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Facultat de Farmàcia

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Uso de procesos convencionales e innovadores para la valorización de los subproductos de la horchata

TESIS DOCTORAL

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CERTIFICAN QUE: la licenciada en Farmacia Dña. Elena Roselló Soto ha realizado, bajo su dirección y en los laboratorios del área, el trabajo que lleva por título: "**Uso de procesos convencionales e innovadores para la valorización de los subproductos de la horchata**" para optar al Título de Doctora en Farmacia.

Y para que así conste, expiden y firman el presente certificado en Burjassot (Valencia), Febrero, 2019.

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  Consejo Regulador de la D.O. Chufa de Valencia.

-La verdadera ciencia enseña, sobre
todo, a dudar y a ser ignorante-
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ABREVIATURAS

AGI: Ácidos Grasos Insaturados
AGMI: Ácidos Grasos Monoinsatirados
AGPI: Ácidos Grasos Poliinsaturadods
AGS: Ácidos Grasos Saturados
CAT: Capacidad Antioxidante Total
CE: Extracción Convencional
CFT (TPC): Compuestos Fenólicos Totales
CRDO: Consejo Regulador de Denominación de Origen
DEAV: Descargas Eléctricas de Alto Voltaje
DPPH: 2,2_difenil-1-picrilhidrazilo
DO: Denominación de Origen
EC: Equivalentes de Catequina
EQ: Equivalentes de Quercetina
FT: Flavonoides Totales
GAE: Equivalentes de Ácido Gálico
GAME: Expresión Mecánica Asistida por Gas
ha: hanegada
HPH: Homogeneización por Altas Presiones
MAE: Microondas
PE: Pulsos Eléctricos
ROS: especies reactivas de oxígeno
rpm: revoluciones por minuto
SC-CO_2 : Fluidos Supercríticos
TEAC: Capacidad Antioxidante en Equivalentes de Trolox
Trolox: Ácido-6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico
UHT: ultra-alta temperatura
UV: ultravioleta
UV-C: ultravioleta de onda corta
UV-B: ultravioleta de onda media
UV-A: ultraviolesta de onda larga

Vit: vitamina
VP: Valor de peróxidos

RESUMEN

La “Horchata de chufa" es una bebida dulce y de aspecto blanquecino obtenida a partir de tubérculos de chufa (*Cyperus esculentus* L. var. *Sativus Boeck*) y consumida fundamentalmente en la Comunidad Valenciana. Durante su proceso de elaboración se producen una gran cantidad de residuos y subproductos, los cuales tradicionalmente han sido considerados como desechos industriales sin valor comercial, pero que sin embargo pueden ser utilizados para diferentes usos debido a su alto contenido en fibra dietética, compuestos antioxidantes bioactivos, además de tener buenas propiedades tecnológicas (capacidad de retención de agua, capacidad de emulsión y estabilidad de emulsión).

El objetivo de la presente Tesis Doctoral es la caracterización de diversos compuestos bioactivos (aceite, nutrientes, y antioxidantes, principalmente) obtenidos a partir de subproductos de la “horchata” mediante el empleo de extracción con métodos convencionales y procesos innovadores como los fluidos supercríticos (SC-CO_2). Los subproductos de la "horchata" son una fuente importante de compuestos bioactivos antioxidantes. En la extracción convencional se consiguió que las temperaturas más altas aumentarán la eficiencia de extracción de los compuestos fenólicos totales, mientras que de la misma manera, las concentraciones de etanol y los tiempos de extracción prolongados mejoran la extracción de éstos compuestos. La extracción con SC-CO_2 fue más eficiente para recuperar compuestos fenólicos lipofílicos con alta capacidad antioxidante en comparación con el procedimiento convencional de extracción mediante método Folch. Se observó una mayor extracción de los diferentes compuestos individuales cuando se aumenta la presión de tratamiento obteniendo los valores más altos cuando la presión fue de 40 MPa. Estos resultados se podrían traducir en beneficios económicos derivados de la valorización de los subproductos frente a su eliminación con el fin de preservar el medio ambiente de

residuos procedentes de la industria horchatera de una manera sostenible, reduciendo la huella de dióxido de carbono.

SUMMARY

"Horchata de chufa" is a sweet and whitish-looking drink obtained from tubers of tiger nut (*Cyperus esculentus* L. var. Sativus Boeck) and consumed mainly in the Valencian Community, during its production process a large quantity of waste and byproducts, which have traditionally been considered as industrial waste without commercial value, but which can nevertheless be used for different uses due to its high content of dietary fiber, antioxidant compounds, bioactive compounds, as well as having good technological properties (capacity water retention, emulsion capacity and emulsion stability).

The objective of this Doctoral Thesis is the characterization of various bioactive compounds (oil, nutrients, and antioxidants, mainly) obtained from tiger nut byproducts through the use of supercritical fluid extraction (SC- CO_2). The by-products of "horchata" are an important source of bioactive antioxidant compounds. In conventional extraction it was observed that higher temperatures increase the extraction efficiency of total phenolic compounds, while in the same way, ethanol concentrations and long extraction times improve the extraction of these compounds. Extraction with SC-CO_2 was more efficient to recover lipophilic phenolic compounds with high antioxidant capacity than the conventional Folch extraction method. A greater extraction of the different individual compounds was observed when the treatment pressure was increased, obtaining the highest values when the pressure was 40 MPa. The results obtained could lead to economic benefits as well as to the valorization of the by-products against their elimination in order to preserve the environment of waste coming from the “horchata” industry in a sustainable way, reducing the carbon footprint.

RESUM

"L´Orxata de xufa" és una beguda dolça i d'aspecte blanquinós obtinguda a partir de tubèrculs de xufa (*Cyperus esculentus* L. var. Sativus Boeck) i consumida fonamentalment en la Comunitat Valenciana. Durant el seu procés d'elaboració es produeixen una gran quantitat de residus i subproductes, els quals tradicionalment han estat considerats com deixalles industrials sense valor comercial, però que no obstant això poden ser utilitzats per a diferents usos pel seu alt contingut de fibra dietètica, compostos antioxidants, compostos bioactius, a més de tenir bones propietats tecnològiques (capacitat de retenció d'aigua, capacitat d'emulsió i estabilitat d'emulsió).

L'objectiu de la present Tesi Doctoral és la caracterització de diversos compostos bioactius (oli, nutrients, i antioxidants, principalment) obtinguts a partir de subproductes de la xufa mitjançant l'ús d'extracció amb fluids supercrítics (SC- CO_2). Els subproductes de "l´orxata" són una font important de compostos bioactius antioxidants. En l'extracció convencional es va observar que les temperatures més altes augmenten l'eficiència d'extracció dels compostos fenòlics totals, mentre que de la mateixa manera, les concentracions d'etanol i els temps d'extracció prolongats milloren l'extracció d'aquests compostos. L'extracció amb SC-CO_2 va ser més eficient per recuperar compostos fenòlics lipofílics amb alta capacitat antioxidant que el procediment convencional d'extracció mitjançant mètode Folch. Es va observar una major extracció dels diferents compostos individuals quan s'augmenta la pressió de tractament obtenint els valors més alts quan la pressió va ser de 40 MPa. Els resultats obtinguts podrien dur a beneficis econòmics així com a la valorització dels subproductes davant la seva eliminació per tal de preservar el medi ambient de residus procedents de la indústria orxatera d'una manera sostenible, reduint la empremta de diòxid de carboni.

1. INTRODUCCIÓN

1. INTRODUCCIÓN

1.1. Horchata de chufa

1.1.1. Origen

La “Horchata de chufa" o más comúnmente conocida como “horchata" es una bebida tradicional producida y consumida fundamentalmente en la Comunidad Valenciana. Esta bebida dulce y de aspecto blanquecino se prepara a partir de tubérculos de chufa (*Cyperus esculentus* L. var. *Sativus Boeck*) y es a menudo consumida durante el verano.

Los orígenes de la chufa, la materia prima a partir de la cual se prepara la “horchata”, son antiguos, así pues ya en el s. V a.C. Herodoto de Halicarnaso, historiador y geógrafo, ya describió en uno de sus libros algo llamado como “biblo”, refiriéndose a la chufa y en el s. IV a.C Teofrasto considerado el “padre de la bótanica” hace referencia a una planta herbácea que se cultivaba en el valle del Nilo y de la cual se obtenía un fruto que se recolectaba, preparaba y era degustado como un manjar (CRDO, 2018).

A partir de aquí podemos encontrar descripciones de la chufa y de sus propiedades en diversas obras. Es en el s. XIII cuando se supone que surgió el nombre de la “horchata” en una leyenda sobre el rey de Aragón Jaime I y una joven que le dió a probar una bebida dulce y blanca. Según cuenta la leyenda el rey dijo: “¿Qué es això? (¿Qué es esto?) y ella respondió: “leche de chufa”, a lo que el rey respondió: “¡Això no es llet, això es or, xata!” (“¡esto no es leche, esto es oro, guapa!”).

Ya en el s. XIV, Arnau de Vilanova introduce por primera vez el término chufa en una de sus obras y a partir de los s. XVIII y s. XIX ya se comienza a describir la chufa, y la bebida que se obtiene de ella la “horchata”, como describe Antonio José Cavanilles botánico valenciano en una de sus obras (CRDO, 2018).

A pesar de toda la información encontrada, todavía no queda claro el orígen de la chufa en Valencia. Unos dicen que la trajeron los musulmanes desde Egipto pasando por África hasta llegar a España en la edad Media, con las conquistas, y por otra parte, otros dicen que ya se cultivaba en Valencia mucho antes y prueba de ello es que la chufa Africana es diferente a la de Valencia (Soriano del Castillo et al., 2014).

1.1.2. La chufa y etapas de su cultivo

La chufa de Valencia (*Cyperus esculentus*) es una planta herbácea de hojas alargadas, con unos 40-50 cm de longitud, áspera al tacto, y en su parte subterránea a los extremos de las raíces se desarrolla este tubérculo llamado chufa (Pascual, Maroto, López-Galarza, Sanbautista, & Alagarda, 2000).

Existen dos variedades de chufa disponibles en el mercado según la procedencia: amarilla y marrón. La variedad amarilla importada seguramente de Africa y conocida como chufa "Africana" se caracteriza por tener un tamaño más grande, y un color más atractivo. Además, esta variedad presenta más rendimiento después de la extracción de la "horchata", contiene menos compuestos grasos y anti-nutricionales, y más proteínas (Okafor, Mordi, Ozumba, Solomon, & Olatunji, 2003) que la variedad marrón, que es la autóctona de la Comunitat Valenciana. Antiguamente, al no estar tipificada según el tipo de suelo, era posible diferenciar básicamente dos formas de chufa autóctona, siendo ésta la alargada (llargueta), la cual está presente en suelos francos y la redondeada, conocida como ametlla y que crece en terrenos arenosos (Soriano del Castillo et al., 2014).

Para obtener la chufa se ha de preparar inicialmente el suelo del campo para que esté suelto, esponjoso y nivelado. Normalmente se planta en abril-mayo, pero cada vez más se adelanta la fecha según la cosecha anterior y así se obtiene mayor cantidad de chufa. Asimismo, la planta es más resistente a posibles plagas al principio de verano. De todas formas

no se debe superar una densidad de siembra de 340.000-350.000 tubérculos plantados/ha, ya que el tubérculo podría no crecer.

La siembra se realiza de manera mecánica y en caballones (montículos de tierra quedando a ambos lados surcos para direccionar el riego). Hay dos sistemas de siembra: en seco como su nombre dice se realiza en terreno llano, seco, trabajado, sin restos vegetales y se riega tras la siembra. La profundidad de siembra debe ser de unos 4-5 cm y require unos 14 riegos de mayo a septiembre.

Por otro lado, siembra a sazón que es la más utilizada, y la tradicional donde la chufa se siembra con sazón de un riego anterior y ya no se riega hasta que brota. A diferencia de la anterior, la profundidad de la siembra ha de estar entre 6-8 cm, se usan tubérculos que se maceran durante 24 horas y se requieren unos 12 riegos de junio a septiembre (Pascual España, 1991; Soriano del Castillo et al., 2014).

Otro punto importante a tener en cuenta en la producción de la chufa es la fertilización, ya que debido al tipo de cutivo y a la gran lixiviación de los fertilizantes, fundamentalmente ocasionado por el tipo de riego (inundación de surcos), se recomienda el uso de abonos orgánicos para no empobrecer el terreno, ya que la planta extrae en cada cultivo nitrógeno, fósforo, potasio y esto podría dar lugar a tubérculos malformados y de mala calidad (Pascual España, 1991).

La recolección se inicia cuando la planta está seca, aproximadamente durante los meses de noviembre a diciembre y normalmente se utiliza una cosechadora que corta la tierra y la transporta a una cinta que la lleva a una tolva del tractor, realizándose por último una quema controlada de la parte aérea de la planta para preparar el terreno de cara a la siguiente cosecha (Soriano del Castillo et al., 2014).

Por último, se prepara el tubérculo para su posterior venta. Para ello se hace pasar por el lavadero que contiene tres bombos para eliminar raíces y tierra, seguidamente el material que queda se hace pasar por una ducha y unas canaletas para separar la grava de la chufa. Finalmente se

elimina la chufa flotante (en mal estado) de la que no flota.

A continuación, la chufa en buen estado se lleva a secado para que pierda la humedad. Se introduce en unas cámaras para realizar el secado lento y controlado durante unos 3 meses. Se extiende la chufa en capas de unos 10-20 cm de espesor y son removidas de forma uniforme periódicamente hasta que va disminuyendo la frecuencia conforme se van secando. Tras este paso se obtiene la llamada "chufa seca del labrador" que ya puede ser vendida o pasar a otro paso más de limpieza y clasificación (destrío) (Pascual España, 1991).

De manera que se pueden obtener seis tipos comerciales de chufa (Soriano del Castillo et al., 2014) a pesar de que en la Orden del 21 de mayo de 1997 por el que se ratifica el Reglamento de la Denominación de Origen "Chufa de Valencia" y su consejo regulador sólo diferencia 5 tipos :

- ***Tierna o verde***: chufa recién lavada sin secar.
- ***Seca del labrador***: chufa seca sin impurezas ni materiales extraños.
- ***Del cosechero***: chufa seca sometida a limpieza, selección y clasificación.
- ***Primera***: chufa que pasa por la criba y tiene un tamaño entre 4-5 mm y 7.5-8 mm de diámetro. No contemplada en la Orden del 21 de mayo de 1997.
- ***Granza***: la chufa con diámetro superior a 7.5-8 mm sin pasar la criba. Son los tubérculos más usados para la siembra.
- ***De destríos, bajos o desechos***: chufa de tamaño menor a 4-5 mm que no son comerciales. Son usados para alimentación del ganado.

1.1.3. Zonas de cultivo y producción de "horchata"

Esta bebida no alcohólica se produce principalmente en la Comunidad Valenciana (España) y especialmente en L´Horta Nord cómo podemos observar en la Figura nº 1, donde se han establecido industrias especializadas en la elaboración de "horchata". Estos términos municipales poseen las condiciones idóneas de clima y suelo para el cultivo de la materia prima para la producción de la "horchata", es decir, la chufa (Soriano del Castillo et al., 2014).

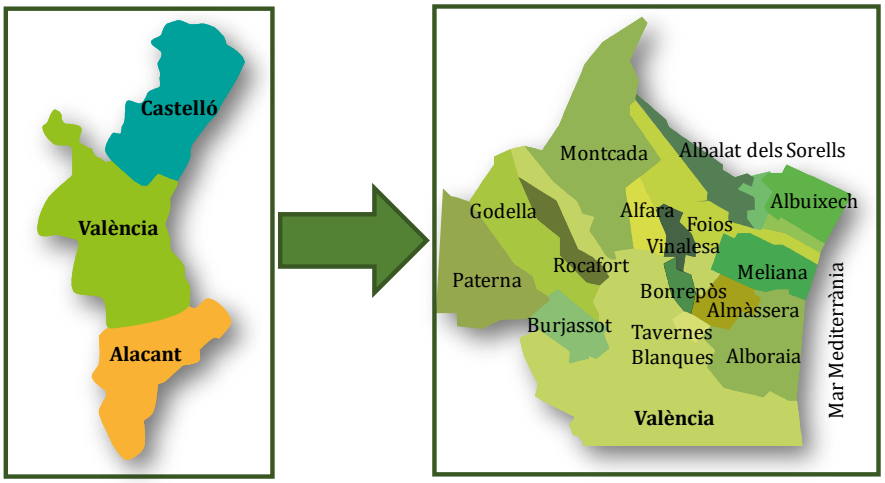

Figura nº 1. Mapa de los términos municipales valencianos productores de chufa.

La chufa y la "horchata" representan el valor cultural y tradicional en la Comunitat Valenciana (Codina Torrella, 2014). Tal vez por ello podemos observar en la Figura nº 2 los valores mantenidos a lo largo de los años en cuanto a extensión de cultivo en L´Horta Nord, mientras que hay un aumento ligero en cuanto al 2018/2019 y esto puede deberse al aumento de un 12.8% del consumo de chufa seca expresada en kg a partir

de los datos aportados por los comercios (CRDO, 2018).

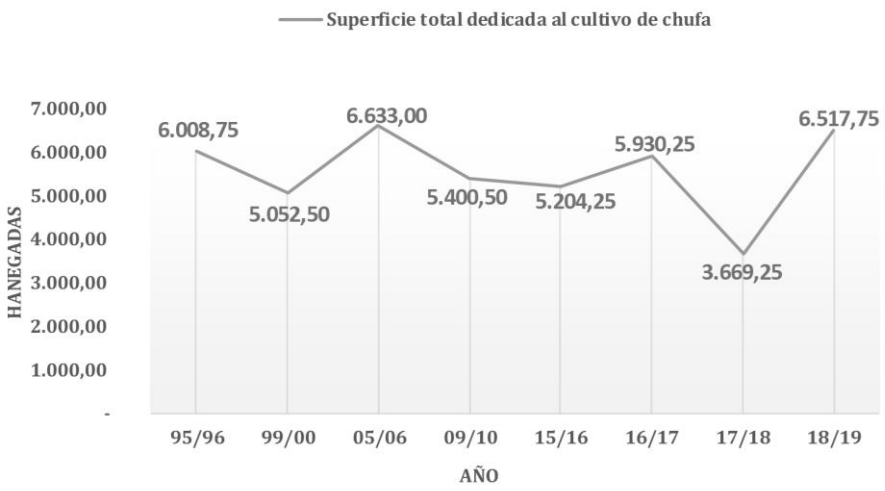

Figura nº 2. Superficie total de cultivo de chufa en la zona de Valencia expresado en hanegadas (1ha = 831m^2) desde el 95/96 hasta el 18/19. Fuente: C.R.D.O. 2018.

Dentro de los términos municipales, también se encuentran grandes diferencias en cuanto a la extension de cultivo, siendo Valencia con 2031.75ha (39%), seguida de Alboraia 1741.50ha (30%) y Almàssera 615.75ha (11%) las que presentan mayores extensiones, tal y como es posible observar en la última actualización de datos del C.R.D.O. En cuanto a Valencia, se tienen en cuenta los términos de Racó Anell, Sant Llorenç, Cases de Bàrcena, Benimaclet, Carpesa, Benifaraig, Borbotó, Poble Nou, Massarrojos, Benicalap, Campanar, Benimàmet, Mahuella, Tauladella, Rafalell y el Palmar.

También se cultiva la chufa en otros países como Burkina Faso, Costa de Marfil, Egipto, EEUU, Francia, Italia, Malí, el norte de Nigeria, Países Bajos, Togo, y Turquía, entre otros (Pascual-Seva et al., 2009).

A su vez, se importa chufa de otros países debido al bajo precio de ésta respecto a la autóctona, a pesar de que su calidad y rendimiento es

inferior. Algunos de estos países son Níger, Burkina Faso y Malí (Codina Torrella, 2014).

En cuanto a la bebida de chufa "horchata" se comercializa además de en España, en México, Panamá, República Dominicana, Estados Unidos y sur de Francia (Martín-Esparza & González-Martínez, 2016).

La "horchata" ha adquirido una gran importancia en la industria alimentaria española desde el punto de vista económico (CRDO, 2018; Rubert, Sebastià, Soriano, Soler, & Mañes, 2011). Por ejemplo, la demanda de "horchata" así como su consumo total anual en España están creciendo durante los últimos años, excediendo los 50 millones de litros (Martín-Esparza & González-Martínez, 2016), lo que significa un valor de mercado al por menor de unos 60 millones euros (CRDO, 2018).

1.1.4. Origen de las chufas

La mayor parte de las chufas producidas en l'Horta Nord está regulada y protegida por la denominación de origen "Chufa de Valencia", desde 1995, remontándose esta trayectoria al año 1985 (CRDO, 2018).

La Denominación de Origen (D.O.) es una garantía de calidad que permite al consumidor diferenciar entre la chufa propia de Valencia de la procedente de otros países cuyas propiedades organolépticas y de calidad son inferiores. Es por esto que la antigua Consellería de Agricultura y Medio Ambiente, con la Orden del 25 de septiembre de 1995, aprobó el Reglamento de la D.O. Chufa de Valencia y su Consejo Regulador.

Este Consejo Regulador de la D.O. Chufa de Valencia actúa como plataforma de información tanto para el sector como para el consumidor final (Soriano del Castillo et al., 2014).

Puesto que ha ido evolucionando este sector, se estableció un nuevo reglamento de la Denominación de Origen Protegida de la Chufa y su consejo regulador, orden 17/2010 del 18 de mayo de 2010, incluyendo

como novedad un registro de los productos alimenticios elaborados con Chufa de Valencia (CRDO, 2018). En este reglamento se establecen las características que deben cumplir las chufas con Denominación de Origen, referidas al peso unitario, morfología de la chufa tierna y composición nutricional (Cuadro nº 1), para garantizar la calidad de las chufas.

Cuadro nº 1. Características de la chufa que establece la denominación de Origen Protegida "Chufa de Valencia". Fuente: DOGV (2017).

PESO UNITARIO	**0.45-0.80 g en fresco/unidad**	
MORFOLOGÍA DE LA CHUFA SECA	**Largo**	**0.9-1.6 cm**
	Ancho	**0.7-1.1 cm**
COMPOSICIÓN NUTRICIONAL (% peso materia seca)	**Azúcares**	**11-17.5 %**
	Grasas	**23-31 %**
	Proteínas	**6.5-12 %**
	Almidón	**25-40 %**

Solo las chufas que adquieren las características señaladas en el reglamento pueden llevar el logotipo de Denominación de Origen "Chufa de Valencia" (Figuras nº 3) o (Figura nº 4) que lleva insertado el símbolo europeo. El símbolo europeo puede introducirse dentro del logotipo de la Denominación de Origen o fuera de éste, siempre que cumpla todos los requisitos establecios en el reglamento.

Figura nº 3. Logotipo de Denominación de Origen "Chufa de Valencia" (CRDO, 2018).

Figura nº 4. Logotipo de Denominación de Origen "Chufa de Valencia" con el símbolo europeo (CRDO, 2018).

Existe otro logotipo de la Denominación de Origen Chufa de Valencia, el cual es de su propiedad, y consiste en una barraca. Éste lo pueden emplear aquellos elaboradores inscritos en el registro de Elaboradores, Envasadores y Expendedores de "horchata" de Chufa de Valencia, que elaboren y, en su caso, envasen, en la zona de producción de la D.O. Chufa de Valencia.

Su uso es opcional, no pudiendo sustituirse el logotipo de la Chufa de Valencia por el de la barraca ni pudiendo ser de dimensiones mayores que ésta última. Este logotipo está constituido por una barraca orlada por la leyenda: "*Horchata de Chufa de Valencia elaborada en la zona de producción de la Denominación de Origen Chufa de Valencia"* que puede

verse de forma completa en la siguiente figura (CRDO, 2018).

Figura nº 5. Logotipo Barraca propiedad de la Denominación de Origen "Chufa de Valencia" (CRDO, 2018).

1.1.5. Proceso de elaboración de la "horchata"

La producción de horchata a partir de chufa requiere de una serie de pasos esenciales y regulados en la Reglamentación Técnico-Sanitaria (RD 1338/1988). A continuación se muestra de forma esquemática el proceso de elaboración de la "horchata" (Figura nº 6). Como puede verse en la figura, éste comienza con el *lavado de las chufas* para así poder eliminar los restos de tierra e impurezas que podemos encontrar en la chufa seca. Para ello, se emplea agua clorada en un recipiente de lavado en agitación. Seguidamente se realiza la *selección de las chufas* y para ello se emplea una disolución de sal a concentración de 15 y 17 ºBaume. De esta forma se pueden diferenciar los tubérculos dañados que flotarán por su menor densidad de los sanos que no flotarán. Posteriormente, las chufas no dañadas son lavadas otra vez para retirar los restos de salmuera de su superficie y las chufas son *rehidratadas* durante un tiempo variable (de 8 a 24h), según las características de los tubérculos y del agua usada, para que la desinfección posterior sea más efectiva, ya que disminuye la rugosidad de la superficie del tubérculo.

Figura nº 6. Representación esquemática del proceso de elaboración de la "horchata" a partir de chufa.

La *desinfección* se realiza con una solución de agua con un mínimo de cloro activo del 1%, en agitación mecánica y durante un tiempo inferior a 30 minutos, luego se realizan varios lavados para quitar todos los posibles restos de germicidas que puedan quedar (BOE, 1988) .

A continuación se realiza la *trituración* mediante un molino en el que se le añade agua (3 litros de agua/kg de chufa seca) para facilitar el proceso y evitar que se pueda parar el molino por apelmazamiento. El siguiente paso de maceración no siempre se realiza, depende del tiempo de remojo empleado previamente. Si ha sido superior a las 8h, se suprime este paso. A continuación del triturado, se prensa, para separar el líquido

del residuo sólido, esta fase se llama *prensado*, y se puede realizar bien en una prensa continua o en una prensa con dispositivo de tamiz. Tras este paso, obtenemos el primer extracto, y se *tamiza*. A continuación se mezcla el residuo del tamiz y del prensado añadiendo 2 litros de agua/kg de chufa, se prensa, tamiza y forma el segundo extracto que se une al anterior obteniendose el extracto final.

Finalmente, llegamos al paso de *mezclado,* donde se adiciona al líquido anteriormente obtenido entre 100 y 150g de azúcar por litro, se agita y se hace pasar por un tamiz, para eliminar cualquier impureza sólida, y se lleva rápidamente a *enfríar,* a una temperatura de aproximadamente 0 ºC. De esta manera la "horchata" ya está preparada para tomar o mantener en enfriadoras a una temperatura ≤2 ºC (Martín-Esparza & González-Martínez, 2016; Soriano del Castillo et al., 2014).

1.1.6. Residuos y subproductos generados durante el proceso de producción de la "horchata"

Durante el proceso de elaboración de la "horchata" se producen una gran cantidad de residuos y subproductos, los cuales tradicionalmente han sido considerados como desechos industriales sin valor comercial (Figura nº 7). Sin embargo, éstos pueden ser utilizados para diferentes usos. Los subproductos pueden separarse en sólidos y líquidos, cuya composición va a limitar su posterior aprovechamiento (Sánchez-Zapata et al., 2012) (Figura nº 8).

Tal y como puede verse en la Figura nº 7, entre los residuos y subproductos más importantes obtenidos durante el proceso de elaboración de la "horchata", es posible destacar la tierra, plantas y chufas dañadas obtenidas durante el proceso de recepción de las materias primas, limpieza y selección de las chufas a utilizar.

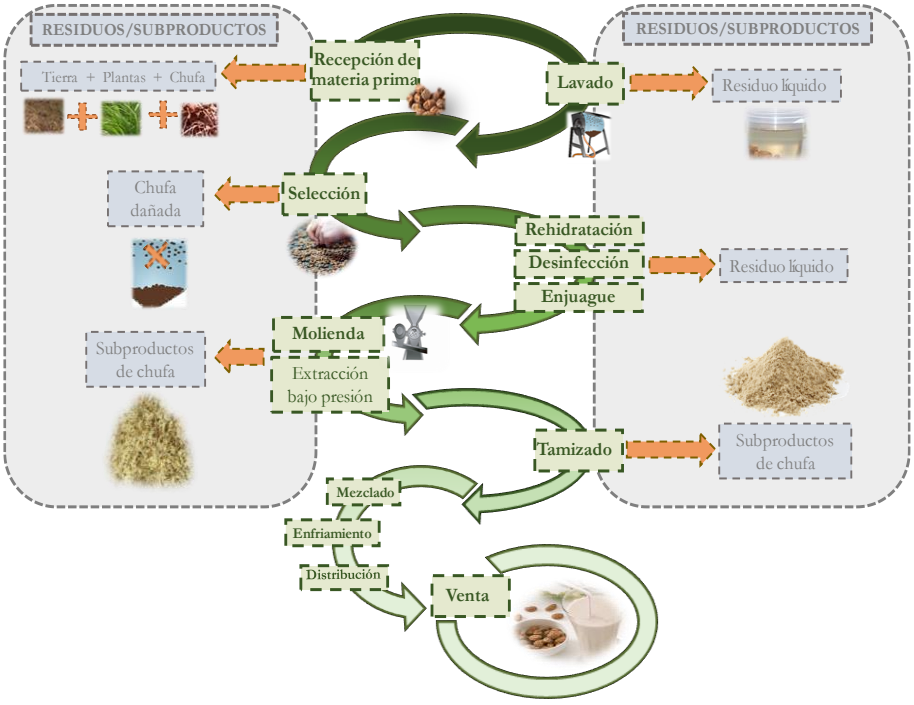

Figura nº 7. Residuos y subproductos obtenidos durante el proceso de elaboración de la "horchata".

Asimismo, durante la fase de lavado, rehidratación, desinfección y enjuague se producen residuos líquidos, fundamentalmente agua de lavado, que puede contener una cantidad importante de compuestos fenólicos o puede ser reutilizada para lavar más materia prima.

Durante las fases de molienda, extracción a baja presión y tamizado se van a generar diferentes subproductos de la chufa, los cuales presentan un alto contenido de fibra y compuestos bioactivos, presentando así un alto potencial para ser valorizados.

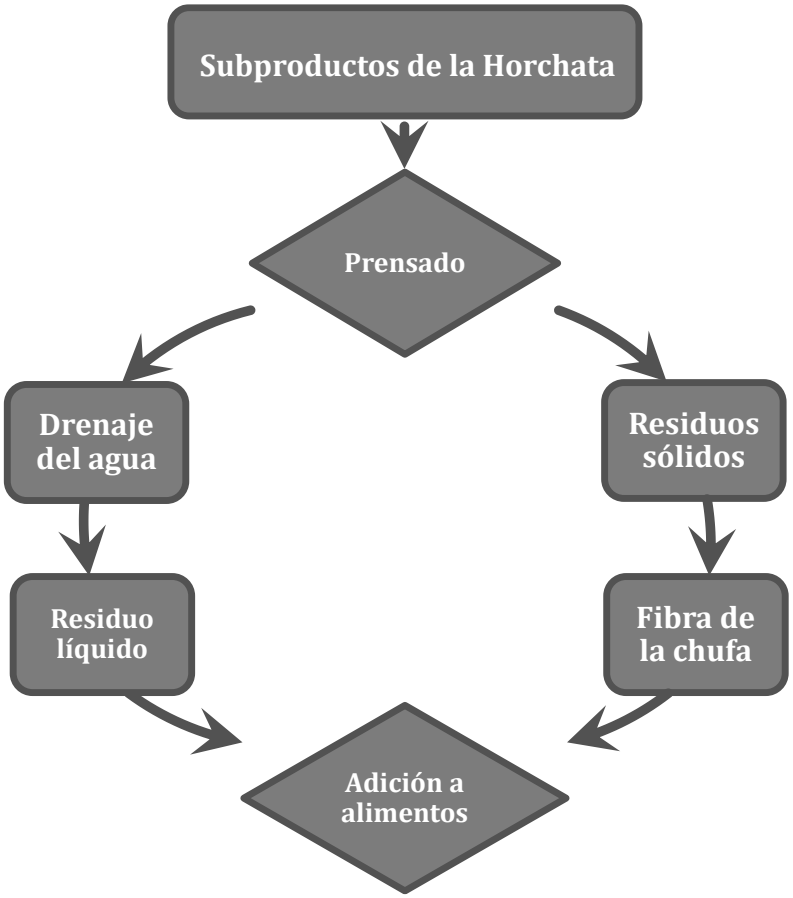

Figura nº 8. Diagrama de flujo de los coproductos de la chufa (sólido y líquido). Fuente: Adaptación de Sánchez-Zapata et al., (2012).

1.1.7. Aspectos nutricionales y compuestos bioactivos de la chufa y subproductos de la "horchata"

La composición nutricional de la chufa es similar a la de otros tubérculos ya que se caracteriza por ser rica en almidón, como hidrato de carbono predominante (Cuadro nº 2).

Cuadro nº 2. Composición centesimal (% materia seca) de la chufa.

Origen	Humedad	Grasa	Proteínas	Fibra	Cenizas	Referencia
Valencia	8.66	35.21	8.45	9.31	1.97	(Codina-Torrella, Guamis, & Trujillo, 2015)
BF	7.75	25.77	7.32	9.07	1.90	
BF	6.38	25.35	5.62	8.42	1.95	
Níger	7.45	28.19	3.28	8.75	1.60	
Egipto	7.30	22.14	4.33	15.47	2.60	(Adel, Awad, Mohamed, & Iryna, 2015)
Camerún	ND	35.32	8.08	10.31	2.39	(Ejoh & Ndjouenkeu, 2006)
Camerún	ND	44.92	7.50	14.49	2.61	
Camerún	ND	26.88	6.99	8.26	2.28	
Camerún	ND	43.50	8.30	14.14	2.60	
Camerún	ND	35.63	6.57	13.70	2.58	
Camerún	ND	31.66	7.54	12.51	2.32	

Nd: No detectado. BF: Burkina Faso.

Además las chufas presentan un alto contenido en fibra (8.26-15.47%) (Sánchez-Zapata, Fernández-López, et al., 2012). A su vez, también presentan un bajo contenido en agua en comparación con otros tubérculos como la patata, lo que facilita su almacenamiento y su

mantenimiento desde el punto de vista nutricional (Soriano del Castillo et al., 2014).

El aporte proteico (3.28-8.45%) de la chufa es inferior al de otros frutos secos como nueces, cacahuetes o pistachos y mayor que el boniato o la yuca (Sánchez-Zapata et al., 2012). La proteína mayoritaria es la albúmina (81.2%) y se encuentran en menor proporción globulinas (4.8%), gluteinas (2.3%) y prolaminas (0.9%) (Soriano del Castillo et al., 2014). El aminoácido que se encuentra en mayor proporción en la chufa es la arginina (1.4%) luego le siguen los ácidos glutámico (0.83%) y aspártico (0.75%), en cambio la metionina (0.16%) y la tirosina (0.12%) son los minoritarios (Soriano del Castillo et al., 2014).

En cuanto al contenido en grasa, es elevado, representando entre el 20-40% el peso del tubérculo, con un valor energético alto, como otros frutos secos (Sánchez-Zapata et al., 2012). El aceite que se obtiene de la chufa es rico en ácidos grasos insaturados (83.8%), de los cuales son mayoritarios el ácido oleico, y linoleico (Figura nº 9). También cabe destacar el alto contenido en otros ácidos grasos saturados como el palmítico (Roselló-Soto et al., 2018; Soriano del Castillo et al., 2014).

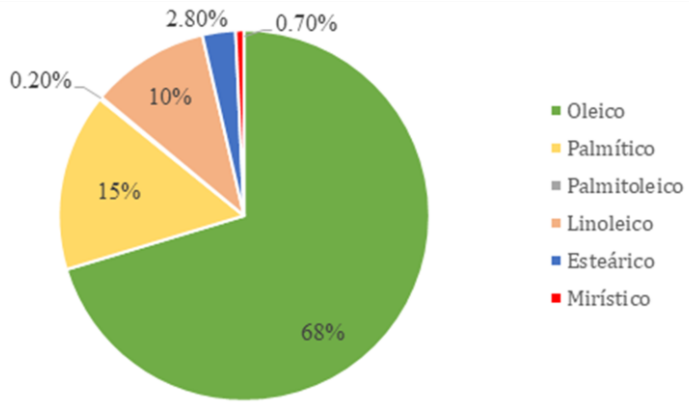

Figura nº 9. Perfil de ácidos grasos (%) del aceite de Chufa de Valencia.

Por otro lado, en lo referente a la cantidad de minerales, el mayoritario es el potasio seguido del fósforo. Por el contrario, calcio, magnesio y sodio son los que se encuentran en menor cantidad, junto con el hierro y el zinc. También se encuentran vitaminas, principalmente vitamina E, así por ejemplo Ezeh et al. (2016) observaron valores de α-tocoferol (146-170 μg/g) y β-tocoferol (30-35 μg/g) en aceites de chufa extraídos por expresión mecánica, asistida por enzimas. Además otros autores han documentado que en la chufa es posible encontrar ácido fólico y, en menor concentración, vitamina B_6, niacina, tiamina, vitamina C y riboflavina (Soriano del Castillo et al., 2014).

