UNIVERSIDAD DE GRANADA

FACULTAD DE CIENCIAS

Departamento de Química Analítica

Programa de Doctorado en Química

Grupo de Investigación AGR-274 “Bioactive ingredients”

Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF)

DEVELOPMENT OF NANOENCAPSULATED PHENOLIC GREEN EXTRACTS FOR FUNCTIONAL FOOD AND NUTRACEUTICAL: *Hibiscus sabdariffa as example*

Memoria presentada por

Sandra Pimentel Moral

Para optar al grado de

Doctor Internacional en Química por la Universidad de Granada

Editor: Universidad de Granada. Tesis Doctorales
Autor: Sandra Pimentel Moral
ISBN: 978-84-1306-106-1
URI: http://hdl.handle.net/10481/54794

Granada, Enero de 2019

Esta Tesis doctoral ha sido realizada gracias a una beca predoctoral de Formación de Profesorado Universitario (FPU) concedida por el Ministerio de Educación, Cultura y Deporte (FPU15/01125) y a la financiación con cargo a fondos del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF) procedentes de diferentes proyectos, contratos y subvenciones de las Administraciones Central y Autonómica, Plan Propio de Investigación de la Universidad de Granada y el grupo TEP-025 del Departamento de Ingeniería Química de la Universidad de Granada.

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por

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Granada, Noviembre de 2018

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Los profesores Dr. Antonio Segura Carretero, Catedrático en el Departamento de Química Analítica de la Universidad de Granada y Director en el Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (CIDAF), y el profesor Dr. Antonio Martínez Férez, Catedrático en el Departamento de Ingeniería Química de la Universidad de Granada,

CERTIFICAN:

Que el trabajo realizado en la presente Tesis doctoral titulada: **"DEVELOPMENT OF NANOENCAPSULATED PHENOLIC GREEN EXTRACTS FOR FUNCTIONAL FOOD AND NUTRACEUTICAL: *Hibiscus sabdariffa* as example"**, se ha realizado bajo su dirección y en los laboratorios del Departamento de Química Analítica e Ingeniería Química de la Universidad de Granada, del Centro Tecnológico de Investigación y Desarrollo del Alimento Funcional (Parque Tecnológico de la Salud), así como también de manera parcial en las instalaciones del Departamento de Tecnología Farmacéutica de la Universidad de Coimbra (Coimbra, Portugal), reuniendo todos los requisitos legales, académicos y científicos para hacer que la doctoranda Dña. Sandra Pimentel Moral pueda optar al grado de Doctor Internacional en Química por la Universidad de Granada presentando la tesis como agrupación de publicaciones.

Y para que así conste, expido y firmo el presente certificado en Granada a 23 de Noviembre de 2018.

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“A veces es difícil dar las gracias en un par de folios cuando en los cuatro años en los que se ha desarrollado la tesis los he pasado rodeada de tantas buenas personas. En primer lugar quiero agradecer a mis directores de Tesis, Dr. Antonio Segura Carretero y Dr. Antonio Martínez Férez por darme la oportunidad de trabajar en sus laboratorios, por apoyarme en mi investigación y dirigir mi camino durante esta etapa. Su tiempo y predisposición han hecho posible que esta Tesis haya culminado con éxito.

También me gustaría dar las gracias a aquellos compañeros que pasaron por CIDAF como Elena, Patricia, Arancha, Maria del Mar, Eli, David, Raquel Márquez, Javi “pequeño ingeniero”, Geovanny… los cuales echo de menos y sé que aunque no estén en este laboratorio me siguen apoyando. Celia, muchísimas gracias por enseñarme a manejarme en este mundo, por ayudarme en mis estancias cuando más perdida estaba y resolver todas mis dudas. Eres una gran persona y te mereces todo el éxito que estás consiguiendo. “Doble V” y Ana gracias por estos años de amistad. ¡Estoy deseando conocer a ese “pequeño Vito” que viene en camino!

