard deviation (n=3)

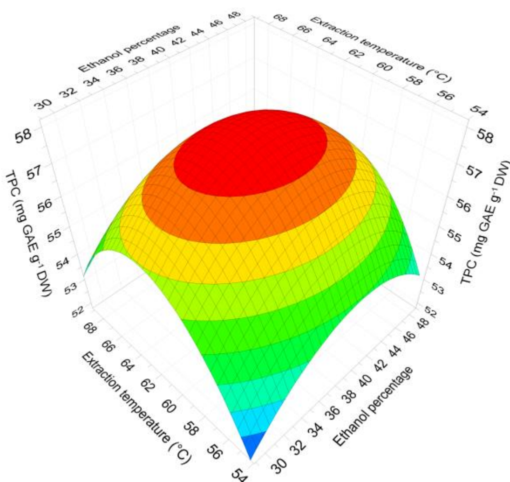
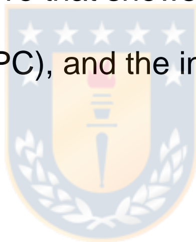


Fig. 1. Response surface curve that shows the optimal extraction conditions for total phenolic compounds (TPC), and the influence of the variables in the extraction yield.



3.3. Antioxidant Capacity (AC) and total phenolic compounds content (TPC)

Using optimized UAE conditions, TPC values were 57.51±0.84 and 578.10±8.26 mg of GAE g^{-1} of DW for RE and PRE, respectively. AC of RE and PRE showed FRAP values of 493.81±19.97 and 4497.33±81.69 µmol TE g^{-1} of DW; and DPPH IC_{50} of 2390.70±1.60 µg mL^{-1} and 186.07±1.28 µg mL^{-1}, respectively. PRE showed TPC, FRAP and DPPH values at least 9.1-fold higher than RE. This value demonstrated the importance of the purification step with XAD-7

column. Currently, two articles report TPC values of arrayán fruit on DW. Simirgiotis et al. (2013) applied an extraction process via maceration with methanol and subsequent purification by solid-phase extraction using C-18 sorbent. Comparing the purified extracts, PRE showed TPC and FRAP mean values 19.6 and 48 times higher than the ones described by the author (29.44±0.10 mg of GAE g^{-1} of DW and 93.4±0.0 μmol TE g^{-1} of DW). Ramirez et al. (2015) applied an UAE with methanol and posterior purification by Amberlite XAD-7 resin. Comparing the purified extracts, PRE showed TPC and FRAP mean values 20 and 48.2 times higher than the ones reported previously (27.61±1.61 mg of GAE g^{-1} of DW and 93.4±4.68 μmol TE g^{-1} of DW). However, DPPH IC_{50} values could not be compared because the reported values in both studies were much lower [Simirgiotis et al. (2013): 10.41±0.02 μg mL^{-1}; Ramirez et al. (2015): 5.88±0.21 μg mL^{-1}], even compared with Trolox standard (165.04±1.44 μg mL^{-1}). Although this unusual result, TPC and FRAP results show the importance of optimizing the extraction conditions using an experimental design. Extraction technology was another factor that contributed to obtaining extracts rich in bioactive compounds since the UAE is characterized by having high extraction yields of phenolic compounds compared to traditional methods and, in some cases, with extraction yields superior to more advanced extraction technologies, such as supercritical fluid extraction and microwave-assisted extraction (Júnior et al., 2006; Khan et al., 2010).

3.4 Chromatography

UHPLC/DAD/MS method has been developed for the detection and identification of following phenolic compounds in arrayán fruit: gallic acid, caffeic acid, vanillic acid, quercetin 3-ß-D-glucoside, *p*-coumaric acid, vanillin, *trans*-ferulic acid, kaempferol 3-glucoside, myricetin, quercetin and kaempferol. Using the chromatographic conditions described in section 2.6 allowed an adequate separation of 11 phenolic compounds in less than 15 minutes (Fig. 2). Seven phenolic compounds were quantified in arrayán fruit samples (Fig. 3), i.e., quercetin 3-ß-D-glucoside, myricetin, quercetin, p-coumaric acid, kaempferol 3-glucoside, gallic acid, and kaempferol (Table 4). The last four are reported for the first time in arrayán fruits, whereas caffeic acid, vanillic acid, vanillin and *trans*-ferulic acid were not detected in this berry.

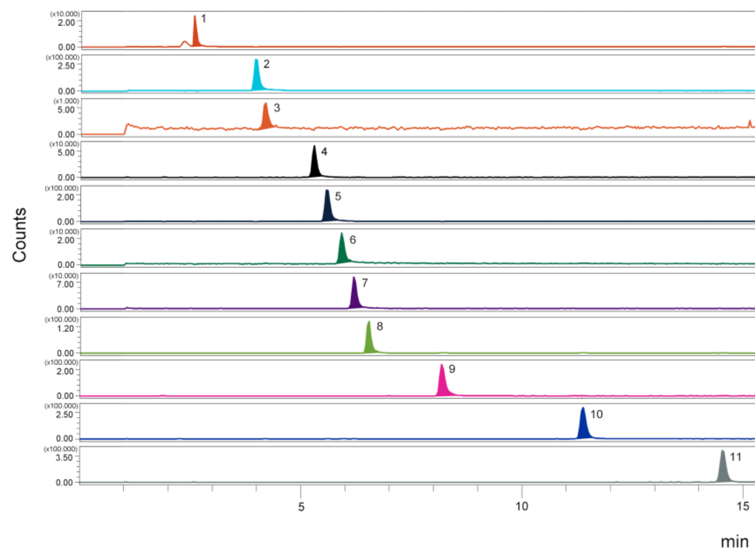


Fig. 2. Mass chromatogram of phenolic compounds analyzed. Gallic acid (1), caffeic acid (2), vanillic acid (3), quercetin 3-β-D-glucoside (4), *p*-coumaric acid (5), vanillin (6), *trans*-ferulic acid (7), kaempferol 3-glucoside (8), myricetin (9), quercetin (10) and kaempferol (11).

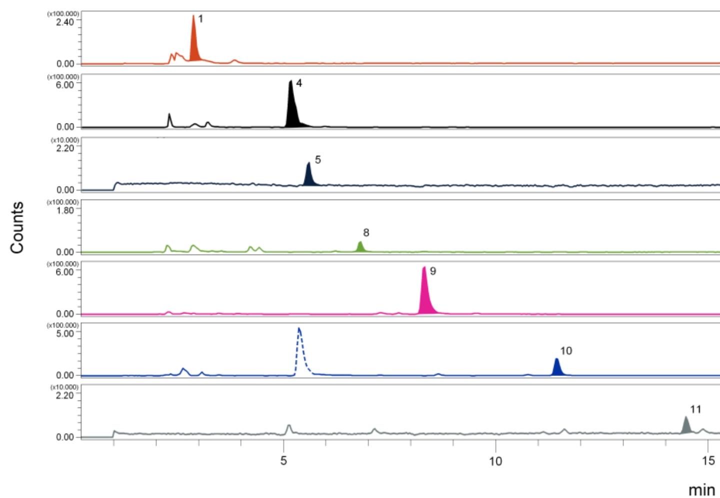


Fig. 3. Mass chromatogram of phenolic compounds found in arrayán fruit samples. Gallic acid (1), quercetin 3-β-D-glucoside (4), p-coumaric acid (5), kaempferol 3-glucoside (8), myricetin (9), quercetin (10) and kaempferol (11).

Table 4. Phenolic compounds profile after optimized ultrasound-assisted extraction of arrayán fruit

Peak	Compounds	t_R[a] (min)	λ max (nm)	m/z[b]	Concentration (mg kg^{-1})[c,d]
1	Gallic acid	2.504	275	169	88.96 ± 2.67
2	Caffeic acid	3.856	322	179	ND
3	Vanillic acid	4.087	263	167	ND
4	Quercetin 3-β-D-glucoside	5.183	353	463	268.49 ± 3.44
5	*p*-coumaric acid	5.506	309	163	2.02 ± 0.05
6	Vanillin	5.823	284	151	ND
7	*trans*-ferulic acid	6.095	320	193	ND
8	Kaempferol 3-glucoside	6.427	265	447	14.25 ± 1.74
9	Myricetin	8.109	372	317	285.01 ± 2.91
10	Quercetin	11.259	372	301	25.64 ± 1.00
11	Kaempferol	14.450	365	285	0.55 ± 0.02

[a]: Retention time (min); [b]: Mass to charge value; [c]: Mean ± standard deviation ($n=3$); ND: not detected in arrayán fruits (<0.01 mg kg^{-1})

Mass chromatogram (Fig. 3), also shows an important signal at retention time (t_R) of 5.238 min with a *m/z* 301, which is the same *m/z* value than quercetin. Based on this chromatographic behavior (t_R and elution order) and *m/z*, it is possible to infer that this signal could correspond to ellagic acid, which was reported in strawberries by Kajdžanoska et al. (2010).

3.5 Antiviral activity

Arrayán fruit extract (30% v/v ethanol) was tested against HAV and MNV, a well-studied human norovirus surrogate, at concentrations of 0.5 and 5 mg mL^{-1}.

Results showed that arrayán fruit extract only has antiviral activity against HAV, reducing the viral titers in a dose-dependent manner (Fig. 4). Incubation of HAV with arrayán fruit extract at concentrations of 5 mg mL^{-1} for 2 h at 37°C reduced titers more than 1 log. This activity is in concordance with the phenolic compounds profile that the extract presents and with early reports that showed antiviral activity of this type of bioactive compound against numerous foodborne viruses, such as HAV (Fabra et al., 2018; Falcó et al., 2018; Li et al., 2013; Seo et al., 2016; Su and D'Souza, 2012).

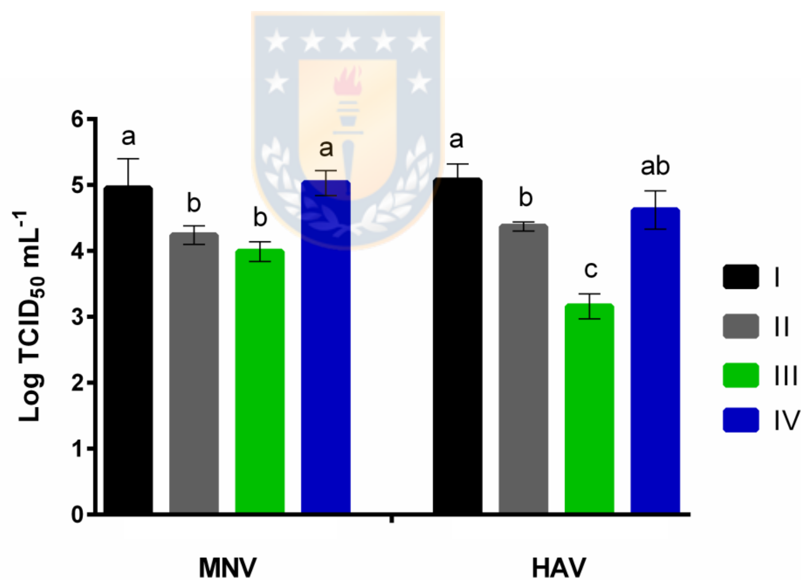


Fig. 4. Reduction of murine norovirus (MNV) and hepatitis A virus (HAV) titers (log $TCID_{50}$ mL^{-1}) treated with arrayán fruit extract at concentrations of 5 and 0.5 mg mL^{-1}. Virus control (I); ethanol control (II); 5 mg mL^{-1} of arrayán fruit extract treatment (III); 0.5 mg mL^{-1} arrayán fruit extract treatment (IV).

Each bar represents the mean of at least triplicate determination.

Different letters denote significant differences between treatments.

4. Conclusion

The present work is the first in which UAE has been optimized for the extraction of phenolic compounds from arrayán fruits (*Luma apiculata*). FFD and CCD were successfully employed to optimize the extraction conditions of TPC. With this optimized UAE process, it was possible to obtain an arrayán fruit extract with TPC and FRAP values 20 and 48-fold higher than the ones described in earlier reports. Extract purification using the non-ionic resin XAD-7 was also demonstrated, increasing TPC, DPPH and FRAP values at least 9.1-fold, compared with the unpurified extract. By means of UHPLC/DAD/MS analysis, it was possible to identify the presence of seven phenolic compounds, reporting for the first time the presence of p-coumaric acid, kaempferol 3-glucoside, gallic acid and kaempferol. Besides, the antiviral effect of arrayán fruit extract against HAV was demonstrated for the first time. These results show that arrayán fruit extracts can be considered as a promising antiviral agent against HAV and a good source of bioactive compounds for nutraceutical and functional foods development.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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References

AOAC, 1990. AOAC (1990). Official Methods of Analysis. (fifteenth ed.), Association of Official Analytical Chemists, Washington, DC (1990).

Azmir, J., Zaidul, I., Rahman, M., Sharif, K., Mohamed, A., Sahena, F., Jahurul, M., Ghafoor, K., Norulaini, N., Omar, A., 2013. Techniques for extraction of bioactive compounds from plant materials: A review. Journal of Food Engineering 117, 426-436.

Brand-Williams, W., Cuvelier, M.-E., Berset, C., 1995. Use of a free radical method to evaluate antioxidant activity. LWT-Food science and Technology 28, 25-30.

Ćujić, N., Šavikin, K., Janković, T., Pljevljakušić, D., Zdunić, G., Ibrić, S., 2016. Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique. Food Chemistry 194, 135-142.

Cuoco, G., Mathe, C., Archier, P., Chemat, F., Vieillescazes, C., 2009. A multivariate study of the performance of an ultrasound-assisted madder dyes extraction and characterization by liquid chromatography-photodiode array detection. Ultrasonics sonochemistry 16, 75-82.

Chemat, F., Grondin, I., Costes, P., Moutoussamy, L., Sing, A.S.C., Smadja, J., 2004. High power ultrasound effects on lipid oxidation of refined sunflower oil. Ultrasonics Sonochemistry 11, 281-285.

Chen, S., 1993. Physicochemical principles for the concentration and freezing of fruit juices. Fruit Juice Processing Technology, edited by Steven Nagy, Chin Shu Chen, Philip E. Shaw. Auburndale, FL: Agscience, Inc.

de Souza, V.R., Pereira, P.A.P., da Silva, T.L.T., de Oliveira Lima, L.C., Pio, R., Queiroz, F., 2014. Determination of the bioactive compounds, antioxidant activity and chemical composition of Brazilian blackberry, red raspberry, strawberry, blueberry and sweet cherry fruits. Food Chemistry 156, 362-368.

Fabra, M.J., Falcó, I., Randazzo, W., Sánchez, G., López-Rubio, A., 2018. Antiviral and antioxidant properties of active alginate edible films containing phenolic extracts. Food hydrocolloids 81, 96-103.