La chufa también presenta un alto contenido en compuestos bioactivos como los polifenoles. Los polifenoles son metabolitos secundarios de las plantas y son un grupo muy numeroso, tal y como puede apreciarse en el Cuadro nº 3. Dependiendo del grado de oxidación del anillo de carbono, es posible diferenciar entre flavonoles, flavonas, isoflavonas, flavan-3-oles, flavanonas y antocianinas (Santhakumar, Battino, & Alvarez-Suarez, 2018).

Los compuestos fenólicos, desempeñan un papel importante en la protección contra las especies reactivas de oxígeno (ROS), ya que el grupo fenólico actúa capturando los electrones desapareados, disminuendo así el riesgo de aparición de diversas enfermedades neurodegenerativas asociadas al estrés oxidativo. En este sentido, diversos estudios han mostrado las propiedades beneficiosas de los polifenoles en la prevención del riesgo de aparición de diferentes enfermedades crónicas como enfermedades cardiovasculares, osteoporosis o diabetes (Costa et al., 2017; Oak et al., 2018; Santhakumar et al., 2018; Scalbert, Manach, Morand, Rémésy, & Jiménez, 2005).

En un estudio llevado a cabo por Luo et al. (2014) estos autores determinaron hasta 20 flavonoides en la chufa, de los cuales seis eran nuevos y se encontraban en las cáscaras de la chufa (eleocarinas) (*Eleocharis tuberosa*). En una investigación posterior, Ezeh et al. (2016)

identificaron y cuantificaron cuatro compuestos fenólicos: ácido transferúlico (3.3-6.2 μg/g), ácido vanílico (1.8-2.2 μg/g), vainillina (1.9-2.2 μg/g) y ácido trans-cinámico (2.1-2.8 μg/g) en aceites de chufa extraídos mediante la ayuda de enzimas y presión.

Asimismo, Koubaa, Barba, et al. (2015) identificaron 78 polifenoles en el aceite de chufa, de los cuales 12 fueron polifenoles comunmente identificados en otras matrices de origen vegetal (ácido p-cumárico, ácido ferúlico, ácido 3-fenilpropiónico, ácido cinámico, ácido vanílico, entre otros).

Cuadro nº 3. Clasificación y estructura de los polifenoles. Fuente: Barba et al. (2014).

Compuestos fenólicos

Ácidos fenólicos	**Ácidos hidroxibenzoicos** (p-hidroxibenzoico, protocatequina, gálico, vanílico, siríngico) **Ácidos hidroxicinámicos** (p-cumárico, cafeico, ferúlico)	
Coumarinas	Umbeliferona, herniarina, psoraleno, imperatonina	
Lignanos	Neolignanos	
Flavonoides	**Flavones** (apigenina, luteolina) **Flavonoles** (kaempferol, quercetina, miricetina) **Flavanonas** (naringenina, eriodictiol, hesperidina) **Flavanonoles** (taxifolina) **Flavanoles** ((+)-catequina, (-)-epicatequina, epigalocatequina, epigalocatequina galato) **Antocianidinas** (pelargonidina, cianidina, delfinidina)	
Estilbenos	Resveratrol	
Isoflavonoides	**Isoflavonas** (daidzeína, genisteína, gliciteína) **Cumestano** (cumestrol)	
Polímeros de polifenoles	**Proantocianidinas** (procianidina, prodelfinidina, propelargonidina) **Taninos hidrolizables** (galotanino, elagitaninos)	

Además, algunos estudios también han documentado la presencia de otros compuestos bioactivos en la chufa como fitoesteroles, siendo el β-sitosterol el predominante seguido del estigmasterol y campesterol (Cuadro nº 4).

Cuadro nº 4. Contenido de fitoesteroles en chufas (mg/100 g).

Origen	β-Sitosterol	Campesterol	Estigmasterol	Referencia
España	60.18	14.01	19.50	(Lopéz-Cortés, Salazar-García, Malheiro, Guardiola, & Pereira, 2013)
Egipto	43.34	11.44	17.85	
Nigeria	60.82	16.63	18.76	
Sud-África	61.15	16.32	17.35	
Ghana	51.70	15.30	20.6	(Yeboah, Mitei, Ngila, Wessjohann, & Schmidt, 2012)

Otras investigaciones también han documentado la presencia de carotenoides en la chufa aunque no existe un estudio detallado sobre el contenido y el perfil de carotenoides existente.

1.2. Aplicaciones y valorización de los subproductos obtenidos durante el proceso de elaboración de la "horchata"

Como se ha mencionado anteriormente, durante el proceso de elaboración de la "horchata de chufa" se generan una cantidad elevada de subproductos que puede representar hasta el 60% del material vegetal, de acuerdo con la información aportada por Sánchez-Zapata, Fuentes-Zaragoza, et al. (2012). De acuerdo al marco legislativo desarrollado en España (Anónimo, 2011) y Europa (Anónimo, 2006, 2008) se establece como prioridad el reciclado y valorización de los residuos y subproductos frente a su eliminación con el fin de preservar los recursos naturales, proteger el medio ambiente y salud del consumidor mediante la prevención o reducción de los impactos globales del uso de los recursos y la mejora de la eficacia de dicho uso.

Es por eso que tanto la industria como los investigadores están mostrando un interés creciente en el estudio de los residuos y subproductos obtenidos durante el proceso de producción de la "horchata".

El residuo sólido puede ser utilizado debido a su alto contenido en fibra dietética, compuestos antioxidantes, compuestos bioactivos, además de tener buenas propiedades tecnológicas (capacidad de retención de agua, capacidad de emulsión y estabilidad de emulsión). Mientras que en el subproducto líquido destaca su alto contenido en prebióticos, antioxidantes y como sustituto del agua en productos cárnicos (Figura nº 10) (Sánchez-Zapata, Fuentes-Zaragoza, et al., 2012).

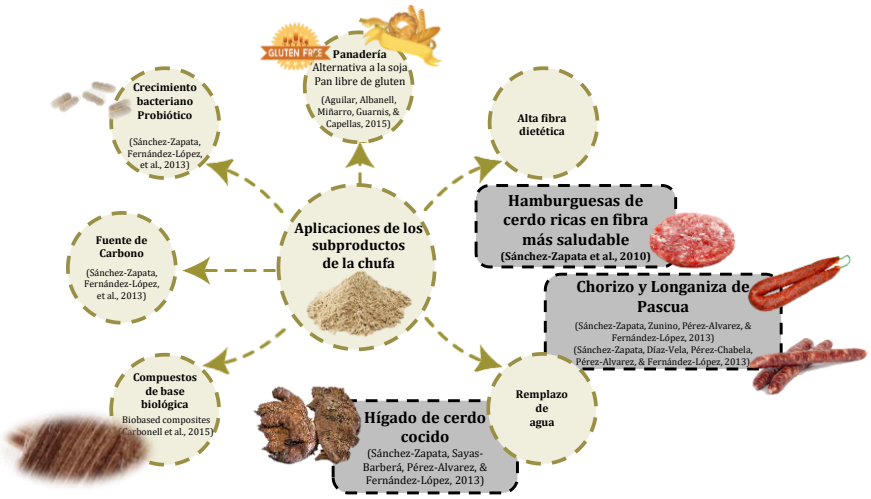

Figura nº 10. Aplicaciones potenciales de los subproductos de la chufa en el desarrollo de nuevos alimentos funcionales.

1.2.1. Uso de los subproductos de la "horchata" como fuente de fibra, productos libres de gluten y para promover el crecimiento de bacterias probióticas

En este sentido, diversos estudios han demostrado el potencial uso de los subproductos sólidos de la chufa en la industria cárnica, especialmente por su alto valor nutricional y contenido en fibra. Así pues, Sánchez-Zapata, Zunino, Pérez-Alvarez, & Fernández-López (2013) determinaron el efecto de la adición de fibra procedente de los subproductos de la chufa (0.5-7.5%) sobre las propiedades fisicoquímicas y sensoriales del chorizo almacenado durante 28 días. Además, también estudiaron las reacciones de oxidación y el crecimiento microbiano de los nuevos productos desarrollados.

Estos autores obtuvieron como resultado un chorizo rico en fibra, con un menor porcentaje de grasa y una mayor humedad. Asimismo, también obtuvieron pequeñas modificaciones en las propiedades fisicoquímicas, como cambios de color, no viéndose afectada la aceptación del

consumidor por los nuevos chorizos obtenidos.

En otro estudio realizado por el mismo grupo de investigación, se evaluó el impacto de la utilización de los diferentes porcentajes de los subproductos de la "horchata" (5, 10 y 15%) en la composición química, propiedades fisicoquímicas, características de cocción y las propiedades sensoriales de hamburguesas de cerdo. Los resultados mostraron que las hamburguesas elaboradas con subproductos de la "horchata" presentaron un mayor contenido en fibra. Además, se obtuvo un mayor rendimiento en la cocción, debido a la capacidad de fijación del agua y aceite. Sin embargo, se produjeron cambios en el color y en la textura de las hamburguesas por la adición de estos subproductos, siendo menos grasas, menos jugosas y con menor sabor a carne que las muestras control (sin adición de subproductos). Los cambios de color no se apreciaron después del proceso de cocción (Sánchez-Zapata et al., 2010).

Otros autores, evaluaron el efecto de los subproductos de la chufa como fuente de ácidos grasos insaturados (AGI) en la *Longaniza de Pascua* (Sánchez-Zapata, Díaz-Vela, Pérez-Chabela, Pérez-Alvarez, & Fernández-López, 2013). Para ello, añadieron diferentes combinaciones de subproductos de "horchata" (1-2%) junto con aceite de nuez (2.5-5%) durante el proceso de fabricación de las longanizas. Los autores observaron el valor más alto de humedad en las *Longanizas de Pascua* con aceite de nuez, sin embargo, cuando se añadió aceite de nuez + subproductos de la "horchata" disminuyó la humedad.

La adición de fibra dietética en los productos cárnicos aumentó la capacidad de retención de agua mientras que el aceite de nuez impidió la pérdida de agua debido a una película impermeable entre la masa de carne y la piel de la *Longaniza de Pascua*. Se observaron que las *Longanizas de Pascua* con subproductos de la "horchata" (con o sin aceite de nuez) mostraron valores de pH más bajos que las muestras control. Respecto al color de las longanizas, cuando ambos ingredientes se

añadieron a la vez (subproductos y aceite de nuez) aumentaron el enrojecimiento y el tono amarillo de las mismas. Se redujo la oxidación de los lípidos probablemente por los compuestos antioxidantes presentes en los subproductos de la "horchata", como son los polifenoles. Por tanto, la utilización de subproductos de la "horchata" puede ser de utilidad en la industria cárnica como fuente de fibra y el aceite de nuez como fuente de ácidos grasos (Sánchez-Zapata, Díaz-Vela, et al., 2013).

Otros investigadores Aguilar, Albanell, Miñarro, Guamis, & Capellas (2015) estudiaron el efecto de los subproductos de la chufa en la elaboración de pan sin gluten. Estos productos libres de gluten muestran una mayor demanda por parte de los consumidores debido al creciente diagnóstico de enfermedad celíaca entre la población. Los autores obtuvieron migas de pan y cortezas más oscuras debido a los aminoácidos y los azúcares que contienen los subproductos de la "horchata", lo que favoreció la reacción de Maillard. Además, se redujo el volumen específico del pan.

Por otro lado, Sánchez-Zapata, Fernández-López, et al. (2013) estudiaron el impacto de los subproductos líquidos de la chufa como fuente de carbono para el crecimiento de bacterias probióticas (*Lactobacillus acidophilus y Bifidobacterium animalis*). Estos subproductos líquidos contienen aproximadamente un 94.4% de agua y un 5.6% de carbohidratos, proteínas, grasas y cenizas. Los resultados indicaron el potencial de estos subproductos de la chufa como fuente de carbono y su posible utilidad en la industria alimentaria como producto fermentable, aunque se necesitan más estudios para determinar la dosis y compatibilidad con otros probióticos.

1.2.2. Extracción de compuestos de interés a partir de los subproductos obtenidos de la elaboración de la "horchata"

1.2.2.1. Técnicas de extracción convencional

Se encuentran en la bibliografía varios tipos de extracción convencional para la extracción de compuestos hidrosolubles y liposolubles en los subproductos obtenidos tras la elaboración de la "horchata" como la extracción mecánica para la obtención de aceites de forma industrial, pero las más empleadas son las extracciones sólido-líquido o líquido-líquido con disolventes orgánicos.

La extracción de compuestos hidrosolubles tales como algunos polifenoles se suele llevar a cabo con mezclas de etanol/agua y en el caso de tocoferoles, carotenoides, fitosteroles, mediante la extracción con Soxhlet y disolventes tipo hexano, éter dietílico, etc.

La extracción en Soxhlet se basa en realizar extracciones continuas de manera automática con el mismo disolvente que se evapora y condensa. Consiste en la utilización de una cantidad apropiada de disolvente en un balón, tal y como se muestra en la Figura nº 11, de manera que antes de vaciar el sifón por primera vez el balón no se quede sin disolvente. Este balón se encuentra encima de una placa calefactora que se pondrá a la temperatura de ebullición del disolvente. La muestra se introduce en un cartucho, que suele ser de celulosa (más económico pero menos duradero) o de porcelana porosa, el cual es un recipiente cilíndrico con base semiesférica para que se apoye en la base el extractor y sea más resistente. Una vez cargada la muestra se coloca un tapón, que normalmente consiste en una torunda de algodón para que ésta no flote, ni pueda salirse del cartucho.

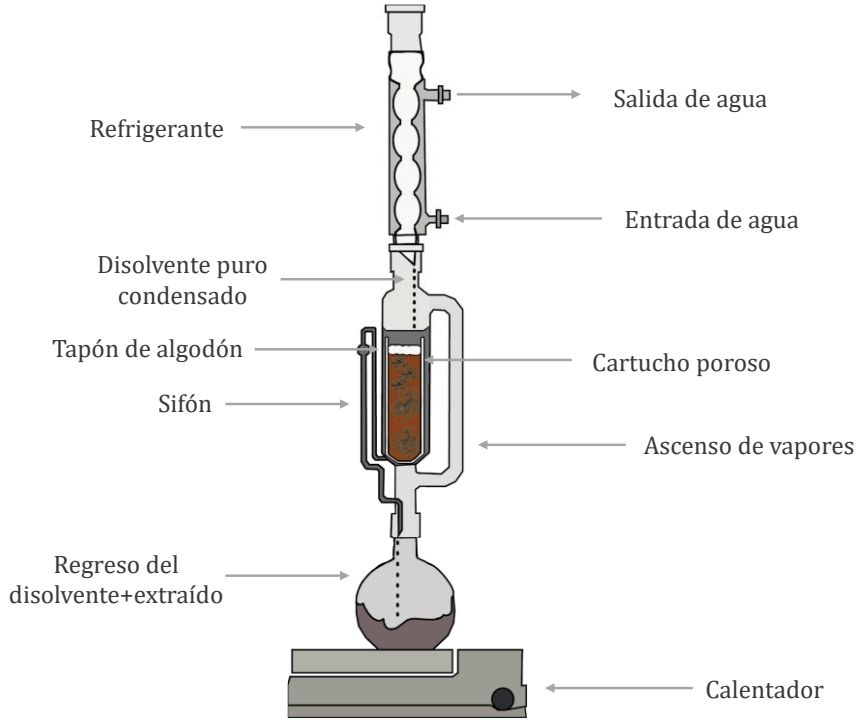

Figura nº 11. Representación esquemática de una extracción sólido-líquido con Soxhlet.

Las extracciones se llevan a cabo con disolventes como hexano, acetona o metanol, que suponen un riesgo para la salud humana ya que son tóxicos (Puértolas & Barba, 2016). En general el número de artículos publicados respecto a la extracción de aceite y compuestos lipófilos a partir de subproductos de la chufa es escaso.

El prensado en frío es la técnica más ampliamente utilizada para la producción industrial de aceite de chufa (Koubaa, Barba, et al., 2015; Lasekan & Abdulkarim, 2012). Varias investigaciones a nivel de laboratorio han demostrado que los lípidos de la chufa se pueden extraer usando los disolventes orgánicos tales como n-hexano, éter de petróleo, etc., con un rendimiento substancial (Ekpe, Igile, Williams, & Eworo, 2016).

En tales casos, las muestras se homogeneizan y se agitan inicialmente con el disolvente seleccionado y después se recupera la fracción lipídica. También se puede utilizar la extracción de Soxhlet. Por ejemplo, un estudio demostró que es posible obtener hasta un 15.9% (w/w) de rendimiento de aceite utilizando una mezcla de disolventes de n-hexano/2-propanol (3:1, v/v) en 6 h mediante un aparato Soxhlet (Yeboah et al., 2012). Recientemente, Ekpe et al. (2016) mostraron que el n-hexano ofrece una eficiencia de extracción superior en comparación con el éter de petróleo. Sin embargo, el uso de hexano se ha cuestionado debido a problemas ambientales y seguridad ocupacional (Lasekan & Abdulkarim, 2012).

1.2.2.2. Técnicas de extracción no convencional

Hoy en día existe una demanda de nuevas técnicas de extracción que reduzcan el consumo de disolventes orgánicos, utilizando tiempos de extracción más cortos y por tanto menor energía y más sostenibles desde el punto de vista medioambiental, ayudando así a reducir la huella de carbono. A este tipo de extracción se le conoce como "extracción verde", ya que trata de proteger el medioambiente y también la salud del consumidor. Algunas de estas tecnologías son los ultrasonidos, pulsos eléctricos (PE), expresión mecánica asistida por gas (GAME), microondas (MAE), fluidos supercríticos (SC-CO_2), entre otros (Farid Chemat, Vian, & Cravotto, 2012).

1.2.2.2.1. Electrotecnologías

La tecnología de los pulsos eléctricos (PE) consiste básicamente en someter el material objeto de estudio a la aplicación intermitente (<300 Hz) de campos eléctricos de moderada-alta intensidad (0.1-20 kV/cm) y corta duración (muy variable, desde pocos μs hasta varios ms). Estos campos eléctricos producen un fenómeno denominado electroporación o

electropermeabilización, consistente en la formación de poros reversible o irreversibles en las membranas celulares tanto eucariotas como procariotas (Teissie, Golzio, & Rols, 2005).

La electroporación irreversible permite mejorar los procesos de extracción (Puértolas et al., 2012). Mientras que en materiales o tejidos vegetales blandos, como el mesocarpio o el pericarpio de la mayoría de las frutas, intensidades de campo eléctrico de entre 0.1 y 10 kV/cm son suficientes para obtener buenos resultados, en materiales duros como las semillas, en los que hay lignificación, es necesaria la aplicación de campos eléctricos más elevados (10-20 kV/cm) (Boussetta et al., 2012; Boussetta, Soichi, Lanoiselle, & Vorobiev, 2014; Sarkis, Boussetta, Tessaro, Marczak, & Vorobiev, 2015).

La eficacia de los PE para mejorar los procesos de extracción no solo depende de los parámetros eléctricos del proceso, sino también de las variables de extracción (temperatura, tipo y concentración del disolvente), de la naturaleza del compuesto a extraer, de su tamaño y de su localización en la célula (vacuolas, citoplasma) (Barba, Grimi, & Vorobiev, 2014; Deng et al., 2015a; Soliva-Fortuny, Balasa, Knorr, & Martín-Belloso, 2009). Además, el efecto de los PE también depende de las características fisicoquímicas de la matriz tratada (pH, conductividad eléctrica) y de las características de los tejidos y de las células que componen la matriz (tamaño, forma, tipo de membrana) (Puertolas et al., 2012; Vorobiev & Lebovka, 2011; Vorobiev & Lebovka, 2008).

La aplicación de descargas eléctricas de alto voltaje (DEAV) se basa en el proceso fisicoquímico de descomposición dieléctrica o arco eléctrico que se produce al entrar en contacto las descargas eléctricas con el agua (Barba, Boussetta, & Vorobiev, 2015; Boussetta et al., 2013). Este proceso es el resultado de la ionización del líquido tras aplicar un pulso de alto voltaje (40 kV) e intensidad (alrededor de 10 kA) de corta duración (µs-ms) entre dos electrodos. El mecanismo por el cual se producen las DEAV se puede resumir en 3 fases: i) generación del pulso eléctrico, ii) descarga

del mismo, iii) formación del arco eléctrico.

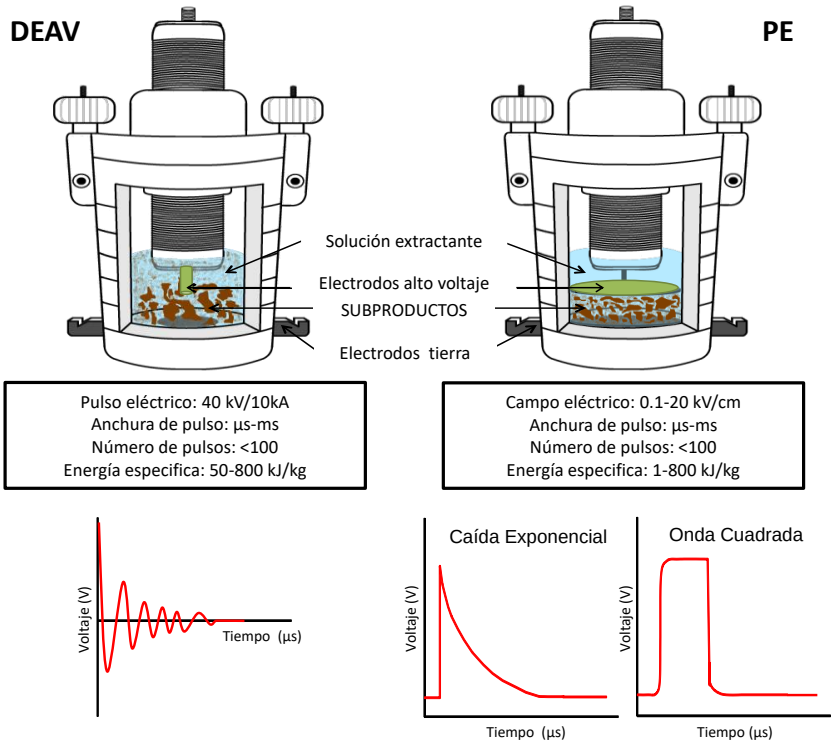

Figura nº 12. Ejemplo de cámaras de procesado y principales parámetros de tratamiento en la tecnología de descargas eléctricas de alto voltaje (DEAV; izquierda) y en la tecnología de pulsos eléctricos (PE; derecha). Adaptado de Roselló-Soto et al. (2015).

Las burbujas que inicialmente están presentes en el agua o que se forman debido al calentamiento localizado que se produce durante este fenómeno, aceleran el proceso. Si el pulso eléctrico es suficientemente intenso, la avalancha de electrones que tiene lugar se convierte en el punto de inicio para propagar la corriente desde el electrodo de aguja hasta el electrodo de tierra (normalmente de geometría plana).

Además del daño eléctrico producido durante este proceso, se producen diferentes fenómenos secundarios (ondas de presión de alta amplitud, cavitación, creación de turbulencia en el líquido, producción de especies reactivas, etc.) con la consiguiente liberación de energía, lo cual provoca la fragmentación de los tejidos celulares (Boussetta & Vorobiev 2014; Koubaa et al. 2015).

Las extracciones asistidas bien por PE o DEAV son métodos prometedores para la recuperación de compuestos valiosos a partir de desechos y subproductos de alimentos (Deng et al., 2015b; Galanakis, 2012; Koubaa, Roselló-Soto, et al., 2015). Sin embargo, hasta la fecha, estas tecnologías no se han aplicado para valorizar subproductos de la "horchata".

1.2.2.2.2. Ultrasonidos

La extracción asistida por ultrasonidos se utiliza cada vez más. Este tratamiento se basa fundamentalmente en la mejora de transferencia de calor y masa a través de las paredes celulares de la planta debido al efecto cavitacional ejercido por este tipo de tratamiento, el cual facilita la disrupción del tejido celular.

Es posible utilizar la extracción asistida por ultrasonidos como un proceso independiente o como parte de un procedimiento gradual para la extracción de los compuestos deseados. El tratamiento por ultrasonidos ofrece como principales ventajas respecto al tratamiento convencional con disolventes, una mayor penetración del disolvente en el material celular, menor tiempo de procesamiento y residencia, mayores rendimientos y reproducibilidad en cuanto a los compuestos obtenidos. Además permite disminuir el consumo de disolventes y emulsionantes, y presenta un alto rendimiento de procesamiento, es útil para la extracción de componentes lábiles al calor, permite ahorros significativos en el mantenimiento, y necesita menos energía para el procesamiento.

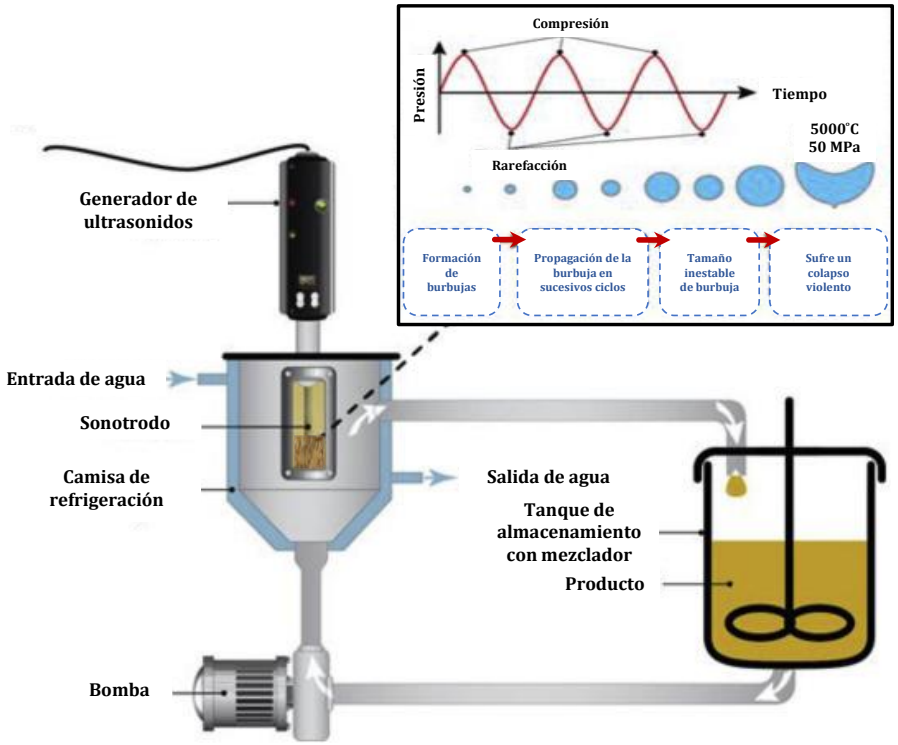

Figura nº 13. Representación de una extracción asistida por Ultrasonidos con explicación del efecto que se genera. Adaptada de Barba et al. (2017) .

Finalmente debido a los hechos anteriormente mencionados, puede considerarse una técnica de extracción más económica (Roselló-Soto et al., 2015). Además, la extracción por ultrasonidos puede integrarse fácilmente con dispositivos ya existentes como parte de la planta tecnológica o, cuando sea necesario, configurarse como una nueva línea de producción (Patist & Bates, 2008). Por lo tanto, esta tecnología puede implementarse fácilmente para mejorar la eficiencia de extracción de polifenoles, flavonoides, flavonoles, azúcares, minerales y carotenoides en diferentes muestras de origen vegetal.

1.2.2.2.3. Microondas

La extracción con microondas es otro método valorización de residuos y subproductos de la industria agroalimentaria y se basa en el impacto directo sobre los compuestos polares. La energía electromagnética, en el intervalo de frecuencia de 300 MHz-300 GHz, se transfiere en forma de calor tras la conducción iónica y rotación del dipolo inducida por el tratamiento (Jain, Jain, Pandey, Vyas, & Shukla, 2009). Se supone que el proceso de extracción por microondas involucra tres pasos secuenciales (Alupului, Calinescu, & Lavric, 2012): i) separación de los solutos de los sitios activos de la matriz de la muestra bajo temperatura y presión incrementadas, ii) difusión del disolvente a través de la matriz de la muestra, y iii) liberación de los solutos de la matriz de la muestra al disolvente.

Debido a las ventajas tales como el calentamiento más rápido para la extracción de sustancias bioactivas de los materiales vegetales, la reducción de los gradientes térmicos, la reducción del tamaño del equipo y el aumento del rendimiento del extracto (Chemat & Cravotto, 2013; Cravotto et al., 2008), el tratamiento con microondas se está realizando de manera habitual para diferentes procesos y esta siendo ampliamente investigado para la valorización de materiales y subproductos vegetales.

La viabilidad de utilizar el proceso de extracción por microondas a escala industrial es de un gran interés de cara a la extracción ecológica de compuestos valiosos de matrices de plantas y subproductos industriales (Díaz-Ortiz et al., 2007; James Mason, Chemat, & Vinatoru, 2011; Li, Radoiu, Fabiano-Tixier, & Chemat, 2012; Tatke & Jaiswal, 2011). Por ejemplo, diferentes autores han propuesto la industrialización de la extracción de aceites esenciales de hierbas aromáticas con microondas (sin disolventes) (Filly et al., 2014) así como la separación de compuestos orgánicos volátiles y no volátiles de las hojas de boldo (Petigny et al., 2014).

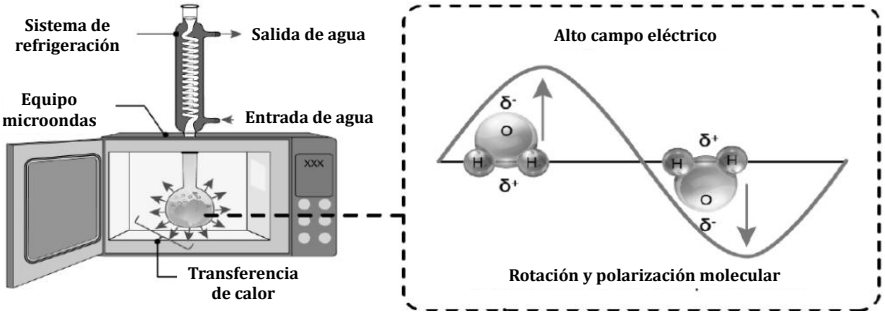

Figura nº 14. Representación de una extracción asistida por Microondas con explicación del efecto que se genera. Adaptada de Barba et al., (2017).

1.2.2.2.4. Extracción con ayuda de alta presión y enzimas

Las enzimas se han convertido en una herramienta popular para la extracción ya que mejoran significativamente el rendimiento de la misma hidrolizando la pared celular y, consecuentemente, ayudando a la extracción de compuestos con un valor añadido (Puri, Sharma, & Barrow, 2012; Roselló-Soto et al., 2016). Para este fin se podrían utilizar varias enzimas celulolíticas o proteolíticas disponibles en el mercado, basándose en el tipo de matriz (Poojary, Orlien, Passamonti, & Olsen, 2017).

Aunque no existen estudios previos evaluando el impacto de este tipo de extracción para valorizar subproductos de la "horchata", algunas investigaciones han estudiado impacto de la extracción asistida por enzimas para la recuperación de aceite de chufa rico en compuestos bioactivos (Ezeh, Niranjan, et al., 2016). Los autores realizaron la extracción enzimática acuosa usando diferentes enzimas celulolíticas tales como alcalasa, α-amilasa Viscozyme® L y Celluclast®, mientras que en otro conjunto de experimentos la extracción asistida por enzimas se combinó con procesos de alta presión. A continuación se muestra una representación esquemática del proceso (Figura nº 15).

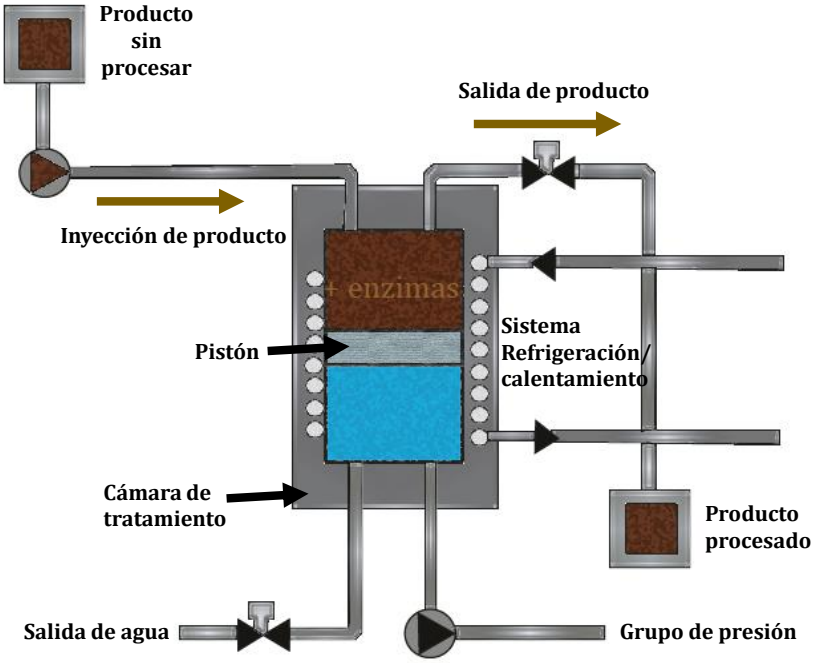

Figura nº 15. Representación esquemática del proceso de alta presión combinado con enzimas para la extracción de compuestos bioactivos.

Los resultados revelaron que la extracción asistida por enzimas permitió extraer cantidades comparables de ácidos grasos, polifenoles y tocoferoles a la obtenida mediante el tratamiento de extracción control por presión, mientras que el tratamiento de alta presión previo a la extracción enzimática mejoró significativamente el contenido de tocoferoles y polifenoles en los aceites. Además, los autores demostraron que los subproductos del proceso de extracción del aceite son una fuente de azúcares conteniendo los extractos metanólicos obtenidos a partir de los residuos utilizados glucosa, sacarosa y ciertos oligosacáridos (Ezeh, Niranjan, et al., 2016). En un estudio similar, los autores encontraron hasta un 90% de recuperación de aceite cuando utilizaron el tratamiento enzimático antes de la extracción mecánica (Ezeh, Gordon, & Niranjan, 2016).

1.2.2.2.5. Fluidos comprimidos

El fluido más utilizado para los procesos de extracción supercrítica es el CO_2. Tiene una temperatura crítica baja (31.1 °C), no tiene toxicidad y es seguro respecto a su uso. Además de estas características, los procesos de extracción que utilizan CO_2 supercrítico se producen en ausencia de luz y aire, minimizando así las reacciones de degradación. Sin embargo, debido a su carácter no polar, el CO_2 no se usa preferentemente solo para extraer polifenoles polares, sino que se mezcla con codisolventes orgánicos (por ejemplo, etanol, metanol, acetona) también llamados modificadores.

Estos disolventes aumentan el poder de solvatación del CO_2 y, por lo tanto, la solubilidad y capacidad de extracción de los polifenoles. Diferentes autores han evaluado la aplicación de la extracción con fluidos supercríticos en la recuperación de compuestos valiosos de diferentes matrices vegetales y han analizado el mecanismo de acción de estas técnicas así como las ecuaciones que rigen el fenómeno de transferencia de masa (Koubaa, Roselló-Soto, et al., 2015; Roselló-Soto et al., 2016).

El etanol es el disolvente más utilizado para reemplazar los convencionales, ya que está permitido en la industria alimentaria, es menos consumido que otros disolventes utilizados tradicionalmente y es fácil de eliminar del extracto por evaporación a temperatura ambiente.

La temperatura crítica de una mezcla de CO_2 y co-disolvente es más alta que la de los disolventes separados, lo que resulta en un menor uso de co-disolvente en la extracción (Adil, Çetin, Yener, & Bayındırlı, 2007).

No se ha encontrado ningún estudio en la literatura disponible evaluando el impacto de los fluidos supercríticos o expresión mecánica asistida por gas para valorizar los subproductos de la "horchata". Si que existentes disponibles algunas investigaciones evaluando el potencial de la extracción de dióxido de carbono en estado supercrítico (SC-CO_2) (20–40 MPa, 40 °C–80 °C, 60–360 min) para recuperar el aceite de chufa

(Lasekan & Abdulkarim, 2012).

Los autores observaron una mayor extracción de aceite al aumentar la presión del SC-CO_2 y el tiempo de tratamiento. Además, también obtuvieron que la composición de los ácidos grasos variaba según las condiciones de extracción.

Para evitar largos tiempos de extracción, es posible utilizar otra tecnología consistente en el uso de la expresión mecánica (EM) asistida por fluidos supercríticos, la cual podría constituir una alternativa interesante para mejorar la recuperación de aceites, compuestos bioactivos, nutrientes, etc. (Koubaa, Barba, et al., 2015). Esta técnica se ha aplicado previamente para extraer aceite de diferentes matrices de origen vegetal (Müller & Eggers, 2014; Venter, Willems, Kuipers, & Haan, 2006; Venter, 2006; Willems & de Haan, 2011; Willems, Kuipers, & de Haan, 2008). Como podemos ver en la Figura nº 16, en el caso de las chufas, Koubaa, Barba, et al. (2015) evaluaron la eficacia de este proceso para la extracción de aceite y polifenoles y los resultados se compararon con la aplicación independiente de SC-CO_2 y EM, obteniendo una reducción significativa en el tiempo de extracción tras utilizar la combinación de SC-CO_2+EM.