Además, también quiero agradecer a los compañeros y amigos de CIDAF, algunos de los cuales me han apoyado desde el comienzo de esta aventura. Mi compañero de fatiga, Álvaro, un amigo que ha ido conmigo de la mano en todo el doctorado (matrículas de doctorado, memorias anuales de FPU, cursos…) sin duda la persona más humilde que conozco. Mil gracias por acompañarme en toda esta etapa. ¡El siguiente eres tú! No me puedo olvidar de Mari Luz y Javi, las personas que más me hacen reír, sus bromas hacen que te olvides de los problemas. Además, todos sabemos que sin Mari Luz esta Tesis nunca hubiese sido depositada ya que eres la mejor secretaria que he podido tener. Javi, mi compi de lab, aún recuerdo tu cara pálida cuando

escuchamos “explotar” el microondas… mientras yo lloraba, tú limpiabas calmándome en todo momento. Gracias por ayudarme en todo. Isa y Jesús, gracias por buscar un camino en esta Tesis. Isa, en estos últimos meses he compartido mucho tiempo contigo, de trabajo, de comidas, de risas y de consejos que me han hecho verte como una amiga más. Mil gracias. Mery, mi “vecina rubia”, gracias por tus conversaciones mañaneras y tus ánimos. Sigue soñando así de bonito. Bea muchas gracias por esos momentos y risas intentando entender el diseño de superficie-respuesta. Rafa gracias por tu generosidad y tus consejos. Rosa muchas gracias por resolver mis dudas analíticas, eres una gran persona y una súper mamá. David Arráez, gracias por tu ayuda con la docencia y por tener ese buen humor todos los días. José Antonio y Raquel del Pino aunque os conozco desde hace menos tiempo, me habéis demostrado el gran corazón que tenéis. Y finalmente Ana Vigil, con tus refranes y frasecillas que tanto nos han enseñado.

No me puedo olvidad de mis compis del Departamento de Ingeniería Química. Muchas gracias Javi y José Antonio por ayudarme en un campo de investigación diferente a mi formación.

On the other hand, I would like to thank to Prof. Eliana to bring me her lab and the opportunity to work in the University of Coimbra. Also, I thank to my workmates Maria du Ceu and Ana Rita their help and our “portuñol” conversations.

En último lugar y el más importante, quiero agradecer a los pilares de mi vida su cariño y su apoyo. Mis padres, los cuales ya saben que significa el término “Under review” han sufrido cuando recibía un “Rejected” y celebrado cada “Accepted”. A ellos les debo el estar hoy aquí. Gracias por aguantarme. Mi hermano, gracias a ti he sabido lo

que es la constancia en el trabajo y el esfuerzo. Tú me has hecho ver lo importante que es el trabajo del día a día para conseguir un objetivo. Sé que muy pronto te veré con el traje de Policía Local puesto. Mis mejores amigas, Regina y Sofía, gracias por llorar, reír, cantar y hacerlo todo conmigo (hasta la portada de esta tesis). Vuestro apoyo ha sido mi mayor consuelo. Y finalmente me gustaría dejar mis últimas líneas a mi compañero de vida, a la persona que ha vivido toda mi carrera profesional desde el primer momento. Nos conocimos cuando comenzábamos nuestras respectivas carreras y 10 años después sigues estando a mi lado, calmando mis nervios, dándome los mejores momentos de mi vida, quitándome cualquier preocupación y haciéndome ver que siempre hay un camino por el que andar. Esta etapa como doctoranda termina, pero en mayo comenzamos juntos una etapa mejor".

"***Dejaría en este libro***
toda mi alma.
Este libro que ha visto
conmigo los paisajes
y vivido horas santas"

(Federico García Lorca, 1918)