Falcó, I., Randazzo, W., Gómez-Mascaraque, L.G., Aznar, R., López-Rubio, A., Sánchez, G., 2018. Fostering the antiviral activity of green tea extract for sanitizing purposes through controlled storage conditions. Food control 84, 485-492.

Frevel, M.A., Pipingas, A., Grigsby, W.J., Frampton, C.M., Gilchrist, N.L., 2012. Production, composition and toxicology studies of Enzogenol® Pinus radiata bark extract. Food and chemical toxicology 50, 4316-4324.

Fuentes, L., Valdenegro, M., Gómez, M.-G., Ayala-Raso, A., Quiroga, E., Martínez, J.-P., Vinet, R., Caballero, E., Figueroa, C.R., 2016. Characterization of fruit development and potential health benefits of arrayan (Luma apiculata), a native berry of South America. Food chemistry 196, 1239-1247.

Galarce-Bustos, O., Pavón-Pérez, J., Henríquez-Aedo, K., Aranda, M., 2019a. An improved method for a fast screening of α-glucosidase inhibitors in cherimoya fruit (Annona cherimola Mill.) applying effect-directed analysis via high-performance thin-layer chromatography-bioassay-mass spectrometry. Journal of Chromatography A 1608, 460415.

Galarce-Bustos, O., Pavón, J., Henríquez-Aedo, K., Aranda, M., 2019b. Detection and identification of acetylcholinesterase inhibitors in Annona cherimola Mill. by effect-directed analysis using thin-layer chromatography-bioassay-mass spectrometry. Phytochemical Analysis 30, 679-686.

Imran, M., Khan, H., Shah, M., Khan, R., Khan, F., 2010. Chemical composition and antioxidant activity of certain Morus species. Journal of Zhejiang University Science B 11, 973-980.

Júnior, D.S., Krug, F.J., Pereira, M.d.G., Korn, M., 2006. Currents on ultrasound-assisted extraction for sample preparation and spectroscopic analytes determination. Applied Spectroscopy Reviews 41, 305-321.

Kajdžanoska, M., Gjamovski, V., Stefova, M., 2010. HPLC-DAD-ESI-MSn identification of phenolic compounds in cultivated strawberries from Macedonia. Macedonian Journal of Chemistry and Chemical Engineering 29, 181-194.

Khan, M.K., Abert-Vian, M., Fabiano-Tixier, A.-S., Dangles, O., Chemat, F., 2010. Ultrasound-assisted extraction of polyphenols (flavanone glycosides) from orange (Citrus sinensis L.) peel. Food Chemistry 119, 851-858.

Konja, G., Lovric, T., 1993. Berry fruit juices. Fruit juice processing technology, 436-514.

Li, D., Baert, L., Uyttendaele, M., 2013. Inactivation of food-borne viruses using natural biochemical substances. Food microbiology 35, 1-9.

Massardo, F., Rozzi, R., 1996. Usos medicinales de la flora nativa chilena. Valoración de la biodiversidad. Ambiente y Desarrollo 12, 76-78.

Mazza, G., 2005. Compositional and Functional Properties of Saskatoon Berry and Blueberry. International Journal of Fruit Science 5, 101-120.

Ne, N., Ubbaonu, C., Alagbaoso, S., Agunwa, I., Eluchie, C., 2016. Proximate, anti-nutritional and phytochemical composition of the yellow variety of the Synsepalum dulcificum (Miracle fruit) berry. American Journal of Food Science and Technology 4, 102-108.

Pineda, A., Carrasco, J., Peña-Farfal, C., Henríquez-Aedo, K., Aranda, M., 2012. Preliminary evaluation of biogenic amines content in Chilean young varietal wines by HPLC. Food Control 23, 251-257.

Pintó, R.M., Diez, J.M., Bosch, A., 1994. Use of the colonic carcinoma cell line CaCo-2 for in vivo amplification and detection of enteric viruses. Journal of medical virology 44, 310-315.

Pompeu, D., Silva, E., Rogez, H., 2009. Optimisation of the solvent extraction of phenolic antioxidants from fruits of Euterpe oleracea using Response Surface Methodology. Bioresource technology 100, 6076-6082.

Prasad, K.N., Hassan, F.A., Yang, B., Kong, K.W., Ramanan, R.N., Azlan, A., Ismail, A., 2011. Response surface optimisation for the extraction of phenolic compounds and antioxidant capacities of underutilised Mangifera pajang Kosterm. peels. Food Chemistry 128, 1121-1127.

Ramirez, J.E., Zambrano, R., Sepúlveda, B., Kennelly, E.J., Simirgiotis, M.J., 2015. Anthocyanins and antioxidant capacities of six Chilean berries by HPLC–HR-ESI-ToF-MS. Food chemistry 176, 106-114.

Rostagno, M.A., Palma, M., Barroso, C.G., 2003. Ultrasound-assisted extraction of soy isoflavones. Journal of Chromatography A 1012, 119-128.

Rozzi, R., Massardo, F., 1994. Las plantas medicinales chilenas II y III. Informativos Nueva Medicina 1, 16-17.

Seo, D.J., Jeon, S.B., Oh, H., Lee, B.-H., Lee, S.-Y., Oh, S.H., Jung, J.Y., Choi, C., 2016. Comparison of the antiviral activity of flavonoids against murine norovirus and feline calicivirus. Food control 60, 25-30.

Simirgiotis, M.J., Bórquez, J., Schmeda-Hirschmann, G., 2013. Antioxidant capacity, polyphenolic content and tandem HPLC–DAD–ESI/MS profiling of phenolic compounds from the South American berries Luma apiculata and L. chequén. Food chemistry 139, 289-299.

Su, X., D'Souza, D.H., 2012. Inactivation of human norovirus surrogates by benzalkonium chloride, potassium peroxymonosulfate, tannic acid, and gallic acid. Foodborne pathogens and disease 9, 829-834.

Wall, M.M., 2006. Ascorbic acid, vitamin A, and mineral composition of banana (Musa sp.) and papaya (Carica papaya) cultivars grown in Hawaii. Journal of Food Composition and analysis 19, 434-445.

Wang, J., Sun, B., Cao, Y., Tian, Y., Li, X., 2008. Optimisation of ultrasound-assisted extraction of phenolic compounds from wheat bran. Food Chemistry 106, 804-810.

Wang, L., Weller, C.L., 2006. Recent advances in extraction of nutraceuticals from plants. Trends in Food Science & Technology 17, 300-312.

Watts, B.M., 1964. Food composition and analysis (Triebold, Howard O.; Aurand, Leonard W.). Journal of Chemical Education 41, A534.

WHO, 2013. Advancing food safety initiatives: strategic plan for food safety including foodborne zoonoses 2013-2022. URL [https://apps.who.int/iris/bitstream/handle/10665/101542/9789241506281_eng.pdf]; accessed March 2020

WHO, 2018. Non communicable diseases. URL [https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases]; accessed March 2020.

Capítulo II Sección 3 Supporting information

Table SI-1. Analysis of variance (ANOVA) for full factorial design

	SS[a]	d.f[b]	MS[c]	F-value	p-value
TPC (mg GAE g^{-1})					
Constant	32737	1	32737		$7.299x10^{-13}$
Factors					
Ethanol concentration [%v/v] (1)	29.535	1	29.535	5.640	0.0449
Extraction time [min] (2)	5.309	1	5.309	1.010	0.343
Extraction temperature [°C] (3)	0.015	1	0.015	0.000	0.958
Mass to solvent ratio [g mL^{-1}] (4)	444.521	1	444.521		$1.559x10^{-5}$
Interaction of two factors					
1 by 2	0.127	1	0.127	0.020	0.880
1 by 3	2.283	1	2.283	0.440	0.526
1 by 4	4.926	1	4.926	0.940	0.360
2 by 3	2.327	1	2.327	0.440	0.524
2 by 4	1.059	1	1.059	0.200	0.665
3 by 4	34.777	1	34.777		0.033
Model	524.883	10	52.488	10.024	0.002
Residual	41.892	8	5.2365		
Corrected total	566.775	18	31.488		

[a] Sum of squares. [b] Degrees of freedom. [c] Mean square.

Table SI-2. Analysis of variance (ANOVA) for central composite design

	SS[a]	d.f[b]	MS[c]	F-value	p-value
TPC (mg GAE g^{-1})					
Constant	27375	1	273745		$1.273x10^{-9}$
Factors					
Ethanol concentration [%v/v] (1)	19.955	1	19.955	5.710	0.048
Extraction temperature [min] (2)	0.469	1	0.469	0.130	0.725
Mass to solvent ratio [g mL^{-1}] (3)	584.266	1	584.266	167.200	$3.837x10^{-7}$
Interaction of two factors					
1 by 1	38.807	1	38.807		0.013
2 by 2	119.860	1	119.860	34.300	$6.248x10^{-4}$
3 by 3	0.908	1	0.908	0.260	0.625
1 by 2	2.673	1	2.673	0.760	0.411
1 by 3	4.435	1	4.435	1.270	0.297
2 by 3	46.944	1	46.944	13.430	0.008
Model	798.609	9	88.734	25.411	0.000
Residual	24.444	7	3.492		
Lack of fit	21.658	5	4.332	3.110	0.261
Pure error	2.7858	2	1.393		
Corrected total	823.053	16	51.441		
	$R^2 = 0.970$	$R^2_{Adj} = 0.932$	CV = 1.869		

[a] Sum of squares. [b] Degrees of freedom. [c] Mean square.

CAPITULO II SECCIÓN 4

Title: Bioaccessibility of main phenolic compounds from arrayán fruit (*Luma apiculata*) after encapsulation in alginate/chitosan-coated zein nanoparticles

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Abstract

This work aimed to evaluate the bioaccessibility of different types of phenolic compounds isolated from the arrayán fruit loaded on different nanoparticulate systems: zein particles, alginate-coated zein particles, and alginate/chitosan complex-coated zein particles. The zein particles were prepared by the nanoprecipitation method and the polysaccharide coating-layers were developed by means of electrostatic deposition. After conducting univariate optimization, the alginate and alginate/chitosan (Alg/Chi) coatings were applied at a concentration of 0.01% w/v of each polysaccharide. Zein, alginate-coated zein, and Alg/Chi complex-coated zein particles loaded with phenolic compounds had a size and ζ-potential of 112.1 nm and +32.5 mV, 164.7 nm and -24.3 mV, and 245.2 nm and +26.2 mV, respectively. The encapsulation efficiency of phenolic compounds loaded on zein (PZ) particles ranged between 95.3 and 98.5%. Alginate and Alg/Chi coating-layers significantly increased the encapsulation efficiency of phenolic acids and glycosylated flavonoids. The bioaccessibility of PZ particles increased by at least 50.2%. The alginate coating-layer increased the phenolic acids and glycosylated flavonoids bioaccessibility, while the Alg/Chi coating produced the best bioaccessibility values for all phenolic compounds (over 65.8%). In the three nanoparticulate systems, the highest bioaccessibility was observed for polar phenolic acids, followed by glycosylated flavonoids. Therefore, the developed encapsulates could be potentially useful to improve

the bioaccessibility of different types of phenolic compounds, especially those with higher polar nature.

Keywords: Phenolic compounds, zein, alginate, chitosan, nanoparticles, coating

1. Introduction

Chronic non-communicable diseases (CNCD) are pathologies characterized for showing long duration and slow progression. The main CNCD are diabetes, cardiovascular diseases, cancer and chronic respiratory diseases. These diseases cause 71 % of all deaths worldwide (41 million deaths per year) and almost three quarters (32 million) of CNCD deaths occur in low- and middle-income countries. Every year near to 15 million CNCD deaths occur between 30 and 69 years old; therefore, the CNCD are not diseases limited to the elderly population (WHO, 2019). Considering this scenario, the scientific community has focused its efforts on the search for bioactive compounds in the plant kingdom capable of preventing or reducing the occurrence of CNCD (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b). Arrayán (*Luma apiculata*) is a native Chilean myrtle tree, also known as temu, widely used in traditional medicine. Its oval leaves are aromatic, while its fruit is a rounded berry, 1-1.5 cm in diameter, reddish at first to bright black when fully ripe (Aguilera et al., 2006; Fuentes et al., 2016). Leaves and fruit have a high antioxidant capacity that could be related to the high content of phenolic compounds (Simirgiotis et al., 2013). Healthy leaf and fruit properties are attributed precisely to this type of bioactive compounds, which could play a critical role in preventing CNCD (Fuentes et al., 2016). Phenolic compounds have the ability to *in vitro* inhibit cancer cell proliferation, reduce vascularization, protect neurons, stimulate vasodilation and

improve insulin secretion (Haminiuk et al., 2012). However, many of these compounds have poor bioaccessibility and are sensitive to extreme environmental conditions such as pH, oxygen, light, or enzymes (Altin et al., 2018). Micro- and nanoencapsulation are plausible solutions to the physical and chemical limitations of phenolic compounds (Atay et al., 2018; Jiang et al., 2020; Yao et al., 2015). In this regard, the encapsulation of phenolic compounds into biopolymeric matrices has been studied in order to improve their bioaccessibility after digestion, allowing these compounds to remain stable, protected from external factors and to be released into a specific location in the gastrointestinal tract to exert its beneficial effect (Ezhilarasi et al., 2013; Jiang et al., 2020; Khan et al., 2019; Sharif et al., 2019; Yao et al., 2015). Among the different forms of encapsulation that use biopolymers, nanoencapsulation stands out, which is defined as a technology to encapsulate substances in miniature and refers to bioactive packing at the nanoscale range (Ezhilarasi et al., 2013; Gharsallaoui et al., 2007).

Zein (main reserve protein in corn), alginate (negatively-charged polymer) and chitosan (cationic biopolymer) are natural biopolymers widely used for encapsulation. These biopolymers have as main characteristic the low cost, high bioavailability and biodegradability, which makes them attractive as wall materials to obtain polymeric nanoparticles (Khan et al., 2019; Luo et al., 2010; Luo et al., 2011). Zein has been widely used in micro- and nanoencapsulation of phenolic compounds (Costamagna et al., 2017; Fabra et al., 2016; Khan et al.,

2019; López de Dicastillo et al., 2019; Neo et al., 2013). Generally, the phenolic compounds are dissolved together with zein in a binary solvent system (ethanol/water) and then added to an antisolvent (water) in order to co-precipitate the compounds together with zein (nanoprecipitation method). However, the protection of bioactive compounds can be enhanced by adding other biopolymers such as alginate and chitosan (Gharsallaoui et al., 2007; Khan et al., 2019; Luo et al., 2012; Luo and Wang, 2014; Luo et al., 2010; Luo et al., 2011). Both polysaccharides have aroused increasing interest as carriers for controlled release of bioactive compounds and drugs, especially chitosan because of its bioadhesivity, low toxicity, permeation enhancing properties, hydrophilicity, biocompatibility, and biodegradability (Hasan et al., 2019; Wittaya-areekul et al., 2006)

Therefore, considering these aspects, the suitability of the different matrices to encapsulate phenolic compounds present in from arrayan fruit was compared, for the first time, in terms of encapsulation efficiency and bioaccessibility after *in-vitro* gastrointestinal digestion of the nanoencapsulates. Specifically, the effect of three nanoparticulate systems [phenolic compounds loaded on zein (PZ), alginate-coated zein (PZ-Alg), and alginate/chitosan complex-coated zein (PZ-Alg/Chi) particles] on the bioaccessibility of main phenolic compounds isolated from arrayán fruit was evaluated, including quercetin 3-ß-D-glucoside, myricetin, quercetin, p-coumaric acid, kaempferol 3-glucoside, gallic acid, and kaempferol.