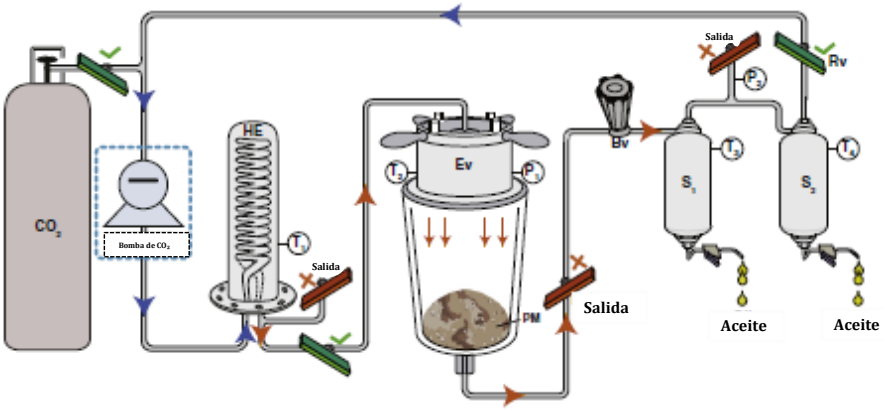

Figura nº 16. Representación esquemática del proceso de SC-CO_2 para la extracción de aceite de chufa. Donde (Bv) es la válvula de contrapresión, (HE) sistema de refrigeración, (P_1 y P_2) son las presiones, (S_1 y S_2) son los separadores, (Rv) es la válvula de recirculación del CO_2, (PM) es la muestra, (Ev) es el equipo de presión y (T_1, T_2 y T_3) son las diferentes temperaturas. Adaptado de Koubaa et al. (2015).

Además, esta técnica permitió la recuperación de una mayor cantidad de compuestos fenólicos en comparación con los procesos de SC-CO_2 y EM cuando se aplicaron de forma independiente.

1.2.2.3. Ventajas y limitaciones de las técnicas innovadoras de extracción

En el Cuadro nº 5 se resumen algunas de las principales ventajas y desventajas respecto al uso de tecnologías innovadoras como ultrasonidos, y SC-CO_2 en comparación con las técnicas de extracción convencionales. Como se muestra, la utilización de la extracción con fluidos supercríticos o ultrasonidos para mejorar la extracción evita o minimiza el uso de disolventes tóxicos y contribuye a ahorrar la energía necesaria para evaporar el disolvente adicional utilizado en la extracción convencional. Además, los disolventes más utilizados en las técnicas de

extracción asistida por ultrasonidos son el etanol y el agua, mientras que el CO_2 en estado supercrítico es el disolvente más utilizado en el caso de la extracción con fluidos supercríticos (SC-CO_2).

Por el contrario, en extracciones convencionales se usan generalmente disolventes orgánicos tóxicos como por ejemplo, hexano, dietil éter, éter de petróleo, metanol, etc. Además, el uso de ultrasonidos y SC-CO_2 bajo las condiciones óptimas puede permitir extraer los compuestos deseados de forma relativamente selectiva. El inconveniente más importante del uso de los ultrasonidos y fluidos supercríticos es el costo de inversión en equipos, que siguen siendo mucho más altos que los utilizados en la extracción convencional.

Cuadro nº 5. Comparativa entre las principales ventajas (+) y desventajas (-) del uso de ultrasonidos, extracción de fluidos supercríticos (SC-CO_2) y técnicas de extracción convencionales.

	Ultrasonidos	**SC-CO_2**	**Extracción convencional**
Uso de disolventes tóxicos	+++	+++	++-
Uso de disolventes "verdes"	+++	+++	+--
Reduce la cantidad de disolvente	+++	+++	---
Reduce el efecto del calentamiento	++-	+++	+--
Reducción del tiempo de extracción	+++	+++	+--
Extracción selectiva	++-	++-	+--
Coste del equipo	---	---	+++
Previene la degradación de compuestos termolábiles	++-	+++	+--
Mejora de la extracción	+++	+++	++-

2. OBJETIVOS

2. OBJETIVOS

El **objetivo general** de la presente investigación es evaluar el impacto de diferentes técnicas de extracción convencional e innovadoras para obtener aceite, nutrientes y compuestos antioxidantes bioactivos a partir de los subproductos de la "horchata" y así evitar su eliminación como residuo.

Para alcanzar este objetivo general se plantean los siguientes **objetivos específicos:**

- Determinar los compuestos bioactivos y caracterizar nutricional y fisicoquímicamente los subproductos de la "horchata".
- Estudiar y optimizar la extracción de aceites a partir de subproductos de la "horchata" mediante procesos convencionales de extracción y tecnologías innovadoras como fluidos supercríticos.
- Extraer los compuestos antioxidantes de los subproductos de la "horchata" e identificarlos y cuantificarlos mediante técnicas espectrofotométricas y cromatográficas. Evaluar la influencia de los tratamientos convencionales y los fluidos supercríticos en la extracción de dichos compuestos.
- Caracterizar la composición de los aceites (ácidos grasos y compuestos fenólicos), así como sus propiedades físico-químicas (resistencia a la oxidación e índice de peróxidos) y biológica (capacidad antioxidante).

3. PLAN DE TRABAJO

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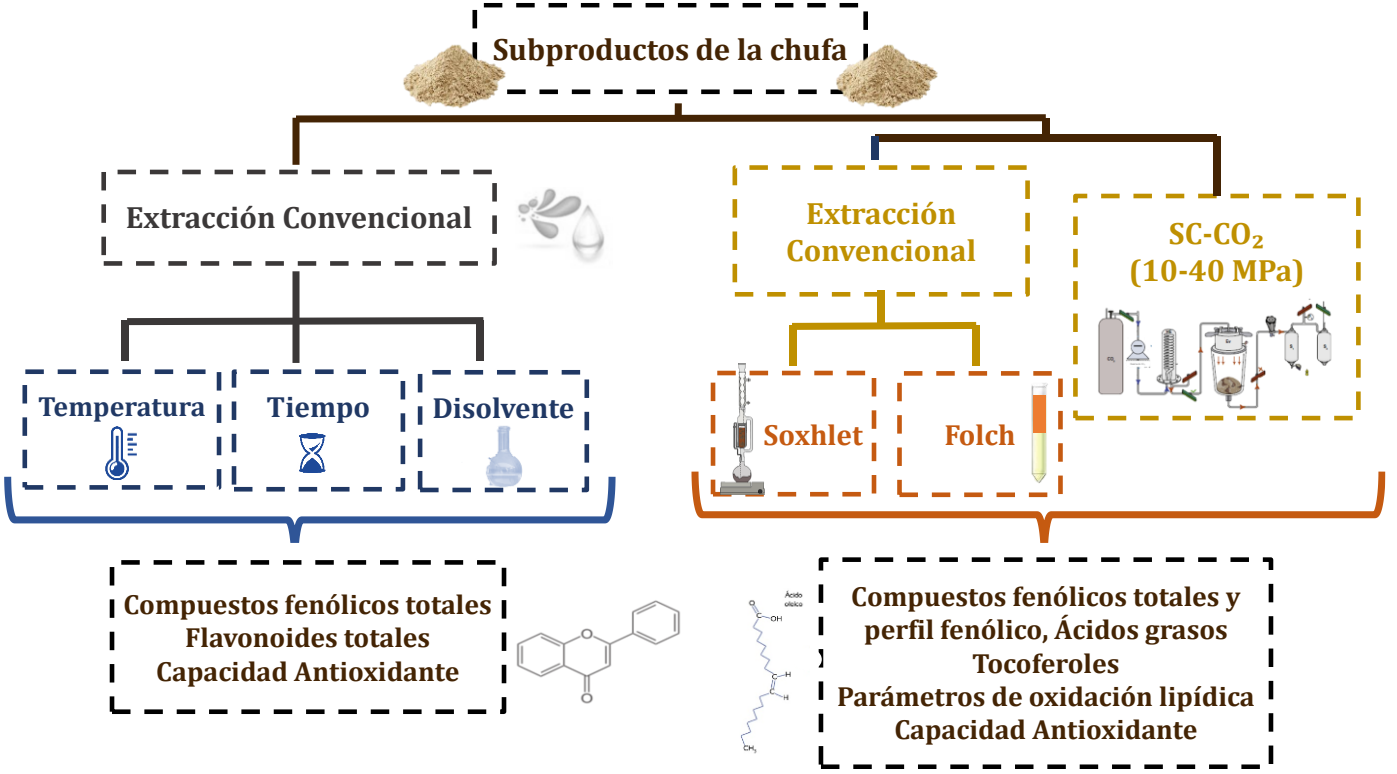
Para la consecución de los objetivos del estudio se propone el siguiente **plan de trabajo**:

1. Revisión de los estudios evaluando el proceso de elaboración de la "horchata" así como los diferentes subproductos obtenidos durante el proceso.
2. Caracterización nutricional, fisicoquímica y determinación de los compuestos antioxidantes bioactivos presentes en los subproductos de la "horchata".
3. Evaluación del impacto de los procesos convencionales (temperatura, tiempo y diferentes mezclas hidroetanólicas) en la extracción de compuestos antioxidantes bioactivos a partir de subproductos de la "horchata".
4. Estudio del efecto de los fluidos supercríticos y procesos convencionales como extracción mediante Soxhlet y método de Folch en la extracción de aceites de los subproductos de la "horchata".
5. Caracterización de los ácidos grasos, tocoferoles, compuestos antioxidantes y parámetros de oxidación lipídica de los aceites obtenidos.
6. Determinación del perfil fenólicos de los aceites mediante el uso de cromatografía líquida-TOF-masa-masa.
7. Evaluación, difusión de los resultados obtenidos y redacción de la Tesis doctoral.

4. DISEÑO

4. DISEÑO


Subproductos de la chufa
Extracción Convencional
Temperatura
Tiempo
Disolvente
Compuestos fenólicos totales
Flavonoides totales
Capacidad Antioxidante
Extracción Convencional
Soxhlet
Folch
SC-CO2 (10-40 MPa)
Compuestos fenólicos totales y perfil fenólico, Ácidos grasos
Tocoferoles
Parámetros de oxidación lipídica
Capacidad Antioxidante

5. PARTE EXPERIMENTAL Y RESULTADOS

5. PARTE EXPERIMENTAL Y RESULTADOS

5.1. Thermal and non-thermal preservation techniques of tiger nuts' beverage "horchata de chufa". Implications for food safety, nutritional and quality properties

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Review

Thermal and non-thermal preservation techniques of tiger nuts' beverage "horchata de chufa". Implications for food safety, nutritional and quality properties

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Keywords: Tiger nuts, "Horchata", Pasteurization, Pulsed electric field, Ultraviolet, High hydrostatic pressure

ABSTRACT

"Horchata de chufa" is a traditional Spanish beverage produced from tiger nuts (*Cyperus esculentus* L.). Due to its richness in nutritional compounds,

it is highly perishable and its conservation by pasteurization and/or adding preservatives is required. Although efficient, conventional thermal treatment for pasteurization induces changes in the nutritional and sensory properties. Replacing conventional pasteurization by non-thermal technologies such as pulsed electric fields, ultraviolet, and high pressure, combined with moderate temperatures (< 40 °C) allows a reduction of energy consumption, along with the preservation of the most thermo-sensitive molecules. Accordingly, this review deals with the description of the most relevant non-thermal technologies applied to preserve "horchata" beverage in order to extend the shelf life and inactivate pathogenic microorganisms as well as to preserve the nutritional and quality properties of this food beverage.

1. Introduction

"Horchata de chufa" or more commonly known as "horchata" is a traditional Spanish beverage produced from tiger nut tubers (*Cyperus esculentus* L.). It is a sweetly milky drink, often consumed during summer, owing to its refreshing properties. This non-alcoholic beverage is mainly produced in the Valencian region of Spain where well-developed "horchata" industries have been established. The beverage is sold not only in Spain but also in Mexico, Panama, Dominican Republic, USA, and South France (Martín-Esparza & González-Martínez, 2016).

Nowadays, two varieties of tiger nuts (yellow and brown) are available in the market. A bigger size, attractive color and fleshier body characterize the yellow variety. Moreover, this variety presents more "milk" upon extraction, containing lower fatty and anti-nutritional compounds, and excess proteins (Okafor, Mordi, Ozumba, Solomon, & Olatunji, 2003) when compared to the brown variety.

The major portion of tiger nuts produced in Valencia are typified, and commercialized as "Chufa de Valencia" (CRDO, 2017), ensuring the origin, consistency and optimum quality (Martín-Esparza & González-Martínez,

2016). The annual value of tiger nuts' (total production of about 8 million kilos with≈70 cents per kilo) is estimated to be around 5.6 million euros.

Moreover, tiger nuts beverage is commercialized and received a great economic importance, particularly in Spanish food industry (Rubert, Sebastià, Soriano, Soler, & Mañes, 2011). For instance, consumer demand for "horchata" is growing remarkably (CRDO, 2017) and the total annual consumption of "horchata" in Spain itself excedes 50 million L (Martín-Esparza & González-Martínez, 2016), signifying a retail market value of about 60 million euros (CRDO, 2017).

"Horchata" has also gained consumers attention due to its high nutritional quality and organoleptic characteristics. It is rich in carbohydrates (12.2 g/100 mL), mainly sucrose (9–10 g/100 mL) and starch (2–3 g/100 mL) and presents moderate fat (2.4–3.1%) and protein (0.6–1.4%) contents (Sánchez-Zapata, Fernández-López, & Angel Pérez-Alvarez, 2012).

In addition, "horchata" presents high amount of oleic (75% of total fat) and linoleic (9% to 10% of total fat) acids. It contains arginine as main amino acid, followed by glutamic and aspartic acids. The amount of essential amino acids (except of histidine) in "horchata de chufa" is higher than that in the proposed model protein systems recommended for adults by the Food and Agriculture Organization (Cortés, Esteve, Frígola, & Torregrosa, 2005). Regarding mineral composition, "horchata" includes potassium, phosphorous, magnesium, and calcium, whereas other essential elements such as iron and zinc are found in minor quantities (Martín-Esparza & González-Martínez, 2016; Sánchez-Zapata et al., 2012).

"Horchata" has been reported to be a healthy drink, as its consumption is linked with the prevention of heart attacks and thrombosis, blood circulation promotion (Chukwuma, Obioma, & Christophe, 2010; Sánchez-Zapata et al., 2012), and decreased colon cancer risk (Adejuyitan, Otunola, Akande, Bolarinwa, & Oladokun, 2009).

Additionally, Alegria-Toran and Farré-Rovira (2003) reported beneficial health properties of "horchata" due to its high content in low-glycemic carbohydrates (mainly starch), and arginine content that stimulates insulin secretion. However, it is necessary to take into account the high sucrose content (minimum 10%, w/v) of "horchata de chufa natural" established by legislation (BOE, 1988).

Traditionally, "horchata" is prepared by grinding water-soaked tiger nuts in water followed by milky juice extraction through sieving (Fig. 1). However, many small- and large-scale industries use automated mills and extractors. Although, it is consumed in households, the industrial scale production requires storage, and downstream processing. Moreover, the implementation of strict legislations for food quality and safety across the globe ensures quality control of beverages before marketing. The processing of tiger nuts-based beverages is critical as raw materials may contain harmful microorganisms, anti-nutrients as well as biological and chemical toxins. Several investigations have revealed that tiger nuts/tubers contain a number of bacteria including Enterobacter aerogenes (Selma, Fernández, Valero, & Salmerón, 2003), Enterobacteriaceae, Shigella and *Escherichia coli* (Sebastià, El-Shenawy, Mañes, & Soriano, 2012), *Listeria monocytogenes* (Selma, Salmerón, Valero, & Fernández, 2006) and fungi including *Fusarium sporotrichioides* and *Fusarium moniliforme* (Mateo & Jiménez, 2000), as well as *Dematophora necatrix* (García-Jiménez, Busto, Vicent, & Armengol, 2004). Moreover, some of these microorganisms are also able to produce toxins such as Fusarium spp. and able to produce mycotoxins including trichothecenes and fumonisins (Mateo & Jiménez, 2000), enniatins and beauvericin (Sebastià et al., 2012). In addition, "horchata" may also contain aflatoxins and ochratoxin A produced by *Aspergillus spp.* (Arranz, Stroka, & Neugebauer, 2006; Rubert et al., 2011; Sebastià, Soler, Soriano, & Mañes, 2010). In this regard, the beverage contains sugar, amino acids, minerals and other nutrients that make it ideal media for microbial

propagation. Consequently, applying certain preservation techniques is crucial for commercial production to avoid microbial contamination.

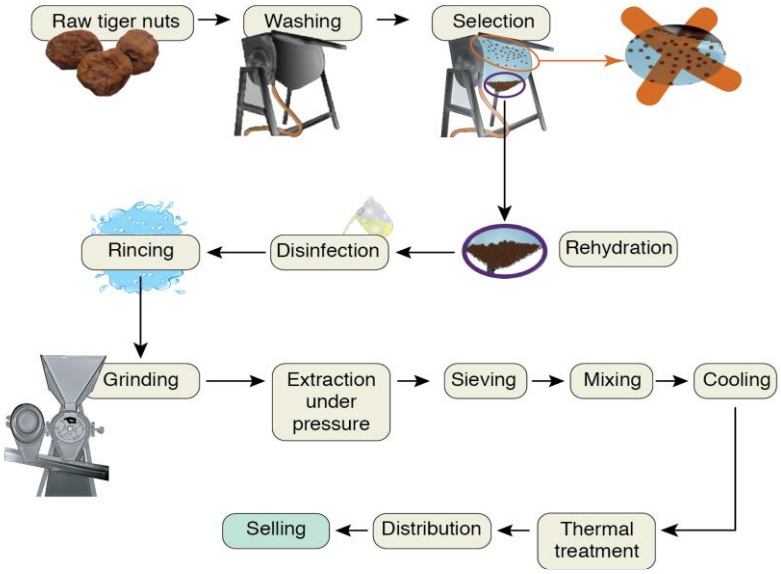

Fig. 1. Schematic representation of the most common process used for the preparation of "horchata" from tiger nuts.

Although, few conventional techniques (e.g. cold storage under refrigeration (0–2 °C) and freezing (≤−18 °C); thermal pasteurization/sterilization and water activity reduction, among others) have been followed for "horchata" preservation, these methodologies present several inherent limitations (Codina-Torrella, 2014). Nowadays, alternatively, several non-conventional processing/preservation techniques are being evaluated for their potential to inactivate spoilage as well as pathogenic microorganisms and retain/improve the quality of "horchata". In this regard, the present review article provides an overview of non-conventional food processing techniques used in "horchata" production and their impact on its food safety, nutritional and quality properties.

2. "Horchata" preservation

2.1. Conventional thermal preservation technologies

Conventional "horchata" processing includes heat treatment or pasteurization. The raw materials, tubers, can be treated by washing with disinfectants (BOE, 1988) or by hot water treatments. Although pasteurization can reduce pathogenic bacteria, spores may remain intact and the nutrients and neutral pH of "horchata" eventually enables spore germination. Consequently, refrigeration is critical during storage, however, low temperature storage for long-term may lead tophase s eparation (Codina, Trujillo, & Ferragut, 2016).

It is well documented that the thermal treatment of beverages (liquid foods) negatively affects the content and concentrations of nutrients, depending on the processing intensity (Barba, Esteve, & Frígola, 2012). Since "horchata" contains significant amounts of starch (≈3%), heating at elevated temperature (> 72 °C) may cause gelatinization and adversely affect the sensory properties (Codina-Torrella, Guamis, Ferragut, & Trujillo, 2017; Corrales, de Souza, Stahl, & Fernández, 2012).

Regarding the physicochemical properties, thermal treatment at 60 °C for 30 min did not affect the pH values, viscosity and total soluble solids of tiger nut milk. However, an increase in conductivity (from 3.2 ms/cm to 4.1 ms/cm) and a decrease in the protein content (from 5.53 to 3.6 mg/L) was noticed between the treated products and the non-treated ones (Elbrhami, 2016). In addition, this thermal treatment decreased vitamin C (from 8.22 mg/100 mL to 5.99 mg/100 mL) and phenolic contents (from 139.14 mg GAE/100 mL to 99.17 mg GAE/100 mL). Finally, the color parameters were also influenced by termal treatment, since tiger nut "milk" was less bright, and redder after thermal treatment.

On the other hand, ultra-high temperature (UHT) techniques have been found to reduce the microbial growth significantly (from 106 CFU/mL to 5× 104 CFU/mL (Selma, Valero, Fernández, & Salmerón,

2002)), however, they lead to lower sensory acceptance (Codina et al., 2016). Therefore, with these limitations and considering significant increase in the demand for "horchata", there is a need for the development of efficient, non-thermal and low-cost processing techniques.

2.2. Non-thermal (non-conventional) preservation technologies

The non-thermal preservation technologies are those in which temperature is not the main factor of inactivation of microorganisms and enzymes, although a slight increase in temperature may occur during the treatment (Barba, Koubaa, do Prado-Silva, Orlien, & de Souza Sant'Ana, 2017). As a result, quality degradative reactions that can be triggered after heat processing are reduced (Barba, Criado, Belda-Galbis, Esteve, & Rodrigo, 2014; Carbonell-Capella, Barba, Esteve, & Frígola, 2013). Some of the most promising emerging nonthermal technologies to extend "horchata" shelf life are pulsed electric fields (PEF) processing, ultraviolet (UV) irradiation, high-pressure homogenization (HPH) and high pressure processing (HPP). Table 1 shows the effect of these treatments on microbial inactivation in tiger nut milk. Some of the most relevant findings are described below.

Table 1. Microbial inactivation in tiger nuts´milk after non-thermal processing.

Technology	Processing conditions	Log_{10} CFU reductions	References
Pulsed Electric Fields			
	25 kV/cm, 300 µs, 150 pulses; 30 kV/cm, 50 µs, 25 pulses; 30 kV/cm, 190 µs, 95 pulses	1.1 log_{10} CFU reductions of *E. aerogenes* by any of the PEF conditions applied	Selma et al. (2003)
	30 kV/cm, 50 µs, 20 pulses; 30 kV/cm, 190 µs, 76 pulses	2.6 log_{10} CFU reductions on *E. coli*	Selma et al. (2004)
	30 kV/cm, 50 µs, 20 pulses; 30 kV/cm, 190 µs, 76 pulses	0.23-0.70 log_{10} reductions on *L. monocytogenes*	Selma et al. (2006)
Ultraviolet			
	UV-C treatment at 253.7 nm, dose range (0.14-4.23 J/cm^2). Estimated fluence rate 2.35 mW/cm^2	3 log_{10} CFU reduction of yeasts and molds, mesophilic flora and psychrotrophic bacteria	Corrales et al. (2012)
	0.107 mW/cm^2 UV incident intensity level, 5 cm distance from the collimated tube, 32 cm from the UV-C lamp	5 log_{10} CFU reductions of *E. coli* P36, *L. innocua* ATCC 51742 and *S. Typhimurium* WG49 after UV dose of 18.4 mJ/cm^2 for 5 cycles.	Elbrhami (2016)
High Pressure Homogenization (HPH)			
	Ultra-HPH (200-300 MPa/40 ºC)	2.37 and 3 log_{10} CFU reduction of mesophilic flora and psychrotrophic bacteria after 200 and 300 MPa ≈3 log_{10} CFU reduction yeasts and molds, *Lactobacillus*, and *Enterobacteriaceae*	Codina-Torrella (2014)
High Pressure Processing			
	400-600 MPa/11 ºC/60-180 s	5.11, 5.74 and 5.49 log10 CFU reduction, for *Listeria*, *E. coli* and *Salmonella*, respectively after 500 MPa for 180 s. 6.14, 7.08 and 6.6 log_{10} CFU reduction, for *Listeria*, *E. coli* and *Salmonella*, respectively after 600 MPa for 180 s	Elbrhami (2016)

2.2.1. Pulsed electric field processing (PEF)

PEF processing for microbial/enzyme inactivation is based on the application of high intensity electric fields (20–50 kV/cm) to the food matrix for a short duration of time (μs to ms) (Álvarez, Condón, & Raso, 2006; Buckow, Ng, & Toepfl, 2013; Poojary et al., 2016). During this process, PEF induces irreversible pores on the microbial cell membranes that may eventually inactivate the microorganisms (Koubaa et al., 2018; Raso et al., 2016; Roohinejad, Koubaa, Sant'Ana, & Greiner, 2018).

Besides, PEF can induce inactivation of undesirable enzymes by causing structural and conformational changes (Poojary et al., 2016). In a previous work, Selma et al. (2003) investigated the effectiveness of PEF to inactivate *E. aerogenes* from "horchata". Although these authors observed only 1.1 log10 CFU reduction of pathogens, PEF treatment significantly delayed the lag phase of bacterial growth. In addition, Selma, Salmerón, Valero, and Fernández (2004) reported a higher efficacy of similar treatments attaining up to 2.6 log10 CFU reductions on *E. coli*. These results concur with some comparative studies showing the detrimental effects of electric pulses, where Gram-positive bacteria were more tolerant than Gram-negative ones (Hülsheger, Potel, & Niemann, 1983). It should be noted that the most important factor to evaluate the potential of non-thermal processing as a preservation technology of "horchata" is its ability to achieve 5 log10 pathogen reduction, as regulated by the FDA (FDA, 2001). However, it should be noted that PEF treatments cause least damage to the quality aspects of the product. In this regard, Cortés et al. (2005) investigated the effect of PEF treatments (20–35 kV/cm for 100–475 μs) on the physicochemical properties such as pH, total fat, peroxide index, peroxidase activity, thiobarbituric acid-reactive substances index, and formol index, of "horchata". They observed that, after PEF treatment, only peroxidase activity decreased significantly ($p < 0.05$). Interestingly, PEF treatment did not cause oxidation of fatty matter, and "horchata" treated by this technique was

oxidatively stable for 5 days under refrigeration. Moreover, PEF treatment did not influence the amino acid profile after the treatment and during subsequent storage. On the other hand, Selma et al. (2006) also noticed that PEF treatment inactivated the growth of *L. monocytogenes* in "horchata", however, the degree of inactivation was not appreciable (0.23–0.70 log10 reductions). The inefficiency of PEF treatment on Listeria could be due to a faster membrane damage selfrepairing ability of the microorganism. Therefore, to prevent the development of pathogenic microorganisms, a combination of PEF treatment with refrigeration during distribution and storage, as well as a low initial load of pathogen in the raw ingredients is required (Selma et al., 2006).

Based on these investigations, it could be concluded that PEF has low but significant influence on microbial inactivation in "horchata", nevertheless, it has least adverse effect on the quality aspect of the beverage after treatment. However, the available articles are relatively longstanding, while the availability of modern/advanced PEF instruments at the present time could enable efficient evaluation of the efficacy of PEF on microbial inactivation. Furthermore, there is a huge research scope for improving PEF treatment efficiency for "horchata" preservation as there is a lot of recent evidence showing that PEF could be successfully employed in extending the shelf life of various beverages (reviewed in (Barba et al., 2015; Buckow et al., 2013; Evrendilek, 2016)). Therefore, future research could be oriented towards changing the treatment parameters and media conditions or employing PEF along with mild heating.

2.2.2. Ultraviolet (UV) treatment

UV radiation preservation technology has emerged as an alternative technique to produce microbiologically safe beverages (Koutchma, Popović, Ros-Polski, & Popielarz, 2016; Patras, 2017). This technology is based on the application of short wave ultraviolet radiation (UV-C, 254

nm) on liquid foods, which is detrimental to microorganisms. As an advantage, this technology does not use/produce thermal energy, consequently has minimal negative effects on the quality of the products (Abdul Karim Shah, Shamsudin, Abdul Rahman, & Adzahan, 2016). Besides, several investigations have revealed that UV-C treated beverages retained the sensory properties and such beverages are preferred over thermally processed products (Abdul Karim Shah et al., 2016; Koutchma et al., 2016). However, the physicochemical properties of treated products should not be overlooked as UV-C at elevated dosages may induce production of toxic compounds (Bule et al., 2010). The effect of UV-C radiation on quality and safety of beverages is reviewed recently by Abdul Karim Shah et al. (2016) and Koutchma et al. (2016).

In case of "horchata", a few authors have investigated the efficacy of UV-C treatment on microbial inactivation and its consequences on quality attributes of the product (Corrales et al., 2012). A UV-C treatment at 253.7 nm having a dose range between 0.14 and 4.23 J/cm^2 did not affect the fat content and its oxidation level in "horchata", while the antioxidant activity of samples decreased with increasing UV-C dose values (Corrales et al., 2012).

Regarding microbial spoilage, UV-C treatment (0.14–4.23 J/cm^2) reduced up to 3 log10 CFU food spoiling microorganisms such as yeasts and molds, mesophilic flora and psychrotrophic bacteria (Corrales et al., 2012). In another study, Elbrhami (2016) evaluated the effect of UV-C light at a distance of 5 cm from the collimated tube, 32 cm from the UV-C lamp and at 0.107 mW/cm2 UV incident intensity level on the inactivation of *E. coli* P36, *L. innocua* ATCC 51742 and *S. Typhimurium* WG49 from tiger nuts milk. 5 log10 CFU reductions of three tested bacteria were achieved at a UV dose of 18.4 mJ/cm^2 for 5 cycles.

Overall, UV irradiation technology was found to be a suitable alternative to thermal preservation technologies for "horchata". However, future investigations should be directed towards its effect on

sensory attributes and formation of artefacts.

2.2.3. High-pressure homogenization (HPH)

Liquid food preservation by high pressure homogenization (HPH) is well documented (Ferragut et al., 2015; Patrignani & Lanciotti, 2016; Pereda, Ferragut, Quevedo, Guamis, & Trujillo, 2007; Poliseli-Scopel, Hernández-Herrero, Guamis, & Ferragut, 2012; Saldo, Suárez-Jacobo, Gervilla, Guamis, & Roig-Sagués, 2009). HPH is a non-thermal technology, where liquid food matrix is forced under high pressure (50–400 MPa) through a narrow orifice (Poojary et al., 2016), which generates a sharp rise in temperature (< 0.7 s), affects the physicochemical properties of the matrix and inactivates the microorganisms (Codina-Torrella et al., 2017; Georget, Miller, Callanan, Heinz, & Mathys, 2014; Patrignani & Lanciotti, 2016). Microbial inactivation under HPH (100–200 MPa) or ultra-HPH (U-HPH) (200–400 MPa) is attributed to disruption of vegetative cells by cavitation, shear stress, turbulence, impingement, and high pressure, among other factors (Dumay et al., 2013; Georget et al., 2014; Patrignani & Lanciotti, 2016). Although, U-HPH has been widely employed for preservation of dairy products, it is relatively less investigated for fruit/vegetable based beverages (Briviba, Gräf, Walz, Guamis, & Butz, 2016; Ferragut et al., 2015).

In a study conducted by Codina-Torrella (2014), the potential of HPH and U-HPH to reduce the microbial population of "horchata" was observed. For instance, she observed a significant decrease (≈2 log10 CFU) of mesophilic flora and psychrotrophic bacteria after the application of HPH+ thermal pasteurization (18 + 4 MPa, 80 °C, 15 s) with and without additives (E-339ii + E331iii, E472C +E-473) and U-HPH (200 and 300 MPa/40 °C) compared to the untreated product, which had an initial load of 5.37 log10 CFU. Moreover, HPH +thermal pasteurization as well as U-HPH reduced the content of yeasts and molds, Lactobacillus, and Enterobacteriaceae from 3 log10 UFC (untreated product) to non-

detectable levels.

On the other hand, several studies have shown that U-HPH can also effect the bioactive compounds of vegetable-based beverages (Briviba et al., 2016; Toro-Funes, Bosch-Fusté, Veciana-Nogués, & Vidal-Carou, 2014). In a recent study, Codina-Torrella et al. (2017) tried to investigate the effect of U-HPH in the physico-chemical stabilization of "horchata". The authors noted that U-HPH improves the colloidal stability by reducing the particle size and inducing new interactions. Moreover, U-HPH treatment at 300 MPa reduced the peroxidase activity significantly, meanwhile, a treatment at 200 MPa provided the highest stability against lipid oxidation compared to conventional homogenization-pasteurization. Overall, the author concluded that UHPH treatment is a suitable alternative to produce stable "horchata" for commercial production.

2.2.4. High pressure processing (HPP)

In the last two decades, HPP has emerged as a potential tool to preserve liquid foods (Balasubramaniam, Martínez-Monteagudo, & Gupta, 2015; Barba, Terefe, Buckow, Knorr, & Orlien, 2015; Misra et al., 2017). Traditionally, HPP has been used in order to satisfy consumer trends demanding more natural/fresh-like products with fewer preservatives and longer shelf life (regulatory requirements demanding the absence of pathogens such as Listeria or Salmonella spp.) and for these reasons, producing high quality products (Bruschi et al., 2017; Ferreira, Almeida, Delgadillo, Saraiva, & Cunha, 2016; Misra et al., 2017). This technology is based on the principle where the application of pressure on food matrix causes the eradication of microorganisms by combined effects of membrane disruption, protein denaturation, enzyme inactivation, oxidative stress, genetic composition modification, etc. A number of researchers have shown the efficiency of HPP for food preservation (Gayán, Torres, & Paredes-Sabja, 2012; Santos et al., 2018;

Serment-Moreno, Barbosa-Cánovas, Torres, & Welti-Chanes, 2014). However, it is established that HPP does not affect low molecular weight nutrients (Barba, Poojary, Wang, Olsen, & Orlien, 2017).

In case of tiger nuts' milk preservation, Elbrhami (2016) evaluated the impact of HPP treatment (400–600 MPa/11 °C/60–180 s) on *E. coli* P36, Salmonella WG 49 and *L. innocua* ATCC 51742 inactivation in tiger nuts' milk. The author observed a 5-log10 CFU reduction of the tested bacteria after applying 500 MPa for 180 s (5.11, 5.74 and 5.49 log10 CFU, for Listeria, *E. coli* and Salmonella, respectively) and 600 MPa for 180 s (6.14, 7.08 and 6.6 log10 CFU, for Listeria, *E. coli* and Salmonella, respectively).

Regarding the physicochemical characteristics, there were no significant ($p > 0.05$) changes in the titratable acidity, pH and total solids after HPP except at 500 MPa during 180 s that decreased the titratable acidity significantly, up to 17.5%. On the other hand, a decrease in the conductivity from 3.2 to 2.6 and 2.3 ms/cm after HPP treatment at 500 and 600 MPa, respectively, was observed. Finally, the protein content was affected by HPP, showing a significant decrease from 5.53 to 4.06 mg/L in tiger nut samples after applying HPP at 600 MPa for a treatment time higher than 90 s.

On the other hand, Elbrhami (2016) did not notice any significant differences ($p < 0.05$) in color parameters between untreated and high pressure-treated samples at 500 MPa for 90 s and 180 s, and at 600 MPa for 90 s. However, the HPP-treated samples at 600 MPa for 180 s were brighter, and less red, whereas a significant ($p < 0.05$) decrease in lightness was observed after the application of HPP (500 MPa for 180 s). Finally, a significant loss in vitamin C (from 8.22 to 5.94 mg/100 mL) and phenolic compound (from 139.14 to 95.85 mg GAE/100 mL) contents was observed after HPP treatment at 600 MPa for 120 s. The same authors also evaluated the shelf life over a period of 8 days at 4 °C, observing that tiger nut milk was shelf stable during all the storage period. Overall, HPP is shown to be a promising technology for improving

the shelf life of "horchata", however, further investigation is required with emphasis given on quality attributes of the treated beverage.

Implementing the above-mentioned non-conventional technologies (PEF, UV, and HPP) in "horchata" processing allows extending the shelf life of the beverage without consuming significant amounts of energy. These technologies could be used to replace the thermal treatment of pasteurization (Fig. 1), mainly when combined with moderate temperatures, as previously reported (Barba et al., 2017).

3. Patents describing the preparation and applications of "Horchata"

The main patents found in the literature describing either the preparation/conservation or the potential applications of "horchata" are presented in Table 2. The first patent (ES216332) found was published in 1954, describing the preparation of fresh beverages including "horchata". Thereafter, other patents were published, which were mainly focused on equipment development for 1) preparation (patents ES139627, ES247349, ES290540, US2015028140, DE3908704, etc.), 2) preservation of "horchata" either by pasteurization and sterilization (patents ES2000355, ES2000035, ES1039158, US2007148288, etc.), or by addition of food preservatives (patent US2007264401) in order to extend the shelf life, 3) formulation of food products by adding other constituents and ingredients to "horchata" as for the manufacturing of ice creams (patent ES2017560), flan (crème caramel) and custard (patent ES2026306) based on tiger nut milk, and 4) the use of "horchata" for the preparation of cosmetic products (patents WO2009013377, ES2367509).

Table 2. Most relevant patents containing "horchata" as search keyword. Source: European Patent Office (Espacenet Patent Search) and www.orbit.com.