Poeta nacido en Fuente Vaqueros, mi pueblo natal

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AG	Arabic gum
ANOVA	One-way analysis of variance
ASE	Accelerated solvent extraction
BPC	Base peak chromatogram
CCD	Central Composite Design
CE	Capillary electrophoresis
CMC	Carboxymethylcellulose
CV	Coefficient of variation
D (1,0)	Number length mean
D (3,2)	Surface area moment mean
D (4,3)	Volume moment mean
DAD	Diode array detector
DLS	Dynamic light scattering
EFSA	European Food Safety Authority
EE	Encapsulation efficiency
ESI	Electrospray ionization
EuroFIR	European Food Information Resource
EVOO	Extra virgin olive oil
FAO	Food and Agriculture Organization
FDA	Food and drug Administration
FD&C Act	Federal Food, Drug and Cosmetic Act
FRAP	Ferric reducing antioxidant power
FTIR	Fourier-transform infrared spectroscopy
GAE	Gallic acid equivalent
GC	Gas chromatography
GIT	Gastrointestinal tract
GRAS	Generally recognized as safe
H. sabdariffa	*Hibiscus sabdariffa*
HLB	Hydrophilic-lipophilic balance
HPLC	High-performance liquid chromatography
HPLC-IR	High performance liquid chromatography coupled infrared spectroscopy
HPLC- NMR	High performance liquid chromatography coupled nuclear magnetic resonance
Hs	*Hibiscus sabdariffa*
IS	Internal standard
Kc	Coalescence rate
LD	Laser diffraction
LMP	Low-methoxyl pectin
LOD	Limit of detection
LOQ	Limit of quantification
LPHNs	Lipid-polymer hybrid nanoparticles
m/z	Mass-to-charge ratio
MAE	Microwave assisted extraction

m_E	Mass of the bioactive ingredient incorporated
m_F	Mass of non-entrapped active material
MIR	Middle infrared region
M_n	Number average molecular weight
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
m_T	Total mass of bioactive ingredient added
M_w	Weight average
NLCs	Nanostructured lipid carriers
O/W	Oil-in-water
O/W/O	Oil-in-water-in-oil
OS:WS	Oil surfactant : water surfactant relation
PBS	Phosphate buffer saline
Pc	Critical pressure
PDI	Polydispersity index
pI	Isoelectric point
PLE	Pressurized liquid extraction
PSD	Particle size distribution
Q	Quadrupole
QE	Quercetin equivalent
QIT	Quadrupole ion trap
QTOF	Quadrupole time-of-flight
ROS	Reactive oxygen species
RP-HPLC	Reversed-phase high performance liquid chromatography
RSD	Relative standard deviation
RSM	Response surface methodology
Rt	Retention time
S/N	Signal-to-noise ratio
SD	Standard deviation
SE	Soxhlet extraction
SEI	Electrostatic interaction
SEM	Scanning electron microscopy
SF	Supercritical fluid
SFE	Supercritical fluid extraction
SLNs	Solid lipid nanoparticles
SO	Sunflower oil
STEP	Space-and time- resolved extinction profiles
Tc	Critical temperature
TEAC	Trolox equivalent antioxidant capacity
TEM	Transmission electron microscopy
TOF	Time-of-flight
TPC	Total phenolic content
TPTZ	2.4.6-trpyridyl-s-triazine
UAE	Ultrasound assisted extraction
UV-Vis	Ultraviolet-Visible
W/O	Water-in-oil
W/O/W	Water-in-oil-in-water

WPC	Whey protein concentrate
WPI	Whey protein isolate
ZP	Zeta potential

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Summary/Resumen

❖ SUMMARY

The current report encompasses all the results found during the work carried out for the PhD Thesis entitled: **"DEVELOPMENT OF NANOENCAPSULATED PHENOLIC GREEN EXTRACTS FOR FUNCTIONAL FOOD AND NUTRACEUTICAL: *Hibiscus sabdariffa* as example".** This Thesis provides data on the developmental timeline for nutraceutical or functional food.

This work is divided into two main sections: Introduction and Experimental Summary.

The **Introduction** section begins by presenting the state of art with regards to nutraceuticals and functional foods. The normal timeline involved in their development is presented, beginning with the search for natural sources of phenolic compounds, their subsequent extraction by green technologies and characterisation using analytical approaches, and finally their encapsulation and incorporation in a food matrix. Firstly, bioactive compounds and phenolic compounds are described and classified according to their chemical structure. Next, the types of green extraction technologies (e.g. microwave assisted extraction, pressurised liquid extraction and supercritical fluid extraction) used for phenolic compounds in plant matrices are reported. Following this, chromatographic analytical techniques used for separation, such as high performance liquid chromatography (HPLC), detection like UV-Vis and mass spectrometry (MS) for the identification and quantification of phenolic compounds are described. Details are then given on the micro and nano-encapsulation of these phenolic compounds and the role of this in ensuring their stability against environmental conditions. In this Thesis, lipid-based nanosystems are detailed, in

addition to the main stability mechanisms of this encapsulation technique. Finally, the present Thesis reports the incorporation of encapsulated phenolic compounds in a food matrix.