2. Materials and methods

2.1. Reagents, chemicals, and standard solutions

Potassium chloride (KCl), anhydrous sodium hydrogen carbonate anhydrous ($NaHCO_3$), monobasic phosphate (KH_2PO_4), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$), ammonium carbonate ($(NH_4)_2CO_3$), calcium chloride dihydrate ($CaCl_2(H_2O)_2$), sodium chloride (NaCl), gallic acid (≥99%), p-coumaric acid (≥98%), quercetin 3-β-D-glucoside (≥90%), quercetin hydrate (95%), kaempferol (≥90%), kaempferol 3-glucoside (≥90%), myricetin (96%), pepsin from porcine gastric mucosa (≥250 U/mg), pancreatin (from porcine pancreas, 4×USP specifications), bile bovine dried unfractionated (microbiology grade), zein from corn (grade Z3625, 22–24 kDa) and alginic acid sodium salt from brown algae (low viscosity) were purchased from Sigma (St. Louis, MO, USA). Chitosan, with a reported degree of deacetylation of 85 ± 2.5% and average molecular weight 25 kDa (commercial grade 85/5), was purchased from Heppe Medical Chitosan GmbH. Acetonitrile and methanol, both HPLC grade, ethyl acetate, and formic acid were obtained from Merck (Darmstadt, Germany). Ethanol (96% v/v purity) was purchased from Panreac Quimica S.A. (Barcelona, Spain). Ultrapure water (18.2 MΩ cm at 25ºC) was produced using a simplicity system from Millipore (Bedford, MA, USA). Seven phenolic compounds stock solutions (gallic acid, p-coumaric acid, quercetin 3-β-D-glucoside, quercetin hydrate, kaempferol,

kaempferol 3-glucoside and myricetin) was prepared in methanol: water (7:3 v/v) for a given concentration of 0.30 μg mL^{-1}

2.2. Preparation of phenolic compounds-loaded nanoparticulate systems

Phenolic compounds loaded on zein (PZ) particles were prepared according to previously described procedures with some modifications (Huang et al., 2017; Khan et al., 2019; Li et al., 2019). Briefly, 2.8 mL of 2.5% w/v zein with 100 μL seven phenolic compounds stock solution (0.30 μg mL^{-1}) in 80% ethanol-water binary mixture was added into 20 mL of distilled water under stirring at 500 rpm for 5 min. PZ particles were coated with alginate and chitosan by using electrostatic deposition technique (Neo et al., 2013). 1.0% w/v alginate (adjusted to pH 7 with NaOH 0.1 N) and chitosan (1% in acetic acid) solutions were prepared in distilled water and under stirring overnight. PZ-Alg particles were obtained by mixing dispersion of PZ particles with 240 μL of alginate solution under stirring at 700 rpm for 5 min. PZ-Alg/Chi particles were obtained by mixing the dispersion of PZ-Alg particles with 240 μL of chitosan solution (adjusted to pH 3 with neat acetic acid) under stirring at 700 rpm for 5 min.

2.3. Encapsulation efficiency of phenolic compounds

First, PZ, PZ-Alg and PZ-Alg/Chi particles were precipitated by adding 15.0 mL of neat ethyl acetate. After vortex-mixing for 10 seconds, precipitated nanoparticles systems were centrifuged at 9000 rpm for 20 min to completely separate phenolic compounds-loaded nanoparticles from free phenolic compounds (organic phase). Free phenolic compounds were quantified by high-performance liquid chromatography (HPLC) according to the procedure described in section 2.5. To this end, 10.0 mL of organic phase (ethyl acetate) was collected and evaporated to dryness under a nitrogen stream (to avoid phenolic compounds oxidation) at 40°C. Prior chromatography, samples were reconstituted with 1.0 mL (10-fold concentrated solution) of methanol: water (7:3 v/v) and filtered through 13-mm polyvinylidene fluoride (PVDF) syringe filter (0.22 µm). Encapsulation efficiency of each phenolic compound was calculated according to the equation (1):

$$\text{Encapsulation efficiency} = \left(1 - \frac{F_{ph}}{T_{ph}}\right) \times 100 \qquad \text{(Equation 1)}$$

Where T_{ph} represents the total amount of phenolic compounds added during nanoparticles production and F_{ph} the free phenolic compounds in the organic phase.

2.4. Size, polydispersity index and ζ-potential measurement

Size, polydispersity index (PDI) and ζ-potential of zein, alginate-coated zein and Alg/Chi complex-coated zein particles were determined by dynamic light scattering (DLS) employing a Malvern Instruments (City, UK) Zetasizer Nano ZS. Each sample was analyzed in a folded capillary cell. Nanoparticulate systems were analyzed, at least, in triplicate.

2.5. Chromatography

HPLC assay was performed in order to determine the encapsulation efficiency and bioaccessibility of both free and encapsulated phenolic compounds, following the protocol described in a recent work (Carrasco-Sandoval et al., 2020), with some minor modifications. To this end, a Shimadzu (Kyoto, Japan) Prominence HPLC system composed of LC-20AT pump, DGU-20AR5R degassing unit, CTO-20AC column oven, CBM-20A communication module, SIL-20A autosampler and SPD-20AV UV/VIS detector was used. Data were acquired, recorded and analyzed by means of Shimadzu LabSolutions 5.54 software. Separation was performed on a Phenomenex (Torrance, CA, USA) Kinetex C18 (150 mm x 4.6 mm, S-5 μm) column preceded by a Kinetex guard column, both set at 35°C, using a binary mobile phase composed of acidified water (0.1% v/v formic acid-A) and acetonitrile (B). The following gradient

program was applied at a flow rate of 0.5 mL min^{-1}: 0-3 min 20%-%20 B (isocratic step), 3-4 min 20%-30% B, 4-6 min 30%-30% B (isocratic step), 6-8 min 30%-75% B, 8-10 min 75%-90% B, 10-14 min 90%-90% B (isocratic step), 14-15 min 90%-20% B, and 15-28 min 20%-20% B (column conditioning). Complete separation was accomplished in less than 18 min for a total run time of 28 min. Detection was performed by UV absorption at 280 nm.

2.6. *In-vitro* digestion

The bioaccessibility of free and encapsulated phenolic compounds was carried out according to the standardized COST Infogest protocol (Minekus et al., 2014), using the gastric and intestinal phases whose compositions are described in Table 1. The oral phase (optional step) was not considered due to the nature of systems studied (solutions and suspensions). Briefly, non-encapsulated phenolic compounds solution (NEP) was previously prepared by adding 100 μL of seven phenolic compounds stock solution (300 mg L^{-1}) in 4.9 mL of distilled water. Suspensions of PZ, PZ-Alg and PZ-Alg/Chi particles were frozen at -70°C for 24 hours and freeze-dried for 48 h using a Genesis 35-EL freeze-dryer (VirTis, USA). Subsequently, an adequate amount of PZ, PZ-Alg and PZ-Alg/Chi particles were resuspended in 5.0 mL of distilled water to obtain suspensions with the same concentration of phenolic compound as NEP.

Table 1. Digestion fluids used for *in vitro* gastrointestinal system simulation

Constituent	Stock concentration (mol L^{-1})	Simulated gastric fluid[a] (SGF) (pH 3)	Simulated intestinal fluid[a] (SIF) (pH 7)
KCl	0.5	6.9 mL	6.8
KH_2PO_4	0.5	0.9 mL	0.8
$NaHCO_3$	1	12.5 mL	42.5
NaCl	2	11.8 mL	9.6
$MgCl_2 (H_2O)_6$	0.15	0.4 mL	1.1
$(NH_4)_2CO_3$	0.5	0.5 mL	–
HCl	6	75.0 uL	85.0 uL

[a] All digestion fluids were made up to 400 ml with distilled water

The model consisted of two steps:

Gastric phase. To simulate gastric digestion phase, 3.75 mL of SGF, 0.8 mL of pepsin solution (*ca.* 25 000 U mL^{-1}) and 2.5 μL of 0.3 mol L^{-1} $CaCl_2$ were added to 5 mL of nanoparticulate systems and NEP. The pH was adjusted to 3.0 using 1 mol L^{-1} HCl. Then, the total mixture volume was made up to 10 mL with distilled water and was incubated at 37 °C for 2 h using a shaking water bath at 120 rpm.

Intestinal phase. To simulate intestinal digestion phase, 5.5 mL of SIF, 2.5 mL of pancreatin (*ca.* 800 U mL^{-1}), 1.25 mL of bile (*ca.* 160 mmol L^{-1}), and 20 uL of 0.3 mol L^{-1} $CaCl_2$ were added to the mixture from gastric phase. The pH was adjusted to 7.0 using 1 mol L^{-1} NaOH. Afterward, the total mixture volume was made up to 20 mL with distilled water and the mixture was incubated in a shaking water bath at 37 °C for another 2 h.

After simulating the digestive process, 15.0 mL of neat ethyl acetate was added to the intestinal digestion phase to extract the bioaccessible phenolic compounds. After vortex-mixing for 10 seconds, the digestion phases were centrifuged at 9000 rpm for 20 minutes to completely separate the organic phase (containing the phenolic compounds) from the aqueous phase. After that, 10.0 mL of organic phase (ethyl acetate) was collected and evaporated to dryness under a nitrogen stream at 40°C. Prior to chromatography, samples were reconstituted with 1.0 mL of methanol: water (7:3 v/v) and filtered through 13-mm polyvinylidene fluoride (PVDF) syringe filter (0.22 μm). All samples were prepared, at least, in triplicate. The bioaccessibility of the phenolic compounds was calculated according to the equation (2):

$$\text{Bioaccessibility}\ (\%) = \left(\frac{PH_{op}}{PH_t}\right) \times 100 \qquad \text{(Equation 2)}$$

Where PH_{op} and PH_t are the phenolics compounds in organic phase and in the initial samples, respectively.

2.7. Statistical analysis

Data are shown as mean values ± standard deviations and analyzed for a significant difference ($p < 0.05$) using GraphPad (San Diego, CA, USA) Prism 6.0 software.

3. Results and discussion

3.1 Stability evaluation of nanoparticulate systems

Different factors interfere with the stability of the colloidal suspensions, so it is relevant to know some properties such as size particle, ζ-potential, PDI and other physicochemical parameters that can be used to evaluate the stability of the colloidal systems (Abellan-Pose et al., 2016).

In order to achieve thermodynamically stable systems, the biopolymer concentration used for Alg-coated zein and Alg/Chi-coated zein particles was optimized through a univariate optimization considering parameters such as particle size, PDI, ζ-potential and precipitate formation in the system (Table 2). As a result, nano-sized particles were obtained in all the reported conditions. Initially, zein particles were made using the nanoprecipitation technique, obtaining suspensions with good stability parameters, i.e, small particle size, low PDI and high ζ-potential value. This last parameter is a measure of the repulsion magnitude or electrostatic attraction between the particles, being one of the most critical parameters that affect the stability of the colloidal suspensions. Absolute ζ-potential values greater than 30 mV certify good colloidal stability due to the high energy barrier between particles (Mora-Huertas et al., 2010). In contrast, PDI is a parameter used to check the homogeneity in particle size distribution and values lower than 0.200 indicate colloidal dispersions without

agglomerates formation or precipitate, ensuring the stability of the colloidal systems. (Luo et al., 2017).

For the preparation of alginate-coated zein and Alg/Chi-coated zein particles, three alginate and chitosan concentrations were evaluated according to previous reports (Hu and McClements, 2015; Khan et al., 2019): 0.010, 0.016 y 0.021% w/v. In alginate-coated zein particles preparation, the lowest alginate concentration tested gave rise to suspensions with the best stability values (Table 2). This suspension presented particle size and ζ-potential values similar to the suspension of intermediate alginate concentration, but with lower PDI ($p < 0.05$), while the highest alginate concentration (0.021% w/v) showed a precipitate in the colloidal suspension and a higher PDI.

In the colloidal suspensions with a precipitate, particle size and ζ-potential were measured on the supernatant liquid (suspension without precipitate) after being centrifuged at 4000 rpm for 10 min. It is worth to mention that the lowest alginate concentration tested did not produce an absolute ζ-potential greater than 30 mV. However, an absolute ζ-potential greater than 20 mV can also provide sufficient stabilization to the particles in solution, due to the presence of biopolymers with high molecular weight that contribute to the steric stabilization (Honary and Zahir, 2013). A similar scenario was observed for the formation of Alg/Chi-coated zein nanoparticles, where the lowest chitosan concentration showed the best stability values. In contrast, the highest chitosan concentrations provided particles with higher PDI values and a precipitate (Table 2). It should be noted

that the systems made with the highest alginate and chitosan concentration had a significant increase in ζ-potential ($p < 0.05$) due to the amount of both biopolymers are higher than the required amount to coat the nanoparticles and thus, they precipitate in the solution. In contrast, the systems made with the lower and intermediate concentration did not present a significant difference in the ζ-potential values ($p > 0.05$). The latter suggests that the lower concentration of both biopolymers (0.01% w/v) was enough to thoroughly coat the surfaces of zein and alginate-coated zein particles with alginate and chitosan, respectively.

Based on the results obtained from the univariate optimization, the alginate-coated zein and Alg/Chi-coated zein particles were produced using a concentration of 0.01% w/v of each polysaccharide. These systems had an increase in particle size ($p < 0.05$) as the covering polymers were added. Thus, the Alg/Chi-coated zein particles presented a larger particle size than the alginate-coated zein particles and the latter system presented a larger particle size than the zein particles. These increases in size can be ascribed to the thicker layer formation with oppositely charged polyelectrolytes (Neo et al., 2013). Regarding the ζ-potential, the negative potential of alginate-coated zein particles indicated that the negatively charged alginate was electrostatically combined with zein nanoparticles, which have a net positive charge, while the positive potential of Alg/Chi-coated zein particles indicated that the positively charged chitosan was electrostatically attached to the alginate-coated zein particles, which has a net negative charge. (Liu et al., 2020). These changes in

size and ζ-potential corroborate the success of the different nanoparticulate systems.