Title	Patent number	Year
Un procedimiento para la fabricación de bebidas refrescantes, granizados horchata y similares	ES216332	1954
Un dispositivo para servir líquidos, especialmente horchata	ES238840	1957
Nueva prensa para la fabricación de horchata	ES234528	1957
Procedimiento de conservación de horchata de chufas envasada	ES-252066	1959
Procedimiento para la obtención de horchata de chufa concentrada	ES274093	1962
Procedimiento para la obtención de horchata de chufa	ES349916	1968
Maquina perfeccionada para elaboración de horchata.	ES139627	1968
Machine centrifugal for obtaining of tiger nut milk of chufa	ES247349	1979
Ground-nut drink	ES518431	1982
Device conservador-dispensador of tiger nut milk	ES290540	1985
Horchata beverage prepn.	ES8704717	1985
Horchata prepn.	ES8704716	1985
Sterilised horchata beverage prodn.	ES2000355	1986
Pasteurised horchata beverage prepn.	ES2000035	1986
Powdered horchata beverage prepn.	ES2005770	1988
Process and apparatus for natural horchata de chufa (tiger nut milk)	DE3908704	1989
Process for manufacturing ice creams based on tiger-nut milk	ES2017560	1989
Process for manufacturing flan (crème caramel) and custard based on tiger-nut milk	ES2026306	1990
Molino-prensa for the manufacture of natural tiger nut milk.	ES1030073	1995
Process to obtain a food composition based on chufas (*cyperus esculentus*) and bananas, and compositions obtained	US5900267	1996
Pasteurization plant.	ES1039158	1997
Preparation method for tiger nut milk.	ES2164001	1999
Long shelf-life Horchata beverage	US2007148288	2005
Tiger nut milk liquor comprises mixture swollen or soaked tiger nuts that are crushed, cinnamon, lemon peel and water, where mixture is pressed and sieved and solid material is retained in sieves until drained	ES2284332	2005
Symbiotic Drink of tiger nut milk of chufas.	ES2304285	2006
Method of obtaining of a fodder with chufa and product obtained with the same	ES2315099	2006
Beverage preservatives	US2007264401	2006
Additive for horchata de chufa (tiger nut milk) free of lactic proteins	WO2008090238	2007
Method for obtaining a cosmetic product from a concentrate of the tiger nut drink	WO2009013377	2007
Soap in exfoliation tablet with crushed chufa and procedure to obtain this cosmetic product.	ES2367509	2010
Device for beverage production	US2015028140	2012

Focusing on disinfection/preservation techniques, as it is the aim of this review, a process of thermal disinfection of the unground tiger nuts either by steam treatment and/or microwave treatment, and apparatuses for the improved production of "horchata" was proposed (patent number DE3908704) (Fig. 2). It represents the only patent found mentioning the use of the non-conventional technology of microwave as disinfection method. This treatment promoted a significant reduction in the pathogenic microorganisms able to grow in "horchata" beverage.

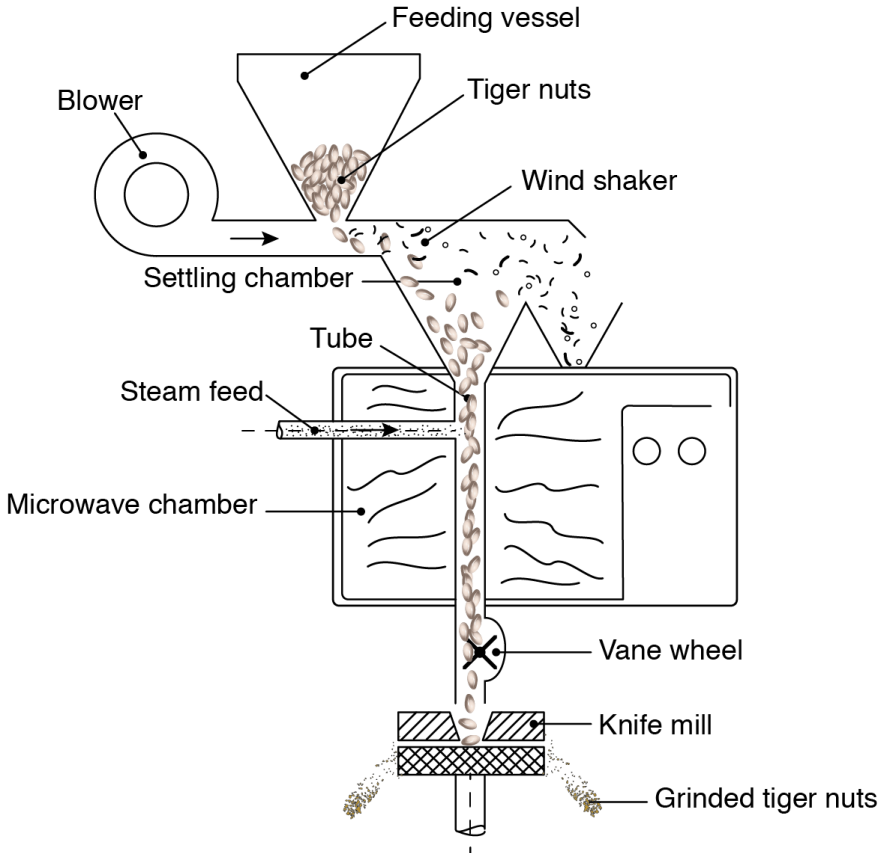

Fig. 2. Schematic representation of tiger nuts disinfection using microwave and steam. Adapted from patent number DE3908704.

4. Conclusion

The use of non-thermal technologies (e.g. pulsed electric field, ultraviolet, and high pressure) for the preservation of "horchata de chufa" beverage presents numerous advantages over conventional pasteurization by heat. However, implementing these technologies in "horchata" processing should be combined with moderate temperature for improved efficiency. Moreover, their environmental effects and impact on the food composition should be further investigated. Overall, there is a huge scope for innovation and research need in non-thermal technologies for preservation of "horchata de chufa" beverage.

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5.2. Tiger nut and its by-products valorization: From extraction of oil and valuable compounds to development of new healthy products

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Tiger nut and its by-products valorization: From extraction of oil and valuable compounds to development of new healthy products

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Tiger nut and its by-products valorization: From extraction of oil and valuable compounds to development of new healthy products

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ABSTRACT

Consumer's growing demand for consumption of “Horchata de chufa”, a Spanish beverage produced from tiger nut tubers, has led to large-scale production of tiger nuts, and its subsequent processing for the food industry. Recent investigations clearly show that tiger nut is a valuable source of stable vegetable oils, rich in monounsaturated fatty acids, and phytosterols as well as high-added value compounds (proteins, carbohydrates, vitamins, minerals and bioactive compounds). Several

conventional (Soxhlet) and alternative innovative (SC-CO_2, enzyme, high pressure, etc.) extraction methods have been developed for the efficient recovery of tiger nut oil and high-added value compounds. Moreover, several authors have shown the potential of tiger nuts by-products in the development of new healthier and functional products such as fiber rich and oxidative stable foods. The present review provides an overview of these investigations and tries to expose potential avenues for future research in commercial exploitation of tiger nuts and its by-products. Industrial relevance: Tiger nuts and their by-products obtained from "horchata de chufa" manufacturing process have a remarkable content of nutrients, fiber and bioactive compounds (eg. polyphenols). Moreover, they have a high amount of oil, rich in monounsaturated fatty acids, tocopherols, and phytosterols, thus being a valuable source to be incorporated into different food matrices in order to modify their technological and functional properties.

At this stage of development, tiger nuts by-products are underutilized at an industrial level. For example, the literature about the oil extraction from tiger nuts by-products is scarce and most of the studies have been focused on the use of conventional (Soxhlet) extraction of oil. This review provides an overview of some of the most relevant innovative processing technologies to allow the industrial sustainability and green recovery of oil from tiger nuts and their by-products and, tries to expose potential avenues for future research in commercial exploitation of tiger nuts and its by-products as source of ingredients to be incorporated in new food matrices to improve their technological and functional aspects.

1. Introduction

Tiger nut is an edible tuber produced by a small weed plant (nowadays, however, cultivated commercially), called *Cyperus esculentus*. Although *C. esculentus* is found across the world, it is distributed mostly in countries such as Spain, Egypt, and Nigeria. In Spain, particularly in

Valencian region, tiger nuts are extensively used to prepare a cold beverage, known as “horchata de chufa” or tiger nut beverage (CRDO, 2009).

Tiger nuts contain substantial amounts of lipids; and the oil is used in cooking as well as a cosmetic ingredient. The nuts are also a good source of carbohydrates and fibers. Despite its rich nutritional values, it is relatively less explored as a food when compared to other edible nuts and tubers. Nowadays, however, the tiger nut processing has received much attention among food industries due to its increasing popularity among Mediterranean consumers (Martín-Esparza & González-Martínez, 2016; Sánchez-Zapata, Fernández-López, & Pérez-Alvarez, 2012).

In food industries, the tiger nut beverages are prepared by mechanical pressing of tubers, followed by extraction of juice (BOE, 1988; Codina-Torrella, Guamis, Ferragut, & Trujillo, 2017). During this process, a large amount of by-products or meal residues are produced (Fig. 1), which are often considered as ‘industrial wastes’ or a product of no commercial value.

In 2007–2008, 4.2 tons of tiger nuts wastes and by-products were produced in the Valencian region, Spain, itself (CRDO, 2009). However, these by-products could be a valuable source of oil and high-added value compounds including nutrients (carbohydrates, proteins, vitamins, and minerals) and bioactive compounds (eg. polyphenols, etc). The nutrients can be used as an energy source, while bioactive compounds have prospective application in nutraceutical industries (Gil-Chávez et al., 2013). Moreover, these by-products also have a high fiber content, which can be used in the development of new products, thus responding to society's demand for potential functional foods (Granato, Nunes, & Barba, 2017).

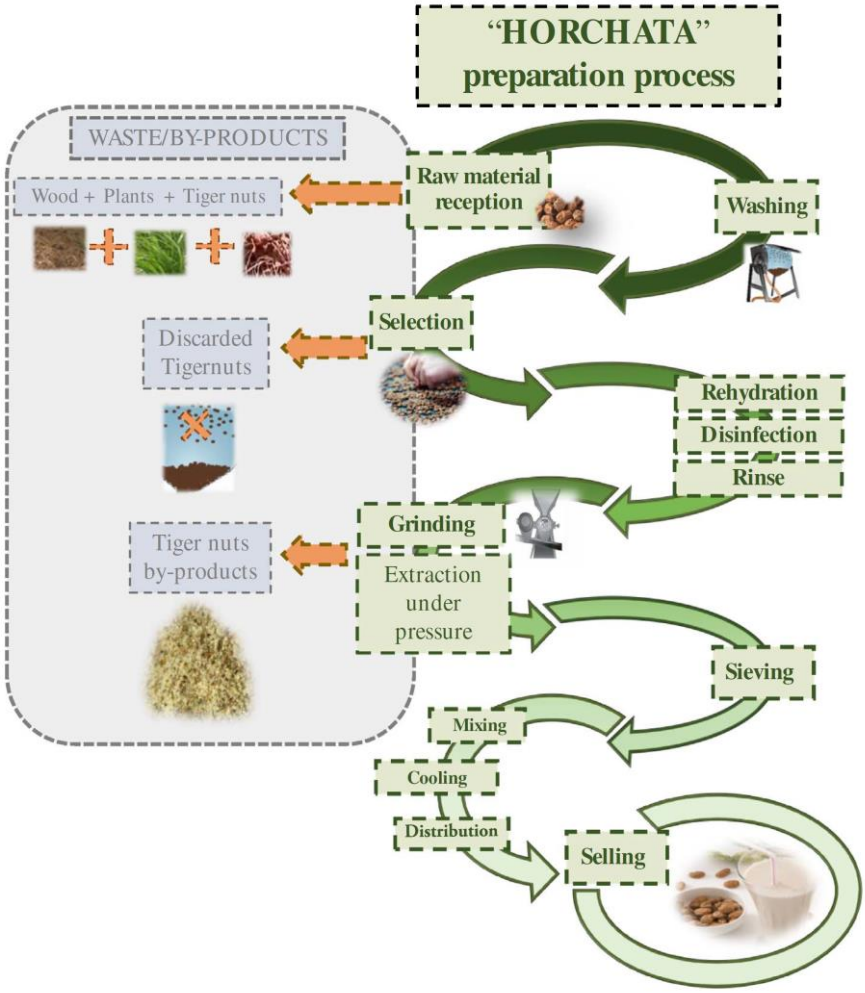

Fig. 1. Wastes and by-products generated during horchata preparation.

Moreover, in recent years, stringent rules have been implemented in many countries for disposal of agricultural wastes considering their associated health and environmental implications (Galanakis, 2015, 2016). The developed countries are promoting prospective utilization of agricultural wastes as a source of energy, nutrient and medicine. For instance, Spanish (Anonymous, 2011) and European (Anonymous, 2006,

2008) legislative frameworks for waste management prioritizes the recycling and recovery of by-products from their disposal in order to preserve natural resources, protect the environment and consumer health.

Extraction of high added-value compounds from the bio-sources remains one of the fastest growing sectors within the food and pharmaceutical industries (Galanakis, 2012; Galanakis, Martinez-Saez, del Castillo, Barba, & Mitropoulou, 2015). The need for more environmental friendly, sustainable and viable processes, has led food scientists and industries to develop alternative processes according to the concept of "green" extraction (Chemat et al., 2017; Koubaa, Rosello-Sotó, et al., 2015). This concept refers to the use of renewable plant resources and "green" solvents, reduction of energy consumption and operations, production of extracts with high quality and purity (non-denatured and biodegradable) and generation of by-products instead of wastes (Chemat, Vian, & Cravotto, 2012).

Over the last decades, substantial research works have evidenced that tiger nut is a good source of oil and its by-products are rich in various nutrients and bioactive compounds (Fig. 2).

Several reports have revealed that the extracts or by-products of tiger nuts exhibit antioxidant activity (Badejo, Damilare, & Ojuade, 2014; Sánchez-Zapata, Fuentes-Zaragoza, et al., 2012), which is ascribed to its polyphenol contents. However, diversity of antioxidant compounds present in tiger nuts has not been thoroughly explored. Nevertheless, a number of investigations have shown the development of attractive food products from tiger nut wastes. In this review, we have tried to provide insights on recovery of oil from tiger nuts and added-value compounds from its by-products. Emphasis has been placed on recent advances in utilization of non-conventional and Green processing techniques used in the recovery process.

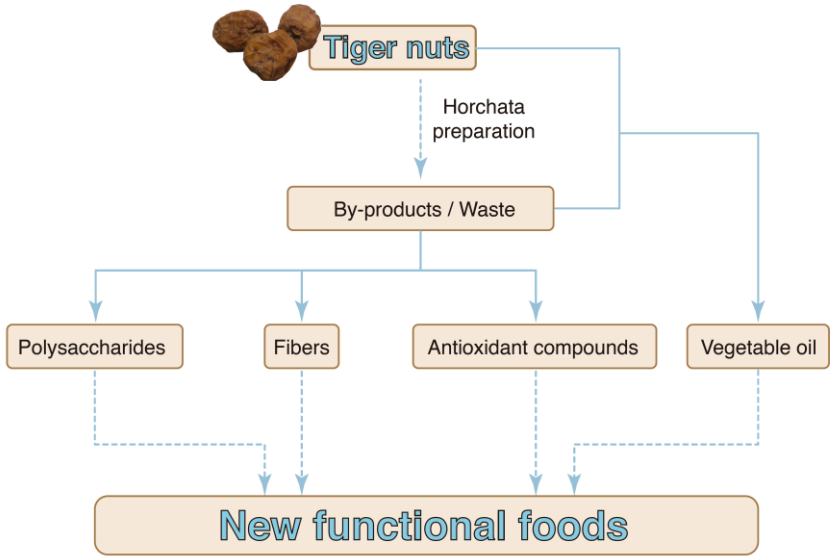

Fig. 2. Possible compounds that could be recovered from by-products of horchata production.

2. Nutritional and physicochemical composition

The comprehensive phytochemical profile of tiger nut is not available. However, the available data reveals that tubers are rich in essential dietary constituents such as proteins (3.28–8.45%), fats (22.14–44.92%), fibers (8.26–15.47%) and ashes (1.60–2.60%) (Adel, Awad, Mohamed, & Iryna, 2015; Codina-Torrella, Guamis, & Trujillo, 2015; Ejoh & Ndjouenkeu, 2006) (Table 1). Among these constituents, the industrially relevant compounds that could be recovered are starch, soluble carbohydrates (mainly in the form of horchata), lipids and fibers. The main carbohydrate in tiger nut beverage is sucrose (Sánchez-Zapata, Fuentes-Zaragoza, et al., 2012). The lipid profiling revealed that the oil has a similar fatty acid composition to olive oil (Adel et al., 2015; Arafat, Gaafar, Basuny, & Nassef, 2009; Lopéz-Cortés, Salazar- García, Malheiro, Guardiola, & Pereira, 2013; Muhammad et al., 2011; Yeboah, Mitei, Ngila, Wessjohann, & Schmidt, 2012; Zhang, Hanna, Ali, & Nan, 1996) (Table 2).

Table 1. Proximate composition (% of dry matter) of tiger nut.

Origin	Moisture	Fat	Protein	Fiber	Ash	Reference
Valencia	8.66	35.21	8.45	9.31	1.97	(Codina-Torrella et al., 2015)
Burkina Faso	7.75	25.77	7.32	9.07	1.90	
Burkina Faso	6.38	25.35	5.62	8.42	1.95	
Niger	7.45	28.19	3.28	8.75	1.60	
Egypt	7.30	22.14	4.33	15.47	2.60	(Adel et al., 2015)
Cameroon	ND	35.32	8.08	10.31	2.39	(Ejoh and Ndjouenkeu, 2006)
Cameroon	ND	44.92	7.50	14.49	2.61	
Cameroon	ND	26.88	6.99	8.26	2.28	
Cameroon	ND	43.50	8.30	14.14	2.60	
Cameroon	ND	35.63	6.57	13.70	2.58	
Cameroon	ND	31.66	7.54	12.51	2.32	

This means that it predominantly consists of oleic acid with values ranging from 59 to 76% compared to values for olive oil from 56 to 85% (Fomuso & Akoh, 2002; Visioli & Galli, 1998). Other major fatty acids in tiger nut oil are palmitic (10–20%), linoleic (8–12%) and stearic (0.3–5.3%) acids. In addition, tiger nuts are rich in α-tocopherol (5–87 μg/g) and phytosterols such as β-sitosterol (43–61 mg/100 g), campesterol (11–17 mg/100 g), and stigmasterol (17–21 mg/100 g) (Lopéz-Cortés et al., 2013; Yeboah et al., 2012) (Table 3). A detailed description on the nutritional composition, physicochemical properties and economic potential of tiger nuts oils is reviewed recently by Ezeh, Gordon, and Niranjan (2014).

3. Extraction

3.1. Conventional extraction

Cold pressing is a widely used technique for industrial production of tiger nut oil (Koubaa, Barba, et al., 2015; Lasekan & Abdulkarim, 2012). Several laboratory level researches have shown that tiger nut lipids can be extracted using organic solvents such as n-hexane, petroleum ether, ethanol etc., with a substantial yield (Ekpe, Igile, Williams, & Eworo, 2016). In such cases, samples are initially homogenized and stirred with a suitable solvent and then the lipid portion is recovered. Soxhlet extraction can also be used. For instance, a study showed that up to 15.9% (w/w) oil yield can be obtained by using n-hexane/2-propanol (3:1, v/v) solvent mixture in 6 h using a Soxhlet apparatus (Yeboah et al., 2012). Recently, Ekpe et al. (2016) showed that n-hexane offers superior extraction efficiency compared to petroleum ether. However, the use of hexane has been questioned due to environmental problems and occupational safety (Lasekan & Abdulkarim, 2012).

3.2. Non-conventional extraction

Nowadays, industries are much concerned towards replacing the use of organic solvents for extraction purposes as it is linked with health, safety, environmental and regulatory issues. Therefore, some innovative alternative techniques have been established for the clean recovery of lipids and other high added-value compounds form tigernuts and its by-products. In this line, some of the reported techniques are described in the following sections.

Table 2. Main fatty acid composition (g/100 g of fatty acids) of tiger nut

Origin	$C_{14:0}$	$C_{16:0}$	$C_{16:1}$	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{18:3}$	$C_{20:0}$	SFA	MUFA	PUFA	Reference
Spain	0.10	15.76	0.27	2.57	72.39	8.22	0.18	0.28	19.01	72.61	8.39	(Lopéz-Cortés et al., 2013)
Egypt	0.07	15.15	0.26	3.35	71.69	8.47	0.04	0.37	19.26	71.98	8.52	
Nigeria	0.16	17.87	0.21	3.79	59.44	8.77	0.12	0.39	22.46	59.71	8.88	
South Africa	0.20	20.38	0.33	4.05	62.95	10.91	0.11	0.31	25.20	63.28	11.02	
Egypt	0.12	15.19	0.29	5.07	69.25	8.37	0.19	ND	20.59	70.66	8.75	(Adel et al., 2015)
Ghana	ND	16.32	ND	5.33	65.55	12.13	ND	0.68	22.33	65.55	12.13	(Yeboah et al., 2012)
Nigeria	1.7	10.4	ND	0.3	76.10	11.8	0.6	6.1	18.50	76.10	12.40	(Muhammad et al., 2011)
Egypt	0.80	14.50	1.50	3.40	69.50	8.80	0.40	0.20	18.90	71.00	9.20	(Arafat et al., 2009)
China	ND	14.99	ND	2.56	69.32	13.11	0.0	ND	17.55	69.32	13.11	(Zhang et al., 1996)

ND: not detected. SFA: Saturated fatty acids. MUFA: Monounsaturated fatty acids. PUFA: Polyunsaturated fatty acids.

3.2.1. Compressed fluids

Supercritical fluid extraction is a promising technology that has been successfully employed in the extraction of diverse compounds from plant materials and other bioresources (Barba, Grimi, & Vorobiev, 2014; Poojary et al., 2016; Roselló-Soto, Koubaa, et al., 2015; Roselló- Soto et al., 2016). This technique minimizes or avoids the use of toxic organic solvents, thus considered as 'green' and environmental friendly (Koubaa, Rosello-Sotó, et al., 2015; Poojary et al., 2016). Moreover, this process offers relatively pure and residual solvent free extracts, which in turn reduces downstream processing. In addition, it frequently involves the use of certain modifiers (also called co-solvents) to improve efficiency and selectivity of extraction (Ezeh et al., 2014; Galanakis et al., 2015; Poojary et al., 2016; Roselló-Soto, Galanakis, et al., 2015; Roselló-Soto, Koubaa, et al., 2015).

In a previous study, the potential of the supercritical carbon dioxide (SC CO_2) extraction to recover fresh chufa oil was evaluated (Lasekan & Abdulkarim, 2012). The authors compared the yield and quality of oils obtained from tiger nuts through supercritical carbon dioxide (SC-CO_2) (20–40 MPa, 40 °C–80 °C, 60–360 min) extraction and traditional Soxhlet extraction. They found that SC-CO_2 pressure and time had a significant influence on oil extraction, obtaining the highest yield of 26.28 g/100 g sample through SC-CO_2 treatment (30.25 MPa, 60 °C, 210 min). Moreover, they also found that fatty acid composition differed based on the processing condition.

To avoid long extraction times, the use of mechanical expression (ME) assisted by supercritical fluids could be used as an alternative to improve the recovery of oil, bioactive compounds, nutrients, etc. (Koubaa, Barba, et al., 2015). One of such process is referred as gas assisted mechanical expression (GAME) and this technique has been previously applied to extract oil from different matrices of vegetable origin (Müller & Eggers, 2014; Venter, 2006; Venter, Willems, Kuipers, & de Haan, 2006; Willems

& de Haan, 2011; Willems, Kuipers, & de Haan, 2008).

In case of tiger nuts, Koubaa, Barba, et al. (2015) evaluated the efficacy of GAME process consisting of both SC-CO_2 and ME for the extraction of oil and polyphenols and the results were compared with independent application of SC-CO_2 and ME. According to the results, pressure levels of 20 and 30 MPa were optimal for SC-CO_2 and ME, respectively. Additionally, GAME process allowed faster oil extraction from nuts, reaching 50% yield oil recovery after 10 min extraction, compared to only 10% and 20% when using SC-CO_2 and ME separately at 20 and 30 MPa pressures, respectively. Furthermore, GAME allowed the recovery of the highest amount of phenolic compounds compared to SC-CO_2 and ME processes when applied independently. Phenolic profiling by HPLC-MS revealed that GAME offers a greater diversity of phenolic compounds (57 compounds) in extracted oils when compared to SC-CO_2 (48 compounds), and ME (27 compounds). Scanning electron microscopy (SEM) images revealed that GAME results in a better cell damage. It was concluded that GAME represents a great opportunity to replace conventional methods for oil extraction from plant matrices.

3.2.2. Enzyme- and high pressure-assisted extraction

Enzymes have become a popular tool in extraction technologies as they significantly improve the extraction yield by hydrolyzing the cellwall components and, consequently, aiding mass transfer of analytes (Puri, Sharma, & Barrow, 2012; Roselló-Soto et al., 2016). Several commercially available cellulolytic or proteolytic enzymes could be used for this purpose based on the type of matrix (Poojary, Orlien, Passamonti, & Olsen, 2017). In case of tiger nuts, the impact of enzymeassisted extraction for the recovery tiger nut oil rich in bioactive compounds was evaluated (Ezeh, Niranjan, & Gordon, 2016). The authors performed aqueous enzymatic extraction using cellulolytic enzymes such as alcalase, α-amylase Viscozyme® L and Celluclast®, while in another set of

experiments enzyme assisted extraction was combined with high pressure processing. Results revealed that aqueous enzyme extraction yielded comparable amounts of fatty acids, polyphenols and tocopherols contents to that obtained through control pressing, while high pressure processing treatment prior to enzymatic extraction enhanced tocopherols and total polyphenolic content in oils significantly. In addition, the authors showed that the by-products of oil extraction process is a source of sugars and; the methanolic extracts of residual meals found to contain glucose, sucrose and certain oligosaccharides (Ezeh, Niranjan, et al., 2016). In a similar study, the authors found up to 90% oil recovery when they used enzymatic treatment prior to mechanical extraction (Ezeh, Gordon, & Niranjan, 2016).

Table 3. Phytosterol concentration (mg/100 g) and α-tocopherol amount (μg/g) of tiger nut.

Origin	β-Sitosterol	Campesterol	Stigmasterol	α-tocopherol	Reference
Spain	60.18	14.01	19.50	4.6	(Lopéz-Cortés et al., 2013)
Egypt	43.34	11.44	17.85	28.7	
Nigeria	60.82	16.63	18.76	ND	
South Africa	61.15	16.32	17.35	ND	
Ghana	51.70	15.30	20.6	86.73	(Yeboah et al., 2012)

ND: not detected

4. Development of new products based on tiger nuts wastes and by-products.

The use of tiger nut beverage by-products (TNBP) obtained during the production of horchata have attracted the interest of both the food industry and the scientific community for the development of healthier

products (Fig. 3) mainly due to their high dietary fiber content (59.71 g/100 g, 99.8% dietary fiber) (Sánchez-Zapata et al., 2009).

They can also be used in the production of biobased composites (Carbonell et al., 2015) or as carbon source for probiotic bacterial growth (Sánchez-Zapata, Fernández-López, et al., 2013). However, it is necessary to use a preservation method prior to their addition to food products due to their high microbial load. Moreover, TNBP have extraordinary water- and oil-holding capacities, high emulsifying ability as well as low water adsorption compared to other dietary fiber sources (Sánchez-Zapata et al., 2009), which make them ideal for improving the functional properties of the developed products.

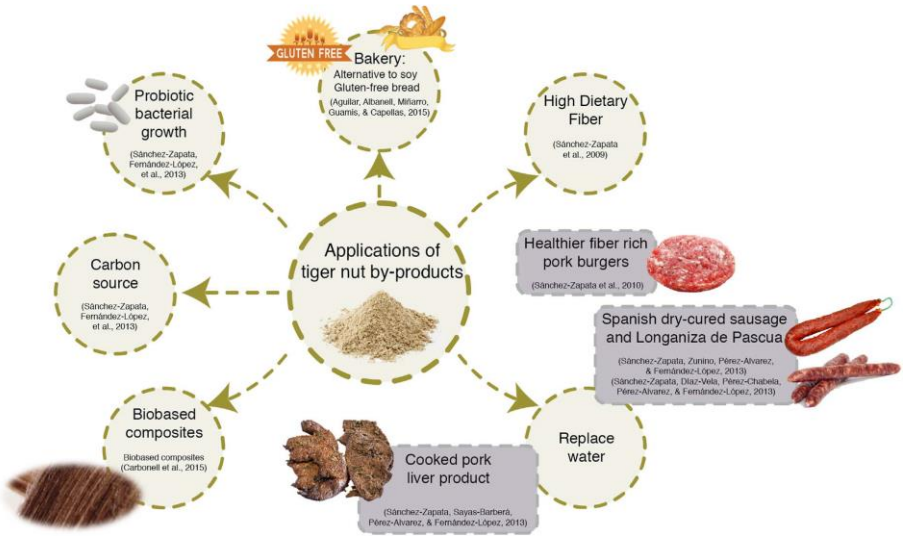

Fig. 3. Potential applications of tiger nuts by-products in the development of new functional foods.

On the other hand, Aguilar, Albanell, Miñarro, Guamis, and Capellas (2015) studied the impact of the use of tiger nut beverage by-products as an alternative to soy, which is a known allergen, for the production of gluten-free bread, obtaining breads with similar composition and

consumer's acceptance to those obtained when soya flours were used. In another study, the influence of the addition of two different tiger nut beverage by-products flours at different percentages (5%, 10% and 20% w/w dry basis) on wheat flour was evaluated (Verdú, Barat, Alava, & Grau, 2017). The authors observed a slight increase in mass loss, although non-significant, in baking process when the by-product flours were added compared to control samples.

Other authors evaluated the potential of tiger nut beverage byproducts, particularly tiger nut fibers (TNF), to be used in the development of healthier fiber-rich meat products. In this line, the impact of increasing levels of TNF (5%, 10% and 15%), to develop fiber rich pork burgers and its effect on nutritional value, cooking characteristics, physicochemical (color and texture) and sensorial properties and consumer acceptability was evaluated (Sánchez-Zapata et al., 2010). As can be expected, the pork burgers elaborated with the tiger nut by-products offered an increased fiber content in burgers. Moreover, the incorporation of these by-products also improved cooking yield, moisture and fat retention compared to control samples. However, the TNF had a negative influence on color and texture, although the changes were not noticeable after cooking process. The sensorial tests and consumer acceptability were positive, thus concluding that the addition of TNF to burgers is a useful tool to increase the dietary fiber of meat products (Sánchez-Zapata et al., 2010). Furthermore, the tiger nuts liquid by-product exhibited the potential to control lipid oxidation in pork burger, possibly due to its high composition in antioxidant compounds (eg. polyphenols) (Sánchez-Zapata, Pérez-Alvarez, & Fernández-López, 2012). Moreover, it enhanced cooking properties by reducing the shrinkage of burger and conveyed better consumer acceptability as they were perceived as less fatty (Sánchez-Zapata, Pérez- Alvarez, et al., 2012).

In a subsequent study, the same research group investigated the impact of TNF addition (0, 5, and 7.5%) on the nutritional,

physicochemical, sensorial characteristics of Spanish dry-cured sausage during the dry-curing process and subsequent 28 days storage, giving emphasis on microbial stability and the oxidation reactions (Sánchez-Zapata, Zunino, Pérez-Alvarez, & Fernández-López, 2013). Overall, the amount of dietary fibers and moisture content of the new product were increased but a reduced fat content was found. The tiger nut fibers concentration did not affect the lipid oxidation stability, however, it changed the color significantly; nevertheless, it did not modify the consumer acceptance (Sánchez-Zapata, Zunino, et al., 2013).

In another work, TNF was used as source of unsaturated fatty acids to improve the nutritional quality of a traditional dry-cured fermented sausage Longaniza de Pascua (Sánchez-Zapata, Díaz-Vela, Pérez- Chabela, Pérez-Alvarez, & Fernández-López, 2013). Together with walnut oil, the addition of TNF enhanced moisture (increased weight), water activity, redness and blueness of the samples, while the lightness value remained unchanged but the pH values was reduced. Moreover, lipid oxidation was prevented, likely due to the high polyphenol content of tiger nut by-products. Although TNF influenced the microbiota, and the microbial populations at the end of ripening were similar to that of control (Sánchez-Zapata, Díaz-Vela, et al., 2013).

Tiger nuts liquid by-products were also used to replace water on a cooked pork liver product (Sánchez-Zapata, Sayas-Barberá, Pérez-Alvarez, & Fernández-López, 2013). The authors replaced 50 or 100% of water with liquid by-product and evaluated the physicochemical properties of cooked pork liver. The results revealed that the by-product enriched products had higher fat and heme iron content and exhibited a lower degree of metmyoglobin formation. Interestingly, the liquid byproduct did not alter the physicochemical properties including color, water activity, reflectance spectrum and pH of the meat product (Sánchez-Zapata, Sayas-Barberá, et al., 2013).

5. Challenges and future perspectives

Development of new foods, natural and healthy ingredients as well as management of wastes derived from food processing have become the major concerns for food industries. Though horchata production provides major marketing and revenue for tiger nut processing industries, the production and use of tiger nut oil or valorization of byproducts is almost neglected in such industries. Interestingly, a number of patents have been granted during the last decades focused on development of tiger nut based products applied in food and cosmetic sectors (MX2010000858 (A) WO2008090238 (A1) ES2284332 (B1); ES2284332 (A1) ES2123447 (A1); ES2123447 (B1) ES2111500 (A1); ES2111500 (B1) ES2031431 (A6) ES2026306 (A6) ES2017560 (A6)) (EPO, 2017), indicating a huge scope in developing new functional foods based on tiger nut by-products (see Section 4).

The lipids, fibers and bioactive compounds obtained during tiger nut processing can be incorporated into different food matrices as it has direct or indirect favorable effect on health as well environment by reducing, reusing and valuing the waste generated during horchata production. However, the valorization of tiger nuts wastes and byproducts constitute a great challenge, especially for Valencian region, but also to the other regions mainly due to unavailability of proper scientific knowledge and by-product processing technologies.

One of the main problems to reuse tiger nuts beverage by-products is the high microbial load that these materials present. Therefore, a pretreatment to reduce the microbial contamination is needed. Ultrasound, pulsed electric fields, microwaves and high pressure processing has been successfully applied to inactivate microorganism (Barba et al., 2015; Barba, Koubaa, do Prado-Silva, Orlien, & de Souza, 2017; Chemat & Ashokkumar, 2017; Zinoviadou et al., 2015) as well as to extract oil (Chemat et al., 2017; Juliano et al., 2017; Koubaa et al., 2016; Puértolas, Koubaa, & Barba, 2016; Sicaire et al., 2016) and valuable

compounds from several plant matrices, wastes and by-products (Barba, Zhu, Koubaa, Sant'Ana, & Orlien, 2016; Jacotet-Navarro et al., 2016; Koubaa, Rosello-Sotó, et al., 2015; Poojary et al., 2017; Putnik et al., 2017; Roselló-Soto, Koubaa, et al., 2015; Roselló-Soto et al., 2016; Tabasso, Carnaroglio, Calcio Gaudino, & Cravotto, 2015), although it depends on processing conditions. Thus, the recovery of oils and highadded value compounds from tiger nuts by-products has dual benefits by addressing both waste and by-products management and societal health.

There is a need to obtain more efficient, fast, and reliable methodologies to obtain oil and high-added value compounds from tiger nuts wastes and by-products. For this purpose, innovative green processes such as ultrasound, microwave, pulsed electric fields, and high pressure are useful tools to be implemented.

There are not exhaustive studies about phytochemical profiling of tiger nuts by-products (eg. polyphenol composition) and most of the works have been focused on their use as source of fiber and vitamins. Therefore, an exhaustive chemical profiling should be conducted prior to their use as source of food additives and/or nutraceuticals. Afterwards the extraction procedures should be optimized and implemented in order to select the most appropriate technique taking into account the targeted compound(s).

6. Conclusions

The available literature confirms that tiger nut is a valuable source for diverse nutrients and bioactive compounds. Moreover, it is a good source of stable oil rich in monounsaturated fatty acids and tocopherols that can be used in cooking. Several investigations revealed that oil could be efficiently extracted using conventional organic solvent extraction, however, recent investigations highlights the advantages of nonconventional extraction techniques, particularly supercritical fluid extraction, in terms of yield and safety. However, a detailed investigation

is required in understanding purity and properties of extracted oil. Furthermore, the abundance of fiber content on tiger nut milk by-products has attracted development of new functional food products. These dietary fibers could be used in preparation of fiber-rich meat products with improved physicochemical and nutritional properties. Since these by-products contain polyphenols, their application could be extended in controlling or inhibiting lipid oxidation in foods.