The **Experimental Summary** is split into three subsections. The first section **(Section A)** deals with the search of new sources of phenolic compounds. In this sense, *Hibiscus sabdariffa* is chosen due to its bioactive composition. The origin, distribution, biological effects, nutritional value and phenolic composition of this plant are also mentioned in this section.

The second section **(Section B)** examines the optimisation of extraction systems for producing extracts rich in phenolic compounds from *Hibiscus sabdariffa*, which can then subsequently undergo characterisation. This section is divided into 3 chapters. In **Chapter 1**, a green extraction technology, namely microwave assisted extraction (MAE), is optimised using different solvent-water ratios, extraction times and temperatures, to obtain the best extraction condition capable of providing the highest phytochemical content. In this regard, all experimental green extracts were characterised by HPLC-MS. In **Chapter 2**, the optimisation of green pressurised liquid extraction (PLE) employing different solvent-water ratios and temperatures to achieve a green extract with the highest phenolic compound content is described. Finally, in **Chapter 3** the optimisation of supercritical fluid extraction (SFE) to obtain the best extraction conditions for phenolic compounds is described. In this technique, different pressures, temperatures and co-solvent amounts were tested. Finally, the characterisation of green extract using HPLC-MS is described.

The third section, **(Section C)** is dedicated to micro- and nano- encapsulation of phenolic compounds from *Hibiscus sabdariffa* and their incorporation into a food

matrix. This section is divided into five chapters in which Chapter 5 and 6 were written in collaboration with the Chemical Engineering Department, whilst Chapter 4, Chapter 7 and Chapter 8 were prepared in collaboration with the University of Coimbra (Portugal), following a 3-month pre-doctoral mobility fellowship named "Estancias breves FPU". In **Chapter 4**, different lipid-based nanosystems are detailed for the entrapment of phenolic compounds. Thus, the main characteristics of emulsions and nanostructured lipid carriers (NLC) (whose techniques are employed in the current Thesis) are reported. In **Chapter 5,** the development of new functional oils containing antioxidants from *Hibiscus sabdariffa* green extract using the emulsion method is detailed. In addition the physical and oxidative stability of these emulsions were evaluated and is reported here. Particle size, coalescence rate, total phenolic content (TPC), antioxidant activity and chemical composition as measured by HPLC-MS are also analysed. **Chapter 6** describes the incorporation of an anthocyanidin-rich commercial *Hibiscus sabdariffa* extract into the inner phase of a water/oil/water emulsion and the improvement of its physical stability through the reinforcement of both interfaces with whey protein concentrate - arabic gum complexes. In **Chapter 7**, the development of an optimum formulation of NLC to protect the phenolic compounds from *Hibiscus sabdariffa*, extracted by MAE and PLE, is described. These particles are characterised according to particle size, polydispersity index and zeta potential in the context of a stable system. In addition, a high encapsulation efficiency of quercetin and anthocyanins was obtained and is discussed. Finally, **Chapter 8** reports the incorporation of *Hibiscus sabdariffa*-loaded nanoparticles into a diary beverage. Following evaluation of a study examining long-term stability, texture properties and *in vitro* release, it is demonstrated that these enriched milks form a stable suspension.

❖ RESUMEN

La presente memoria recoge los resultados obtenidos durante el periodo de realización de la Tesis doctoral titulada **"DESARROLLO DE EXTRACTOS FENÓLICOS NANOENCAPSULADOS PARA ALIMENTACIÓN FUNCIONAL Y NUTRACÉUTICA: *Hibiscus sabdariffa* como ejemplo".**

Este trabajo se divide en dos secciones principales: Introducción y parte Experimental.