After the optimization process, loaded-nanoparticles were prepared by adding the phenolic compounds to the zein solution before particles formation. As can be observed in Table 3, there were no significant differences ($p>0.05$) neither in particle size nor in the ζ-potential values between loaded and non-loaded particles. The alginate-coated zein and zein particles loaded with phenolic compounds showed similar values than that previously reported for capsules loaded with phenolic compounds prepared with the nanoprecipitation technique (Chen et al., 2018; Hu and McClements, 2015; Li et al., 2019). Interestingly, loaded-alginate-coated zein and Alg/Chi coated zein particles presented larger particle size and lower ζ-potential than those reported by Khan et al. (2019) to encapsulate resveratrol. These differences can be ascribed to different factors such as the methodology used for capsules formation, the chemical nature of encapsulated compounds, potential interactions between the core (active compounds) and shell (biopolymers) materials, the intrinsic characteristic of the biopolymers used (i.e. molecular weight) and the physicochemical properties of the suspensions (Teeranachaideekul et al., 2007).

Table 2. Mean size, PDI and ζ-potential of zein particles coated with alginate and chitosan at various concentrations

	Concentration of alginate–chitosan (w/v%)						
	0.000–0.000	0.010–0.000	0.016–0.000	0.021–0.000*	0.010–0.010	0.010–0.016*	0.010–0.021**
Size (d, nm)	110.7 ± 1.3^{A}	166.6 ± 1.5^{B}	167.7 ± 1.8^{B}	181.2 ± 2.1^{C}	242.5 ± 0.9^{D}	244.8 ± 4.0^{D}	257.0 ± 3.1^{E}
PDI	0.135 ± 0.006^{A}	0.145 ± 0.005^{A}	0.164 ± 0.008^{B}	0.191 ± 0.012^{C}	0.134 ± 0.004^{A}	0.163 ± 0.013^{B}	0.208 ± 0.011^{C}
ζ-Potential (mV)	+33.1 ± 0.5^{A}	-25.9 ± 0.3^{B}	-26.9 ± 0.8^{B}	-30.6 ± 1.1^{C}	+24.8 ± 0.3^{D}	+26.2 ± 1.2^{D}	+31.6 ± 0.8^{E}

Values with different letters are significantly different ($p < 0.05$). * Slight precipitate formation. ** Moderate precipitate formation

Table 3. Mean size, PDI and ζ-potential of loaded and non-loaded particles with phenolic compounds

	Zein particles[a]	PZ particles	Alg-coated zein particles[a]	PZ-Alg particles	Alg/Chi-coated zein particles[a]	PZ-Alg/Chi particles
Size (d, nm)	110.7 ± 1.3^{A}	112.1 ± 1.9^{A}	166.6 ± 1.5^{B}	164.7 ± 2.3^{B}	242.5 ± 0.9^{C}	245.2 ± 1.6^{C}
PDI	0.135 ± 0.006^{A}	0.144 ± 0.013^{A}	0.145 ± 0.005^{A}	0.139 ± 0.005^{A}	0.134 ± 0.004^{A}	0.141 ± 0.012^{A}
ζ-Potential (mV)	+33.1 ± 0.5^{A}	+32.5 ± 0.7^{A}	-25.9 ± 0.3^{B}	-24.3 ± 0.9^{B}	+24.8 ± 0.3^{C}	+26.2 ± 1.2^{C}

Values with different letters are significantly different ($p < 0.05$). [a] non-loaded particles with phenolic compounds

3.2 Encapsulation efficiency

In order to ascertain the type of phenolics incorporated within the nanoparticles, the entrapment efficiency of the systems for each phenolic compound was calculated and the results are summarized in Table 4.

In general, high encapsulation efficiencies were observed in all evaluated nanoparticulated systems, although it increased with the amount of coating layers, being higher in those prepared with both alginate and chitosan. Table 4 gathers a detailed analysis of each phenolic compound encapsulated into the biopolymer matrices. Differences among samples can be attributed to the fact that the encapsulation efficiency is affected by different factors, including the chemical structure, polarity and nature of both the shell and core material (Silva-Buzanello et al. (2015).

Specifically, the encapsulation efficiency of quercetin and gallic acid were similar to those reported by Neo et al. (2013) and Rodríguez-Félix et al. (2019), who prepared zein particles using the electrospraying method. It should be highlighted that the encapsulation efficiency was significantly higher ($p < 0.05$) for flavonoid aglycones (i.e. quercetin, kaempferol and myricetin) than for phenolic acids (gallic acid and p-coumaric acid) and glycosylated flavonoids (quercetin 3-ß-D-glucoside and kaempferol 3-glucoside). This effect can be ascribed to the fact that zein, which contains many non-polar amino acids that makes it a hydrophobic protein, present a higher binding affinity to less polar

phenolic compounds (flavonoid aglycones). However, the encapsulation efficiency of phenolic acids and glycosylated flavonoids increased when zein nanoparticles were coated with alginate and chitosan layers, reaching values similar to that found for flavonoin aglycones, thus suggesting that these polar compounds were preferentially entrapped into the polysaccharide layers (with high hydrophilic character) during the formation of nanoparticulate systems. Interestingly, the presence of the alginate layer significantly increased the encapsulation efficiency of glycosylated flavonoids and phenolic acids ($p < 0.05$), while the incorporation of chitosan layer increased the encapsulation efficiency of phenolic acids, probably ascribed to the acid solution used to dissolve the chitosan. In contrast, flavonoid aglycones did not show a significant increase in the encapsulation efficiency ($p > 0.05$) after coating the zein nanoparticles neither with alginate nor with chitosan layers. A similar trend has been recently reported by Khan et al. (2019), who encapsulated resveratrol (phenolic aglycone) in zein particles coated with alginate and chitosan, using the nanoprecipitation technique.

Table 4. Encapsulation efficiency of phenolic compounds in nanoparticulate systems

Encapsulation efficiency (%)	Zein particles	Alg-coated zein particles	Alg/Chi-coated zein particles
Gallic acid	95.3 ±0.7^{a,B}	97.4 ± 0.6^{b,A}	99.1 ± 0.5^{c,A}
p-coumaric acid	95.6 ± 0.9^{a,B}	97.5 ± 0.3^{b,A}	99.3 ± 0.6^{c,A}
Quercetin 3-ß-D-glucoside	96.1 ± 0.4^{a,B}	98.5 ± 0.6^{b,A}	98.2 ± 1.1^{b,A}
Quercetin	98.4 ± 0.6^{a,C}	98.5 ± 0.7^{a,A}	98.7 ± 1.4^{a,A}
Kaempferol 3-glucoside	96.2 ± 0.6^{a,B}	98.2 ± 0.7^{b,A}	99.0 ± 0.8^{b,A}
Kaempferol	98.5 ± 0.4^{a,C}	98.6 ± 1.2^{a,A}	98.0 ± 0.3^{a,A}
Myricetin	98.3 ± 0.7^{b,C}	98.2 ± 1.3^{b,A}	97.6 ± 1.5^{b,A}

Values with different lowercase letters in the same row are statistically different ($p<0.05$). Values with different uppercase letters in the same column are statistically different ($p<0.05$).

3.3 Bioaccessibility

Although the encapsulation has proven to be efficient in preventing degradation of bioactive compounds, it can also have an impact on their bioaccessibility. Free and nanoencapsulated phenolic compounds were subjected to *in-vitro* digestion in order to study the impact of the encapsulating matrices on their bioaccessibility. The digestive process consists of enzymatic stages series that produce the main components hydrolysis of the food with the consequent numerous nutrients release. The tool for evaluating bioavailability is bioaccessibility, which corresponds to the relationship between the bioaccessible or soluble concentration of a substance and total substance

concentration present in the sample. (Cilla et al., 2018). Bioaccessibility can be defined as the maximum amount that can be absorbed through the intestinal epithelium and reach the systemic circulation and, therefore, it is used as an indicator of maximum oral bioavailability. (Versantvoort et al., 2005). In this context, the health-promoting effects of phenolic compounds is limited due to their physicochemical properties such as low solubility and instability in a biological environment, low bioavailability and rapid systematic elimination (Deng et al., 2018; Gao and Hu, 2010). Therefore, to ensure the phenolic compounds bioaccessibility and functionality, it is necessary that they will be stable and do not undergo modifications in the gastrointestinal tract. In this scenario, the development of nanoparticulate systems has been proposed as effective solution for this purpose. To evaluate the effect of different nanoparticulate systems on the bioaccessibility of the main phenolic compounds present in the arrayán fruit, the bioaccessible fraction of free and encapsulated phenolic compounds were compared after *in-vitro* digestion (Fig. 1). Table 5 gathers the bioaccessible fraction of each phenolic compound.

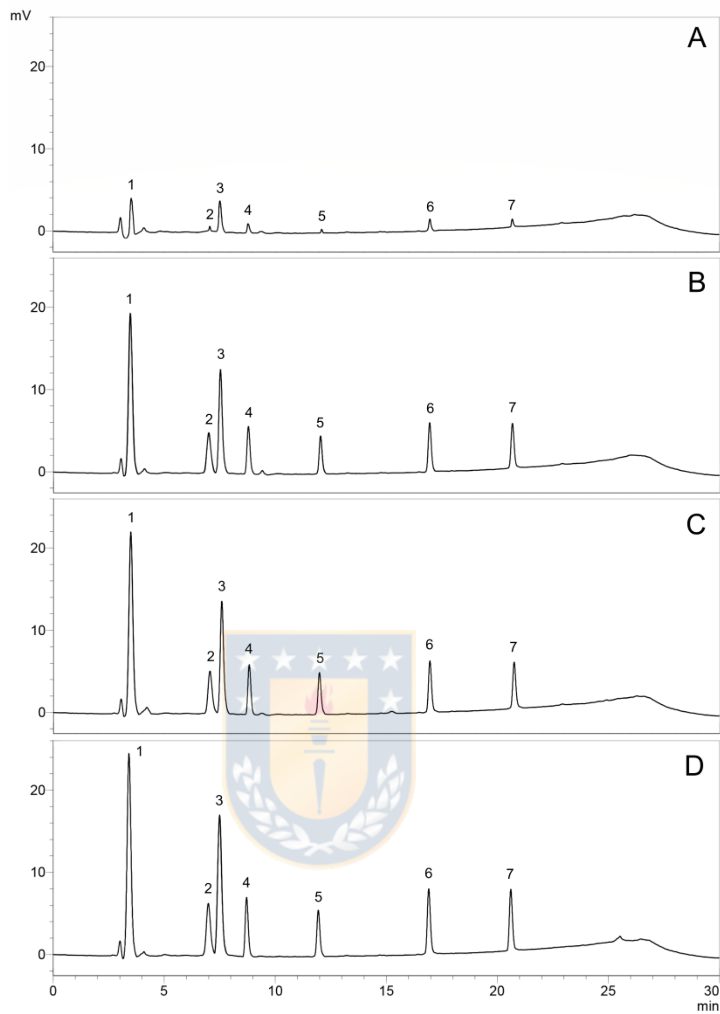


Fig. 1. HPLC chromatograms of phenolic compounds after *in-vitro* gastrointestinal digestion procedure at 280 nm. Free phenolic compounds (A), phenolic compounds loaded on zein particles (B), phenolic compounds loaded on alginate-coated zein particles (C), phenolic compounds loaded on alginate/chitosan complex-coated zein particles (D). Gallic acid (1), quercetin 3-β-D-glucoside (2), p-coumaric acid (3), kaempferol-glucoside (4), myricetin (5), quercetin (6), kaempferol (7).

Table 5. Phenolic compounds bioaccessibility in different nanoparticulate systems after the gastrointestinal digestion.

Bioaccessibility (%)	Non-encapsulated	Zein particles	Alg-coated zein particles	Alg/Chi-coated zein particles
Gallic acid	13.3 ± 0.9^{a,B}	65.3 ± 1.6^{b,A}	70.4 ± 0.8^{c,A}	81.8 ± 0.8^{d,A}
p-coumaric acid	16.1 ± 1.4^{a,C}	63.9 ± 1.3^{b,A}	67.7 ± 0.5^{c,B}	84.0 ± 0.6^{d,B}
Quercetin 3-ß-D-glucoside	8.2 ± 1.2^{a,D}	59.6 ± 1.4^{b,C}	63.3 ± 0.7^{c,D}	78.6 ± 0.7^{d,C}
Quercetin	6.4 ± 0.7^{a,E}	55.6 ± 0.8^{b,D}	57.0 ± 1.3^{b,E}	73.9 ± 1.2^{c,D}
Kaempferol 3-glucoside	10.6 ± 0.8^{a,F}	61.5 ± 1.1^{b,C}	64.0 ± 0.8^{c,D}	76.0 ± 0.8^{d,E}
Kaempferol	5.0 ± 1.1^{a,E}	52.6 ± 0.6^{b,E}	53.7 ± 0.9^{b,F}	72.6 ± 0.9^{c,D}
Myricetin	3.4 ± 0.7^{a,G}	50.2 ± 0.9^{b,F}	57.4 ± 1.1^{c,E}	65.8 ± 0.7^{d,F}

Values with different lowercase letters in the same row are statistically different ($p<0.05$). Values with different uppercase letters in the same column are statistically different ($p<0.05$).

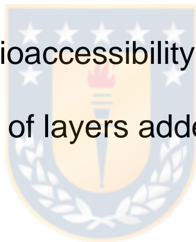
As observed in Table 5, free phenolic compounds presented limited bioaccessibility, which varied between 3.4 and 16.1%, which is mainly explained by the low oral bioavailability, poor water solubility and the phenolic compounds chemical instability. (Gao and Hu, 2010). After loading the phenolic compounds into the zein nanoparticles, the bioaccessibility of phenolic compounds successfully increased up to 50.2%. These results are consistent with previous reports, in which zein nanoparticles were proved to enhance the bioaccessibility of different bioactive compounds. (Chen et al., 2020; Khan et al., 2019; Rodríguez-Félix et al., 2019; Yao et al., 2018; Zou et al., 2016). Concerning to the effect of each type of phenolic compound on the bioaccessibility, a similar trend was observed for all structures, which was not dependent on the

composition of the encapsulating matrices, showing phenolic acids the higher bioaccessibility followed by glycosylated flavonoids, and the less polar compounds-flavonoid aglycones. However, the presence of each layer significantly improved the bioaccessibility of phenolic compounds, being accentuated when both alginate and chitosan layer took part of the encapsulating shell material. Specifically, a significant increase ($p<0.05$) in the bioaccessibility of phenolic acids and glycosylated flavonoids was observed when PZ particles were coated with alginate layer. However, except for myricetin, flavonoid aglycones did not have a significant bioaccessibility increase ($p>0.05$). This effect is consistent with Khan et al. (2019) report, which described no significant differences in resveratrol (phenolic aglycone) bioaccessibility after being loaded on zein particles and zein particles coated with alginate using nanoprecipitation method. Interestingly, PZ-Alg/Chi nanoparticles showed the best bioaccessibility values (over 65.8%), probably due to phenolic compounds greater release in the simulated intestinal fluid compared to PZ and PZ-Alg particles (Khan et al., 2019).