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5.3. Enhancing Bioactive Antioxidants' Extraction from "Horchata de Chufa" By-Products

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Enhancing Bioactive Antioxidants' Extraction from "Horchata de Chufa" By-Products

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Abstract: During the production of a traditional drink produced from the tubers of *Cyperus esculentus* L. also known as "horchata de chufa," a high quantity of by-products are generated. These byproducts are rich with valuable biological compounds, hence, there is a need to report their

extraction conditions for further use in food production as raw materials. Therefore, the objective of this study was to evaluate and improve the conventional extraction process, applied for recovery of phenolic compounds, total flavonoids, and total antioxidant capacity from the by-products. Independent variables for extraction were: (i) Solvent type (mixtures of ethanol-water (v/v) at 0%, 25% and 50%); (ii) temperature (40, 50 and 60˚C), and (iii) extraction time (1, 2 and 3 h). The obtained results showed that solvent type, temperature, and time significantly influenced ($p < 0.05$) all investigated parameters. The highest content of total polyphenols (16.02 mg GAE/100 g of dry matter; d.m.), and total flavonoids (30.09 mg CE/100 g d.m.) was achieved by ethanol at 25% (v/v), after 3 h of extraction with temperatures of 60˚C and 50˚C, respectively. The highest value of antioxidant capacity (1759.81 μM Trolox equivalents/g d.m.) was observed with 50% aqueous ethanol (v/v), at 60˚C, and 3 h of extraction. From the obtained results, it can be concluded that the by-products of "Horchata de Chufa" are an important source of antioxidant bioactive compounds.

Keywords: horchata de chufa; tiger nuts; by-products; antioxidant capacity; flavonoids; phenolic compounds

1. Introduction

"Horchata de chufa" is a typical refreshing beverage from Spain, having a milky appearance, white color, and pleasant flavor. This drink is obtained from the tubers of the tiger nuts (*Cyperus esculentus* L.). The industrial production of horchata is of great economic importance for the Spanish food industry, and in particular for Valencian Community, representing an annual consumption of 50 million liters per year or having an estimated retail value of 60 million euros [1,2]. During the production, a high quantity of by-products is generated, which can represent up to 60% of the raw material [3].

Due to large production of by-products in the food industry [4–6], measures have been established for their waste management with the aim to reduce the environmental footprint and to promote further uses in other industrial processes as sustainable raw materials [7]. That is why the scientific community have shown an increased interest in horchata by-products [8].

By-products from the production of "horchata de chufa" can be separated into solid and liquid phases that have suitable compositions for different uses. Due to high content of dietary fiber, antioxidants, and bioactive compounds (polyphenols), the solid residue can be used for functional food production and tackling problem with widespread nutritional deficiencies [9]. Liquid by-product stands out due to its high content of prebiotics and antioxidant compounds, with good technological properties. For instance, water retention and emulsification capacities, while being adequate water-substitute for meat industries [3]. On the down side, Sánchez-Zapata et al. Indicated presence of high microbial load in tiger nuts, hence it is necessary to carry out a heat treatment before their addition to the food products [10].

One of the most common methods of processing in food industry is conventional extraction (CEx) by solvent systems, e.g., liquid–liquid or solid–liquid [8]. The CEx involves the use of organic solvents, many of them toxic, such as methanol, hexane or acetone. They potentially pose risks to public health and the environment [11,12]. Luckily a large number of acceptable solvents exist (e.g., ethanol), that are very suitable for the food production [13,14]. According to Mokrani and Madani [15], acetone is the best solvent for the extraction of proanthocyanidins and tannins, whereas ethanol is more efficient for the extraction of flavonoids and their glycosides [16]. Even though methanol is appropriate for extracting phenolic acids and catechin, it is a toxic solvent.

Polyphenols constitute a large group of bioactive compounds (BAC) [17,18]. For example, flavonoids are divided into different subgroups

depending on the degree of oxidation of the carbon rings, the main ones being flavonols, flavones, isoflavones, flavan-3-ols, flavanones, and anthocyanins [19]. In the group of non-flavonoids are phenolic acids, water-soluble tannins and stilbenes, with phenolic acids being the most common in dieting [20].

Although a large amount of tiger nuts are produced each year, optimization of a CEx to recover antioxidant BACs for industrial purposes from this matrix is rather scarce. Therefore, this study aimed to evaluate the influence of the solvent type, temperature, and time for recovery of the BACs from by-products remained after production of "horchata de chufa."

2. Materials and Methods

2.1. Chemical and Reagents

Acetone, sodium carbonate (Na2CO3), and sodium hydroxide (NaOH) were purchased from J.T.Baker, Deventer, Netherlands. ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)), aluminum chloride (AlCl3), catechin, ethanol p.a. (99.5%), Folin-Ciocalteau 1 N, gallic acid, sodium nitrite (NaNO2), potassium persulfate (K2S2O8) and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were obtained from Sigma-Aaldrich, Steinheim, Germany. Deionized H2O was purchased from Millipore, Bedford, MA, USA.

2.2. Samples

Tiger nuts (*Cyperus esculentus* L.) were purchased from a local supermarket in Valencia (Spain), with the designation of origin "Chufa de Valencia." Analyzed by-products in this study were obtained during the laboratory production of "Horchata de Chufa." Laboratory scale equipment was used to acquire the beverage. For this purpose, 250 g of tiger nuts from Valencia, previously soaked for 8 h, were weighed. Then,

the container was filled with 1 dm3 of distilled water, the filter vessel was placed, and the tiger nuts were poured. The hand blender was introduced and a slight pressure was applied with the mortar, after pressing the by-products that remained and were subsequently used.

In order to obtain a dehydrated by-product, it was placed in glass trays of a drying oven for 24 h at a constant temperature of 100˚C. Next, the percentage of moisture was determined by the loss of weight due to the evaporation of the water. The procedure was as follows: The porcelain crucibles were placed in the oven for 1 h. Then, it was taken out and left to cool in the desiccator for 20 min, and weighed. On analytical balance, 5 g of the by-products was weighed and placed in different crucibles, then dried in the oven at 100˚C for 24 h. After this, the crucibles were placed inside the desiccator for 20 min until a constant weight. The same procedure was used with fresh tiger nuts with two repetitions for each examination. The moisture was calculated by following Equation (1):

$$Moisture\ = \frac{(M1 - M2)}{M} \times 100$$

where: M1 = weight of the crucible with wet sample; M2 = weight of the crucible with dry sample; M = sample weight.

2.3. Extraction

For the determination of the total phenolic compounds, total flavonoids and total antioxidant capacity, a solid–liquid extraction was carried out. Firstly, 1 g of dehydrated by-product was weighed and 15 mL of the ethanol:water mixtures were added at different concentrations (0%, 25% and 50%, v/v), according to a previously established protocol [21]. Then, the beakers with the samples were placed and stirred on a plate with magnetic stirrer. The speed (RPM) was adjusted, to avoid spilling during the stirring. The samples were covered with aluminum foil to avoid the evaporation of the solvent during the extraction. In each of

the stirring plate's rows, the temperature was adjusted to 40, 50 and 60˚C. These conditions were set for all extractions with varying the extraction time (1, 2 and 3 h). The obtained samples (n = 27) were filtered and used for determination of the total phenolic compounds (TPC), total flavonoids (TF), and the total antioxidant capacity (TEAC). 2.4. Determination of Total Phenolic Compounds (TPC) to determine the TPC, a previously established method was used with some modifications [22], originally reported by Singleton et al. [23]. Briefly, 0.5 mL of extract was mixed with 4.5 mL of distilled water and afterwards 1 mL of the 2% sodium carbonate solution (w/v), and 0.25 mL of the Folin-Ciocalteau reagent (1 N) were added. The mixture was left to stand for 1 h at room temperatura in darkness. Subsequently, the absorbance was measured at 765 nm. The determination of TPC was performed by interpolating the values in a calibration line of the gallic acid standard (10 g/mL) at different concentrations between 0–5 g/mL. The results were expressed as mg equivalents of gallic acid (GAE)/100 g of dry matter. All spectrophotometric measurements were conducted on a Perkin-Elmer Lambda 2 UV/Vis spectrophotometer (Perkin-Elmer, Jügesheim, Germany). All determinations were carried out in duplicates.

2.5. Determination of Total Flavonoids (TF)

For TFs determination, the protocol established by Jara-Palacios et al. was used, with some modifications [24]. The AlCl3 method used consisted of the formation of chelates with orthodihydridoxylated, 3-hydroxylated and 5-hydroxylated flavanoids in basic medium. These chelates have a pink coloration that can be determined spectrophotometrically quantified at λ = 510 nm. In the analysis, 5 mL of diluted extract (1:5, v/v or 1:25, v/v) was mixed with 5 mL of distilled water, and 1 mL of 5% NaNO2 (w/v). Then, the closed mixture was left to stand at room temperature for 5 min. After this time, 1.5 mL of 10% AlCl3 (w/v) was added, and the mixture was left at the room temperature for

an additional 5 min. Afterwards, 5 mL of 1 M NaOH was added, and the mixture was brought up to 25 mL with distilled water. Then, the absorbance of the mixtures was measured with a wavelength of λ = 510 nm. The concentration of TF was established by calibration line prepared with the catechin standard (50 μg/mL), in concentrations between 0 and 10 μg/mL. The results were expressed as mg equivalents of catechin (CE)/100 g of dry matter.

2.6. Determination of Total Antioxidant Capacity (TEAC Method)

The total antioxidant capacity was measured by the modified TEAC method (Trolox equivalent antioxidant capacity) according to the protocol established by Roselló-Soto et al. [22]. The radical ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) was obtained by mixing 25 mL of 7 mM ABTS with 440 L of 140 mM potassium persulfate (K2S2O8). The mixture was left to stand at the room temperature for 12–16 h in darkness, while being stable for two days. For the preparation of the working solution, the radical ABTS was diluted with ethanol, until its absorbance was 0.70 0.02 at 734 nm. Then, 2 mL of the working ABTS solution was added to the cuvette and the initial absorbance (A0) was measured at a wavelength of = 734 nm. When the absorbance values were in required range, then, 0.1 mL of diluted extract (1:2, 1:5, and 1:10 v/v) was added, and the absorbance was measured at 20 min (Af). To calculate the inhibition percentage (%) of the samples, the following equation was used:

$$\% \text{ Inhibition} = (1 - A_f/A_0) \mathbf{x} 100$$

where A_0 is the absorbance at initial time and A_f is the absorbance obtained after 20 min.

The results were expressed as micromolar (M) Trolox equivalent (TE)/g of dry matter. In order to obtain the values in μM TE/g of dry matter for the samples, the values of inhibition percentage were

interpolated in a previously prepared calibration curve. This was done by using different concentrations of a Trolox® standard as x-axis, and the percentage of inhibition for these Trolox concentrations as y-axis.

2.7. Statistical Analyses

For the study of significant differences in the results obtained, an analysis of variance (ANOVA) was carried out. Pearson's correlations measured associations among the total antioxidant capacity, total phenolic compounds, and total flavonoids according to previous research [25]. For all statistical data, $p < 0.05$ was considered statistically significant. The statistical analysis was performed with the Statgraphics Centurion XVI® program (StatPoint Technologies, Inc., Warrenton, VA, USA).

3. Results and Discussion

3.1. Total Phenolic Compounds (TPC)

The TPC in the extracts obtained under different extraction conditions (i.e., temperature, time and different percentages of ethanol:water) was evaluated. From Figure 1 it can be seen that TPC values ranged from 2.86 to 16.02 mg GAE/100 g of dry matter, and were dependent on the temperatures, times, and concentrations of used ethanol. All of these three factors had significant effect ($p < 0.05$) on the content of TPC from extracts.

The highest value of phenolic compounds (16.02 mg GAE/100 g of dry matter) was observed at 60 ˚C with 25% (v/v) of ethanol in water, and an extraction time of 3 h. In fact, seen in Figure 1, the extraction efficiency for TPC increased with higher temperatures, judging by the values of 7.50, 7.80, and 14.46 mg GAE/100 g of dry matter, at 40, 50 and 60˚C, respectively. All of these were measured at the same concentration of ethanol and extraction time (25% ethanol, 2 h). These results may be

explained by the increase in the solubility of phenolic compounds at higher temperaturas with increased speed of diffusion which favors mass transfer phenomena [15,26]. However, this is outweighed by the polyphenolic thermal stability, as after maximum increases of temperature, their thermal degradation will occur in numerous plant material [26–30].

In a similar study by Yuan et al., for hazelnut shells, higher TPC was found with increased temperature, i.e., for 30 ˚C and 50 ˚C that had values of 801 mg and 1050 mg GAE/100 g of hazelnut shell, respectively [26]. This indicated that as the temperature increases, the surface tension and the viscosity of the solvent decreases, thus, favoring the inclusion of the solvent to matrix. Dorta et al. suggested that higher temperatures can affect antioxidant activity and reduce the stability of the phenolic compounds contained in the extracts [31]. On the contrary, Mokrani and Madani showed that TPC yield in peaches decreased, when the temperature increased from 25 to 70 ˚C with values of 363 mg and 284 mg of GAE/100 g of dry weight, respectively [15].

Regarding the experimental ethanol concentrations, increased TPC content was observed with higher concentrations of ethanol, although extracting with 25% ethanol was not statistically different from extracting with 50% ethanol (Figure 1). In other words, up to an 82% increase of TPC was observed with 50% ethanol vs. aqueous extracts (no ethanol), at 60 ˚C and 2 h of extraction time. However, this trend was not as clear after 1 h of extraction at 40 and 50˚C, since 25% ethanol improved extraction of TPC, compared to those from 50% ethanol. In a similar way, this tendency was also observed at a temperature of 60˚C, with an extraction time of 3 h. Therefore, the use of ethanol in mixtures with water contributed towards a polar medium and improved the extraction of phenolic compounds.

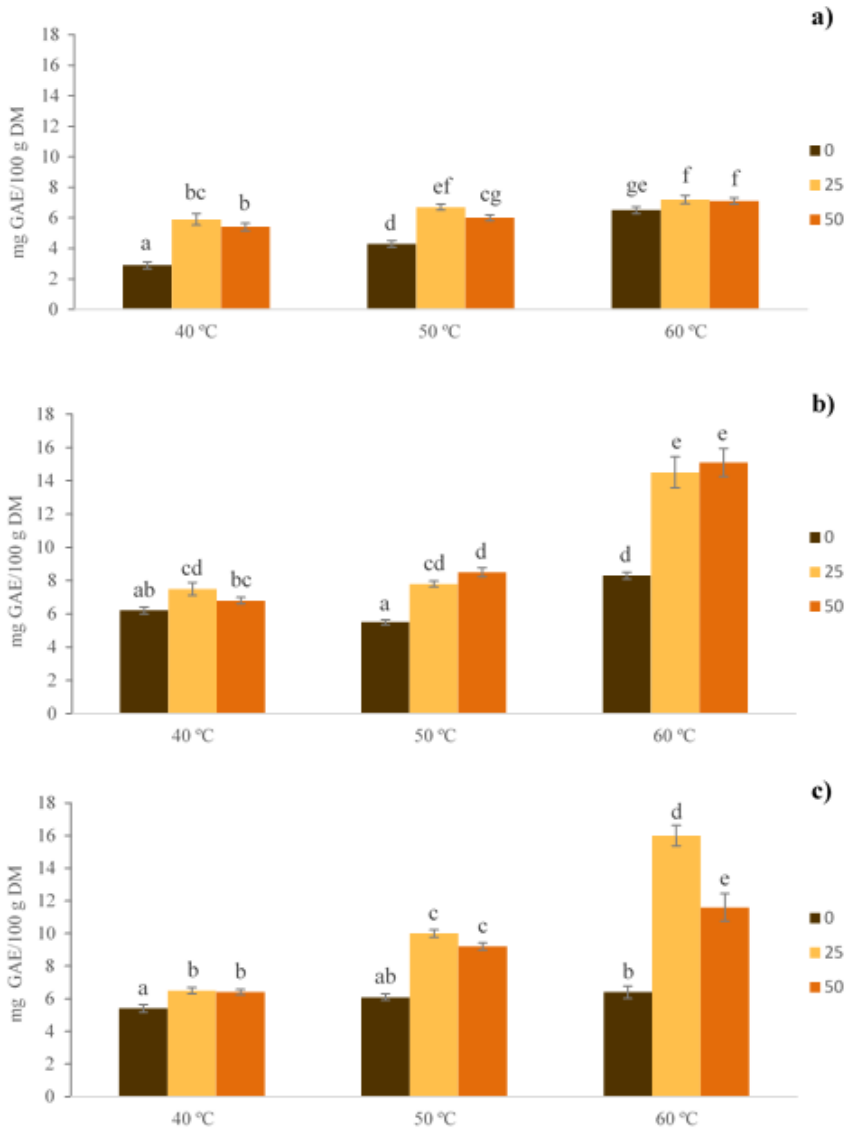

Figure 1. Content of total phenolic compounds, after extraction with different hydroethanolic mixtures (0%, 25% and 50% ethanol:water, v/v); temperature (40, 50 and 60˚C); and extraction time. (a) 1 h; (b) 2 h; and (c) 3 h. Different letters show statistically significant differences ($p < 0.05$) for temperaturas and concentrations of ethanol used. DM: dry matter.

The studies carried by Naczk and Shahidi [32], showed that the solubility of phenolic compounds depends on the polarity of solvent, as well as the formation of insoluble complexes, and the interaction with other components from foods. Yuan et al. used methanol, ethanol and acetone in concentrations of 20%, 50% and 80% for the extraction of TPC in the hazelnut shells [26]. The highest values of TPC were extracted with 50% of acetone. Socaci et al. showed that the most effective solvent for the extraction of TPC in beer by-products was acetone:water at 60% (v/v), with values of 114 mg GAE/100 g of dry weight [33]. Meneses et al. also obtained similar results in beer by-products, having value of 990 mg GAE/100 g of dry weight when 60% (v/v) acetone was used [34].

In another study, Mokrani and Madani evaluated the influence of the solvent type at different concentrations in the extraction of TPC from peach [15]. The optimal extraction was observed after using 60% acetone (v/v), with a value of 363 mg GAE/100 g of dry weight. This was as twice as high as the extraction made by water or with 60% ethanol (v/v), when it equaled to 182 mg and 178 mg GAE/100 g of dry weight, respectively. Tournour et al. reported the TPC from various grape marcs in a range of 6.93–13.17 mg GAE/100 g of dry residue, that were extracted with 80% aqueous etanol (v/v) over 48 h [35]. Caldas et al., used ethanol in concentrations of 8%, 20%, 50%, 80%, and 92% for the extraction of TPC from grape skins [36]. Here, in the comparison to other concentrations, results showed a greater quantity of phenolic compounds for extracts with 50% ethanol (v/v).

The extraction time significantly influenced the amount of obtained TPC. The concentration of phenolic compounds increased with prolonged extraction, when using 25% ethanol (v/v) at T = 60 ˚C. For 1 h of extraction, the value was 7.22 mg GAE/100 g of dry matter, whereas for 2 h and 3 h they were 14.46 mg and 16.02 mg of GAE/100 g of dry matter, respectively. Studies conducted by Yuan et al., showed similar results after extracting TPC from hazelnut shells [26].

TPC obtained from tiger nut samples from Nigeria had values of 115.70 mM GAE/100 g of tiger nut in roasted tubers [37]. These values were higher, compared to those obtained in raw or dried nuts, and equaled to 80.70 mM GAE/100 g of chufa. Koubaa et al., used alternative methods for the extraction of TPC in tiger nuts' oil [38]. The results obtained by these authors showed values of 4.53 mg and 6.21 mg GAE/100 g of oil for supercritical fluids (SC-CO_2) extraction conducted at 20 and 30 MPa. Similar results were obtained after mechanical expression (ME), using similar treatment conditions, where obtained values were 4.71–5.29 mg GAE/100 g of oil. In this sense, Ezeh et al. also obtained values of 16.50 mg GAE/100 g of oil after applying SC-CO_2 [39]. Mokrani and Madani, obtained the optimum TPC after 3 h extraction from the peach samples, and with a value of 363 mg GAE/100 g of dry weight. However, the use of longer times did not improve the extraction of phenolic compounds, indicating that too long of an extraction time can increase phenolic oxidation [15].

3.2. Total Flavonoids

The content of TF in the extracts was between 1.80 and 30.09 mg CE/100 g of dry matter, depending of the used temperature, time, and concentration of ethanol. The optimal value of extraction was observed at 50 ˚C, with 25% of ethanol, and 3 h of extraction time. Both, the time and percentage of ethanol had a significant effect on the TF content, while temperature showed no influence (Figure 2). In general, higher values were with 25% of ethanol, compared to the mixtures without ethanol and with 50% ethanol. These results can be explained by different polarities of the solvent, since the mixtures of ethanol:water, have a greater polarity and therefore greater efficiency in the extraction of these components [31].

The researchers evaluated the effect of the solvent in determination of TF in beer by-products [33]. The highest values of TF was obtained after

using a mixture of ethanol:water (80:20, v/v) (≈461 mg equivalents of quercetin (QE)/100 g of dry weight), compared to the aqueous extraction (2 mg QE/100 g of dry weight) under similar extraction conditions. Moreover, using ethanol in the range of those used in this study (40–60% and 60:40, v/v, ethanol:water), the recovery yield was ≈50 and 130 times higher than the aqueous extraction, respectively. Another study, showed that 60% ethanol (v/v) was the most efficient (57 mg QE/100 g of dry weight) for TF extraction from peach samples, obtaining values ≈3.3-fold higher, as compared to the aqueous extraction (17 mg QE/100 g of dry weight) [15]. Similar results for TF were obtained in beer by-products, with a value ≈50 mg QE/100 g of dry weight after using a mixture of ethanol:water (60:40, v/v) (≈50 mg QE/100 g of dry weight), obtaining ≈3-fold higher values, compared to the aqueous extraction under similar extraction conditions [33].

Regarding the influence of temperature on TF, a greater content of TF at 50 vs. 60 ˚C was observed, reaching an increase of up to 17% during 3 h of extraction (Figure 2). However, these results may be caused by higher temperatures that reduced the stability of these compounds. The studies on peach samples showed an increased yield of TF with the temperature increase from 25 to 60˚C, and values of 38 to 81 mg QE/100 g of dry weight [15]. However, temperatures above 60˚C reduced the value of TF in the samples.

In general, there is no clear trend in the results regarding the time for the extraction of TF. However, the extraction time of 3 h (25% ethanol (v/v); T = 50 ˚C) had the most TF, with values of 30.09 mg CE/100 g of dry matter, as compared to 7.20 and 5.87 mg CE/100 g of dry matter, after 1 and 2 h of extraction, respectively.

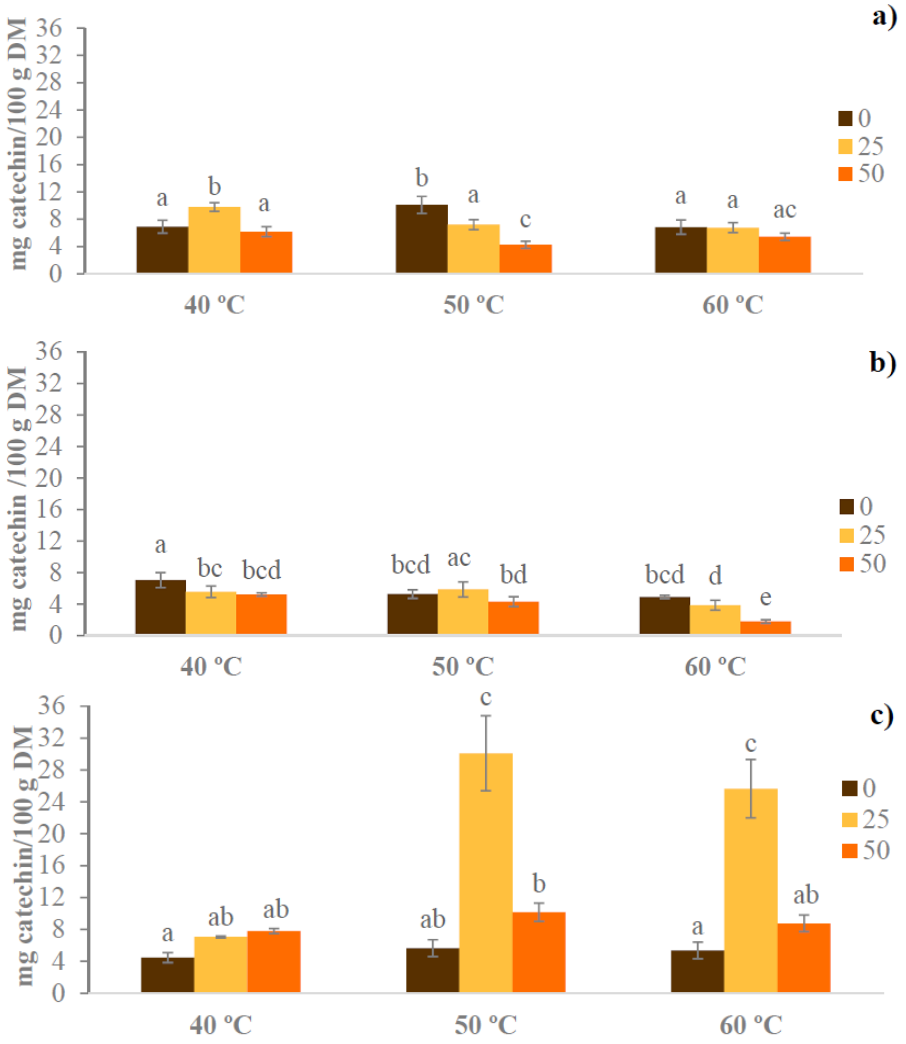

Figure 2. Total flavonoid content, after extraction with different hydroethanolic mixtures (0, 25 and 50% ethanol:water, v/v), temperature (40, 50 and 60˚C) and extraction time: (a) 1 h; (b) 2 h; and (c) 3 h. Different letters show statistically significant differences ($p < 0.05$) for temperatures and concentrations of ethanol used.

3.3. Total Antioxidant Capacity (TEAC)

Analysis of variance showed that temperature, ethanol percentage and time had a statistically significant effect ($p < 0.05$) on the total antioxidant capacity in the extracts.

As seen in Figure 3, generally, the antioxidant capacity showed a tendency to increase with higher concentrations of ethanol. This may be due to the fact that the use of ethanol in mixtures with wáter created a more polar medium, thus, favoring the extraction of these compounds [34].

The highest values of TEAC (1759.81 μM Trolox/g of were observed with the highest ethanol concentration (50%, v/v), longest extraction time (3 h), and temperature T = 60 ˚C.

The application of high temperature can favor the extraction of antioxidant compounds and increase the solubility.

3.4. Relationship between Total Antioxidant Capacity, Total Phenols and Total Flavonoids

To identify whether there was any correlation between total antioxidant capacities, total phenols and total flavonoids, a Pearson's correlation analysis was performed (Table 1).

Table 1. Pearson's correlation coefficients between antioxidant capacity (TEAC), phenolic compounds (TPC) and total flavonoids (TF).

	TPC	**TF**
TEAC	0.712 *	0.194
TPC	-	0.314*

* Statistically significant correlations

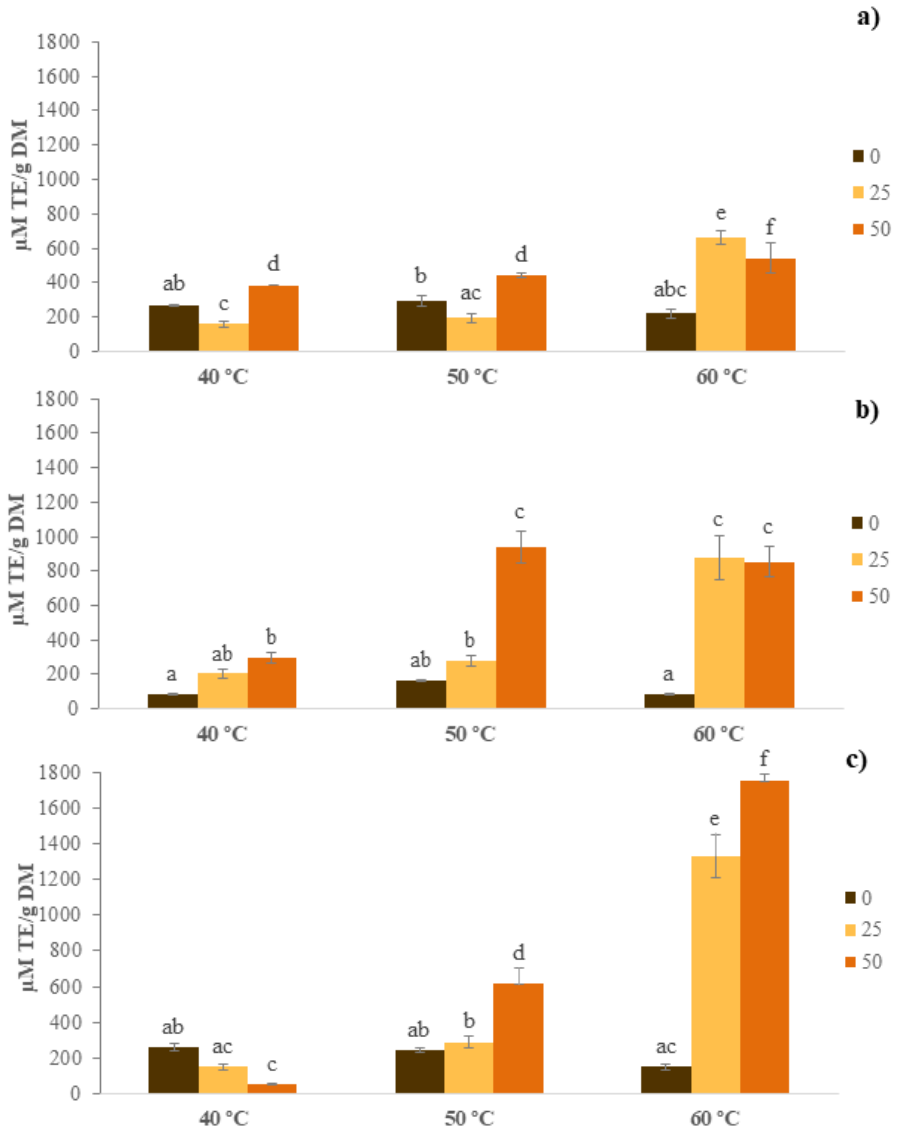

Figure 3. Total antioxidant capacity µM Trolox equivalents (TE)/g of dry matter (DM), after extraction with different hydroethanolic mixtures (0, 25 and 50% (v/v) ethanol:water,), temperature (40, 50 and 60°C) and extraction time: (a) 1 h; (b) 2 h; and (c) 3 h. Different letters show statistically significant differences ($p < 0.05$) for temperatures and concentrations of ethanol used.

As it can be seen in Table 1, a strong positive correlation was observed between TEAC and TPC ($r = 0.712$, $p < 0.05$). Medium positive pairwise correlations were observed for TPC and the TF ($r = 0.314$, $p < 0.05$). However, the correlations between TF and TEAC were not significant ($r = 0.194$, $p > 0.05$). Similar results were obtained in peach extracts, where positive correlations between total phenolic compounds and antioxidant capacity ($r = 0.76$, $p < 0.001$) were reported. On the other hand, the correlation between TF and antioxidant capacity was negative ($r = -0.82$) [15].

The study by Socaci et al. showed a positive correlation between the DPPH antioxidant activity and the content of polyphenols/flavonoids in beer by-products [33]. Here, coefficients were much stronger and equaled to 0.908 for flavonoids, and 0.980 for phenolic compounds. Tournour et al. obtained similar results, with a positive correlation between phenolic compounds and DPPH method ($r = 0.944$) in wine by-products [35].

4. Conclusions

From the results of the present study, it can be concluded that by-products from the production of "horchata de chufa" are an important source of bioactive antioxidants. Moreover, temperature, time, and ethanol concentration presented a statistically significant influence on the recovery of total BACs. Higher temperatures increased the extraction efficiency of TPC, while in the same way, etanol concentrations and prolonged extraction times improved TPC yields. Maximum extraction values of 16.02 mg GAE/100 g of dry matter were observed after using 25% ethanol (v/v), at 60˚C with an extraction duration of 3 h.

The concentration of ethanol and time showed a significant influence on the recovery of total flavonoids ($p < 0.05$). Generally, TF values were higher after using 25% ethanol (v/v), as compared to 50% of ethanol (v/v) and its complete absence. The TF values increased with

temperature and reached the optimum value of 30.09 mg CE/100 g of dry matter at 50˚C, extracted with 25% ethanol (v/v) after 3 h of extraction.

A statistically significant influence of temperature and ethanol concentration was found for the recovery of antioxidant compounds ($p < 0.05$). In general, the antioxidant capacity will increase with higher concentrations of ethanol. This same trend was observed with respect to the time and temperature. The maximum yield of 1759.81 μM Trolox/g of dry matter was found with 50% etanol (v/v), 60˚C and an extraction time of 3 h. TAEC and TPC had positive correlation similar to TPC and the TF. Further studies of by-products from the "horchata" should focus on the use of alternative methods for the extraction of antioxidant compounds aiming to reduce the time, usage of minimally toxic (in)organic solvents, and lower the processing temperatures.

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5.4. Evaluating the impact of supercritical-CO_2 pressure on the recovery and quality of oil from "horchata" by-products: Fatty acid profile, α-tocopherol, phenolic compounds, and lipid oxidation parameters

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Evaluating the impact of supercritical-CO_2 pressure on the recovery and quality of oil from "horchata" by-products: Fatty acid profile, α-tocopherol, phenolic compounds, and lipid oxidation parameters

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A R T I C L E I N F O

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A B S T R A C T

The effect of supercritical carbon dioxide (SC-CO_2) (10–40 MPa) and conventional extraction (CE) to recover oil from by-products obtained during "horchata" production was assessed. To evaluate both extraction techniques, the fatty acid composition, polyphenols, α-tocopherol,

antioxidant capacity and lipid oxidation parameters of the extracts obtained were analysed. A linear relationship between extraction pressure and oil yield was observed. However, the highest oil yield was obtained under conventional extraction. The by-products from "horchata" presented a profile where monounsaturated fatty acids (MUFA) were the predominant, representing ≈ 70% of total fatty acids. The amount of saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA) was higher and MUFA lower at 10 MPa samples compared to the oils extracted using SC-CO_2 at 20, 30 or 40 MPa, where no differences were detected. The content of α-tocopherol was significantly higher after SC-CO_2 treatments compared to conventional extraction, independently of the applied treatment. On the other hand, the values of phenolic compounds and total antioxidant activity (TAC) increased as the pressure conditions of the SC-CO_2 extraction increased, presenting a linear adjustment of the data. Regarding lipid oxidation, the lower oxidation indexes were obtained when the SC-CO_2 pressure increased. Finally, our results confirmed that the application of SC-CO_2 could be a potential alternative to conventional extraction in order to obtain oils from "horchata" by-products rich in high-added value compounds without the use of organic solvents which can be toxic.

1. Introduction

"Horchata de chufa" is the nutritious product of milky aspect, obtained mechanically by aqueous extraction using pressure from the tiger nuts (*Cyperus esculentus* L.) (BOE, 1988; Roselló-Soto et al., 2018). "Horchata" is sold not only in Spain but also in France, Dominican Republic, Mexico, Panama and USA (Martín-Esparza & González-Martínez, 2016), with an important impact from an economic point of view.

During "horchata" manufacturing, a great content of by-products are generated. "Horchata" by-products have been traditionally considered as an industrial waste with no commercial value. However, among other

applications, these by-products could constitute a source of oil with potential to be used for food and/or cosmetics, among other applications (CRDO, 2009; Roselló-Soto et al., 2018).

Moreover, "horchata" by-products are also a good source of highadded value compounds such as nutrients (vitamins, minerals, proteins and carbohydrates) and bioactive compounds (eg. polyphenols). Some of these compounds have been associated with beneficial properties on health, including the blood circulation activation as well as the prevention of heart diseases and thrombosis (Chukwuma, Obioma, & Christophe, 2010), and the reduction in the risk of appearing colon cancer (Adejuyitan, Otunola, Akande, Bolarinwa, & Oladokun, 2009).

Therefore, "horchata" by-products could be used as a source of natural food additives and/or for the development of nutraceuticals (Gil-Chávez et al., 2013; Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018). In addition, these by-products also contain a great fiber amount, which can be employed in the manufacture of new products, thus responding to society's demand for new foods that reduce chronic diseases and achieve optimal health (Granato, Nunes, & Barba, 2017).

In this way, the oils and natural compounds obtained can be incorporated into different dietary matrices. This could have a potential positive effect on health and well-being and on the environment (Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018). Moreover, this strategy is also in full correspondence with the current legislative framework developed in several countries (Anonymous, 2006, 2008). These regulations establish as a priority the recycling and valorisation of by-products against their elimination. All this in order to preserve natural resources, protect the environment and consumer health, through the prevention or reduction of the global impacts of the use of resources and the improvement of the effectiveness of such use.

Traditionally, the use of extraction by mechanical expression in batch (less content of material used during a short period of time) or

continuously have been used to obtain oil from different matrices of plant origin (eg. palm oil). Cold pressing combined with n-hexane is the main employed methodology to obtain oil from fresh tiger nuts. However, cold pressing alone cannot extract all the oil (Ali Rehab & El Anany, 2012; Puértolas, Koubaa, & Barba, 2016; Yeboah, Mitei, Ngila, Wessjohann, & Schmidt, 2012). Likewise, the use of n-hexane has been questioned due to environmental and safety problems (Lasekan & Abdulkarim, 2012).