La primera parte de la **Introducción** muestra el estado actual de los nutracéuticos y alimentación funcional, así como su desarrollo desde la búsqueda de fuentes naturales de compuestos fenólicos, su extracción mediante tecnologías que respetan el medio ambiente, su caracterización mediante técnicas analíticas y finalmente su encapsulación e incorporación a matrices alimentarias. En primer lugar, en la primera etapa, se describen y se clasifican los compuestos bioactivos y compuestos fenólicos en función de su estructura química. La segunda etapa consiste en la extracción de estos compuestos mediante tecnologías llamadas "Green" (extracción asistida por microondas, extracción por fluidos presurizados y extracción por fluidos supercríticos). A continuación, en la tercera etapa, se describen las técnicas analíticas que se llevan a cabo para la separación de estos compuestos como la cromatografía líquida de alta resolución (HPLC) y sistemas de detección como el detector UV-Vis y espectrofotometría de masas (MS) para la identificación y cuantificación de los mismos. La siguiente sección descrita se basa en la micro- y nano-encapsulación de estos compuestos para asegurar su estabilidad frente a las condiciones ambientales. En esta Tesis, se describe las técnicas de encapsulación

basadas en sistemas lipídicos así como los mecanismos de estabilidad que este sistema necesita. La última etapa de este trabajo es la incorporación de compuestos fenólicos encapsulados en una matriz alimentaria evaluando su interacción con los componentes de la misma.

La parte **Experimental** se divide en tres secciones. La **Sección A** está basada en la búsqueda de nuevas fuentes de compuestos bioactivos. En este sentido, *Hibiscus sabdariffa* es escogido por su composición bioactiva. Por lo tanto, el origen, distribución, los efectos biológicos, el valor nutricional y su composición en compuestos fenólicos, son descritos en esta sección.

La **Section B** está dedicada a la optimización de los sistemas de extracción para obtener un extracto rico en compuestos fenólicos procedentes de *Hibiscus sabdariffa*. Estos extractos serán posteriormente caracterizados. Esta sección se divide en 3 capítulos. En el **Capítulo 1,** se optimiza una de las técnicas de extracción "Green" llamada extracción asistida de microondas (MAE), usando diferentes ratios de disolvente-agua, diferentes tiempos de extracción y diferentes temperaturas. Así se obtendrá la mejor condición de extracción que consigue el mayor contenido en compuestos fenólicos. Para ello, todos los ensayos experimentales fueron caracterizados mediante HPLC-MS. En el **Capítulo 2**, la extracción mediante fluidos presurizados (PLE) es también optimizada, empleando diferentes ratios de disolvente-agua y temperaturas para obtener un extracto "Green" con el mayor contenido en compuestos fenólicos. Finalmente, en el **Capítulo 3**, la optimización de la extracción por fluidos supercríticos (SFE) permitió obtener la mejor condición de extracción para

cada familia de compuestos fenólicos. En esta técnica, se utilizaron diferentes presiones, temperaturas y cantidades de co-solvente.

Por otro lado, **Sección C** es dedicada a la micro-nano encapsulación de compuestos fenólicos procedentes de *Hibiscus sabdariffa* y su incorporación a matrices alimentarias. Esta sección está dividida en 5 capítulos, de los cuales los capítulos 5 y 6 se llevaron a cabo en el Departamento de Ingeniería Química de la Universidad de Granada y los capítulos 4, 7 y 8 se desarrollaron en colaboración con la Universidad de Coimbra (Portugal) a través de una estancia predoctoral de 3 meses financiada por "Estancias Breves FPU". En el **Capítulo 4**, se describen diferentes nano sistemas basados en sustancias lipídicas que se aplican en la encapsulación de compuestos fenólicos. Así, se comentan las principales características de las emulsiones y de nano sistemas lipídicos (NLC) (las cuales son empleadas en esta Tesis). En el **Capítulo 5**, se lleva a cabo la formulación de nuevos aceites funcionales nuevos que contienen antioxidantes procedentes de un extracto de *Hibiscus sabdariffa* a través de la tecnología de emulsiones. Además se analiza la estabilidad física y oxidativa mediante el tamaño de partícula, ratio de coalescencia, contenido total de compuestos fenólicos, actividad antioxidante y composición química medida por HPLC-MS. En el **Capítulo 6**, se incorpora un extracto comercial de *Hibiscus sabdariffa* enriquecido en antocianidinas en la fase interna de una emulsión agua/aceite/agua, y su estabilidad física se mejora con el reforzamiento de ambas interfaces a través de complejos proteína – goma arábiga. En el **Capítulo 7**, se optimiza una formulación de NLC para proteger los compuestos fenólicos procedentes de *Hibiscus sabdariffa* que han sido extraídos mediante MAE y PLE. Estas nanopartículas se caracterizaron