4. Conclusions

For the first time, different classes of phenolic compounds present in the arrayán fruit were encapsulated simultaneously in different nanoparticulate systems, prepared by nanoprecipitation method and electrostatic deposition of different

charged biopolymers (alginate and chitosan). The zein particle loaded with phenolic compounds had the smallest particle size (~ 112.1 nm) and ζ-potential of +32.5 mV, while Alg/Chi-coated zein particles loaded with phenolic compounds had the largest particle size (~ 245.2 nm) and ζ-potential of +26.2 mV. Zein nanoparticles increased all phenolic compounds bioaccessibility, while Alg and Alg/Chi coatings significantly increased encapsulation efficiency and bioaccessibility of phenolic acids and glycosylated flavonoids. Further, it was observed a clear trend regarding compounds polarity, showing polar compounds higher bioaccessibilities. These results proved that encapsulation process significantly increased the bioaccessibility of phenolic compounds, which was accentuated with the number of layers added.



Conflicts of interest

All authors declare that they have no conflict of interest.

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References

Abellan-Pose, R., Teijeiro-Valiño, C., Santander-Ortega, M.J., Borrajo, E., Vidal, A., Garcia-Fuentes, M., Csaba, N., Alonso, M.J., 2016. Polyaminoacid nanocapsules for drug delivery to the lymphatic system: Effect of the particle size. International Journal of Pharmaceutics 509, 107-117.

Aguilera, M.A., Casanueva, M.E., Hernández, C.E., 2006. Composición de la araneofauna en dos especies de árboles nativos Peumus boldus Mol. y Luma apiculata (DC) Burret en el parque botánico Pedro del Río Zañartu (Hualpen), Concepción, VIII Región, Chile. Gayana (Concepción) 70, 176-185.

Altin, G., Gültekin-Özgüven, M., Ozcelik, B., 2018. Chitosan coated liposome dispersions loaded with cacao hull waste extract: Effect of spray drying on physico-chemical stability and in vitro bioaccessibility. Journal of Food Engineering 223, 91-98.

Atay, E., Fabra, M.J., Martínez-Sanz, M., Gomez-Mascaraque, L.G., Altan, A., Lopez-Rubio, A., 2018. Development and characterization of chitosan/gelatin electrosprayed microparticles as food grade delivery vehicles for anthocyanin extracts. Food Hydrocolloids 77, 699-710.

Carrasco-Sandoval, J., Rebolledo, P., Peterssen-Fonseca, D., Fischer, S., Wilckens, R., Aranda, M., Henríquez-Aedo, K., 2020. A fast and selective method to determine phenolic compounds in quinoa (Chenopodium quinoa Will) seeds applying ultrasound-assisted extraction and high-performance liquid chromatography. Chemical Papers, 1-8.

Cilla, A., Bosch, L., Barberá, R., Alegría, A., 2018. Effect of processing on the bioaccessibility of bioactive compounds – A review focusing on carotenoids, minerals, ascorbic acid, tocopherols and polyphenols. Journal of Food Composition and Analysis 68, 3-15.

Costamagna, M.S., Gómez-Mascaraque, L.G., Zampini, I.C., Alberto, M.R., Pérez, J., López-Rubio, A., Isla, M.I., 2017. Microencapsulated chañar phenolics: A potential ingredient for functional foods development. Journal of Functional Foods 37, 523-530.

Chen, S., Sun, C., Wang, Y., Han, Y., Dai, L., Abliz, A., Gao, Y., 2018. Quercetagetin-Loaded Composite Nanoparticles Based on Zein and Hyaluronic Acid: Formation, Characterization, and Physicochemical Stability. Journal of Agricultural and Food Chemistry 66, 7441-7450.

Chen, Y., Zhao, Z., Xia, G., Xue, F., Chen, C., Zhang, Y., 2020. Fabrication and characterization of zein/lactoferrin composite nanoparticles for encapsulating 7,8-dihydroxyflavone: Enhancement of stability, water solubility and bioaccessibility. International Journal of Biological Macromolecules 146, 179-192.

Deng, J., Yang, H., Capanoglu, E., Cao, H., Xiao, J., 2018. 9 - Technological aspects and stability of polyphenols, in: Galanakis, C.M. (Ed.), Polyphenols: Properties, Recovery, and Applications. Woodhead Publishing, pp. 295-323.

Ezhilarasi, P.N., Karthik, P., Chhanwal, N., Anandharamakrishnan, C., 2013. Nanoencapsulation Techniques for Food Bioactive Components: A Review. Food and Bioprocess Technology 6, 628-647.

Fabra, M.J., Flores-López, M.L., Cerqueira, M.A., de Rodriguez, D.J., Lagaron, J.M., Vicente, A.A., 2016. Layer-by-layer technique to developing functional nanolaminate films with antifungal activity. Food and bioprocess technology 9, 471-480.

Fuentes, L., Valdenegro, M., Gómez, M.-G., Ayala-Raso, A., Quiroga, E., Martínez, J.-P., Vinet, R., Caballero, E., Figueroa, C.R., 2016. Characterization of fruit development and potential health benefits of arrayan (Luma apiculata), a native berry of South America. Food Chemistry 196, 1239-1247.

Galarce-Bustos, O., Pavón-Pérez, J., Henríquez-Aedo, K., Aranda, M., 2019a. An improved method for a fast screening of α-glucosidase inhibitors in cherimoya fruit (Annona cherimola Mill.) applying effect-directed analysis via high-performance thin-layer chromatography-bioassay-mass spectrometry. Journal of Chromatography A 1608, 460415.

Galarce-Bustos, O., Pavón, J., Henríquez-Aedo, K., Aranda, M., 2019b. Detection and identification of acetylcholinesterase inhibitors in Annona

cherimola Mill. by effect-directed analysis using thin-layer chromatography-bioassay-mass spectrometry. Phytochemical Analysis 30, 679-686.

Gao, S., Hu, M., 2010. Bioavailability challenges associated with development of anti-cancer phenolics. Mini Rev Med Chem 10, 550-567.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., Saurel, R., 2007. Applications of spray-drying in microencapsulation of food ingredients: An overview. Food Research International 40, 1107-1121.

Haminiuk, C.W.I., Maciel, G.M., Plata-Oviedo, M.S.V., Peralta, R.M., 2012. Phenolic compounds in fruits – an overview. International Journal of Food Science & Technology 47, 2023-2044.

Hasan, M., Elkhoury, K., Kahn, C.J.F., Arab-Tehrany, E., Linder, M., 2019. Preparation, Characterization, and Release Kinetics of Chitosan-Coated Nanoliposomes Encapsulating Curcumin in Simulated Environments. Molecules 24, 2023.

Honary, S., Zahir, F., 2013. Effect of zeta potential on the properties of nano-drug delivery systems-a review (Part 1). Tropical Journal of Pharmaceutical Research 12, 255-264.

Hu, K., McClements, D.J., 2015. Fabrication of biopolymer nanoparticles by antisolvent precipitation and electrostatic deposition: Zein-alginate core/shell nanoparticles. Food Hydrocolloids 44, 101-108.

Huang, X., Dai, Y., Cai, J., Zhong, N., Xiao, H., McClements, D.J., Hu, K., 2017. Resveratrol encapsulation in core-shell biopolymer nanoparticles: Impact on antioxidant and anticancer activities. Food Hydrocolloids 64, 157-165.

Jiang, T., Liao, W., Charcosset, C., 2020. Recent advances in encapsulation of curcumin in nanoemulsions: A review of encapsulation technologies, bioaccessibility and applications. Food Research International 132, 109035.

Khan, M.A., Yue, C., Fang, Z., Hu, S., Cheng, H., Bakry, A.M., Liang, L., 2019. Alginate/chitosan-coated zein nanoparticles for the delivery of resveratrol. Journal of Food Engineering 258, 45-53.

Li, H., Wang, D., Liu, C., Zhu, J., Fan, M., Sun, X., Wang, T., Xu, Y., Cao, Y., 2019. Fabrication of stable zein nanoparticles coated with soluble soybean polysaccharide for encapsulation of quercetin. Food Hydrocolloids 87, 342-351.

Liu, Q., Han, C., Tian, Y., Liu, T., 2020. Fabrication of curcumin-loaded zein nanoparticles stabilized by sodium caseinate/sodium alginate: Curcumin solubility, thermal properties, rheology, and stability. Process Biochemistry 94, 30-38.

López de Dicastillo, C., Piña, C., Garrido, L., Arancibia, C., Galotto, M.J., 2019. Enhancing Thermal Stability and Bioaccesibility of Açaí Fruit Polyphenols through Electrohydrodynamic Encapsulation into Zein Electrosprayed Particles. Antioxidants 8, 464.

Luo, X., Zhou, Y., Bai, L., Liu, F., Zhang, R., Zhang, Z., Zheng, B., Deng, Y., McClements, D.J., 2017. Production of highly concentrated oil-in-water emulsions using dual-channel microfluidization: Use of individual and mixed natural emulsifiers (saponin and lecithin). Food Research International 96, 103-112.

Luo, Y., Teng, Z., Wang, Q., 2012. Development of Zein Nanoparticles Coated with Carboxymethyl Chitosan for Encapsulation and Controlled Release of Vitamin D3. Journal of Agricultural and Food Chemistry 60, 836-843.

Luo, Y., Wang, Q., 2014. Zein-based micro- and nano-particles for drug and nutrient delivery: A review. Journal of Applied Polymer Science 131.

Luo, Y., Zhang, B., Cheng, W.-H., Wang, Q., 2010. Preparation, characterization and evaluation of selenite-loaded chitosan/TPP nanoparticles with or without zein coating. Carbohydrate Polymers 82, 942-951.

Luo, Y., Zhang, B., Whent, M., Yu, L., Wang, Q., 2011. Preparation and characterization of zein/chitosan complex for encapsulation of α-tocopherol, and

its in vitro controlled release study. Colloids and Surfaces B: Biointerfaces 85, 145-152.

Minekus, M., Marie, A., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Brodkorb, A., 2014. A standardised static in-vitro digestion method suitable for food – an international consensus. Food & Function.

Mora-Huertas, C.E., Fessi, H., Elaissari, A., 2010. Polymer-based nanocapsules for drug delivery. International Journal of Pharmaceutics 385, 113-142.

Neo, Y.P., Ray, S., Jin, J., Gizdavic-Nikolaidis, M., Nieuwoudt, M.K., Liu, D., Quek, S.Y., 2013. Encapsulation of food grade antioxidant in natural biopolymer by electrospinning technique: A physicochemical study based on zein–gallic acid system. Food Chemistry 136, 1013-1021.

Rodríguez-Félix, F., Del-Toro-Sánchez, C.L., Javier Cinco-Moroyoqui, F., Juárez, J., Ruiz-Cruz, S., López-Ahumada, G.A., Carvajal-Millan, E., Castro-Enríquez, D.D., Barreras-Urbina, C.G., Tapia-Hernández, J.A., 2019. Preparation and Characterization of Quercetin-Loaded Zein Nanoparticles by Electrospraying and Study of In Vitro Bioavailability. Journal of Food Science 84, 2883-2897.

Sharif, N., Fabra, M.J., López-Rubio, A., 2019. 9 - Nanostructures of zein for encapsulation of food ingredients, in: Jafari, S.M. (Ed.), Biopolymer Nanostructures for Food Encapsulation Purposes. Academic Press, pp. 217-245.

Silva-Buzanello, R.A.d., Ferro, A.C., Bona, E., Cardozo-Filho, L., Araújo, P.H.H., Leimann, F.V., Gonçalves, O.H., 2015. Validation of an Ultraviolet–visible (UV–Vis) technique for the quantitative determination of curcumin in poly(l-lactic acid) nanoparticles. Food Chemistry 172, 99-104.

Simirgiotis, M.J., Bórquez, J., Schmeda-Hirschmann, G., 2013. Antioxidant capacity, polyphenolic content and tandem HPLC–DAD–ESI/MS profiling of phenolic compounds from the South American berries Luma apiculata and L. chequén. Food Chemistry 139, 289-299.

Teeranachaideekul, V., Müller, R.H., Junyaprasert, V.B., 2007. Encapsulation of ascorbyl palmitate in nanostructured lipid carriers (NLC)—Effects of formulation parameters on physicochemical stability. International Journal of Pharmaceutics 340, 198-206.

Versantvoort, C.H.M., Oomen, A.G., Van de Kamp, E., Rompelberg, C.J.M., Sips, A.J.A.M., 2005. Applicability of an in vitro digestion model in assessing the bioaccessibility of mycotoxins from food. Food and Chemical Toxicology 43, 31-40.

WHO, 2019. Noncommunicable diseases Fact sheet. 2018 https://www. who. int/en/news-room/fact-sheets/detail/noncommunicablediseases. Accessed March 2020.

Wittaya-areekul, S., Kruenate, J., Prahsarn, C., 2006. Preparation and in vitro evaluation of mucoadhesive properties of alginate/chitosan microparticles containing prednisolone. International Journal of Pharmaceutics 312, 113-118.

Yao, K., Chen, W., Song, F., McClements, D.J., Hu, K., 2018. Tailoring zein nanoparticle functionality using biopolymer coatings: Impact on curcumin bioaccessibility and antioxidant capacity under simulated gastrointestinal conditions. Food Hydrocolloids 79, 262-272.

Yao, M., McClements, D.J., Xiao, H., 2015. Improving oral bioavailability of nutraceuticals by engineered nanoparticle-based delivery systems. Current Opinion in Food Science 2, 14-19.

Zou, L., Zheng, B., Zhang, R., Zhang, Z., Liu, W., Liu, C., Xiao, H., McClements, D.J., 2016. Enhancing the bioaccessibility of hydrophobic bioactive agents using mixed colloidal dispersions: Curcumin-loaded zein nanoparticles plus digestible lipid nanoparticles. Food Research International 81, 74-82.