The need to obtain more ecological, sustainable and viable processes has led the food industries and scientists to develop alternative processes according to the concept of "green" or "ecological" extraction (Koubaa et al., 2015). This concept refers to the use of renewable plant resources and "green" solvents, reduction of energy consumption and operations, production of extracts with high quality and purity (nondenatured and biodegradable) and the generation of by-products instead of waste (Chemat, Vian, & Cravotto, 2012).

In this line, previous studies evaluated the potential of extraction of oil rich in antioxidant bioactive compounds from tiger nuts assisted by supercritical carbon dioxide (SC-CO_2), obtaining good results (Koubaa et al., 2015; Lasekan & Abdulkarim, 2012). In fact, the extraction with supercritical fluids has been used in different food and agricultural applications, fuels, analysis/chromatography, pharmaceutical products, environmental contaminants, metal extractions and pesticides.

During the last decades, this technology has acquired a great relevance for the extraction of nutritionally valuable and bioactive compounds from different matrices (Barba, Grimi, & Vorobiev, 2014). This technique can decrease (close to zero) the use of toxic organic solvents. In addition, the reduction of the main supercritical fluid (CO_2) is achieved without waste, giving an extract free of solvent, and the operation can be controlled for recycling. However, depending on the targeted compounds to extract, a great extraction time could be required compared to other techniques. The use of modifiers to increase extraction and selectivity is common.

Despite the great potential of "horchata" by-products as source of oil with high content in antioxidant bioactive compounds, at this stage of development, there is no available information about the impact of SC-CO_2 on "horchata" by-products oil recovery. Moreover, fatty acid composition, polyphenols, α-tocopherol, antioxidant capacity and lipid oxidation parameters were not studied.

For instance, this study is of great importance as traditionally tiger nuts by-products have been used only as source of fiber (Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018) and this work could open the door to its useful application as oil and lipophilic compounds source. Therefore, the main objectives of the present work are i) to compare the impact of SC-CO_2, and conventional extraction (CE) to recover oil from by-products obtained during "horchata" production, and ii) to evaluate the fatty acid composition, polyphenols, α-tocopherol, antioxidant capacity and lipid oxidation parameters of the extracts obtained after SC-CO_2 and CE.

2. Material and methods

2.1. Samples

"Horchata" by-products were provided by the Regulatory Council D.O. Tiger nut of Valencia (Valencia, Spain). They were obtained after a conventional process for obtaining "horchata" from tiger nuts (*Cyperus esculentus*) with denomination of origin "Chufa de Valencia". They were dried in an air-circulating oven (Memmert UFP 600, Schwabach, Germany) at 60 °C for 72 h. This material was ground using a homogenizer (IKA, Mod. A11 basic) and sieved for uniformity of particle size and vacuum packed.

2.2. Chemicals and reagents

The n-hexane (HPLC grade; 96%), Folin-Ciocalteau, phenol reagent,

2,2,4-trimethylpentane (reagent grade;>99.5%), 2-propanol (HPLC grade; 99.9%), sodium metal (extra pure; 99%), ethanol, metanol (reagent grade; 99.9), gallic acid monohydrate (extra pure; 99.5%), sodium hydrogen carbonate (reagent grade; 99.7%) were obtained from Scharlau (Barcelona, Spain). Chloroform (Normapur; 99%) and glacial acetic acid (Normapur) were obtained from Prolabo (VWR; Barcelona, Spain). Potassium iodide (PA;>99.5%), FAME 37 Mix, cis-vaccenic acid (> 97%), α-tocopherol, ABTS radical and Trolox® were supplied from Sigma-Aldrich (Madrid, Spain). p-anisidine (reagent grade; 99%) was obtained from Acros organics (Madrid, Spain).

2.3. Supercritical CO_2 and conventional extractions

The supercritical carbon dioxide (SC-CO_2) extraction was used to recover chufa oil from "horchata" by-products. Fifty grams of "horchata" by-products were used to carry out the extractions assisted by supercritical carbon dioxide system (SFE-1000; Thar Process Inc. Pittsburgh, PA), applying different pressures (10, 20, 30 and 40 MPa) at 40 °C for 2 h. The CO_2 flow was adjusted at 20 g/min and the oil was recovered with 30 mL of ethanol. The conditions were selected based on a previous study (Koubaa, Barba, et al., 2015). All extractions were carried out in triplicate.

The conventional extraction was carried out following the Folch, Lees, and Sloane Stanley (1957) procedure with some modifications. Fifty grams of dried "horchata" by-products were re-hydrated with 15 mL of NaCl (1%). Then, the mixture was mixed with 60 mL of $CHCl_3$:MetOH (2:1) at 10,000 rpm with one UltraTurax during 2 min. Subsequently, 20 mL of NaCl (1%) were added and homogenized again for 30 s. Samples were centrifuged at 3,000 ×g for 3 min and the $CHCl_3$ layer (containing the oil) was recovered and dried with sodium sulphate anhydrous. Finally, $CHCl_3$ was removed using a rotatory evaporator at 55 °C and oil was stored at −30 °C until analysis.

2.4. Determination of the total phenolic content

Total phenolic compound determinations were performed according to the method previously described by Singleton, Orthofer, and Lamuela-Raventos (1998) with some modifications (Koubaa, Barba, et al., 2015). Results were expressed in gallic acid equivalents (mg GAE/100 g extract). Nine gallic acid concentrations were used to build the calibration curve. Samples (500 µL) were added of 2.5 mL Folin-Ciocalteu reagent (10%) and 2 mL of saturated Na_2CO_3 solution (7.5%). Reaction occurred in a water bath at 50 °C for 15 min, and absorbancies were read in a UV spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan) at 760 nm.

2.5. Fatty acids

Total fatty acids were quantified according to the method previously described by Domínguez, Borrajo, and Lorenzo (2015). Three hundred microliters of ethanolic extract (SC-CO_2 extraction) or fifty milligrams of chufa oil (Folch extraction) from "horchata" by-products were used to determine the fatty acid profile. For the fatty acids transesterification, 4 mL of a sodium methoxide (2%) solution were added to the oil samples, vortexed every 5 min during the 15 min at room temperature, then 4 mL of a H_2SO_4 solution (in methanol at 33%) were added, vortexed for a few seconds and vortexed again before adding 2 mL of distilled water. The organic phase (containing fatty acid methyl esters) was extracted with 2.5 mL of hexane.

Separation and quantification of the FAMEs was carried out using a gas chromatograph (GC-Agilent 7890B; Agilent Technologies Spain, S.L., Madrid, Spain), equipped with a flame ionization detector and an autosampler PAL-RTC 120. Moreover, a Supelco SP-2560 fused silica capillary column (100 m, 0.25mm i.d., 0.2 µm film thickness; Supelco Inc., Bellafonte, PA, USA) was also used. The chromatographic conditions described by Domínguez et al. (2015) were followed. Individual FAMEs

were identified by comparing their retention times with those of authentication standards, and the results were expressed as g/100 g of total fatty acids identified.

2.6. Vitamin E (tocopherols determination)

In order to identify and quantify tocopherols, the method previously described by Cilla et al. (2012), with some modifications, was used. One hundred milligrams of chufa oil from "horchata" by-products were dissolved in 1.8 mL of n-hexane and then filtered through a 0.45 μm hydrophobic membrane into an amber screw-cap vial with teflón septum.

The HPLC systems used was an Alliance 2695 model (Waters, Milford, USA) equipped with a 2475 Multi-λ Fluorescence Detector (Waters Milford, USA). Empower 3TM advanced software (Waters, Milford, USA) was used to control system operation and results management. The analysis of tocopherols was performed using a normalphase silica column (SunFireTM Prep Silica, 4.6mm ID × 250 mm, 5 μm particle size, Waters, Milford, MA, USA). The solvent (2% v/v 2-propanol in n-hexane) flow rate was 1 mL/min, the run last for 15 min, and the temperature of the column oven was adjusted at 30 °C. From each sample, 10 μL was injected and the detection of tocopherols was carried out using a fluorescence detector (λ-excitation 290 nm/λ-emission 330 nm). The content of tocopherols in chufa oil from "horchata" by-products was calculated, in duplicate for each sample, based on external standard technique, from a standard curve of peak area vs. concentration.

2.7. Oxidation parameters

2.7.1. Peroxide value

Peroxide value was determined following the AOAC (2007). In order to dissolve the oil, 10 mL of trichloromethane were added to 0.5 g of oil sample. Then, 15 mL of acetic acid and 1 mL of saturated aqueous solution

of potassium iodide were added. The sample was stirred one minute with a light rotation movement and was kept five minutes under darkness conditions. Once incubation was finished, 75 mL of distilled water were added, and the sample was vigorously shaken. Finally, free iodine was titrated with sodium thiosulfate 0.01 N in an automatic titrator (905 Titrando, Metrohm Ion Analysis, Herisau, Switzerland). Peroxide value, expressed as milliequivalents O2/kg oil.

2.7.2. p-anisidine

The determination of p-anisidine value of the oil samples was carried out following an IUPAC method (IUPAC, 1987). The oil samples (0.5 g) were dissolved in isooctane in a volumetric flask of 25 mL. Then, the sample was mixed with a p-anisidine solution in acetic acid (0.25% w/v) for 10 min to produce a coloured complex. The samples with and without p-anisidine solution were measured in an UV spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan) at 350 nm.

2.7.3. Totox value

The Totox value indicates the overall oxidation state of the oil. This value was calculated according to the formula:

$$TV = AV + 2PV$$

where TV is the totox value, AV is the anisidine value and PV is the peroxide value.

2.8. Measurement of the antioxidant activity

The determination of the total antioxidant capacity was carried out according to the method previously described by Re et al. (1999), based on the capacity of a sample to inhibit the ABTS radical cation compared with a reference antioxidant standard (Trolox®). The radical was generated using 440 µL of potassium persulfate (140 mM). The solution was diluted with ethanol until obtaining an absorbance value of 0.70 ±

0.02 at 734 nm. Once the radical was obtained, 2 mL of ABTS•+ were mixed with 100 μL of appropriately diluted sample and the absorbance was measured at 734 nm for 20 minmM TE. The values of percentage of inhibition obtained were interpolated in a calibration curve of Trolox® and the results were expressed in milliMolar of Trolox equivalents (mM TE).

2.9. Statistical analysis

A total of 15 samples (five different extraction conditions x three replicates) were used to estimate the statistical differences in extraction yield, phenolic content and antioxidant activity among the extraction conditions used in "horchata" by-products. These parameters were examined using an ANOVA test. Least-squares means were compared among extraction conditions using the Duncan's post hoc test (significance level $P < 0.05$). The values were given in terms of mean values±standard deviations. All statistical analysis were performed using IBM SPSS Statistics® 21 software.

3. Results and discussion

3.1. Oil extraction

Firstly, the impact of SC-CO_2 pressure on oil yield from "horchata" by-products was evaluated and compared to the yield obtained after using conventional extraction. As can be seen in Fig. 1, the oil yield increased from 0.61g/100 g of "horchata" by-products after applying 10 MPa compared to 7.36 g/100 g of "horchata" by-products after using 40 MPa. In fact, a linear relationship between extraction pressure and oil yield (g oil/100 g of by-product=2.192·Pressure−1.8479; R^2=0.9736) was found. This result is in close agreement with the classical observations after using SC-CO_2 extractions, where multiple studies concluded that pressure had a positive and linear effect on oil yield. In this line, Lasekan and

Abdulkarim (2012) and Koubaa, Barba, et al. (2015) obtained similar findings when they evaluated the impact of SC-CO_2 to extract oil from tiger nuts. Similar results were also found after applying SC-CO_2 (10–50 MPa) in milk thistle (Ben Rahal, Barba, Barth, & Chevalot, 2015), grape seeds (Rombaut et al., 2014), and rapeseed hulls (Mhemdi, Koubaa, El Majid, & Vorobiev, 2016). The increase in oil yield when pressure was increased could be related to an increased density of CO_2 with pressure, thus resulting in high oil solubility (Mhemdi et al., 2016; Xu, Gao, Liu, Wang, & Zhao, 2008). For instance, it is well known that pressure affects the density of CO_2, and in the present work the operational temperature was constant (40 °C), thus the density of CO_2 at this temperature varies between 0.629 g/L (10 MPa) and 0.956 g/L (40 MPa) (NIST, 2018).

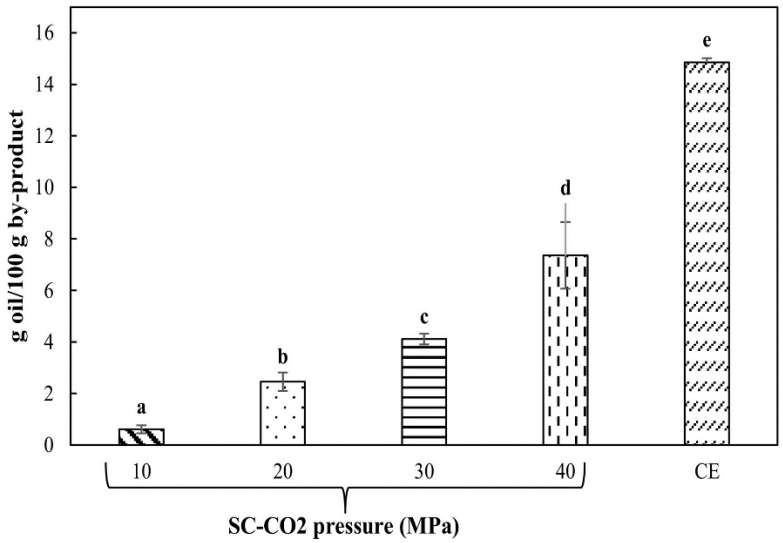

Fig. 1. Oil extraction (g/100 g) from "horchata" by-products assisted by supercritical (SC)-CO_2 at different pressures (10–40 MPa) and conventional extraction (CE).

Besides the aforementioned, the conventional extraction showed the highest yield oil values (14.85 g/100 g of "horchata" by-products), which

confirmed that the use of organic solvents increased the oil yield. Similar results were obtained by Ndayishimiye, Getachew, and Chun (2017) in citrus seed, who also observed that conventional extraction (using n-hexane) doubled the oil yield compared to SC-CO_2 extraction (20 MPa; 45°C), as occured in the present study.

3.2. Impact of SC-CO_2 and conventional extraction on fatty acids

The fatty acid profiles of oil from "horchata" by-products extracted using SC-CO_2 at different pressures (10–40 MPa) and conventional extraction (Folch) are shown in Table 1. Chomatographic analysis revealed that the predominant fatty acids in the oils obtained were C18:1n-9 (62.4–69.0%), C16:0 (13.8–17.9%), C18:2n-6 (10.9–12.1%) and C18:0 (3.0–4.3%). Therefore, the oils obtained from "horchata" byproducts presented a profile where monounsaturated fatty acids (MUFA) were the predominant, representing ≈70% of total fatty acids. Regarding polyunsaturated fatty acids (PUFA), the C18:2n-6 was the most representative fatty acid, although the C18:3n-3 fatty acids were also detected, representing 0.47–0.79% of the total fatty acids. These findings are in agreement to the fatty acids profile of the tiger nuts described in other studies (Ezeh & Gordon, 2016; Lasekan & Abdulkarim, 2012; Sánchez-Zapata, Fernández-López, & Angel Pérez-Alvarez, 2012; Yeboah et al., 2012) who reported values of C16:0 between 13.5 and 16.6%, C18:0 from 3.2 to 6.3%, C18:1n-9 between 65.5 and 72.6%, C18:2n-6 from 8.9 to 13.4% and C18:3n-3 between not detected and 0.4%.

C18:1n-9 can provide nutritional and dietary values, thus being interesting for its use for cardiovascular and skin diseases (Calani, Brighenti, Bruni, & Del Rio, 2012). Moreover, it should be noted the important content of C18:2n-6, an essential fatty acid of omega-6 family involved in several physiological functions (Denis, Potier, Vancassel, Heberden, & Lavialle, 2013). Therefore, being the oils obtained from

"horchata" by-products a useful tool in the development of new functional foods or nutraceuticals.

Table 1. Fatty acid content after supercritical fluid extraction (SC-CO_2) and conventional extraction techniques.

Fatty acids	**SC-CO_2 pressure (MPa)**				**Conventional Extraction**
	10	20	30	40	
C14:0	0.29±0.04^{a}	0.13±0.01^{b}	0.10±0.01^{bc}	0.09±0.01^{c}	0.08±0.00^{c}
C16:0	17.97±1.30^{a}	14.82±0.04^{b}	14.30±0.07^{b}	13.85±0.14^{b}	14.17±0.38^{b}
C16:1*n-7*	0.54±0.02^{a}	0.37±0.01^{b}	0.33±0.02^{bc}	0.31±0.01^{cd}	0.29±0.01^{d}
C17:0	nd.	0.09±0.09	0.09±0.01	0.08±0.00	0.08±0.00
C17:1*n-7*	nd.	0.08±0.00^{a}	0.07±0.00^{ab}	0.06±0.00^{b}	0.06±0.00^{b}
C18:0	4.32±0.52^{a}	3.09±0.06^{bc}	3.07±0.09^{b}	3.05±0.05^{b}	3.50±0.09^{c}
C18:1*n-9*	62.42±1.93^{a}	67.42±0.30^{b}	68.33±0.14^{b}	69.08±0.35^{b}	67.34±0.88^{b}
C18:1*n-7*	1.12±0.02^{a}	1.02±0.02^{b}	1.03±0.02^{b}	1.03±0.00^{b}	1.31±0.05^{c}
C18:2*n-6*	12.11±0.03^{a}	11.60±0.17^{b}	11.22±0.03^{bc}	10.94±0.26^{c}	11.29±0.30^{bc}
C20:0	0.44±0.04^{a}	0.46±0.00^{a}	0.46±0.02^{a}	0.52±0.02^{b}	0.63±0.01^{c}
C20:1*n-9*	nd.	0.24±0.00	0.26±0.00	0.26±0.00	0.31±0.01
C18:3*n-3*	0.79±0.05^{a}	0.56±0.02^{b}	0.53±0.02^{bc}	0.47±0.02^{cd}	0.47±0.02^{d}
C22:0	nd.	nd.	0.08±0.00	0.10±0.00	0.13±0.01
C24:0	nd.	0.13±0.00	0.13±0.00	0.15±0.01	0.25±0.01
SFA	23.02±1.91^{a}	18.72±0.10^{b}	18.23±0.16^{b}	17.83±0.06^{b}	18.83±0.49^{b}
MUFA	64.08±1.92^{a}	69.12±0.29^{b}	70.02±0.11^{b}	70.75±0.34^{b}	69.34±0.81^{b}
PUFA	12.89±0.02^{a}	12.16±0.19^{b}	11.75±0.05^{bc}	11.42±0.28^{c}	11.81±0.32^{bc}
n-3	0.79±0.05^{a}	0.56±0.02^{b}	0.53±0.02^{bc}	0.47±0.02^{c}	0.52±0.02^{bc}
n-6	12.11±0.03^{a}	11.60±0.17^{b}	11.22±0.03^{bc}	10.94±0.26^{c}	11.29±0.30^{bc}
n-6/n-3	15.41±1.03^{a}	20.56±0.48^{b}	21.22±0.64^{bc}	23.18±0.64^{d}	21.81±0.35^{c}

SFA: Saturated fatty acids; MUFA: Monounsaturated fatty acids; PUFA: Polyunsaturated fatty acids; nd.: Non-detected. $^{a\text{-}d}$ Different letters in the same row indicate significant differences in function of the applied treatment.

Regarding the effect of SC-CO_2 pressure, samples extracted using SC-CO_2 at 10 MPa showed the lowest values of C18:1n-9 and the highest of C16:0, C18:0, C18:2n-6 and C18:3n-3. Likewise, the content of SFA and PUFA was higher and MUFA lower in 10 MPa samples compared to the oils extracted using SC-CO_2 at 20, 30 or 40 MPa, where no significant differences ($P > 0.05$) were detected. Probably, the differences between the fatty acid profile of the oils extracted at 10 MPa compared to 20–40 MPa can be attributed to the low amount of oil found in the ethanolic extracts of 10 MPa samples, which means that not all the fatty acids can be detected, thus affecting to their fatty acid profile.

On the other hand, no significant differences were observed for SFA, MUFA and PUFA when the results obtained with SC-CO_2 (20, 30 and 40 MPa) and conventional extraction were compared. However, some significant differences were found in some individual minor fatty acids. For instance, samples extracted with conventional extraction had lower content of C16:1n-7 (0.29% vs. ~0.35%) and higher content of C18:0 (3.50% vs. ~3.07%), C18:1n-7 (1.31% vs. ~1.03%) and C20:0 (0.63% vs. ~0.48%) compared to the samples extracted with SC-CO_2.

3.3. Impact of SC-CO_2 on vitamin E (tocopherols) and total phenolic compounds (TPC)

Moreover, the effect of SC-CO_2 on tocopherols and phenolic compounds recovery from "horchata" by-products was also evaluated and compared to conventional extraction (Fig. 2). First of all, it should be noted that among the different tocopherols which can be identified following the method used in the present work, it was only possible to identify and quantify α-tocopherol in the oils obtained from "horchata" by-products. The values of α-tocopherol in our work (4.1–12.4 mg/ 100 g of oil) were similar to those reported by other authors in tiger nuts, who found values ranging between 8.67 mg/100 g of oil (Yeboah et al., 2012) and 14.57 mg/100 g of oil (Ezeh & Gordon, 2016). Interestingly, one-way

ANOVA analysis showed a decreased in the amount of α-tocopherol when SC-CO_2 pressure was increased. This value decreases linearly (α-tocopherol amount=−0.2174·Pressure+14.26; R^2=0.9814) as the pressure increases, from 12.4 mg/100 g of oil in 10 MPa samples to 5.8 mg/100 g of oil in 40 MPa samples. This could be due to the fact that tocopherols were diluted with oil when their recovery increased. In fact, when the results of α-tocopherol were expressed as mg/100 g of "horchata" by-product, the tendency was the opposite. The values obtained were 0.076 (10 MPa), 0.233 (20 MPa), 0.307 (30 MPa), 0.433 (40 MPa) and 0.609 mg/100 g of "horchata" byproduct after CE extraction. Similarly to our results, Xu et al. (2008) described that vitamin E extraction of sea buckthorn increased as increased SC-CO_2 pressure (results expressed as mg/kg of dry berry). However, these authors found that the values of α-tocopherol were significantly higher after SC-CO_2 compared to conventional extraction, independently of the applied treatment. The α-tocopherol contributes to the stability of the oil as tocopherols acts as antioxidant (Ezeh & Gordon, 2016). In fact, it was observed a direct and significant correlation between the amount of α-tocopherol and PUFA (r=0.7165; P=0.0197) and especially with omega-3 content (r=0.7886; P=0.0067). This fact suggested that α-tocopherol is important in protecting them against oxidation (Quiles, Ramírez-Tortosa, Gómez, Huertas, & Mataix, 2002).

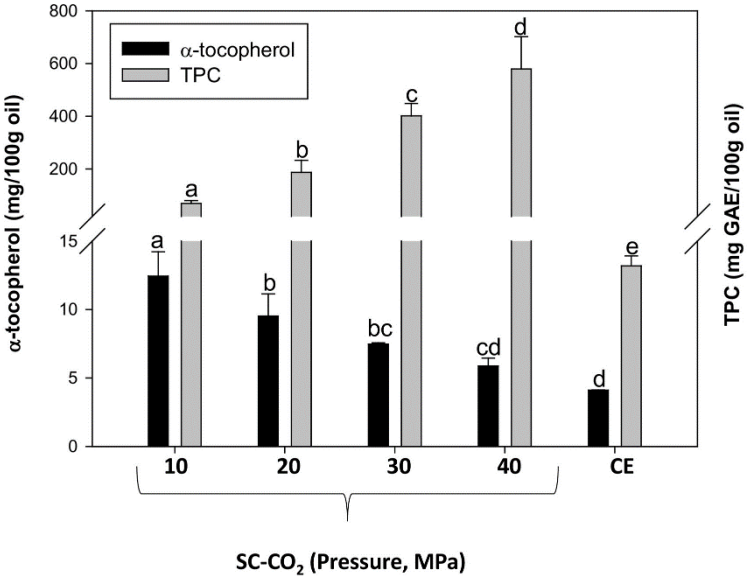

Fig. 2. Total phenolic compounds (TPC) and α-tocopherol content after SC-CO_2 (10–40 MPa) and conventional extraction (CE).

On the other hand, the TPC values were significantly increased as the SC-CO_2 pressures were increased. The TPC value increased linearly (TPC=17.447·Pressure+126.76; R^2=0.9885) from 69.3 mg GAE/ 100 g of oil in 10 MPa samples to 579.6 mg GAE/100 g of oil in 40 MPa samples. Our values were higher than those described by Koubaa, Barba, et al. (2015) who found values about 110 mg GAE/100 g of tiger nuts using SC-CO_2 and about 320 mg GAE/100 g of tiger nuts using gas assisted mechanical expression (GAME) extractions. These differences in TPC amounts were also influenced by different results units used in this study (mg GAE/100 g oil) and in other researches (mg GAE/100 g of product). In addition, our results also contrast with those obtained by Koubaa, Barba, et al. (2015) because they did not find any significant (SC-CO_2; 10–30 MPa).

However, the results obtained in our study were in close agreement with those obtained by Koubaa, Lepreux, Barba, Mhemdi, and Vorobiev

(2017) when they studied the TPC extraction from olive kernel, a byproduct from olive oil production, assisted by SC-CO_2. These authors attributed the differences in TPC extraction yields to the different permeability of the press-cakes after SC-CO_2. It is possible that due to damage structure during production process the TPC can be easily extracted.

The amount of TPC of oil from conventional extraction was lower ($P < 0.05$) (13.2 mg GAE/100 g of oil) than in SC-CO_2. The amount of TPC found in conventional extraction was in close agreement with the results previously published by Ali Rehab and El Anany (2012) in tiger nuts, who reported values of 16.5 mg GAE/100 g of oil. The low value of TPC in conventional extraction is related with the fact that the phenolic compounds are in the methanolic phase instead of in the $CHCl_3$-fat phase. To verify this information, the TPC analysis was also made in the methanolic phase obtaining 173.97 ± 3.06 mg GAE/100 g (data not shown). In this regard, the phenolic compounds of oil are important in assessing its antioxidant activity, thus protecting oil against lipid oxidation, and improving their stability (Ezeh & Gordon, 2016).

3.4. Effects of SC-CO_2 on lipid oxidation and antioxidant capacity

The effect of SC-CO_2 at different pressures (10–40 MPa) and CE on lipid oxidation parameters and antioxidant capacity of oil extracted from "horchata" by-products are shown in Fig. 3. The figure reflects the comparison between the relative percentage for CE (C0) and alternative extraction method SC-CO_2 (C). Lipid oxidation is one of the most important processes that occur in food systems, which results in the quality deterioration of the product (Suja, Abraham, Thamizh, Jayalekshmy, & Arumughan, 2004). The generation of off-flavours, loss of nutrient value, and the accumulation of toxic compounds are the most common changes that occur in oil (Arabshahi, Vishalakshi Devi, & Urooj, 2007). Peroxide value is one of the methods that are used to measure the

oxidative stability of oils, giving a clear indication of lipid oxidation (Suja et al., 2004). For instance, CE showed mean values of 15.2 meq/kg oil, which were similar to those found in samples obtained from SC-CO_2 at 10 MPa. The values found for CE were higher than those obtained for other authors (Sobhani, Mohammed, Ghobakhlou, & Ghazali, 2018; Yeboah et al., 2012) in tiger nut oil extracted with another conventional technique (15.2 vs. 5.5 and 3.1 meq/kg oil, for CE and Soxhlet extraction during 6 and 8 h, respectively), and similar to the values (15.2 meq/kg oil vs. 16.3 meq/kg oil, for CE and Soxhlet extraction, respectively) found by Hu et al. (2018). Moreover, significant lower values ($P < 0.05$) were found when the pressure conditions of the SC-CO_2 extraction increased (15.5, 11.1, 10.6 and 10.5 meq/kg oil for SC-CO_2 at 10 MPa, 20 MPa, 30 MPa and 40 MPa, respectively). In contrast, Hu et al. (2018) found lower values in tiger nut oil extracted with other non-conventional technique (MAE).

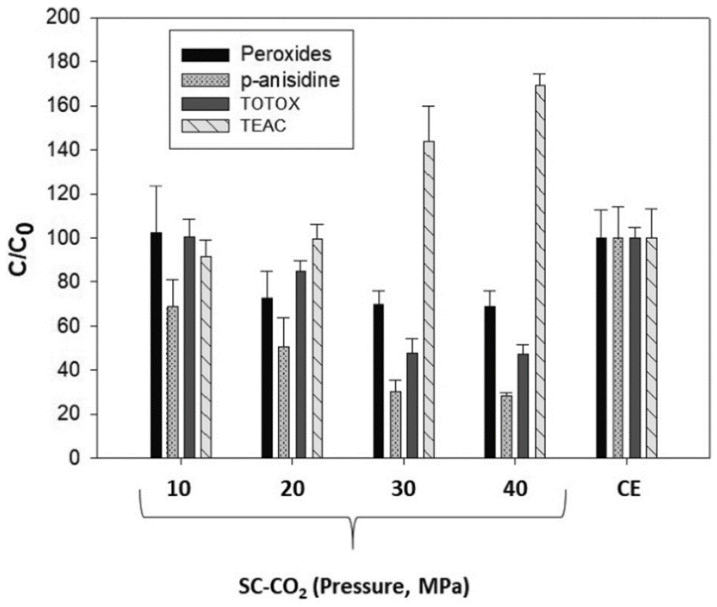

Fig. 3. Impact of SC-CO_2 (10–40 MPa) and conventional extraction (CE) on lipid oxidation parameters (peroxides, p-anisidine, TOTOX) and antioxidant capacity (TEAC).

In all cases, the values were higher than the limit (10 meq/kg oil) considered as acceptable for sensory attributes (CODEX, 1999), although the values obtained in SC-CO_2 extractions with higher pressure conditions (SC-CO_2 at 30 MPa and 40 MPa) offered the best results, approaching to the aforementioned limit.

Regarding p-anisidine index, its value reflects the formation of compounds derived from secondary oxidation (McGinley, 1991). The secondary stage of oxidation occurs when the hydroperoxides decompose to form carbonyls and other compounds, in particular aldehydes, which confer undesirable flavours to the oils (rancid odours). In this regard, lower p-anisidine value means better quality of the oil. Both, the type of lipid and oxidation time are two of the main factors having influence on this parameter (Kim, Yeo, Kim, Kim, & Lee, 2013). The results found in the oil samples obtained from "horchata" by-products extracted with SC-CO_2 at the lowest pressures displayed higher p-anisidine values compared to those obtained with higher pressures (14.7, 10.8, 6.5 and 6.0 for SC-CO_2 at 10 MPa, 20 MPa, 30 MPa and 40 MPa, respectively). This fact could be related to the higher amount of tocopherols in the oil extracted at high pressures. In fact, a direct and significant correlation was found between p-anisidine and tocopherols ($r=0.843$; $P < 0.05$). These values were lower than those found in the oil obtained with CE (Fig. 3), which could be attributed to the different conditions of the extraction. The values obtained by Sobhani et al. (2018) in tiger nut oil extracted with Soxhlet were much lower tan those found in the present study with CE (21.4 vs. 0.32, respectively). Values of p-anisidine index below 2.0 are related to good quality oils (Choo, Birch, & Dufour, 2007). In the present study, the values obtained exceed the aforementioned value, being the samples extracted with SC-CO_2 at 40 MPa those showing the best values for this index.

Totox value indicates an oil's overall oxidation state. Similarly to peroxide values, CE showed mean values close to those found in the

samples obtained from SC-CO_2 at 10 MPa (57.2 vs. 57.4, respectively). Regarding SC-CO_2 extraction, the values of Totox decreased as the pressure increased (57.4, 48.5, 27.2 and 26.9, respectively). Furthermore, the lower was Totox value, the better was the quality of oil, so that samples extracted with SC-CO_2 at 40 MPa would have higher quality. Lower values of this parameter were found by other authors in tiger nut oil (Sobhani et al., 2018).

Regarding the total antioxidant capacity (TEAC values) of the oil obtained from "horchata" by-products, the samples extracted with SC-CO_2 at 40 MPa showed the highest values. As expected, the values of antioxidant activity increased as the pressure conditions of the SC-CO_2 extraction increased. In fact, this behaviour was reflected in a linear adjustment of the data as the pressure increased (TEAC value=1.051·Pressure+21.675; R^2=0.945), from 34.88mM Trolox in 10 MPa samples to 64.35mM Trolox in 40 MPa samples. The comparison of SC-CO_2 with CE showed similar values at lower pressures (34.88mM Trolox and 37.94mM Trolox vs. 38.05mM Trolox, for SC-CO_2 at 10 MPa and 20 MPa vs. CE, respectively). These results confirmed the potential application of SC-CO_2 to recover oils rich in antioxidant bioactive compounds from "horchata" by-products. As can be seen in Fig. 3, an inverse relationship was found between lipid oxidation parameters and antioxidant capacity (TEAC values) of the SC-CO_2 extracts. Therefore, a significant correlation was found between the peroxide value ($r=-0.64$; $P < 0.05$), p-anisidine ($r=-0.75$; $P < 0.05$) and Totox value ($r=-0.91$; $P < 0.01$).

The main constituents found in SC-CO_2 extracts are relatively nonpolar (eg. fatty acids and α-tocopherol, among others), however phenolic compounds (Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018), some of them relatively polar in nature, seem to be the main responsable of anti-lipid peroxidative properties. In fact, a strong positive correlation was found between antioxidant capacity and TPC ($r=0.95$; $P < 0.01$). As it is

well known, phenolic hydroxyl groups can act as hydrogen donors and hence possess antioxidant potential, acting as radical scavengers potential, and protecting extracts against lipid peroxidation (Sánchez-Zapata et al., 2012).

4. Conclusions

The oils obtained from "horchata" by-products could be used as a useful tool in the development of new functional foods or nutraceuticals. The oils obtained from "horchata" by-products constitute an important source of unsaturated fatty acids, being monounsaturated fatty acids (MUFA) the predominant (70%). Moreover, C18:2n-6 was the most representative polyunsaturated fatty acids (PUFA). The amount of SFA, MUFA and PUFA differed according SC-CO_2 pressure, being SFA as well as PUFA higher and MUFA lower at 10 MPa samples compared to the oils extracted using SC-CO_2 at 20, 30 or 40 MPa, where no differences were detected. The content of α-tocopherol was significantly higher after SC-CO_2 compared to conventional extraction, independently of the applied treatment. In addition, a decrease in the amount of α-tocopherol was observed when SC-CO_2 pressure was increased, showing a linear decrease as the pressure increased. Regarding lipid oxidation, the better results were obtained when the SC-CO_2 pressure was increased. On the other hand, the values of phenolic compounds and antioxidant activity increased as the pressure conditions of the SC-CO_2 extraction increased. Finally, our results confirmed the potential application of SC-CO_2 to recover oils rich in bioactive compounds from "horchata" by-products.

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5.5. Phenolic profile of oils obtained from "horchata" by-products assisted by supercritical-CO_2 and its relationship with antioxidant and lipid oxidation parameters: Triple TOF-LC-MS-MS characterization

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ABSTRACT

In this study, the effect of different supercritical CO_2 (SC-CO_2) pressures (10–40 MPa) on phenolic compounds extraction in oils obtained from "horchata" by-products was evaluated, and the results were compared to those obtained after conventional oil extraction (CE). Moreover, the relationship between the individual phenolic compounds and the total antioxidant capacity as well as oil oxidative quality

parameters was compared. The phenolic profile and contents were largely influenced by extracting conditions. The main phenolic compound obtained by SC-CO_2 was the isohydroxymatairesinol, particularly at 30 and 40 MPa, while 3-vinylphenol was the predominant compound in oils extracted by CE procedure. Increasing SC-CO_2 extraction pressures enhanced the extraction of phenolic compounds, along with improving the antioxidant capacity and oxidative quality of extracted oil. The principal component analysis indicated that the main phenolic compounds associated with TEAC values were those extracted by SC-CO_2, which were inversely correlated to oxidative indexes.

1. Introduction

Oils obtained from nuts have been traditionally used for human consumption (e.g. food and cooking) with important nutritional purposes as well as in the pharmaceutical industry (Koubaa et al., 2016; Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018; Sakai et al., 2010). For instance, tiger nuts oil, is well known by its health related benefits due to its important content in oleic acid, polyunsaturated fatty acids (linoleic and linolenic acids), vitamins C, and E (especially α-tocopherol), minerals such as potassium, calcium and magnesium, which are necessary for bones and muscles (Belewu & Belewu, 2007), and also to its high oxidative stability compared to other oils (Ezebor, Igwe, Owolabi, & Okoh, 2006).

Moreover, tiger nuts oils also contain alkaloids, saponins and tannins, which exhibit antimicrobial and anti-inflammatory properties (Evans, 2005; Salem, Zommara, & Imaizumi, 2005) as well as other phenolic compounds associated with beneficial effects on health such as blood circulation activation, prevention of heart diseases and thrombosis (Chukwuma, Obioma, & Christophe, 2010), and the reduction in the risk of appearing colon cancer (Adejuyitan, Otunola, Akande, Bolarinwa, & Oladokun, 2009).