mediante el tamaño de partícula, el índice de polidispersidad y potencial zeta, mostrando un sistema estable. Además, se consiguió una alta eficiencia de encapsulación de quercetina y antocianinas. Finalmente, en el **Capítulo 8**, estas nanopartículas que contienen los compuestos fenólicos procedentes de *Hibiscus sabdariffa* son incorporadas en una matriz láctea. La evaluación en el tiempo y a diferentes temperaturas, la textura y la liberación *in vitro* demostraron que esta matriz enriquecida es una suspensión estable.

Objetives/Objetivos

❖ OBJECTIVES

Dietary habits have progressively changed over the last decade as consumers become more aware of the relationship between diet and health and its role in preventing chronic diseases such as diabetes, cancer and obesity. As a result, the search of bioactive compounds within natural sources for the development of functional foods and nutraceuticals, is becoming increasingly useful in helping consumers to maintain or improve their quality of life.

With this in mind, the **overall goal** of the present Thesis is:

- ❖ To evaluate the different steps (search of natural sources, green extraction, analytical characterisation, encapsulation and incorporation in a food matrix) involved in the development of nano-encapsulated phenolic green extracts for their application in functional food and nutraceuticals, using *Hibiscus sabdariffa* due to evidence of its high bioactivity against several diseases.

To achieve this goal the following specific objectives were established:

- ✧ To search for phenolic compounds in *Hibiscus sabdariffa* using an evaluation of the bioactivity and phenolic composition of this plant as a potential candidate for the development of nutraceuticals and functional foods.
- ✧ To use multi-response surface methodology to optimise the extraction of phenolic compounds from *Hibiscus sabdariffa* using green extraction technologies such as microwave-assisted extraction, pressurised liquid extraction and supercritical fluid extraction systems.

- ✧ To use high-performance liquid chromatography coupled with time-of-flight mass spectrometry (HPLC-ESI-TOF-MS) or quadruple-time-of-flight mass spectrometry (HPLC-ESI-QTOF-MS) to characterise the phenolic composition of the new extracts of *Hibiscus sabdariffa,* obtained previously by green extraction technologies.
- ✧ To develop and optimise different encapsulation technologies based on lipid systems to protect the green phenolic extracts from *Hibiscus sabdariffa*. To this end, simple water-oil emulsions, double emulsions and lipid nanoparticles are employed and their physical and chemical stability evaluated.
- ✧ To incorporate the encapsulated phenolic compounds from *Hibiscus sabdariffa* into a dairy product in order to evaluate and compare the delivery and stability of such compounds when incorporated into a food matrix in an encapsulated or free form.

❖ OBJETIVOS

Los hábitos en la alimentación han ido evolucionando a lo largo de la última década. Esto es debido a que los consumidores se preocupan cada vez más por tener una dieta saludable y así poder prevenir enfermedades muy comunes como son la diabetes, cáncer, obesidad, entre otras. En este sentido, la búsqueda de compuestos bioactivos procedentes de fuentes naturales para la producción de alimentos funcionales y nutracéuticos está aumentando considerablemente, con el objetivo de ayudar a los consumidores a mantener o mejorar su calidad de vida.