CAPITULO II SECCIÓN 5

Title: Evaluation of the antioxidant and inhibitory activity against α-glucosidase and acetylcholinesterase from arrayán (*Luma apiculata*) applying directed effect analysis via high-performance thin-layer chromatography-(bio)autography-mass spectrometry

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Abstract

Antioxidants are compounds widely distributed in the plant kingdom whose interest has increased in recent times because their consumption has been related to the decrease in chronic-degenerative diseases such as type 2 diabetes (T2D) and Alzheimer's (AD). T2D and AD are chronic non-communicable diseases (CNCD) whose prevalence has been growing in parallel in recent decades, and currently represent a significant concern due to high morbidity and mortality rates worldwide. In this context, alpha-glucosidase (α-G) and acetylcholinesterase (AChE) inhibitors are attractive bioactive compounds to be used in the T2D and AD treatment, respectively. The objective of this work was to develop a high throughput methodology to detect and tentatively identify compounds with antioxidant and inhibitory activity against α-G and AChE in fruits and leaves from arrayán (*Luma apiculata*), applying effect-directed analysis via high-performance thin layer-chromatography (HPTLC) linked with (bio)autography and mass spectrometry (MS). The proposed methodology decreased in at least 1.3 and 4-fold the concentration of α-G and AChE required per assay. Further, a new substrate and enzyme impregnating method enhance the contrast between the inhibitory bands and plate purple background. Applying this methodology, coupled to HPTLC-MS, it was possible for the first time to detect and identify a compound with antioxidant and inhibitory activity against α-G and AChE in arrayán fruit (*Luma apiculata*). The compound was tentatively

assigned as fraxetin (*m/z* 207 $[M–H]^–$), a hydroxycoumarin reported for the first time in arrayán fruit.

Keywords: bioautography; α-glucosidase inhibitors; acetylcholinesterase inhibitors; antioxidant activity; Alzheimer's; diabetes, arrayán; fraxetin

1. Introduction

Arrayán is an evergreen tree species of Myrtaceae family found in southern Chile and Argentina (Puntieri et al., 2018). It has been used in traditional medicine as antiasthmatic, antidiarrheal and antiseptic (Araya-Contreras et al., 2019). Its leaves are simple, round, or oval (Aguilera et al., 2006), while its fruit is an edible purplish-black berry with an intense flavor and aroma (Fuentes et al., 2016). The fruits and the leaves have a high antioxidant capacity, that could be related to the large concentration of phenolic compounds that they possess, including flavonoids, anthocyanins and phenolic acids (Simirgiotis et al., 2013). Some pharmacological effects of the arrayán fruit and leaf are attributed precisely to the presence of phenolic compounds, which are attributed to the ability to reduce the risk of chronic non-communicable diseases (CNCD) (Fuentes et al., 2016). Among these diseases, type 2 diabetes (T2D) and Alzheimer's disease (AD) represent a significant concern due to their high morbimortality rates worldwide (Martín-Timón et al., 2014; Nichols et al., 2019). T2D and AD are two CNCD whose prevalence has been growing in parallel in recent decades (Li et al., 2015). T2D is a metabolic disease characterized by hyperglycemia and an oxidative imbalance that, in the long term, lead to the appearance of a series of pathophysiological complications, which result in high social and economic costs (Zhang et al., 2012). This disease comprises a heterogeneous group of disorders characterized by variable degrees of insulin

resistance, lower hormone secretion and higher glucose production in the liver. Therefore, a therapeutic approach to diabetes treatment is to decrease hyperglycemia (Inocente Camones et al., 2019), which can be achieved by inhibiting enzymes that hydrolyze carbohydrates such as α-glucosidase (α-G) (Bhandari et al., 2008). Different epidemiological studies have revealed that T2D is a risk factor for the development of AD, although the mechanisms that link both pathologies are still unknown. According to some authors, dementia is much more frequent in diabetic patients (Abdelhafiz et al., 2016; Hsu et al., 2011; Strachan et al., 2008). Furthermore, T2D-related conditions such as obesity, metabolic syndrome and hyperinsulinemia may also be risk factors in the development of AD (Li et al., 2015). AD is a progressive neurodegenerative disorder characterized by brain nerve cell degeneration. Among the most notable basic manifestations are memory loss, temporal-spatial disorientation, and intellectual decline (Cass, 2017). The impairment of cholinergic activity is one of the main findings in this pathology and it is related to the acetylcholine synthesis and hydrolysis, the later catalyzed by acetylcholinesterase (AChE) and butyrylcholinesterase (BuChE) enzymes (Ito et al., 2010). AChE inhibition, a key enzyme in acetylcholine degradation, and oxidative stress reduction are currently the main therapeutic strategies to treat AD. (Konrath et al., 2013). Current enzymatic inhibitors for the treatment of both diseases cause unwanted adverse effects, such as gastrointestinal problems and liver toxicity. (Lordan et al., 2013). For this reason, the scientific community has focused its efforts on

finding new bioactive natural compounds from natural sources (Galarce-Bustos et al., 2019b; Kolawole and Akanji, 2013; Lordan et al., 2013). Considering the therapeutic strategies of both diseases, the hyphenation of high-performance thin-layer chromatography (HPTLC) and effect directed analysis (EDA) is an excellent alternative for detection of bioactive compounds, i.e autography (DPPH radical assay), and bioautography (α-G and AChE inhibitors) (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b). This approach coupled with different detection systems such as ultraviolet (UV) and visible (Vis) spectrophotometry, fluorescence detection (FLD), nuclear magnetic resonance (NMR) and mass spectrometry (MS) allows a rapid identification of bioactive compounds (Morlock, 2014). The objective of the present work was to detect and identify compounds of phenolic nature with antioxidant activity and inhibitory capacity against α-G and AChE in arrayán (*Luma apiculata*) fruits and leaves, applying EDA through HPTLC-(bio)autography-MS.

2. Materials and methods

2.1 Reagents, chemicals, and standard solutions

α-glucosidase from *Saccharomyces cerevisiae* (E.C. number 3.2.1.20), AChE from *Electrophorus electricus* (electric eel) (E.C number 3.1.17), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Fast Blue B salt, 1- naphthyl acetate (AChE substrate), caffeic acid (≥98%), donepezil hydrochloride (pharmaceutical secondary standard) and gallic acid (≥99%) were purchased from Sigma (St. Louis, MO, USA). 2-naphthyl-D-glucopyranoside (α-glucosidase substrate) was obtained from Glycosynth (Warrington, Cheshire, UK). Ultrapure water was produced (18 MΩ cm) using a Simplicity system from Millipore (Bedford, MA, USA). Analytical and chromatographic grade organic solvents (acetonitrile, ethanol, methanol and isobutanol), di-potassium hydrogen phosphate (≥99.0%), potassium dihydrogen phosphate (≥99.5%), potassium hydroxide hydrate (≥99.9%), ferric chloride (≥99.0%), aluminum chloride (≥99.0%) and formic acid (98%) were purchased from Merck (Darmstadt, Germany). α-glucosidase solution was prepared at a concentration of 250 U mL^{-1} in 100 mM potassium phosphate buffer at pH 7.4. AChE solution was prepared at a concentration of 300 U mL^{-1} in 50 mM Tris-hydrochloric acid (HCl) buffer at pH 7.8. Both enzymatic solutions were divided into 100 μL aliquots and kept at -18°C until use.

2.2 Sample and standard preparation

Arrayán fruits and leaves were collected in Concepción, Biobio region, Chile (36º 50´ 0´´S and 73º 3´ 0´´W) in May 2019. Leaves and fruits samples without blemishes or damage were washed with deionized water and frozen at-24 ºC for 48h. Then, separately, fruits and leaves were ground and homogenized using an IKA (Staufen, Germany) A11 basic analytical mill. After a second frozen step for 24 h, samples were freeze-dried for 48 h at -55°C using a Martin Christ (Osterode am Harz, Germany) Alpha 1-2 LD plus freeze-dryer. Raw extracts of arrayán fruits and leaves were obtained by ultrasound-assisted extraction (UAE) using conditions previously optimized for the extraction of phenolic compounds (capítulo II sección 2 y 3) (Table 1). UAE was carried out using an ultrasonic bath (Getidy, China) with a frequency of 40 kHz and a potency of 200 W. Samples were centrifuged for 30 min at 16162 x *g,* and a small volume of clear supernatant (*ca.* 1.5 mL) was collected and filtered through 13 mm polyvinylidene fluoride (PVDF) syringe filter (0.22 µm). This filtrate was used for HPTLC (bio)autographic assays. Caffeic acid (positive control for α-G assay), donepezil (positive control for AChE assay), gallic acid (positive control for antioxidant activity assay) were prepared in methanol/water (7:3, v/v) at a concentration of 150 mg L^{-1}.

Table 1. UAE conditions for phenolic compounds extraction from *Luma apiculata*

Factor	Fruits	Leaves
Extraction temperature (°C)	62	50
Ethanol percentage in hydroethanolic mixture*	39	42
Extraction time (min)	10	27
Mass to solvent ratio (g mL^{-1})	1:33.4	1:33.4

* acidified with formic acid at 1%, v/v

2.3 High-performance thin-layer chromatography

Standard solutions (positive controls) and samples were applied on 10 × 10 cm HPTLC plates coated with a 0.2 mm layer of silica gel 60 F254 employing an automatic TLC Sampler 4 (CAMAG, Muttenz, Switzerland) using the following settings: band length 6 mm, track distance 10.0 mm, application x-axis and y-axis at 10.0 mm, dosage speed 150 nL s^{-1}; predosage volume, 200 nL; rinsing solvent methanol and two rinsing cycles. Application volume for samples (raw extract of arrayán fruit and leaves) and positive controls were 3 µL. Chromatography was performed in a 10 × 10 cm twin-trough chamber (CAMAG) up to a migration distance of 70 mm using a mobile phase composed of acetonitrile, isobutanol, formic acid and water (7:1:1:1 v/v/v/v). Raw extracts were applied in triplicate dividing the HPTLC plate into three sections: the first

section was used for (bio)autography, the second section for chemical derivatization, and the third one for MS analysis.

2.4 HPTLC (bio)autographic assays

2.4.1 α-G and AChE inhibition bioautography

The HPTLC bioautography method for α-G and AChE inhibitory compounds detection were developed following the methods proposed previously (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b) with some modifications. After chromatography, the mobile phase was removed at 100°C for 30 min on the TLC plate heater. To detect α-G and AChE inhibitors, the plate first sections were horizontally dipped in a glass tank (10 x 6 cm) containing 10.0 ml of 2-naphthyl-D-glucopyranoside (1 mg mL^{-1}) and 10 mL of 1-naphthyl acetate (1.5 mg mL^{-1}), respectively. In both assays, the plates were dried at 50°C for 10 min on the TLC plate heater, and then horizontally dipped in 10 mL enzymatic solution (α-G:1.25 U mL^{-1} in 100 mM phosphate buffer, pH 7.4; AChE: 0.75 U mL^{-1} in 50 mM Tris-HCl buffers at pH 7.8). Liquid excess was partially removed by contacting the plates with absorbent paper for 3 seconds. Incubation was carried out into closed moisture chamber (containing a proper volume of water) at 37°C for 10 min, avoiding direct contact between water and the plate. Thereafter, both HPTLC plate sections were sprayed with freshly prepared Fast

Blue B salt solution (1.5 mg/mL) by means of TLC sprayer from Merck. The presence of inhibitory compounds were observed as colorless bands on purple background. Plate images were documented under illumination (reflectance) with white light using a CAMAG Reprostar 3 documentation system. All instruments were controlled via WinCats1.4.7 software from CAMAG.

2.4.2 DPPH autography

For the detection of compounds with antioxidant activity, HPTLC-DPPH autographic assay was performed following a previous method with minor modification (Agatonovic-Kustrin and Morton, 2017). A 0.4% DPPH solution was prepared in methanol, stored protected from light at 2–8 °C. Derivatization was accomplished by dipping the corresponding HPTLC plate section into the derivatizing agent for 2 s and with an immersion speed of 3 cm s^{-1} using the CAMAG Immersion Device. Plates derivatized with DPPH solution were stored in dark for 15 min and then photographed. Compounds that possess radical scavenging activity were detected as bright yellow bands under purple background.

2.5 Chemical identification of bioactive compounds

The chemical identification of compounds with antioxidant and inhibitory activity against α-G and AChE was carried out by means of a phytochemical screening on HPTLC plates following the methods proposed by Maya et al. (2019). After chromatography, the second plate section was visualized under visible light, 254 nm-UV or 366 nm-fluorescence. Further the HPTLC plates was sprayed with detection reagents for peptides, alkaloids, phenols, coumarins, flavonoids, and tannins, and observing again under visible light, 254 nm-UV or 366 nm-fluorescence (Debenedetti, 2009; Maya et al., 2019).

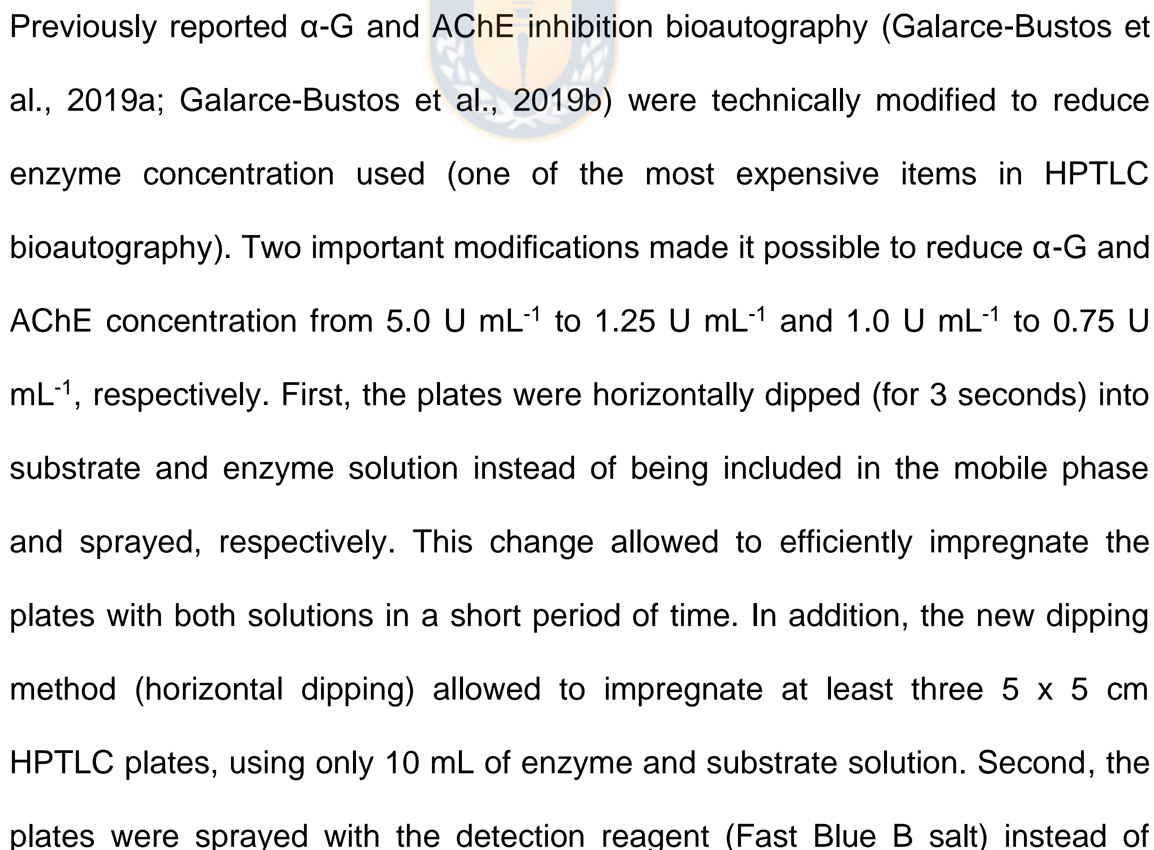
2.6 Mass spectrometry identification of bioactive compounds

Bands with antioxidant and inhibitory activity against α-G and AChE were selected and marked using a graphite pencil on the HPTLC plate based on hR_F value visualized in CAMAG cabinet (254 and 366 nm). Mass spectra were obtained by compound elution into MS by means of TLC/MS interface assembled with oval elution head (4.0 x 2.0 mm) using an ethanol and water mixture (1:1 v/v) at a flow rate of 0.2 mL min^{-1} for 60 s. MS analysis was performed in Shimadzu (Kyoto, Japan) LCMS-8030 triple quadrupole mass spectrometer with electrospray ionization (ESI) source using the following settings: ESI in negative mode, capillary voltage: 3.0 kV, nebulizer gas (N_2) flow:

3 L min^{-1}, drying gas (N_2) flow: 15 L min^{-1}, desolvation line temperature 250°C, and heat block temperature 400 °C. Mass spectra were acquired in full scan mode (*m/z* 100-2000). Background signals of TLC plates were subtracted for each analysis. Data were acquired and recorded by Shimadzu Lab-Solution software version 5.51.