For instance, in the last two decades the consumption of oil from nuts has grown considerably. In 2012/2013, the world's consumption of vegetable oils reached 155.95 million tons, which represented an increase of 6 million tons (4%) compared to that recorded in 2011/2012 (USDA, 2013). In this increasing market, improvement of the production process and the search for new sources is always pursued, which can be obtained in four areas: oil yield increasing, oil quality improvement, production costs reduction and environment respect (Koubaa et al., 2016).

Tiger nuts are an expensive raw material, which are traditionally used to prepare "horchata", a traditional beverage, mainly commercialized in Spain (Roselló-Soto, Poojary, Barba, Koubaa, et al., 2018). During "horchata" manufacturing, a great content of by-products are generated, being traditionally discarded. However, among other applications, these by-products could constitute a source of oil with potential to be used for food and/or cosmetics, among other applications (CRDO, 2009; Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018).

The extraction process is one of the key stages to obtain oil from tiger nuts by-products. Although, there are no studies evaluating the recovery of oil from tiger nuts by-products, the common extraction way, which is used to obtain oil from raw tiger nuts should be mechanical expression and/or solvent extraction (e.g. using n-hexane). Before commercialization, the extracted oils should be refined in order to improve their nutritional and sensorial properties. Mechanical expression using hydraulic or screw presses is by far the easier and the cheapest way to obtain good quality oil (Koubaa et al., 2015). Despite significant recent advances in the field of press design, these processes do not allow a satisfactory exhausting of the raw material thus decreasing the economical profit. Conventional solvent extraction using organic solvents is very efficient for oil extraction. However, concerns about the solvent residues in the oleoresin products, the new regulations of volatile

organic solvent emissions in the air, and the extent of further refining that is required after the extraction step restrain the use of this technology (Chemat, Vian, & Cravotto, 2012; Koubaa et al., 2016; Rombaut, Tixier, Bily, & Chemat, 2014). Alternatively, oil extraction can be assisted by either microwave or ultrasound technology (Koubaa et al., 2016). While microwave technology is fast and require small amount of sample, ultrasound technology can reduce the time of extraction, improves solvent penetration and interaction with solute. Although both technologies are more environmentally friend than CE, both technologies still demands the use of appropriate solvents such as ethanol and methanol and microwave technology (without solvent) is suitable for samples with high fat content (Da Porto, Porretto, & Decorti, 2013; Koubaa et al., 2016).

The use of SC-CO_2 as extraction solvent is one of 12 principals of Green Chemistry, more specifically in full correspondence with the concept of using safer solvents (Anastas & Eghbali, 2009). The main advantages of SC-CO_2 as extraction technique are the lack of residual solvent after depressurization (solvent-free extract) and low operating cost. The use of CO_2 also has other advantages compared to conventional solvents: it is nontoxic, noninflammable, environmentally safe, widely available at high purity and low cost, high volatility, and is considered as versatile and suitable for extraction of low volatility, polarity and heat liable compounds (Koubaa et al., 2016; Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018). The main mechanisms involved in the improved capacity to extract target compounds is due to the combination of both liquid and gas properties: density similar to the liquid (solubilizes target compounds as a liquid) while viscosity and diffusivity are between liquid and gas values (fluid diffuses like a gas), which occurs in a short time. SC-CO_2 can be coupled to other operation and analytical techniques in order to improve performance of pressurized extraction, nanofiltration and chromatography techniques (Chemat et al., 2017; Formato, Gallo,

Ianniello, Montesano, & Naviglio, 2013; Sánchez-Camargo, Parada-Alfonso, Ibáñez, & Cifuentes, 2017).

In this context and in order to produce oil of high quality in full correspondence with green concept, the development of new extraction methods based on the use of supercritical fluids was intensively studied (Balvardi et al., 2015). Some previous studies have evaluated the potential of conventional and innovative approaches to extract oil from raw tiger nuts (Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018). However, despite the great potential of "horchata" by-products as source of oil with high content in antioxidant bioactive compounds, at this stage of development, there is no available information about the impact of SC-CO_2 on "horchata" by-products oil phenolic profile recovery.

For instance, this study is of great importance as traditionally tiger nuts by-products have been used only as source of fiber (Roselló-Soto, Poojary, Barba, Lorenzo, et al., 2018 and this work could open the door to its useful applications. Therefore, the main objectives of the present work are i) to compare the impact of SC-CO_2, and conventional extraction (CE) on the phenolic profile of oils obtained from by-products obtained during "horchata" production, and ii) to study the relationship between the phenolic profile and the antioxidant capacity and lipid oxidation parameters of the extracts obtained after SC-CO_2 and CE.

2. Material and methods

2.1. Samples

"Horchata" by-products were provided by the Regulatory Council D.O. Tiger nut of Valencia (Valencia, Spain). They were obtained after a conventional process for obtaining "horchata" from tiger nuts (*Cyperus esculentus*) with denomination of origin "Chufa de Valencia". They were dried in an air-circulating oven (Memmert UFP 600, Schwabach, Germany) at 60 °C for 72 h. This material was ground for uniformity of

particle size and vacuum packed.

2.2. Chemicals and reagents

The n-hexane (HPLC grade; 96%), Folin-Ciocalteau, phenol reagent, 2,2,4-trimethylpentane (reagent grade;>99.5%), 2-propanol (HPLC grade; 99.9%), sodium metal (extra pure; 99%), ethanol, metanol (reagent grade; 99.9), gallic acid monohydrate (extra pure; 99.5%), sodium hydrogen carbonate (reagent grade; 99.7%) were obtained from Scharlau (Barcelona, Spain). Chloroform (Normapur; 99%) and glacial acetic acid (Normapur) were obtained from Prolabo (VWR; Barcelona, Spain). Potassium iodide (PA;>99.5%), FAME 37 Mix, cis-vaccenic acid (> 97%), α-tocopherol, ABTS radical and Trolox® were supplied from Sigma-Aldrich (Madrid, Spain). p-Anisidine (reagent grade; 99%) was obtained from Acros organics (Madrid, Spain).

2.3. Supercritical CO_2 and conventional extractions

The supercritical carbon dioxide (SC-CO_2) extraction was used to recover chufa oil from "horchata" by-products. Fifty grams of "horchata" by-products were used to carry out the extractions assisted by supercritical carbon dioxide system (SFE-1000; Thar Process Inc. Pittsburgh, PA), applying different pressures (10, 20, 30 and 40 MPa) at 40 °C for 2 h. The CO_2 flow was adjusted at 20 g/min and the oil was recovered with 30 mL of ethanol. The conditions were selected based on a previous study (Koubaa et al., 2015).

The conventional extraction was carried out following the (Folch, Lees, & Sloane Stanley, 1957) procedure with some modifications. Fifty grams of dried "horchata" by-products were re-hydrated with 15 mL of NaCl (1%). Then, the mixture was mixed with 60 mL of $CHCl_3$:MetOH (2:1) at 10,000 rpm with one UltraTurax during 2 min. Subsequently, 20 mL of NaCl (1%) were added and homogenized again for 30 s. Samples were centrifuged at 3000g for 3 min and the $CHCl_3$ layer (containing the

oil) was recovered and dried with sodium sulphate anhydrous. Finally, $CHCl_3$ was removed using a rotatory evaporator at 55 °C and oil was stored at -30 °C until analysis.

2.4. Triple TOF-LC-MS-MS characterization of phenolic compounds

An Agilent 1260 Infinity (Agilent, Waldbronn, Germany) with a Waters UPLC C18 column 1.7 μm (2.1×50 mm) Acquity UPLC BEH.C18 from Waters (Cerdanyola del Vallès, Spain) was used to separate the main phenolic compounds in oil extracted by either SC-CO_2 or CE procedure from "horchata" by-products. The mobile phase was composed by water (solvent A; 0.1% formic acid) and methanol (solvent B, 0.1% formic acid) that was eluted (flow rate of 0.4 mL/min) as follows: 0 min 90% A; 13 min 100% (B); 15 min 90% A. The injection volume and flow rate used were 5 μL and 0.4 mL/min, respectively.

This system was coupled to a TripleTOF™ 5600 (AB SCIEX) LC/MS/MS system that was used to identify the main phenolic compounds. Prior to sample analysis, a calibration was performed by infusing a solution by an external calibration delivery system (CDS). The acquisition of MS data was carried out in the range between 80 and 1200 m/z on negative mode. The IDA acquisition method was performed in the survey scan type (TOF-MS) and the dependent scan type (product ion) using −50 V of collision energy. The MS analysis was performed with the following parameters:−4500 V ion spray voltage; 90 V declustering potential (DP); −50 V collision energy (CE); 400 °C temperature with 25 psi curtain gas (CU); 50 psi for both ion source gas 1 (GC1) and ion source gas 2 (GS2).

The IDA MS/MS analysis was carried with ions that had>100 CPS, with ion tolerance of 50 mDa, 25 V collision energy and activated dynamic background subtract. The data were acquired and processed by software analyst PeakView1.1 and its applications (XIC Manager and Formula Finder). The quantification of phenolics compounds was performed by an external calibration curve using resveratrol as standard.

2.5. Antioxidant and lipid oxidation parameters

The determination of the total antioxidant capacity was carried out according to the method previously described by Re et al. (1999) with some modifications (Barba, Esteve, Tedeschi, Brandolini, & Frígola, 2013). The samples were appropriately diluted using ethanol and the results were expressed in millimolar of Trolox equivalents (mM TE). Peroxide value (PV) was determined following the procedure previously described by AOAC (2007). PV was expressed as milliequivalents O2/kg oil. The determination of p-Anisidine value of the oil samples was carried out following an IUPAC method IUPAC (1987). The samples with and without p-Anisidine solution were measured in an UV spectrophotometer (UV-1800, Shimadzu Corporation, Kyoto, Japan) at 350 nm. The Totox value, which indicates the overall oxidation state of the oil, was determined according to Wanasundara, Shahidi, and Jablonski (1995).

2.6. Statistical analysis

The values were given in terms of mean values ± standard deviations. The differences in the parameters studied in relation to the extraction conditions were examined using an ANOVA test. Least-squares means were compared among extraction conditions using the Duncan's post hoc test (significance level $P < 0.05$). A study was conducted to determine whether there were correlations between a pair of variables (Pearsońs test). Moreover, a principal component analysis (PCA) was applied to the whole dataset to gain insight into the data structure, in order to detect the most important factors of variability and to describe the relations between variables and observations. The (I×J) data matrix was pre-treated using the unit variance standardization prior to PCA. Factor loadings analysis was also performed and graphs were developed using the first two principal components (PC1 vs PC2). All statistical analyses were performed using the softwares IBM SPSS Statistics® 21 and Statgraphics® Centurion XV (Statpoint Technologies, Inc., USA).

3. Results and discussion

3.1. Phenolic profile

The impact of SC-CO_2 pressure (10–40 MPa) on individual phenolic compounds extracted from "horchata" by-products' oil was evaluated and compared to CE procedure. As can be seen in Table 1, the phenolic profile differed according to the extraction conditions, obtaining different profile depending on the treatment applied (SC-CO_2 or CE) as well as the different pressure used.

The lignin isohydroxymatairesinol was the predominant compound found in the SC-CO_2 samples, followed by scopoletin and caffeic acid, particularly at 40 MPa. A remarkable increase in the number and amount of phenolic compounds was observed for SC-CO_2 protocol due to increasing pressure from 10 to 40 MPa. Differently, the main phenolic compounds obtained with CE procedure were 4-vinylphenol, pcoumaric acid and p-hydroxybenzoic acid.

The variety of phenolic compounds presented in Table 1 was also reported in other studies. For instance, Parker, Ng, Smith, and Waldron (2000) obtained p-coumaric, ferulic, p-hydroxybenzoic acid, p-hydroxybenzaldehyde, and vanillin on both "chufa" skins and tuber. The authors also stated that "chufa" skins are highly lignificated and rich in p-coumaric acid, in comparison to tubers. Similar to our study, Oladele, Adebowale, and Bamidele (2017) reported the variety of phenolic compounds in yellow and brown tiger nuts. While ferulic and p-hydroxybenzoic acids (58.38 and 29.12 mg/100 g, respectively) were the main phenolic compounds in yellow tiger nuts, vanillic and p-coumaric acids (15.20 and 17.25 mg/100 g, respectively) were the predominant in the brown variety. The authors also reported the presence of p-hydroxybenzaldehyde, caffeic and sinapinic acids, and flavonoids. In another study about the phenolic composition of tiger nuts, Ezeh, Niranjan, and Gordon (2016) reported the presence of trans-cinnamic

acid as one of the main phenolic compounds, along with ferulic and vanillic acids and vanillin.

The structure integrity of "horchata" by-products seems to influence the extraction of phenolic compounds. It was stated by Parker et al. (2000) that integrity of lignin matrix was associated to release of phenolic compounds, particularly for p-coumaric and ferulic acids. The continuous degradation of lignin structure was indicated by these authors as an important factor that influenced phenolic content of final extracts. In our case, the differences in the extraction protocol (grinding for SC-CO_2 vs homogenization at 10000 rpm with UltraTurax for CE) evaluated in the present study can contribute to explain the differences observed between SC-CO_2 and CE phenolic profile. The severe damage to vegetal matrix of "horchata" by-products by high-speed homogenization in solvent (CE) facilitated the release of trapped phenolic compounds, mainly p-coumaric and ferulic acids. Differently, grinding operation prior to SC-CO_2 extraction was limited to reduce particle size and expose part of trapped compounds, mainly p-coumaric acid (Table 1). This partial degradation explains the lower extraction yield of phenolic compounds with exception of isohydroxymatairesinol, scopoletin, and caffeic acid in the phenolic profile and content obtained from SC-CO_2 protocol compared to CE phenolic profile.

In addition to that, the use of additional technologies to destabilize the integrity of tiger nuts matrix for the extraction of phenolic compounds was also reported in literature. Ezeh et al. (2016) explored the use of pressure processing prior to enzymatic treatment (combination of amylase, cellulose, carbohydrases, and proteases) to improve oil extraction. The authors also obtained significant higher phenolic content in oils extracted by combining pressure and enzymes than pressing extraction. The authors also argued that enzymatic hydrolysis of ester bounds between phenolic compounds and cell wall components were associated with the increasing yield of extraction. In addition, the impact

of enzymatic treatment (protease, α-amilase and Viscozyme) in the structure of tiger nuts was previously evaluated by Ezeh, Gordon, and Niranjan (2016) who observed (by means of confocal image analysis) extensive cell wall damage and improved oil extraction yield.

On the other hand, CE procedure is believed to facilitate the extraction of phenolic compounds from "horchata" by-product. The higher content of p-coumaric and ferulic acids obtained by CE procedure (Table 1) seems to support this consideration. A reasonable explanation for this outcome is the intense degradation promoted by this procedure that subjected "horchata" by-product to a homogenizer at 10,000 rpm in contact with a mixture of solvents (NaCl, $CHCl_3$, and MetOH mixture).

Pure SC-CO_2 is not commonly applied to extract phenolic compounds due to differences in the polarity of SC-CO_2 (suitable for the recovery of non-polar and moderately polar compounds) and phenolic compounds (hydrophilic character) (Woźniak, Marszałek, Skąpska, & Jędrzejczak, 2017). In this line of thought, improving the solvating power of SC-CO_2 is necessary to enhance the recovery of polyphenols. For instance, more polar co-solvents can interact with phenolic compounds (by means of hydrogen binding and dipole-dipole, for instance) and facilitate the release of compounds in comparison to pure SC-CO_2 extraction. In this line of thought, ethanol and water have been proven to enhance the extraction yield of phenolic compounds from chokeberry pomace (Woźniak et al., 2017).

Although SC-CO_2 can recover phenolic compounds from vegetable matrix at mild conditions (suitable for heat liable compounds), some authors associated SC-CO_2 treatment with hydrolysis of complex phenolic compounds into simple phenolic compounds. Marszałek et al. (2018) evaluated the impact of SC-CO_2 treatment on apple juice phenolic profile. The authors obtained significant reductions on both total phenolic and individual phenolic content between fresh and SC-CO_2 processed juice, which were attributed to processing pressure, increased acidity, and

partial inactivation of oxidative enzymes.

Table 1. Individual phenolic compounds (ppb) of oils extracted from "horchata" by-products assisted by supercritical (SC)–CO_2 at different pressures (10–40 MPa) and conventional solvent extraction (CE).

Compounds	SC-CO_2 pressure				CE
	10	20	30	40	
Caffeic acid[A]	3.90±0.47[a]	44.99±4.78[bc]	83.85±36.27[bd]	102.19±6.65[d]	38.43±5.81[c]
Cinnamic acid[A]	ND	ND	ND	ND	40.66±6.02
p-Coumaric acid[A]	ND	5.37±2.61[a]	14.79±1.15[b]	18.38±0.57[c]	126.76±18.53[d]
Ferulic acid[A]	ND	ND	ND	ND	22.33±2.83
Ferulic acid-4-*O*-glucoside[A]	ND	ND	ND	ND	46.95±0.14
24-Methylcholestanol ferulate[A]	45.40±2.45	ND	ND	ND	ND
4-Vinylphenol	ND	ND	ND	ND	1084.48±112.06
p-Hydroxybenzoic acid[G]	2.52±0.88[a]	3.10±0.82[a]	5.06±0.60[b]	8.69±1.83[bc]	67.70±4.52[d]
Ethyl vanillin[G]	ND	ND	ND	ND	25.38±2.49
Vanillic acid[G]	ND	ND	ND	ND	10.84±0.14
Sesamin[E]	ND	ND	ND	ND	28.67±13.70
Sinensetin[F]	ND	2.57±1.02[a]	4.08±0.01[b]	16.07±1.30[c]	ND
Isohydroxymatairesinol[E]	ND	274.18±6.13[a]	756.22±72.42[b]	1331.45±10.00[c]	ND
Scopoletin[D]	19.35±1.94[a]	142.56±7.67[b]	209.35±26.33[c]	310.80±49.09[d]	ND
Homovanillyl alcohol[C]	ND	4.54±0.27[a]	2.38±0.33[b]	4.43±0.31[a]	ND
Peonidin[B]	ND	ND	7.81±3.27	ND	ND
trans-Resveratrol-3-*O*-glucoside[H]	ND	ND	ND	ND	25.68±7.10

[a–d] Mean values in the same row (corresponding to the same compound) not followed by a common letter differ significantly ($P < 0.05$). ND: not detectable. [A] Hydroxycinnamic acid, [B] Anthocyanin, [C] Metabolite of hydroxytyrosol, [D] Coumarin, [E] Lignan, [F] Flavone, [G] Dihydroxybenzoic acid, [H] Stilbene.

3.2. Relationship between individual phenolic compounds, antioxidant capacity and lipid oxidation parameters

The correlations (Pearsońs test) obtained from data set are presented in Table 3. A positive correlation between total phenolic compounds (TPC) and TEAC was obtained, which indicates that phenolic compounds play a central role as antioxidants. This result is in accordance with the results reported by other authors. For instance, Sanjaya et al. (2014) obtained a positive correlation between TPC of Myrmecodia pendans SC-CO_2 extract and DPPH assay (pressure in the range of 9–22.5 MPa and temperature between 40 and 70 °C). Similarly, Wang et al. (2011) obtained a positive correlation ($r > 0.850$) between TPC and DPPH assay for *Ampelopsis grossedentata* stems extract by SC-CO_2 technique (pressure between 150 and 250 bar and temperature in the range of 40–60 °C).

Negative correlations between TPC with p-Anisidine and with TOTOX and between TEAC and all oxidation indexes were also obtained. These relations strengthen the role of extracted phenolic compounds from "horchata" by-products as active compounds against oxidative reactions. A similar outcome was obtained by Gharibzahedi, Mousavi, Hamedi, and Khodaiyan (2014) who observed that TPC and Rancimat assay (an accelerated method to characterize the oxidative status of oils) were negatively correlated ($r=-0.92$) in Persian walnut oil.

Caffeic acid, isohydroxymatairesinol, scopoletin, and sinensetin were the compounds that displayed positive correlations with TPC and TEAC ($r > 0.850$). These compounds were also negatively correlated with PV, p-Anisidine and TOTOX. This outcome indicated that such compounds play a major role as antioxidant compounds from "horchata" by-products. Moreover, the use of SC-CO_2 technology was crucial to extract these compounds since CE procedure displayed significant lower capacity to extract them (Table 1).

Table 2. Linear correlations for individual and Total Phenolic Content (TPC), Trolox Equivalent Antioxidant Capacity (TEAC), Peroxide value, p-Anisidine, and TOTOX values.

	TPC	TEAC	Peroxide value	*p*-Anisidine	TOTOX
4-Vinylphenol	-0.288	-0.344	0.469	0.811**	0.486
24-Methylcholestanol ferulate	-0.426	-0.464	0.536	0.246	0.495
Caffeic acid	0.863***	0.928***	-0.690*	-0.686*	-0.895***
Cinnamic acid	-0.287	-0.347	0.459	0.814**	0.482
p-Coumaric acid	-0.161	-0.219	0.369	0.731*	0.362
Ethyl vanillin	-0.288	-0.343	0.470	0.811**	0.487
Ferulic acid	-0.287	-0.346	0.464	0.813**	0.484
Ferulic acid 4-*O*-glucoside	-0.288	-0.334	0.489	0.800**	0.493
p-Hydroxybenzoic acid	-0.213	-0.250	0.466	0.747*	0.436
Homovanillyl alcohol	0.472	0.563	-0.782**	-0.740*	-0.631
Isohydroxymatairesinol	0.936***	0.943***	-0.683*	-0.801**	-0.918***
Peonidin	0.355	0.399	-0.383	-0.454	-0.579
trans-Resveratrol-3-*O*-glucoside	-0.283	-0.354	0.429	0.817**	0.468
Scopoletin	0.878***	0.887***	-0.802**	-0.883***	-0.927***
Sesamin	-0.273	-0.359	0.378	0.806**	0.439
Sinensetin	0.859**	0.879***	-0.575	-0.676*	-0.754*
Vanillic acid	-0.288	-0.333	0.491	0.799**	0.494
Tocopherol	-0.448	-0.391	0.054	-0.118	0.280
TEAC	0.949***				
Peroxide value	-0.586	-0.637*			
p-Anisidine	-0.694*	-0.749*	0.682*		
TOTOX	-0.894***	-0.911***	0.787***	0.818**	

Sig: * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$).

This inversed proportion between individual phenolic content and oxidative status indexes was studied by Zheng, Yang, Zhou, Liu, and Huang (2014) who evaluated the effect of canolol (phenolic compound naturally found in rapeseed oil). The authors obtained positive correlations between DPPH assay with TPC and with canolol content (both with $r > 0.890$) while negative correlations between canolol content and oxidation indexes (PV and p-Anisidine values; both with $r < -0.890$) were also observed.

Table 3. Scores of variation for the first three principal components (PC).

	PC1	PC2	PC3
4-Vinylphenol	-0.250	-0.149	0.003
24-Methylcholestanol ferulate	-0,017	0,330	-0,024
Caffeic acid	0.151	-0.313	0.100
Cinnamic acid	-0.250	-0.149	0.003
p-Coumaric acid	-0.233	-0.197	0.012
Ethyl vanillin	-0.250	-0.149	0.003
Ferulic acid	-0.250	-0.149	0.003
Ferulic acid-4-*O*-glucoside	-0.249	-0.147	0.002
p-Hydroxybenzoic acid	-0.240	-0.173	-0.023
Homovanillyl alcohol	0.198	-0.129	-0.293
Isohydroxymatairesinol	0.200	-0.243	-0.136
Peonidin	0.093	-0.087	0.824
Scopoletin	0.225	-0.211	-0.083
Sesamin	-0.240	-0.145	0.005
Sinensetin	0.173	-0.231	-0.384
Trans-resveratrol-3-*O*-glucoside	-0.247	-0.149	0.004
Vanillic acid	-0.249	-0.147	0.002
α-Tocopherol	0.086	0.361	0.023
TPC	0.160	-0.277	0.010
TEAC	0.175	-0.272	0.027
PV	-0.185	0.166	-0.067
p-Anisidine	-0.256	0.073	-0.078
TOTOX	-0.205	0.232	-0.196

Total Phenolic Content (TPC). Trolox Equivalent Antioxidant Capacity (TEAC). Peroxide value (PV).

Interestingly, most of the compounds extracted by CE procedure (r in the range of 0.799–0.817) were positively correlated to p-Anisidine assay. Seems reasonable to infer that phenolic compounds extracted by CE procedure displayed lower potential to prevent/inhibit the oxidation of "horchata" by-product oil than compounds obtained by SC-CO_2 technology.

3.3. Principal component analysis (PCA)

The PCA protocol was applied to standardize the data obtained from SC-CO_2 and CE extractions and determination of TPC, TEAC, PV, p-Anisidine, and TOTOX value. The number of principal components was determined by selecting principal components with Eigenvalor higher than 1, which yielded 3 principal components (Fig. 1). The analysis revealed that PC1, PC2 and PC3 explained 59.91%, 87.51%, and 92.81% of cumulative variance, respectively.

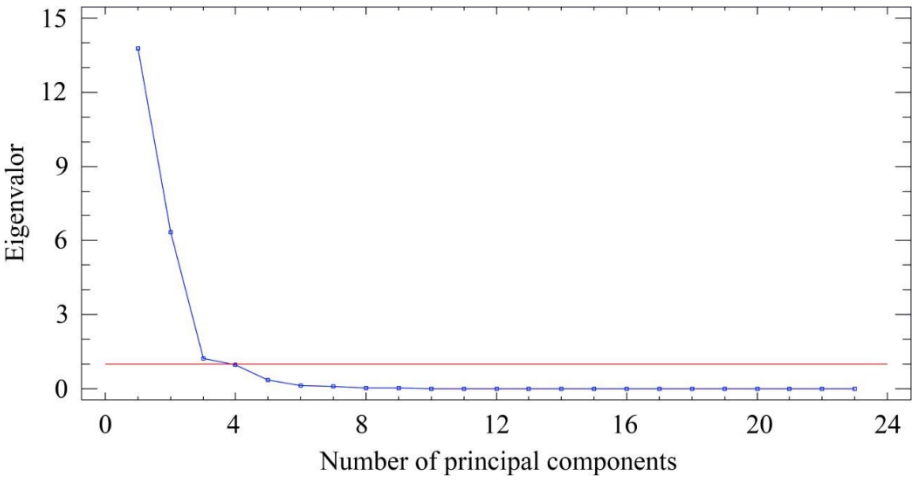

Fig. 1. Sedimentation graphic to elucidate the number of principal components.

The loading plots are displayed in Fig. 2a (PC1 vs PC2) and b (PC1 vs PC2 vs PC3). The variation on PC1 (Fig. 2a) can be attributed to most of phenolic compounds extracted by CE procedure (e.g. ethyl vanillin, vinylphenol, and ferulic acid), and scopoletin (extracted only by SC-CO_2 protocol). Regarding PC2, this component can be related to α-tocopherol, PV, caffeic acid, TPC, and TEAC. On PC3, the variation in this component was attributed to sinensetin, homovanillyl alcohol, and peonidin. In addition, the three-dimensional disposition of variables (Fig. 2b) also

indicates that samples obtained by SC-CO_2 at 30 MPa explain an important portion of data set variation.

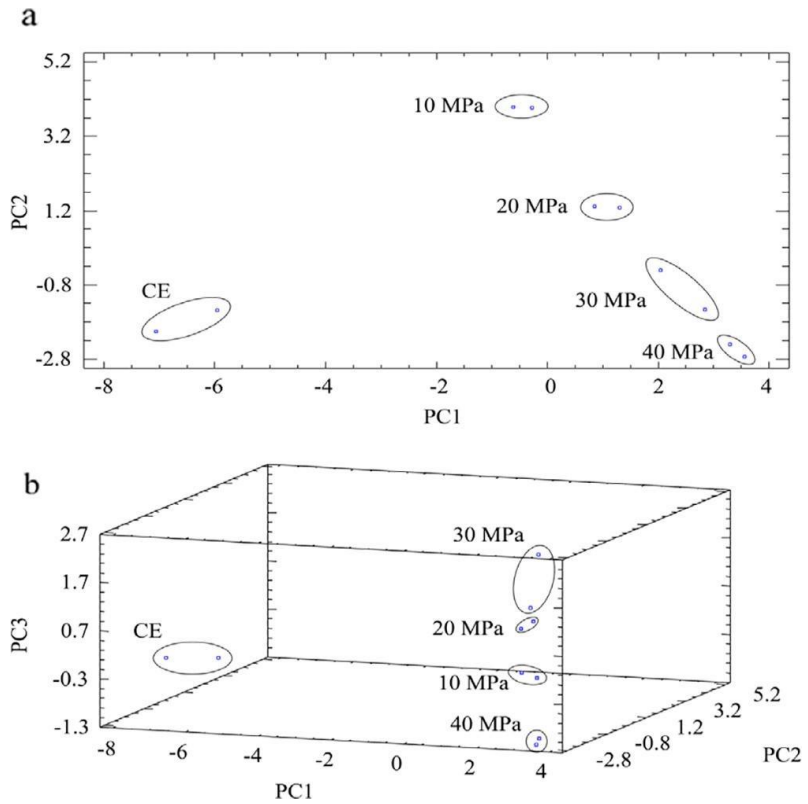

Fig. 2. Loading plots for each extraction conditions. Pressure (MPa). Principal component (PC).

The score of variation for each phenolic compound and the analysis of TPC, TEAC, PV, p-Anisidine, and TOTOX value are presented in Table 3 and Fig. 3a and b. The data distribution in Fig. 3a indicates that 4 clusters were formed. The first cluster is formed by the main phenolic compounds extracted from SC-CO_2, TPC, and TEAC; a second cluster that has α-tocopherol and 24-methylcholestanol ferulate; a third cluster composed by p-Anisidine, PV, and TOTOX values; and a fourth cluster formed by

phenolic compounds extracted by CE protocol.

In Fig. 3a, it is reasonable to infer that extraction of phenolic compounds by SC-CO_2 (particularly sinensetin, isohydroxymatairesinol, and caffeic acid) was located in the same quadrant as TPC and TEAC (PC1 and PC2 > 0). Moreover, this cluster is in opposite direction to the cluster of oxidative indexes (PC1 and PC2 < 0). Previous studied about the antioxidant capacity of sinensetin (Kim et al., 2009), isohydroxymatairesinol (Eklund et al., 2005) and caffeic acid (Sato et al., 2011) strengthen the role of such compounds as main antioxidant compounds.

A cluster formed by α-tocopherol and 24-methylcholestanol ferulate was also observed (Fig. 3a). This outcome can be explained by the unique condition of α-tocopherol. Although SC-CO_2 protocol was more efficient to extract α-tocopherol than CE procedure (PC1 > 0), increasing pressure in SC-CO_2 protocol reduced the efficiency of extraction (from 12.44 to 5.88 mg/100 g oil for 10 and 40 MPa, respectively). In this condition, the α-tocopherol cluster was located in a different quadrant and cluster than other variables. Due to its relative close position to α-tocopherol, the same interpretation was attributed to 24-methylcholestanol ferulate.

The relative position of the group formed by phenolic compounds extracted by CE procedure highlights the differences between SC-CO_2 and CE procedures on the extraction of phenolic profile from "horchata" by-products. The relative position of this group of phenolic compounds on the loading plots strengthens the hypothesis that SC-CO_2 can produce extracts of higher antioxidant potential than CE procedure, as indicated in Table 2.

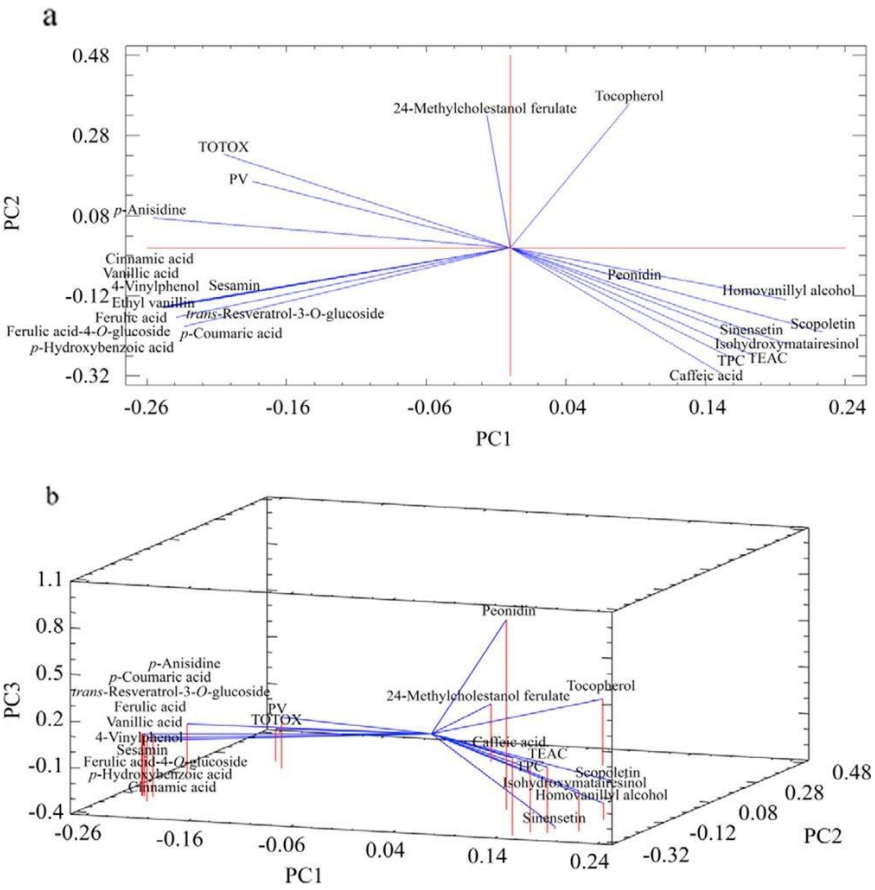

Fig. 3. Principal component analysis (PCA) plots for individual phenolic compounds, total phenolic compounds (TPC), trolox equivalent antioxidant capacity (TEAC), and oxidation indexes.

4. Conclusions

SC-CO_2 technology was more efficient to recover lipophilic phenolic compounds with high antioxidant activity than CE procedure. The oxidative stability of extracted oil was improved after carrying SC-CO_2 extraction at both 30 and 40 MPa, in comparison to extractions executed at either lower pressures or CE procedure. Therefore the use of SC-CO_2 is advised in the extraction of lipophilic antioxidant compounds from

"horchata" by-products. The extraction of phenolic compounds from "horchata" by-products can be explored by both cosmetic and food industries.

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6. RESUMEN DE RESULTADOS Y DISCUSIÓN GENERAL

6. RESUMEN DE RESULTADOS Y DISCUSIÓN GENERAL

6.1. Efecto de la temperatura, mezclas hidroetanólicas y tiempo de extracción en la recuperación de compuestos antioxidantes

Tras evaluar el efecto de la temperatura, tiempo de extracción y los diferentes porcentajes de etanol/agua en la extracción de compuestos fenólicos totales (CFT) a partir de subproductos de la "horchata", se observó que los tres factores tenían un efecto significativo ($p<0.05$) en cuanto al contenido de CFT en los extractos obtenidos.

Así pues el valor más alto de compuestos fenólicos totales (16.02 mg equivalentes de ácido gálico (GAE)/100 g de materia seca) se obtuvo tras utilizar durante 3 horas una temperatura de 60 ºC con un porcentaje del 25% (v/v) de etanol en agua. Se observó un aumento de la eficiencia de extracción de los CFT a medida que se incrementó la temperatura, obteniendo valores de 7.50, 7.80 y 14.46 mg GAE/100 g materia seca a 40, 50 y 60 °C, respectivamente medidos tras utilizar la misma concentración de etanol y tiempo de extracción (25% etanol, 2 horas). Estos resultados pueden explicarse por el aumento en la solubilidad de los compuestos fenólicos a temperaturas más altas aumentando así la velocidad de difusión, y favoreciendo el fenómeno de transferencia de masa (Bursać-Kovačević et al., 2018; Mokrani & Madani, 2016). Sin embargo, tras utilizar temperaturas muy altas, se puede producir la degradación de algunos polifenoles termolábiles (Putnik et al., 2017).

En este sentido, en un estudio similar realizado por Yuan, Lu, Eskridge, Isom, & Hanna (2018), estos autores evaluaron el efecto de la temperatura en la extracción de CFT a partir de cáscaras de avellana,

observando valores más altos de CFT al aumentar la temperatura de 30 ºC (801 mg de GAE/100 g) a 50 ºC (1050 mg de GAE/100 g). Estos resultados manifiestan que a medida que aumenta la temperatura, la tensión superficial y la viscosidad del disolvente disminuyen, favoreciendo así la inclusión del disolvente en la matriz. En esta misma línea, (Dorta, Lobo, & Gonzalez, 2012) sugirieron que las temperaturas más altas pueden afectar a la actividad antioxidante de los extractos y reducir la estabilidad de los compuestos fenólicos presentes en los mismos.