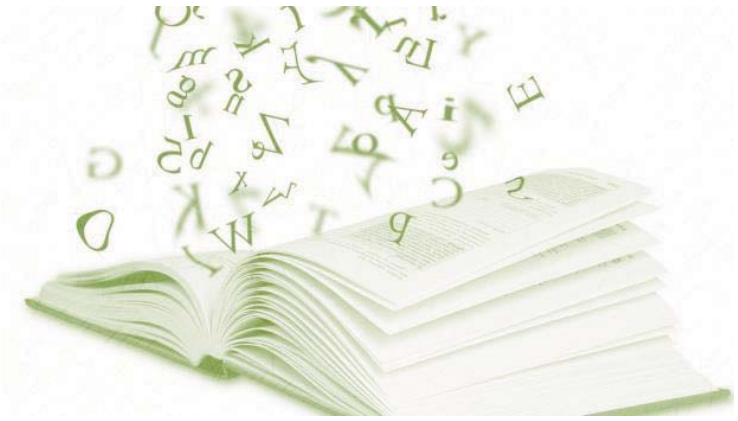
Así, el principal objetivo de esta Tesis es:

- ❖ La evaluación de las diferentes etapas (búsqueda de compuestos fenólicos procedentes de fuentes naturales, extracción, caracterización analítica, encapsulación e incorporación a una matriz alimentaria) para el desarrollo de extractos fenólicos nanoencapsulados con aplicación en alimentación funcional y nutracéutica, usando *Hibiscus sabdariffa* como un ejemplo interesante debido a su alta bioactividad frente a varias enfermedades.

Para conseguir este objetivo general, se establecieron los siguientes objetivos específicos:

- ✧ Búsqueda de compuestos fenólicos procedentes de *Hibiscus sabdariffa*. Una revisión se llevó a cabo para evaluar la bioactividad y la composición fenólica de esta planta para ser una buena candidata en el desarrollo de nutracéuticos y alimentos funcionales.

- ✧ Optimización mediante la metodología superficie-respuesta, de la extracción de los compuestos fenólicos de *Hibiscus sabdariffa* a través de técnicas de extracción "verdes", tales como extracción asistida por microondas, extracción por fluidos presurizados y extracción por fluidos supercríticos.
- ✧ Caracterización mediante cromatografía líquida acoplada a tiempo de vuelo (HPLC-ESI-TOF-MS) o quadrupolo tiempo de vuelo (HPLC-ESI-QTOF-MS), de la composición fenólica de los nuevos extractos de *Hibiscus sabdariffa* obtenidos anteriormente mediante técnicas de extracción "verdes".
- ✧ Desarrollo y optimización de diferentes técnicas de encapsulación basadas en sistemas lipídicos para proteger los compuestos fenólicos de *Hibiscus sabdariffa*. Así, emulsiones simples agua-aceite, emulsiones dobles y nanopartículas lipídicas se desarrollaron para este objetivo, evaluando su estabilidad física y química.
- ✧ Incorporación de los compuestos fenólicos encapsulados procedentes de *Hibiscus sabdariffa* en una matriz láctea, para evaluar y comparar su liberación y estabilidad cuando estos compuestos son incorporados a una matriz alimentaria de forma libre o encapsulada.



Introduction

1. FUNCTIONAL FOOD AND NUTRACEUTICAL

Nutrition, coined from the Latin word *nutrire* meaning "to nourish". This word encapsulates food intake, absorption, assimilation, biosynthesis, catabolism and excretion. Nutrients are metabolised and utilised to support all of life´s processes[1]. Hence, the nutrition state of each individual person depends on his or her dietary habits. In the past, healthy dietary habits were established to provide the energy needed to perform daily functions and maintain normal metabolic processes. Thus, macronutrients, such as carbohydrates, fats and proteins, and micronutrients such as vitamins and minerals, are the basis of all life activities. They provide the required energy through oxidative decomposition for the proper functioning of the body. In addition, foods also contain non-nutrient substances that provide healthy properties and are essential in preventing diseases.

Increasing knowledge with respect to the impact of foods and dietary habits on metabolic regulation means that dietary habits have progressively changed over the last decade, with new dietary strategies being developed. In this way, functional foods have increased in demand by consumers who are more and more interested in the functional properties of some natural compounds with a view to incorporating more foodstuffs with added-value. Such interests can be explained by the increasing cost of healthcare, the steady increase of life expectancy, and the desire of older people for improved quality of life in their later years.