3. Results and Discussion

3.1 Development of HPTLC-(bio)autography method



Previously reported α-G and AChE inhibition bioautography (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b) were technically modified to reduce enzyme concentration used (one of the most expensive items in HPTLC bioautography). Two important modifications made it possible to reduce α-G and AChE concentration from 5.0 U mL^{-1} to 1.25 U mL^{-1} and 1.0 U mL^{-1} to 0.75 U mL^{-1}, respectively. First, the plates were horizontally dipped (for 3 seconds) into substrate and enzyme solution instead of being included in the mobile phase and sprayed, respectively. This change allowed to efficiently impregnate the plates with both solutions in a short period of time. In addition, the new dipping method (horizontal dipping) allowed to impregnate at least three 5 x 5 cm HPTLC plates, using only 10 mL of enzyme and substrate solution. Second, the plates were sprayed with the detection reagent (Fast Blue B salt) instead of

being dipped. This modification avoided small clumps formation (excess colored complex) on HPTLC plate, resulting in a more homogeneous plate coloration. With this new methodology, the inhibitory bands were easily visualized against the purple background after 1 to 2 min reaction time.

To the best of our knowledge, this is the lowest enzyme concentration reported up to now. The proposed method reduces by 1.3-fold the AChE concentration reported by Galarce-Bustos et al. (2019b), 3.9-fold the one used by Mroczek (2009) and 8.7-fold the one used by Marston et al. (2002). At the same time, α-G concentration is 4-fold lower than that reported by Galarce-Bustos et al. (2019a), and 8-fold lower the one used by Simões-Pires et al. (2009).

3.2 Identification of antioxidant and inhibitory compounds against α-G and AChE

Currently, numerous analytical tools have been coupled to HPTLC to facilitate bioactive compounds identification from the HPTLC plate (Galarce-Bustos et al., 2019a; Galarce-Bustos et al., 2019b; Móricz et al., 2018). In this sense, the TLC-MS interface is an important tool for identification of unknown compounds, since it allows the direct elution of a band (compound) from the HPTLC plate to mass spectrometer (Aranda and Morlock, 2006, 2007). This strategy is widely used for rapid identification of new bioactive compounds detected by direct bioautography (Krüger et al., 2017; Móricz et al., 2018). Using this analytical

strategy, the fruit and arrayán leaves extracts showed adequate chromatographic separation. After HPTLC (bio)autography, the leaves extract only showed bands with antioxidant activity (Fig 1), while the fruit extract showed bands (hR_F = 19) with antioxidant and inhibitory activity against α-G and AChE (Fig 2). Subsequently, a first structural approximation of the compound with multiple bioactivities was performed by visualizing the band at 254 and 366 nm and spraying the second plate section (which was studied in parallel to the HPTLC (bio)autography) with various detection reagents [aluminum chloride, ninhydrin, Dragendorff's reagent, KOH (10% ethanol solution) and $FeCl_3$ (80% ethanol solution)]. Under 366 nm-fluorescence, the inhibitory band detected in arrayán fruit, showed a great light blue fluorescence, which was intensified when sprayed with the KOH and $AlCl_3$ reagents (Fig 2). These results suggest that the compound with antioxidant and inhibitory activity of both enzymes could be a coumarin, phenolic substance widely distributed in the plant kingdom. (Debenedetti, 2009; Kovač-Bešović and Durić, 2003; Maya et al., 2019). After structural approach, the MS analysis was performed on the third plate section in order to identify the bioactive compound tentatively. The band mass spectrum showed an deprotonated molecule $[M–H]^-$ at m/z = 207 (Fig 2). Considering the phytochemical screening and MS analysis (m/z value) results, the compound could be tentatively identified as fraxetin (7,8-dihydroxy-6-methoxychromen-2-one, Fig. 2), the only compound reported in literature with $[M−H]^-$ parent ion at value m/z 207 (Gómez et al., 2019; Saleem et al., 2019; Zhao et al., 2016; Zhou

et al., 2008). Fraxetin is reported for the first time in arrayán or any other berry fruit, as well as its inhibitory activity against α-G and AChE. This coumarin has been reported to possess antioxidative, anti-inflammatory, antiviral, antitumor, antihyperglycemic and neuroprotective effects (Murali et al., 2013; Witaicenis et al., 2014; Zhou et al., 2008). Very recently, its presence was reported in fruits of *Solanum virginianum*, a wild growing Solanaceae from Asia (Ke-peng et al., 2020) .The present work would support the antihyperglycemic fraxetin effect through its inhibitory capacity against α-G. This result is complementary to those obtained by Murali et al. (2013), who reports that fraxetin also can improve the glycemic profile in diabetic rats by modulating the glycolytic enzymes involved in glucose metabolism at hepatic and renal level. Therefore, the affinity of fraxetin on the enzymes involved in carbohydrate metabolism makes it an excellent alternative for treating T2D. Another biological activity supported in this work is the neuroprotective effect of fraxetin through its antioxidant and inhibitory activity against AChE. The inhibition of this enzyme is currently the first strategy used in the treatment of AD (Asghari et al., 2018).

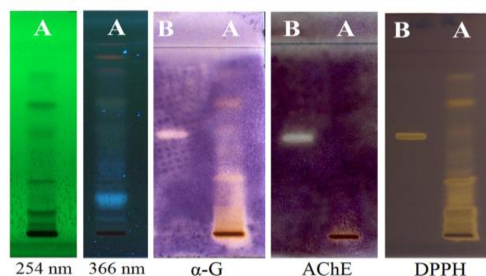


Fig 1. HPTLC chromatograms of arrayán leaf extract on silica gel F254 plates. (A) HPTLC-(Bio)autography of arrayán leaf extract, 89.82 µg applied. (B) Positive controls (caffeic acid and gallic acid: 0.90 µg applied; donepezil: 0.03 µg applied).

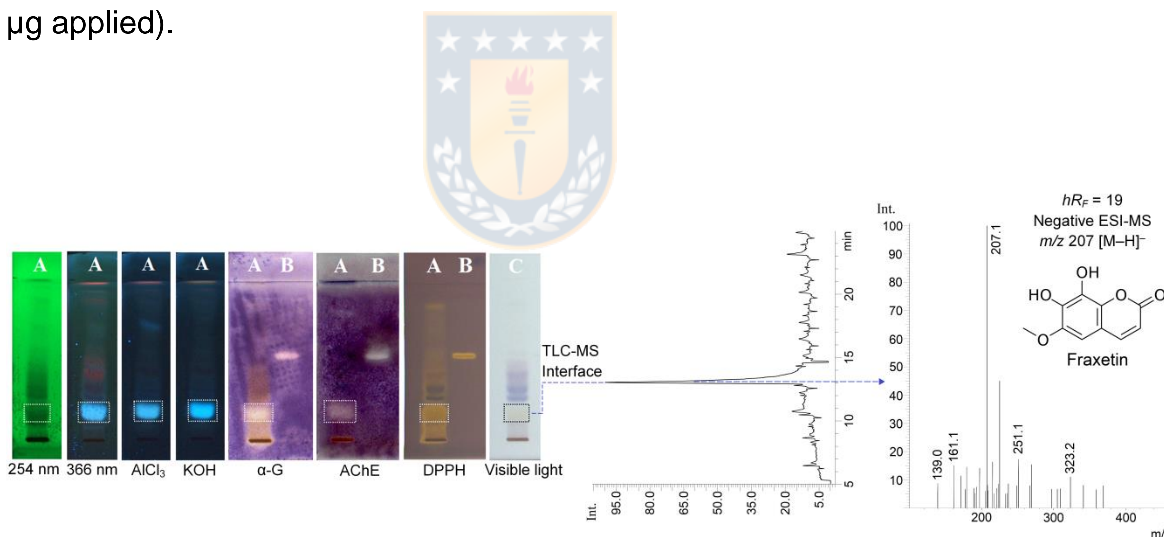


Fig 2. HPTLC chromatograms of arrayán fruit extract on silica gel F254 plates. (A) HPTLC-(Bio)autography of arrayán fruit extract, 89.82 µg applied. (B) Positive controls (caffeic acid and gallic acid: 0.90 µg applied; donepezil: 0.03 µg applied). (C) HPTLC-ESI-MS mass spectra of selected band and tentative compound identification.

4. Conclusions

For the first time, the presence of a compound with antioxidant and inhibitory activity against α-G and AChE applying a rapid effect-directed analysis via an HPTLC-(bio)autography-MS is reported in arrayán berries (*Luma apiculata*). HPTLC-(bio)autography conditions were improved through a new impregnation method of enzyme and substrate on the HPTLC plate (horizontal plate dipping) and reduction of enzyme concentration (an α-G and AChE concentration 1.3 and 4-fold lower than the ones described in recent reports) which resulted in a successful and cheaper methodology capable of detecting bioactive compounds potentially beneficial for health. This analytical approach allowed the detection and tentative identification of fraxetin, a compound reported for the first time in the arrayán berries or in any other berry fruit with promising activity on AD and T2D.

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Conflicts of interest

All authors declare that they have no conflict of interest.

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References

Abdelhafiz, A.H., McNicholas, E., Sinclair, A.J., 2016. Hypoglycemia, frailty and dementia in older people with diabetes: Reciprocal relations and clinical implications. Journal of Diabetes and its Complications 30, 1548-1554.

Agatonovic-Kustrin, S., Morton, D.W., 2017. High-performance thin-layer chromatography-direct bioautography as a method of choice for alpha-amylase and antioxidant activity evaluation in marine algae. Journal of Chromatography A 1530, 197-203.

Aguilera, M.A., Casanueva, M.E., Hernández, C.E., 2006. Composición de la araneofauna en dos especies de árboles nativos Peumus boldus Mol. y Luma apiculata (D.C.) Burret en el parque botánico Pedro del Río Zañartu (Hualpen), Concepción, VIII Región, Chile. Gayana (Concepción) 70, 176-185.

Aranda, M., Morlock, G., 2006. Simultaneous determination of riboflavin, pyridoxine, nicotinamide, caffeine and taurine in energy drinks by planar chromatography-multiple detection with confirmation by electrospray ionization mass spectrometry. Journal of Chromatography A 1131, 253-260.

Aranda, M., Morlock, G., 2007. Simultaneous Determination of Caffeine, Ergotamine, and Metamizol in Solid Pharmaceutical Formulation by HPTLC-UV-FLD with Mass Confirmation by Online HPTLC-ESI-MS. Journal of Chromatographic Science 45, 251-255.

Araya-Contreras, T., Veas, R., Escobar, C.A., Machuca, P., Bittner, M., 2019. Antibacterial Effect of <i>Luma apiculata</i> (DC.) Burret Extracts in Clinically Important Bacteria. International Journal of Microbiology 2019, 7803726.

Bhandari, M.R., Jong-Anurakkun, N., Hong, G., Kawabata, J., 2008. α-Glucosidase and α-amylase inhibitory activities of Nepalese medicinal herb Pakhanbhed (Bergenia ciliata, Haw.). Food Chemistry 106, 247-252.

Cass, S.P., 2017. Alzheimer's Disease and Exercise: A Literature Review. Current Sports Medicine Reports 16, 19-22.

Debenedetti, S., 2009. TLC and PC in Isolation, Identification and Characterization of Allelochemicals, pp. 103-133.

Fuentes, L., Valdenegro, M., Gómez, M.-G., Ayala-Raso, A., Quiroga, E., Martínez, J.-P., Vinet, R., Caballero, E., Figueroa, C.R., 2016. Characterization of fruit development and potential health benefits of arrayan (Luma apiculata), a native berry of South America. Food Chemistry 196, 1239-1247.

Galarce-Bustos, O., Pavón-Pérez, J., Henríquez-Aedo, K., Aranda, M., 2019a. An improved method for a fast screening of α-glucosidase inhibitors in cherimoya fruit (Annona cherimola Mill.) applying effect-directed analysis via high-performance thin-layer chromatography-bioassay-mass spectrometry. Journal of Chromatography A 1608, 460415.

Galarce-Bustos, O., Pavón, J., Henríquez-Aedo, K., Aranda, M., 2019b. Detection and identification of acetylcholinesterase inhibitors in Annona cherimola Mill. by effect-directed analysis using thin-layer chromatography-bioassay-mass spectrometry. Phytochemical Analysis 30, 679-686.

Gómez, J., Simirgiotis, M.J., Lima, B., Paredes, J.D., Villegas Gabutti, C.M., Gamarra-Luques, C., Bórquez, J., Luna, L., Wendel, G.H., Maria, A.O., 2019. Antioxidant, gastroprotective, cytotoxic activities and UHPLC PDA-Q orbitrap mass spectrometry identification of metabolites in Baccharis grisebachii decoction. Molecules 24, 1085.

Hsu, C.-C., Wahlqvist, M.L., Lee, M.-S., Tsai, H.-N., 2011. Incidence of Dementia is Increased in Type 2 Diabetes and Reduced by the Use of Sulfonylureas and Metformin. Journal of Alzheimer's Disease 24, 485-493.