Respecto a las concentraciones de etanol experimentadas, se observó un mayor contenido de CFT cuando se utilizaron concentraciones del 25% y 50% de etanol según las condiciones de tiempo y temperatura. La extracción mediante el uso de etanol al 25% no fue estadísticamente diferente de la extracción tras utilizar etanol al 50%. En otras palabras, se observó hasta un aumento del 82% en el contenido de CFT tras utilizar mezclas hidroetanólicas (50:50, etanol:agua, v/v) frente a los extractos acuosos (sin etanol), a 60 °C y 2 h de tiempo de extracción. Sin embargo, esta tendencia no fue tan clara después de 1 h de extracción a 40 y 50 °C, ya que el etanol al 25% mejoró la extracción de CFT en comparación con las mezclas hidroetanólicas con etanol al 50%. De manera similar, esta tendencia también se observó a una temperatura de 60 °C, con un tiempo de extracción de 3 h. Por lo tanto, el uso de etanol en mezclas acuosas contribuyó a un medio polar, mejorando así la extracción de compuestos fenólicos.

Los estudios llevados a cabo por Naczk & Shahidi (2004), mostraron que la solubilidad de los compuestos fenólicos depende de la polaridad del disolvente, así como de la formación de complejos insolubles, y de la interacción con otros componentes en el alimento. En otro estudio, Yuan et al. (2018) utilizaron mezclas de metanol, etanol y acetona en concentraciones de 20, 50 y 80% para la extracción de CFT a partir de las cáscaras de avellana, obteniendo los valores más altos de CFT tras utilizar

un 50% de acetona. Socaci et al. (2018) observaron que el disolvente más eficaz para la extracción de los CFT en los subproductos de la cerveza era la mezcla acetona:agua al 60% (v/v), obteniendo valores de 114 mg de equivalentes de ácido gálico (GAE)/100 g de materia seca. Meneses, Martins, Teixeira, & Mussatto (2013) también obtuvieron resultados similares tras evaluar la extracción de CFT a partir de subproductos de cerveza, encontrando valores de 990 mg de GAE/100 g de materia seca cuando se usó acetona al 60% (v/v) mientras que Tournour et al. (2015) encontraron valores de CFT en un intervalo de 6.93-13.17 mg GAE/100 g de residuo seco tras utilizar mezclas hidroetanólicas (80:20, v/v) en orujos de uva durante 48 horas.

Por otra parte, el tiempo de extracción influyó significativamente en la cantidad de CFT obtenidos. Así pues, la concentración de CFT aumentó con el tiempo de extracción tras utilizar etanol al 25% y temperatura de 60 °C. Tras una hora de extracción, el valor de CFT fue de 7.22 mg GAE/100 g de materia seca, mientras que para 2h y 3h los valores obtenidos fueron de 14.46 mg y 16.02 mg de GAE/100 g de materia seca, respectivamente. En esta misma línea, el trabajo realizado por Yuan et al. (2018) mostró resultados similares al extraer CFT a partir de cáscaras de avellanas.

Respecto al contenido de flavonoides totales (FT), se observaron valores entre 1.80 y 30.09 mg equivalentes de catequina (EC)/100 g de materia seca, dependiendo de la temperatura, tiempo de extracción y concentración de etanol utilizados. El valor óptimo de extracción se observó tras utilizar temperaturas de 50 °C, 25% de etanol y 3 h de tiempo de extracción. Tanto el porcentaje de etanol como el tiempo mostraron un efecto significativo en el contenido de FT, mientras que la temperatura no tuvo ninguna influencia. En general, los valores más altos se obtuvieron tras utilizar 25% de etanol en comparación con las mezclas sin etanol y con un 50% de etanol. Estos resultados pueden explicarse por las diferentes polaridades de los disolventes, ya que las mezclas de

etanol:agua tienen una mayor polaridad y, por lo tanto, una mayor eficiencia en la extracción de estos compuestos (Dorta et al., 2012).

Meneses et al. (2013) evaluaron el efecto del disolvente en la extracción de FT en subproductos de cerveza. Estos autores obtuvieron los valores más altos (≈461 mg equivalentes de quercetina (EQ)/100 g de materia seca) tras utilizar una mezcla de etanol:agua (80:20, v/v), en comparación con la extracción acuosa (2 mg EQ/100 g de materia seca) bajo condiciones de extracción similares. Además, utilizando porcentajes de etanol en los intervalos utilizados en la presente tesis (40-60 y 60:40, v/v, etanol:agua), el rendimiento de extracción fue de aproximadamente 50 y 130 veces más alto que la extracción acuosa, respectivamente.

Otro estudio, mostró que la extracción con etanol al 60% (v/v) fue la más eficiente (57 mg EQ/100 g de peso seco) para extraer los FT de muestras de melocotón, obteniendo valores más ≈3.3 veces mayores en comparación con la extracción acuosa (17 mg EQ/100 g de materia seca) (Mokrani y Madani, 2016). Se obtuvieron resultados similares para FT en subproductos de cerveza, con un valor de ≈50 mg EQ/100 g de materia seca tras utilizar una mezcla de etanol:agua (60:40, v/v) (50 mg EQ/100 g de materia seca), obteniendo valores 3 veces mayores en comparación con la extracción acuosa en condiciones de extracción similares (Socaci et al., 2018).

Con respecto a la influencia de la temperatura en los FT, se observó una mayor extracción de FT a 50 °C respecto a 60 °C, alcanzando un aumento de hasta el 17% durante las 3 h de extracción. Sin embargo, estos resultados pueden deberse a que temperaturas más altas pueden reducir la estabilidad de estos compuestos.

En términos generales, no hay una tendencia clara en los resultados con respecto al tiempo para la extracción de FT. Sin embargo, la extracción durante 3 h (25% de etanol (v/v) a 50 °C) presentó la mayor extracción de FT, con valores de 30.09 mg EC/100 g de materia seca, en comparación con los 7.20 y 5.87 mg EC/100 g de materia seca obtenidos

tras 1 y 2 h de extracción, respectivamente.

Por otra parte, tras evaluar el efecto de la temperatura, porcentaje de etanol y tiempo de extracción en la capacidad antioxidante total (CAT) de los extractos obtenidos, se observó que los 3 factores tenían un efecto significativo ($p<0.05$). En general, la CAT aumentó cuando se utilizaron mayores concentraciones de etanol. Esto puede deberse al hecho de que el uso de etanol en mezclas con agua creó un medio más polar, favoreciendo la extracción de diferentes compuestos antioxidantes solubles en este tipo de disolventes (Meneses et al., 2013).

Los valores más altos de CAT (1759.81 μM de Ácido-6-hidroxi-2,5,7,8-tetrametilcroman-2-carboxílico (Trolox)/g de materia seca) se observaron cuando se utilizaron las concentraciones más altas de etanol (50%, v/v), el tiempo de extracción más largo (3h) y la temperatura más alta 60 ° C. De esta forma, puede observarse como la aplicación de temperaturas altas en este tipo de subproductos puede favorecer la extracción de compuestos antioxidantes y aumentar la solubilidad.

6.2. Impacto de los fluidos SC-CO_2 en la extracción de aceite, ácidos grasos, y vitamina E de los subproductos de chufa y comparación con extracción convencional

Tras aplicar la extracción por fluidos supercríticos (10-40 MPa) para extraer aceite de los subproductos de "horchata" se observó una relación lineal entre la presión de extracción y el rendimiento de aceite (g de aceite/100 g de subproducto = 2.192 X Presión – 1.8479; R^2 = 0.9736), obteniendo la mayor extracción 7.36 g/100 g de subproducto de "horchata" a la presión más alta (40 MPa). Este resultado coincide con lo previamente observado por otros autores tras aplicar SC-CO_2 en diferentes matrices, los cuales concluyeron que la presión tenía un efecto positivo y lineal en el rendimiento de extracción de aceite.

En este sentido, Lasekan & Abdulkarim (2012) y Koubaa, Barba, et al. (2015) obtuvieron resultados similares cuando evaluaron el impacto de SC-CO_2 para extraer el aceite de chufa. También se encontraron resultados similares después de aplicar SC-CO_2 (10-50 MPa) en cardo mariano (Ben Rahal, Barba, Barth, & Chevalot, 2015), semillas de uva (Rombaut et al., 2014) y cortezas de colza (Mhemdi, Koubaa, El Majid, & Vorobiev, 2016). El mayor rendimiento de extracción de aceite con presiones más altas podría estar relacionado con un aumento en la densidad del CO_2, lo que resulta en una mayor solubilidad del aceite en el CO_2, facilitando así la extracción (Mhemdi et al., 2016; Xu, Gao, Liu, Wang, & Zhao, 2008). Por ejemplo, es bien sabido que la presión afecta a la densidad del CO_2, y en el presente trabajo la temperatura operativa fue constante (40 ºC), por lo que la densidad del CO_2 a esta temperatura varía entre 0.629 g/L (10 MPa) y 0.956 g/L (40 MPa) (NIST, 2018).

Además de lo mencionado anteriormente, la extracción convencional mediante el método de Folch mostró los valores más altos de aceite

(14.85 g/100 g de subproductos de "horchata"), lo que confirmó que el uso de disolventes orgánicos aumentó el rendimiento del aceite. En este sentido, Ndayishimiye, Getachew, & Chun (2017) obtuvieron resultados similares al utilizar estos procesos convencionales para extraer aceite de semillas de cítricos, observando que la extracción convencional (usando n-hexano) duplicó el rendimiento de aceite en comparación con la extracción de SC-CO_2 (20 MPa; 45 ºC), como ocurrió en el presente estudio.

Los aceites obtenidos a partir de subproductos de "horchata" presentaron un perfil en el que predominaron los ácidos grasos monoinsaturados (AGMI), que representan aproximadamente el 70% del total de ácidos grasos. Con respecto a los ácidos grasos poliinsaturados (AGPI), el C18:2n-6 fue el ácido graso más representativo, aunque también se detectó C18:3n-3, que representó el 0.47-0.79% del total de ácidos grasos. Estos hallazgos están de acuerdo con el perfil de ácidos grasos obtenidos por otros autores para la chufa (Ezeh, Gordon, et al., 2016; Lasekan & Abdulkarim, 2012; Sánchez-Zapata, Fernández-López, & Angel Pérez-Alvarez, 2012; Yeboah et al., 2012), los cuales obtuvieron valores de C16:0 entre 13.5 y 16.6%, C18:0 de 3.2 a 6.3%, C18:1n-9 entre 65.5 y 72.6%, C18:2n-6 de 8.9 a 13.4% y C18:3n-3 entre no detectado y 0.4%.

No se observaron diferencias significativas para AGS, AGMI y AGPI tras aplicar SC-CO_2 en comparación con la extracción convencional. Sin embargo, se encontraron algunas diferencias significativas en algunos ácidos grasos. Por ejemplo, las muestras extraídas con métodos convencionales tuvieron menor contenido de C16:1n-7 (0.29% vs. ~ 0.35%) y mayor contenido de C18:0 (3.50% vs. ~ 3.07%), C18:1n-7 (1.31 % vs. ~ 1.03%) y C20:0 (0.63% vs. ~ 0.48%) en comparación con las muestras extraídas con SC-CO_2.

Por otra parte, los valores de α-tocoferol (4.1-12.4 mg/100 g de aceite) fueron similares a los obtenidos por otros autores en la chufa, quienes

encontraron valores que oscilaron entre 8.67 mg/100 g de aceite (Yeboah et al., 2012) y 14.57 mg/100 g de aceite (Ezeh, Gordon, et al., 2016). Se observó una disminución en la cantidad de α-tocoferol cuando la presión de SC-CO_2 aumentó. Este valor disminuye linealmente (cantidad de α-tocoferol = -0.2174 X Presión + 14.26; R^2 = 0.9814) a medida que aumenta la presión, de 12.4 mg/100 g de aceite en muestras de 10 MPa a 5.8 mg/100 g de aceite en muestras tratadas a 40 MPa.

Esto podría deberse al hecho de que los tocoferoles se diluyeron en el aceite cuando aumentó su recuperación. De hecho, cuando los resultados de α-tocoferol se expresaron como mg/100 g de subproducto de "horchata", la tendencia fue la opuesta. Los valores obtenidos fueron de 0.076 (10 MPa), 0.233 (20 MPa), 0.307 (30 MPa), 0.433 (40 MPa) y 0.609 mg/100 g de subproducto de "horchata" después de la extracción convencional. Sin embargo, Xu et al. (2008) describieron que la extracción de vitamina E del espino amarillo (*Hippophae Rhamnoides L.*) aumentaba a medida que aumentaba la presión de SC-CO_2 (los resultados expresados como mg/kg de baya seca). En cualquier caso, estos autores encontraron que los valores de α-tocoferol fueron significativamente más altos tras aplicar SC-CO_2 en comparación con la extracción convencional, independientemente del tratamiento aplicado.

6.3. Impacto de la extracción convencional (CE) y por fluidos supercríticos (SC-CO_2) en los polifenoles de los extractos lipófilos obtenidos

Los valores de compuestos fenólicos totales (CFT) aumentaron significativamente a medida que aumentó la presión de SC-CO_2 utilizada. El valor de CFT aumentó linealmente (CFT = 17.447 X Presión + 126.76; R2 = 0.9885) de 69.3 mg de equivalentes de ácido gálico (GAE)/100 g de aceite en muestras de 10 MPa a 579.6 mg GAE/100 g de aceite en muestras de 40 MPa. Nuestros valores fueron más altos que los descritos por Koubaa, Barba, et al. (2015), los cuales obtuvieron valores de aproximadamente 110 mg GAE/100 g de chufa tras utilizar SC-CO_2 y aproximadamente 320 mg de GAE/100 g de chufa tras utilizar expresión mecánica asistida por gas (GAME). Estas diferencias en las cantidades de CFT también se deben a las diferentes unidades de resultados utilizadas en este estudio (mg de GAE/100 g de aceite) y en otras investigaciones (mg de GAE/100 g de producto). Además, nuestros resultados también contrastan con los obtenidos por Koubaa, Barba, et al. (2015) donde estos autores no encontraron diferencias significativas en la extracción de CFT, independientemente del tratamiento aplicado (SC-CO_2; 10-30 MPa).

Sin embargo, los resultados obtenidos en nuestro estudio se mostraron de acuerdo con los obtenidos por Koubaa, Lepreux, Barba, Mhemdi, & Vorobiev (2017) cuando estudiaron la extracción de CFT de orujo de oliva, un subproducto de la producción de aceite de oliva, tras aplicar SC-CO_2. Estos autores atribuyeron las diferencias en los rendimientos de extracción de CFT a la diferente permeabilidad de las tortas de prensado obtenidas tras aplicar SC-CO_2. Es posible que debido a la estructura dañada durante el proceso de producción, los compuestos fenólicos se puedan extraer más fácilmente.

La cantidad de CFT en los aceites obtenidos tras extracción

convencional fue menor ($P<0.05$) (13.2 mg GAE/100 g de aceite) que tras aplicar SC-CO_2. La cantidad de CFT encontrada en la extracción convencional coincidió con los resultados publicados previamente por Ali Rehab & El Anany (2012) en las chufas, los cuales obtuvieron valores de 16.5 mg de GAE/100 g de aceite. Los valores más bajos de CFT tras la extracción convencional se relacionan con el hecho de que los compuestos fenólicos se encuentran preferentemente en la fase metanólica en lugar de en la fase grasa $CHCl_3$. Para verificar esta información, el análisis de CFT también se realizó en la fase metanólica obteniendo 173.97±3.06 mg de GAE/100 g. En este sentido, los compuestos fenólicos del aceite son importantes para evaluar su actividad antioxidante, protegiendo así el aceite contra la oxidación de los lípidos y mejorando su estabilidad (Ezeh, Gordon, et al., 2016).

Respecto al perfil y el contenido de polifenoles individuales obtenido tras aplicar el tratamiento por SC-CO_2 se observó un aumento notable en el número y la cantidad de compuestos fenólicos a medida que aumentó la presión aplicada, siendo predominante el isohidroximatairesinol. Mientras que los compuestos fenólicos obtenidos con el procedimiento convencional de extracción de aceite (método de Folch) fueron: 4-vinilfenol, ácido p-cumárico y ácido p-hidroxibenzoico.

El perfil obtenido se asemeja en cualquier caso al encontrado por otros autores como Parker et al. (2000), los cuales observaron un contenido importante de ácido p-cumárico, ferúlico, p-hidroxibenzoico, p-hidroxibenzaldehído y vainillina tanto en la piel de chufa como en el tubérculo. Oladele et al. (2017) observaron una diferencia en cuanto al contenido de compuestos fenólicos en función del tipo de chufa analizada obteniendo ácidos ferúlico y p-hidroxibenzoico (58.38 y 29.12 mg/100 g, respectivamente) como los principales compuestos fenólicos en las chufas de color amarillo, mientras que los ácidos vanílico y p-cumárico (15.20 y 17.25 mg/100 g, respectivamente) fueron los predominantes en la variedad marrón. Estos autores también observaron la presencia de p-

hidroxibenzaldehído, ácidos cafeico y sinapínico y flavonoides. En otro estudio sobre la composición fenólica de productos de la chufa, Ezeh et al. (2016) mostraron la presencia de ácido trans-cinámico como uno de los principales compuestos fenólicos, junto con los ácidos ferúlico y vanílico y la vainillina.

La integridad de la estructura de los productos derivados de la "horchata" parece influir en la extracción de compuestos fenólicos. En este sentido, Parker et al. (2000) observaron una estrecha relación entre la integridad de la matriz lignificada y la extracción de compuestos fenólicos, en particular para los ácidos p-cumárico y ferúlico. La continua degradación de la estructura de lignina se mostró como un factor muy importante que influyó en el contenido fenólico de los extractos finales obtenidos por estos autores. En nuestro caso, las diferencias en el protocolo de extracción (molienda para SC-CO_2 frente a la homogeneización a 10.000 rpm con UltraTurrax® para CE) evaluadas en el presente estudio pueden contribuir a explicar las diferencias observadas entre el perfil fenólico de SC-CO_2 y CE. El daño severo a la matriz vegetal de los subproductos de la "horchata" tras la homogeneización a gran velocidad en el disolvente (CE) facilitó la liberación de compuestos fenólicos atrapados, principalmente ácidos p-cumáricos y ferúlicos.

Por contra, la molienda antes de la extracción por SC-CO_2 se limitó a reducir el tamaño de partícula y exponer parte de los compuestos atrapados, principalmente ácido p-cumárico. Esta degradación parcial explica el menor rendimiento de extracción de los compuestos fenólicos con excepción del isohidroximatairesinol, la escopoletina y el ácido cafeico en el perfil fenólico y el contenido obtenido del protocolo SC-CO_2 en comparación con el perfil fenólico tras aplicar CE.

Además de eso, el uso de tecnologías adicionales para desestabilizar la integridad de la chufa para la extracción de compuestos fenólicos también se ha estudiado por otros autores. En este sentido, Ezeh et al.

(2016) investigaron el uso de altas presiones antes del tratamiento enzimático (combinación de amilasa, celulosa, carbohidrasas y proteasas) para mejorar la extracción de aceite a partir de chufa. Estos autores obtuvieron un contenido fenólico más alto en los aceites extraídos combinando presión y enzimas en comparación con la expresión mecánica convencional. Ezeh et al. (2016) atribuyeron el aumento del rendimiento de extracción de polifenoles a la hidrólisis de la unión entre el extremo éster de los compuestos fenólicos y los componentes de la pared celular facilitada por las enzimas. Además, el impacto del tratamiento enzimático (proteasa, α-amilasa y Viscozyme®) en la estructura de las chufas fue evaluada previamente por Ezeh, Gordon, et al. (2016), los cuales observaron un daño importante en la pared celular inducido por el tratamiento, observando así una mayor extracción de aceite y posiblemente de compuestos fenólicos.

Por otro lado, se cree que el procedimiento de CE facilita la extracción de compuestos fenólicos de los subproductos de la "horchata". El mayor contenido de ácidos p-cumáricos y ferúlicos obtenidos por el procedimiento de CE parece apoyar esta consideración. Una explicación razonable para este resultado es la intensa degradación promovida por este procedimiento al homogeneizar los subproductos de "horchata" a 10.000 rpm en contacto con una mezcla de sales y disolventes (NaCl, $CHCl_3$ y methanol).

El SC-CO_2 puro no se aplica comúnmente para extraer compuestos fenólicos debido a las diferencias en la polaridad del SC-CO_2 (adecuado para la recuperación de compuestos no polares y moderadamente polares) y compuestos fenólicos (carácter hidrófilo) (Woźniak et al., 2018). Teniendo esto en cuenta, es necesario mejorar el poder de solvatación del SC-CO_2 con el objetivo de mejorar la recuperación de los polifenoles. Por ejemplo, más codisolventes polares pueden interactuar con compuestos fenólicos (por ejemplo, mediante la unión de hidrógeno y dipolo-dipolo) y facilitar la liberación de compuestos en comparación

con la extracción de SC-CO_2 puro. Así pues, algunos estudios han demostrado que el etanol y el agua mejoran el rendimiento de extracción de compuestos fenólicos de subproductos de *Aronia sp.* (Woźniak et al., 2018).

6.4. Evaluación de los parámetros de oxidación lipídica y capacidad antioxidante de los aceites tras SC-CO_2 y extracción convencional (CE)

La oxidación lipídica es uno de los procesos más importantes que ocurren en los sistemas alimentarios, resultando en el deterioro de la calidad del producto (Suja, Abraham, Thamizh, Jayalekshmy, & Arumughan, 2004). La generación de sabores desagradables, la pérdida de valor nutritivo y la acumulación de compuestos tóxicos son los cambios más comunes que ocurren en el aceite (Arabshahi-D, Vishalakshi Devi, & Urooj, 2007). El índice de peróxidos es uno de los métodos que se utilizan para medir la estabilidad oxidativa de los aceites, dando una clara indicación de la oxidación lipídica (Suja et al., 2004). Por ejemplo, los aceites obtenidos mediante extracción convencional mostraron valores medios de 15.2 meq oxígeno activo/kg de aceite, que fueron similares a los encontrados en muestras obtenidas tras aplicar SC-CO_2 a 10 MPa. Los valores encontrados para los aceites obtenidos mediante extracción convencional fueron más altos que los obtenidos por otros autores (Sobhani, Mohammed, Ghobakhlou, & Ghazali, 2018; Yeboah et al., 2012) en aceite de chufa extraído con otra técnica convencional (15.2 vs. 5.5 y 3.1 meq oxígeno activo/kg de aceite, para extracción de CE y Soxhlet durante 6 y 8 horas, respectivamente), y similares a los valores (15.2 meq oxígeno activo/kg de aceite frente a 16.3 meq oxígeno activo/kg de aceite, para la extracción de CE y Soxhlet, respectivamente) encontrados por Hu et al. (2018). Además, se encontraron valores significativamente más bajos ($P<0.05$) cuando las condiciones de presión de la extracción SC-CO_2 aumentaron (15.5, 11.1, 10.6 y 10.5 meq/kg de aceite para SC-CO_2 a 10 MPa, 20 MPa, 30 MPa y 40 MPa, respectivamente). En contraste, Hu et al. (2018) encontraron valores más bajos en el aceite de chufa extraído con otra técnica no convencional (microondas).

En todos los casos, los valores fueron superiores al límite (10 meq oxígeno activo/kg de aceite) considerado aceptable para los atributos sensoriales (CODEX, 1999), aunque los valores obtenidos en extracciones de SC-CO_2 con condiciones de presión más altas (SC-CO_2 a 30 MPa y 40 MPa) ofrecieron los mejores resultados, acercándose al límite antes mencionado.

Con respecto al índice de p-anisidina, su valor refleja la formación de compuestos derivados de la oxidación secundaria (McGinley, 1991). La etapa secundaria de oxidación ocurre cuando los hidroperóxidos se descomponen para formar carbonilos y otros compuestos, en particular aldehídos, que confieren sabores indeseables a los aceites (olores rancios). En este sentido, un valor más bajo de p-anisidina está relacionado con una mejor calidad del aceite. Ambos, el tipo de lípido y el tiempo de oxidación son dos de los factores principales que influyen en este parámetro (Kim, Yeo, Kim, Kim, & Lee, 2013). Los resultados encontrados en las muestras de aceite obtenidas a partir de subproductos de "horchata" extraídos con SC-CO_2 a las presiones más bajas mostraron valores más altos de p-anisidina en comparación con los obtenidos con presiones más altas (14.7, 10.8, 6.5 y 6.0 para SC-CO_2 a 10 MPa, 20 MPa, 30 MPa y 40 MPa, respectivamente). Este hecho podría estar relacionado con la mayor cantidad de polifenoles en el aceite extraído a altas presiones. De hecho, se encontró una correlación inversa y significativa entre la p-anisidina y los CFT ($r = -0.694$; $P < 0.05$). Estos valores fueron más bajos que los encontrados en el aceite obtenido con extracción convencional, lo que podría atribuirse a las diferentes condiciones de la extracción. Los valores obtenidos por Sobhani et al. (2018) en aceite de chufa extraído con Soxhlet fueron mucho más bajos que los encontrados en el presente estudio tras extracción convencional con el método de Folch (21.4 vs. 0.32, respectivamente). Los valores del índice de p-anisidina por debajo de 2.0 están relacionados con aceites de buena calidad (Choo, Birch, & Dufour, 2007). En el presente estudio, los valores

obtenidos superan el valor mencionado anteriormente, siendo las muestras extraídas con SC-CO_2 a 40 MPa las que muestran los mejores valores para este índice.

El valor de Totox indica el estado de oxidación general de un aceite. De manera similar al índice de peróxidos, los aceites extraidos mediante el método de Folch mostraron valores medios cercanos a los encontrados en las muestras obtenidas por SC-CO_2 a 10 MPa (57.2 vs. 57.4, respectivamente). Con respecto a la extracción de SC-CO_2, los valores de Totox disminuyeron a medida que aumentaba la presión (57.4, 48.5, 27.2 y 26.9, respectivamente). Además, cuanto menor era el valor de Totox, mejor era la calidad del aceite, de modo que las muestras extraídas con SC-CO_2 a 40 MPa tendrían una mayor calidad. Otros autores hallaron valores más bajos de este parámetro en el aceite de chufa (Sobhani et al., 2018).

Con respecto a la capacidad antioxidante total medida mediante el test ABTS (valores de capacidad antioxidante en equivalentes Trolox (TEAC)) del aceite obtenido a partir de subproductos de "horchata", las muestras extraídas con SC-CO_2 a 40 MPa mostraron los valores más altos. Como se esperaba, los valores de la actividad antioxidante fueron mayores a medida que aumentaron las condiciones de presión de la extracción de SC-CO_2. De hecho, este comportamiento se reflejó en un ajuste lineal de los datos a medida que aumentaba la presión (valor TEAC = 1.051 X Presión + 21.675; R^2 = 0.945), desde 34.88 mM Trolox en muestras de 10 MPa a 64.35 mM Trolox en muestras de 40 MPa. La comparación de SC-CO_2 con CE mostró valores similares a presiones más bajas (34.88 mM Trolox a 10 MPa, 37.94 mM Trolox a 20 MPa y 38.05 mM Trolox en CE). Estos resultados confirmaron la potencial aplicación de SC-CO_2 para recuperar aceites ricos en compuestos bioactivos antioxidantes de los productos derivados de la "horchata".

Se obtuvo una correlación positiva entre los compuestos fenólicos totales (CFT) y la capacidad antioxidante total medida mediante el test

ABTS (TEAC), lo que indica que los compuestos fenólicos desempeñan un papel central como posibles antioxidantes. Estos resultados se muestran de acuerdo con los resultados obtenidos por otros autores. Por ejemplo, Sanjaya et al. (2014) obtuvieron una correlación positiva entre los CFT del extracto SC-CO_2 de Sarang semut (*Myrmecodia pendans)* y el ensayo con el radical 2,2-difenil-1-picrilhidrazilo (DPPH) (al utilizar intervalos de presión de 9-22.5 MPa y temperaturas comprendidas entre 40 y 70 °C). Del mismo modo, Wang et al. (2011) obtuvieron una correlación positiva (R>0.850) entre el ensayo CFT y DPPH para los extractos obtenidos a partir de tallos de Moyeam *(Ampelopsis grossedentata)* tras aplicar SC-CO_2 (15 y 25 MPa/40-60 °C).

Por otra parte, se obtuvieron correlaciones negativas entre compuestos fenólicos totales con los test de la p-anisidina y TOTOX, así como entre la capacidad antioxidante total medida mediante el test ABTS y todos los índices de oxidación. Estas relaciones refuerzan el papel de los compuestos fenólicos extraídos de los subproductos de la "horchata" como compuestos activos contra las reacciones oxidativas. En este sentido, Gharibzahedi, Mousavi, Hamedi, & Khodaiyan (2014) obtuvieron resultados similares, observando que el ensayo de compuestos fenólicos totales y el test Rancimat (un método acelerado para caracterizar el estado oxidativo de los aceites) se correlacionaron negativamente (R = -0.92) en el aceite de nuez persa (*Juglans regia L.*).

El ácido cafeico, el isohidroximatairesinol, la escopoletina y la sinensetina fueron los compuestos que mostraron correlaciones positivas con CFT y TEAC (R>0.850). Estos compuestos también se correlacionaron negativamente con el valor de peróxidos (VP), p-anisidina y TOTOX. Este resultado indicó que tales compuestos desempeñan un papel importante como compuestos antioxidantes de los productos derivados de la "horchata". Además, el uso de la tecnología SC-CO_2 fue crucial para extraer estos compuestos, ya que el procedimiento de CE mostró una capacidad significativamente menor para extraerlos.

Zheng, Yang, Zhou, Liu, & Huang (2014) estudiaron esta proporción inversa entre el índice de contenido fenólico individual y el índice de estado oxidativo, quienes evaluaron el efecto del canolol (compuesto fenólico que se encuentra naturalmente en el aceite de colza). Estos autores obtuvieron correlaciones positivas entre el ensayo DPPH con los CFT y con el contenido de canolol (ambos con R> 0.890), mientras que también se observaron correlaciones negativas entre el contenido de canolol y los índices de oxidación (valores de VP y p-anisidina; ambos con R <-0.890).

Curiosamente, la mayoría de los compuestos extraídos por el procedimiento CE (R en el intervalo de 0.799-0.817) se correlacionaron positivamente con el ensayo de p-anisidina. Parece razonable inferir que los compuestos fenólicos extraídos por el procedimiento de CE mostraron un potencial más bajo para prevenir/inhibir la oxidación del aceite derivado de "horchata" que los compuestos obtenidos por la tecnología SC-CO_2.

Además también se observó una correlación directa y significativa entre la cantidad de α-tocoferol y AGPI (R = 0.7165; P = 0.0197) y especialmente con el contenido de omega-3 (R = 0.7886; P = 0.0067). Este hecho sugiere que el α-tocoferol es importante para protegerlos contra la oxidación (Quiles, Ramírez-Tortosa, Gómez, Huertas, & Mataix, 2002).

7. CONCLUSIONES

7. CONCLUSIONES

Del estudio realizado se pueden establecer las siguientes conclusiones:

1. Los subproductos de la "horchata" son una fuente importante de compuestos bioactivos antioxidantes.

2. Respecto a la extracción convencional utilizando diferentes porcentajes de mezclas hidroetanólicas, modificando la temperatura de extracción y la duración de la misma, se observa que la temperatura, el tiempo y la concentración de etanol presentan una influencia estadísticamente significativa en la recuperación de los compuestos fenólicos totales. Las temperaturas más altas aumentan la eficiencia de extracción de los compuestos fenólicos totales, mientras que de la misma manera, las concentraciones de etanol y los tiempos de extracción prolongados mejoran la extracción de estos compuestos. Se observan valores de extracción máximos de 16.02 mg de equivalentes de ácido gálico (GAE)/100 g de materia seca tras la extracción con etanol al 25% (v/v), a 60 °C, durante 3 horas.

3. En la extracción convencional de flavonoides totales, la concentración de etanol y el tiempo de extracción influyeron significativamente en la recuperación. La extracción fue mayor después de usar etanol al 25% (v/v), en comparación con el 0% o el 50% de etanol (v/v). Los valores de flavonoides totales aumentaron con la temperatura y alcanzaron un valor máximo de 30.09 mg equivalente de catequina (CE)/100 g de materia seca a 50 °C, al extraerse con etanol al 25% (v/v) durante 3 horas.

4. En la recuperación de compuestos antioxidantes la temperatura y la concentración de etanol influyeron significativamente. La capacidad antioxidante aumentó con mayores concentraciones de etanol, con un

mayor tiempo de extracción y a mayor temperatura. El rendimiento máximo de 1759.81 μM Trolox/g de materia seca se alcanzó con etanol al 50% (v/v), 60 ° C y un tiempo de extracción de 3 h. Asimismo, se estableció una correlación positiva entre la capacidad antioxidante y los compuestos fenólicos totales, así como entre las concentraciones de compuestos fenólicos totales y flavonoides totales.

5. La tecnología de extracción con fluidos supercríticos (SC-CO_2) fue más eficiente para recuperar compuestos antioxidantes lipófilos, en particular compuestos fenólicos con alta capacidad antioxidante que el procedimiento convencional de extracción mediante método Folch. Asimismo, el aceite extraído presentó una mayor estabilidad oxidativa tras aplicar SC-CO_2 a 30 y 40 MPa, en comparación con las extracciones realizadas a presiones más bajas o al procedimiento de extracción convencional.

6. Se observa una mayor extracción de los diferentes compuestos individuales tras aplicar extracción con fluidos supercríticos cuando se aumenta la presión de tratamiento obteniendo los valores más altos cuando la presión fue de 40 MPa. Sin embargo para determinados compuestos (etilvanilina, sinensetina, 4-vinilfenol, ácidos cinámicos, cumárico, ferúlico, p-hidroxibenzoico y vanílico) se obtuvieron los valores más altos de extracción cuando se aplicó el procedimiento convencional. Por lo tanto se debe seleccionar la técnica de extracción de acuerdo al compuesto fenólico diana que se pretenda recuperar.

Estas conclusiones plantean retos futuros ya que existe una necesidad de realizar estudios adicionales utilizando otros métodos alternativos para la extracción de compuestos a partir de subproductos de la "horchata", que deberían centrarse en reducir el tiempo de extracción, disminuir o minimizar el uso de disolventes tóxicos y reducir la temperatura de extracción. En ese sentido otras tecnologías de extracción innovadoras, como electrotecnologías, ultrasonidos y procesamiento por altas presiones hidrostáticas, podrían resultar útiles para valorizar los subproductos líquidos y sólidos. Asimismo, también sería necesario evaluar la presencia de otros compuestos bioactivos y nutrientes de interés que pudieran estar presentes en los subproductos de la chufa tras la elaboración de la “horchata” como fitoesteroles, carotenoides, etc., que pueden ser de interés para las industrias cosméticas y alimentarias.

8. BIBLIOGRAFÍA

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ANEXO 1.CONTRIBUCIONES

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La presente tesis doctoral ha dado lugar a 5 artículos, publicados o que se publicaran en las siguientes revistas:

1. **Roselló-Soto, E., Poojary, M.M., Barba, F.J., Koubaa, M., Lorenzo, J.M., Mañes, J., Moltó, J.C. (2018).** Thermal and non-thermal preservation techniques of tiger nuts' beverage "horchata de chufa". Implications for food safety, nutritional and quality properties. Food Research International, 105, 945-951.

2. **Roselló-Soto, E., Poojary, M.M., Barba, F.J., Lorenzo, J.M., Mañes, J., Moltó, J.C. (2018).** Tiger nut and its by-products valorization: From extraction of oil and valuable compounds to development of new healthy products. Innovative Food Science and Emerging Technologies, 45, 306-312.

3. **Roselló Soto, E., Barba, F.J., Putnik, P., Bursac Kovacevic, D., Lorenzo, J.M., Cantavella-Ferrero, Y. (2018).** Enhancing bioactive antioxidants' extraction from "horchata de chufa" by-products. Foods, 7, 161.

4. **Roselló-Soto, E., Barba, F.J., Lorenzo, J.M., Domínguez, R., Pateiro, M., Mañes, J., Moltó, J.C. (2018).** Evaluating the impact of supercritical-CO_2 pressure on the recovery and quality of oil from "horchata" by-products: Fatty acid profile, α-tocopherol, phenolic compounds, and lipid oxidation parameters. Food Research International, En prensa.

5. **Roselló-Soto, E., Barba, F.J., Lorenzo, J.M., Munekata, Paulo E. S., Gómez, B., Moltó, J.C. (2019).** Phenolic profile of oils obtained from "horchata" by-products assisted by supercritical-CO_2 and its

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CONGRESOS

Comunicaciones orales

Roselló-Soto, E., Cantavella Ferrero, Y., Barba, F.J. (2018). Innovative alternative approaches to recover oil and high-added value compounds from by-products obtained from vegetable-based beverages production process: Horchata de chufa. 9th International Congress of Food Technologists, Biotechnologists and Nutritionists "INTEGRATING NEW APPROACHES TO PRODUCE SAFE, NUTRITIOUS AND SUSTAINABLE FOOD". Zagreb, Croacia. ISBN: 978-953-99725-7-6.

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Abstracts

Roselló-Soto, E., Cantavella Ferrero, Y., Marti-Quijal, F.J., Roohinejad, S., Annor, G.A., Dohrmann, D.D., Munekata, P.E.S., Lorenzo, J.M., Barba, F.J. (2018). SC-CO_2 extraction and triple TOF–LC–MS–MS characterization of polyphenols from oils extracted of tiger nuts by-products. Conference of Food Engineering (COFE). Minneapolis, Minnesota, USA.

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