Inocente Camones, M.A., Jurado Teixeira, B., Ramos Llica, E., Alvarado Chávez, B., Fuertes Ruiton, C., Cárdenas Montoya, L., Rivera Castillo, B., 2019. Actividad hipoglucemiante in vitro de los polisacáridos digeridos de Nostoc sphaericum Vaucher ex Bornet & Flahault (cushuro). Horizonte Médico (Lima) 19, 26-31.

Ito, K., Ahadieh, S., Corrigan, B., French, J., Fullerton, T., Tensfeldt, T., 2010. Disease progression meta-analysis model in Alzheimer's disease. Alzheimer's & Dementia 6, 39-53.

Ke-peng, D., Wei, L., Kang-lin, X., Lin, Z., Sheng-jun, D., 2020. A new flavonoid isolated from fruits of Solanum virginianum. Tianjin Journal of Chinese Herbal Medicine 51, 3845-3849.

Kolawole, O.T., Akanji, M.A., 2013. Inhibitory effect of leaf extract of Newbouldia laevis on the metabolic activities of α-glucosidase and α-amylase. ||| Bangladesh Journal of Pharmacology 8, 371-377.

Konrath, E.L., Passos, C.d.S., Klein-Júnior, L.C., Henriques, A.T., 2013. Alkaloids as a source of potential anticholinesterase inhibitors for the treatment of Alzheimer's disease. Journal of Pharmacy and Pharmacology 65, 1701-1725.

Kovač-Bešović, E.E., Durić, K., 2003. Thin layer chromatography-application in qualitative analysis on presence of coumarins and flavonoids in plant material. Bosnian Journal of Basic Medical Sciences 3, 19-26.

Krüger, S., Hüsken, L., Fornasari, R., Scainelli, I., Morlock, G., 2017. Effect-directed fingerprints of 77 botanical extracts via a generic high-performance thin-layer chromatography method combined with assays and mass spectrometry. Journal of Chromatography A 1529, 93-106.

Li, X., Song, D., Leng, S.X., 2015. Link between type 2 diabetes and Alzheimer's disease: from epidemiology to mechanism and treatment. Clin Interv Aging 10, 549-560.

Lordan, S., Smyth, T.J., Soler-Vila, A., Stanton, C., Ross, R.P., 2013. The α-amylase and α-glucosidase inhibitory effects of Irish seaweed extracts. Food Chemistry 141, 2170-2176.

Marston, A., Kissling, J., Hostettmann, K., 2002. A rapid TLC bioautographic method for the detection of acetylcholinesterase and butyrylcholinesterase inhibitors in plants. Phytochemical Analysis 13, 51-54.

Martín-Timón, I., Sevillano-Collantes, C., Segura-Galindo, A., del Cañizo-Gómez, F.J., 2014. Type 2 diabetes and cardiovascular disease: have all risk factors the same strength? World journal of diabetes 5, 444.

Maya, M., Ramanaiah, I., Venkatakrishna, K., Rameshkumar, K., Veeramanikandan, V., Eyini, M., Balaji, P., 2019. Investigation of Bioactive Compounds of Capsicum Frutescence and Annona Muricata by Chromatographic Techniques. Journal of Drug Delivery and Therapeutics 9, 485-495.

Móricz, Á.M., Ott, P.G., Yüce, I., Darcsi, A., Béni, S., Morlock, G.E., 2018. Effect-directed analysis via hyphenated high-performance thin-layer chromatography for bioanalytical profiling of sunflower leaves. Journal of Chromatography A 1533, 213-220.

Morlock, G.E., 2014. Chromatography Combined with Bioassays and Other Hyphenations – The Direct Link to the Compound Indicating the Effect, Instrumental Methods for the Analysis and Identification of Bioactive Molecules. American Chemical Society, pp. 101-121.

Mroczek, T., 2009. Highly efficient, selective and sensitive molecular screening of acetylcholinesterase inhibitors of natural origin by solid-phase extraction-liquid chromatography/electrospray ionisation-octopole-orthogonal acceleration time-of-flight-mass spectrometry and novel thin-layer chromatography-based bioautography. Journal of Chromatography A 1216, 2519-2528.

Murali, R., Srinivasan, S., Ashokkumar, N., 2013. Antihyperglycemic effect of fraxetin on hepatic key enzymes of carbohydrate metabolism in streptozotocin-induced diabetic rats. Biochimie 95, 1848-1854.

Nichols, E., Szoeke, C.E.I., Vollset, S.E., Abbasi, N., Abd-Allah, F., Abdela, J., Aichour, M.T.E., Akinyemi, R.O., Alahdab, F., Asgedom, S.W., Awasthi, A., Barker-Collo, S.L., Baune, B.T., Béjot, Y., Belachew, A.B., Bennett, D.A., Biadgo, B., Bijani, A., Bin Sayeed, M.S., Brayne, C., Carpenter, D.O., Carvalho, F., Catalá-López, F., Cerin, E., Choi, J.-Y.J., Dang, A.K., Degefa, M.G., Djalalinia, S., Dubey, M., Duken, E.E., Edvardsson, D., Endres, M., Eskandarieh, S., Faro, A., Farzadfar, F., Fereshtehnejad, S.-M., Fernandes, E., Filip, I., Fischer, F., Gebre, A.K., Geremew, D., Ghasemi-Kasman, M., Gnedovskaya, E.V., Gupta, R., Hachinski, V., Hagos, T.B., Hamidi, S., Hankey, G.J., Haro, J.M., Hay, S.I., Irvani, S.S.N., Jha, R.P., Jonas, J.B., Kalani, R., Karch, A., Kasaeian, A., Khader, Y.S., Khalil, I.A., Khan, E.A., Khanna, T., Khoja, T.A.M., Khubchandani, J., Kisa, A., Kissimova-Skarbek, K., Kivimäki, M., Koyanagi, A., Krohn, K.J., Logroscino, G., Lorkowski, S., Majdan, M., Malekzadeh, R., März, W., Massano, J., Mengistu, G., Meretoja, A.,

Mohammadi, M., Mohammadi-Khanaposhtani, M., Mokdad, A.H., Mondello, S., Moradi, G., Nagel, G., Naghavi, M., Naik, G., Nguyen, L.H., Nguyen, T.H., Nirayo, Y.L., Nixon, M.R., Ofori-Asenso, R., Ogbo, F.A., Olagunju, A.T., Owolabi, M.O., Panda-Jonas, S., Passos, V.M.d.A., Pereira, D.M., Pinilla-Monsalve, G.D., Piradov, M.A., Pond, C.D., Poustchi, H., Qorbani, M., Radfar, A., Reiner, R.C., Robinson, S.R., Roshandel, G., Rostami, A., Russ, T.C., Sachdev, P.S., Safari, H., Safiri, S., Sahathevan, R., Salimi, Y., Satpathy, M., Sawhney, M., Saylan, M., Sepanlou, S.G., Shafieesabet, A., Shaikh, M.A., Sahraian, M.A., Shigematsu, M., Shiri, R., Shiue, I., Silva, J.P., Smith, M., Sobhani, S., Stein, D.J., Tabarés-Seisdedos, R., Tovani-Palone, M.R., Tran, B.X., Tran, T.T., Tsegay, A.T., Ullah, I., Venketasubramanian, N., Vlassov, V., Wang, Y.-P., Weiss, J., Westerman, R., Wijeratne, T., Wyper, G.M.A., Yano, Y., Yimer, E.M., Yonemoto, N., Yousefifard, M., Zaidi, Z., Zare, Z., Vos, T., Feigin, V.L., Murray, C.J.L., 2019. Global, regional, and national burden of Alzheimer's disease and other dementias, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet Neurology 18, 88-106.

Puntieri, J., Torres, C., Magnin, A., Stecconi, M., Grosfeld, J., 2018. Structural differentiation among annual shoots as related to growth dynamics in Luma apiculata trees (Myrtaceae). Flora 249, 86-96.

Saleem, H., Zengin, G., Locatelli, M., Mollica, A., Ahmad, I., Mahomoodally, F.M., Zainal Abidin, S.A., Ahemad, N., 2019. In vitro biological propensities and chemical profiling of Euphorbia milii Des Moul (Euphorbiaceae): A novel source for bioactive agents. Industrial Crops and Products 130, 9-15.

Simirgiotis, M.J., Bórquez, J., Schmeda-Hirschmann, G., 2013. Antioxidant capacity, polyphenolic content and tandem HPLC–DAD–ESI/MS profiling of phenolic compounds from the South American berries Luma apiculata and L. chequén. Food Chemistry 139, 289-299.

Simões-Pires, C.A., Hmicha, B., Marston, A., Hostettmann, K., 2009. A TLC bioautographic method for the detection of α- and β-glucosidase inhibitors in plant extracts. Phytochemical Analysis 20, 511-515.

Strachan, M.W., Reynolds, R.M., Frier, B.M., Mitchell, R.J., Price, J.F., 2008. The relationship between type 2 diabetes and dementia. British medical bulletin 88.

Witaicenis, A., Seito, L.N., da Silveira Chagas, A., de Almeida, L.D., Luchini, A.C., Rodrigues-Orsi, P., Cestari, S.H., Di Stasi, L.C., 2014. Antioxidant and intestinal anti-inflammatory effects of plant-derived coumarin derivatives. Phytomedicine 21, 240-246.

Zhang, L., Chen, X., Liu, J., Zhu, Q., Leng, Y., Luo, X., Jiang, H., Liu, H., 2012. Discovery of novel dual-action antidiabetic agents that inhibit glycogen phosphorylase and activate glucokinase. European Journal of Medicinal Chemistry 58, 624-639.

Zhao, M., Ding, W., Wang, S., Wang, C., Du, Y., Xu, H., Wang, Q., Jin, S., 2016. Simultaneous determination of nine coumarins in rat plasma by HPLC–MS/MS for pharmacokinetics studies following oral administration of Fraxini Cortex extract. Journal of Chromatography B 1025, 25-32.

Zhou, L., Kang, J., Fan, L., Ma, X.-C., Zhao, H.-Y., Han, J., Wang, B.-r., Guo, D.-A., 2008. Simultaneous analysis of coumarins and secoiridoids in Cortex Fraxini by high-performance liquid chromatography–diode array detection–electrospray ionization tandem mass spectrometry. Journal of Pharmaceutical and Biomedical Analysis 47, 39-46.

CAPÍTULO III: Conclusiones finales

Conclusiones

La presente tesis doctoral tuvo como base el desarrollo de metodologías químico-analíticas para obtener datos científicos que demuestren y sustenten las características funcionales del arrayán (frutos y hojas) y de quinua (semillas). De los resultados obtenidos en el presente trabajo se puede concluir lo siguiente:

1. Se implementó, optimizó y validó satisfactoriamente el proceso de extracción de compuestos fenólicos desde semillas de quinua mediante extracción asistida por ultrasonido

2. Basándose en la metodología desarrollada para quinua, fue posible optimizar vía diseño experimental el proceso de extracción de compuestos fenólicos desde el fruto y hoja de arrayán mediante extracción asistida por ultrasonido. Para la hoja de arrayán las condiciones óptimas de extracción fueron 42% v/v etanol/agua, t= 27 min, T°= 50ºC y relación masa/solvente=1:33.4 g mL^{-1}, mientras que para el fruto fueron 39% v/v etanol/agua, T°= 62°C, t=10 min y relación masa/solvente=1:33.4 g mL^{-1}. Bajo estas condiciones, se obtuvo una concentración de 57.51±0.84 y 128.16±1.18 mg AGE/g en base seca en frutos y hojas, respectivamente.

3. Con las condiciones óptimas de extracción, se obtuvo extractos crudos de fruto y hoja de arrayán con una alta capacidad antioxidante según los valores obtenidos en los ensayos FRAP y DPPH. Mediante el proceso de concentración y purificación de los extractos crudos con la resina Amberlite XAD-7, el

contenido de compuestos fenólicos y capacidad antioxidante se incrementaron al menos en 9.1 veces para el fruto y en 4.6 veces para la hoja.

4. Se implementó satisfactoriamente un método UHPLC/MS para establecer el perfil de compuestos fenólicos en los extractos crudos de fruto y hoja de arrayán. En el extracto de fruto se identificaron dos flavonoides glicosilados (quercetina 3-ß-D-glucósido y kaempferol 3-glucósido), tres agliconas de flavonoides (miricetina, quercetina y kaempferol) y dos ácidos fenólicos (ácido p-cumárico y ácido gálico), mientras que en el extracto de hoja se identificaron dos flavonoides glicosilados (quercetina 3-ß-D-glucósido y kaempferol 3-glucósido), dos agliconas de flavonoides (miricetina y quercetina), y un ácido fenólico (ácido gálico).

5. Los extractos de fruto y hoja de arrayán (30% v/v etanol/agua) presentaron actividad antiviral de manera dosis-dependiente sobre los principales virus entéricos asociados a contaminación alimentaria. El extracto de hoja tuvo un efecto significativo sobre el virus de la hepatitis A y norovirus murino, mientras que el extracto de fruto presentó un efecto moderado sobre el virus de la hepatitis A. Esta actividad antiviral se atribuye al perfil fenólico de ambos extractos.

6. Los compuestos fenólicos (estándares puros) presentaron una limitada bioaccesibilidad después de ser sometidos a un proceso de digestión *in vitro*, la cual varió entre un 3.4 y 16.1%.

7. Tres sistemas nanoparticulados fueron preparados exitosamente mediante los métodos de nanoprecipitación y deposición electrostática. Estos sistemas aumentaron significativamente la bioaccesibilidad de todos los compuestos fenólicos. La bioaccesibilidad de los fenólicos cargados en nanopartículas de zeína aumentó en al menos un 50.2%. La bioaccesibilidad de los compuestos fenólicos cargados en nanopartículas de zeina recubiertas con alginato varió entre un 53.7 y 70.4%, mientras que los fenólicos cargados en nanoparticulas de zeina recubiertas con complejo alginato/quitosano varió entre un 65.8 y 84.0%. En todos los sistemas nanoparticulados, los compuestos más polares (ácidos fenólicos y flavonoides glicosilados) fueron más bioaccesibles que los menos polares (agliconas de flavonoides).

8. Se implementó una plataforma científica-tecnológica por HPTLC/(bio)autografía/MS para detectar e identificar compuestos bioactivos en el fruto y hoja de arrayán con actividad antioxidante e inhibitoria sobre las enzimas α-glucosidasa y acetilcolinesterasa. El extracto crudo de fruto de arrayán presentó un compuesto con marcada actividad antioxidante e inhibitoria sobre ambas enzimas, el cual fue identificado tentativamente como fraxetina (hidroxicumarina), mientras que el extracto crudo de hoja sólo presentó bandas con actividad antioxidante.