[1] Stephen Adeniyi Adefegha, "Functional Foods and Nutraceuticals as Dietary Intervention in Chronic Diseases; Novel Perspectives for Health Promotion and Disease Prevention," *Journal of Dietary Supplements* 15, no. 6 (2018): 977–1009.

According to a Euromonitor survey, Japan is the world's largest market, followed by the United States, with the European market remaining less developed. These three dominant markets contribute to over 90% of nutrient sales worldwide[2].

In response, consumer requirements are changing with regards to food production[3]. As a consequence, the food industry and the scientific community have collaborated to increase the development of more and more specific products with different ingredients, formats, colours, tastes, smells, textures and mixtures in the pursuit of fulfilling consumer's expectations. As such, functional foods represent one of the most interesting areas of research and innovation in the food industry[4].

"Functional food" and "*nutraceutical"* are two terms used to describe health-promoting foods or their extracted components. However, at present, there is no statutory definition for "functional food" or "nutraceutical" by the FD&C Act (Federal Food, Drug, and Cosmetic Act) or related provisions, despite the FDA (Food and Drug Administration) acknowledging that the two categories are regulated by the FD&C Act[5]. A worldwide conceptual definition of **functional food** established by the European Commission is presently accepted. This defines functional foods as foods that have *"satisfactorily demonstrated to affect beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to*

[2] Barbara Bigliardi and Francesco Galati, "Innovation Trends in the Food Industry: The Case of Functional Foods," Trends in Food Science and Technology 31, no. 2 (2013): 118–29.
[3] Riikka Puhakka, Raisa Valve, and Aki Sinkkonen, "Older Consumers' Perceptions of Functional Foods and Non-Edible Health-Enhancing Innovations," International Journal of Consumer Studies 42, no. 1 (2018): 111–19
[4] Rao Sanaullah Khan et al., "Functional Food Product Development - Opportunities and Challenges for Food Manufacturers," Trends in Food Science and Technology 30, no. 1 (2013): 27–37
[5] Alice Yuen Ting Wong, Julie Ming Chu Lai, and Albert Wai Kit Chan, "Regulations and Protection for Functional Food Products in the United States," Journal of Functional Foods 17 (2015): 540–51.

either an improved state of health and well-being and/or reduction of risk of disease"[6]. A more modern definition is provided by the Functional Food Center, which classifies a functional food as follows: "*Natural or processed foods that contain known or unknown biologically-active compounds; which in defined, effective non-toxic amounts, provide a clinically proven and documented health benefit for the prevention, management, or treatment of chronic disease*"[7].

Many natural foods, mainly plant-based foods, are therefore considered functional foods because of their active substance content. Examples of sources of bioactive compounds include olive oil, fruits and vegetables.

The term ***"nutraceutical"*** was coined from *"nutrition"* and *"pharmaceutical"* in 1989, and can be defined as *"a food (or part of a food) that provides medical or health benefits, including the prevention and/or treatment of a disease"*. Although debate continues regarding the difference between *"functional foods"* and *"nutraceuticals"* it is convenient to consider nutraceuticals as healthful products elaborated from foods that are formulated and consumed in defined dosages, usually in the form of capsules or tablets. In contrast, functional foods are consumed as food and not in dosage form[8].

Functional foods and nutraceuticals in the human diet lead to the promotion of a healthy lifestyle and the prevention of various age and lifestyle-related chronic diseases. Indeed, the use of functional foods and nutraceuticals in the treatment of

[6] M. Viuda-Martos, J. Fernández-López, and J.A. Pérez-Álvarez . "Pomegranate and Its Many Functional Components as Related to Human Health : A Review," Comprehensive Reviews in Food Science and Food Safety, 9 (2010): 635–54

[7] Danik M Martirosyan, Bryan Singharaj, and Functional Foods, "Health Claims and Functional Food : The Future of Functional Foods under FDA and EFSA Regulation," no. August 2016 (2017).

[8] S. El Sohaimy, "Functional Foods and Nutraceuticals-Modern Approach to Food Science," World Applied Sciences Journal 20, no. 5 (2012): 691–708

disease is gaining more and more attention, especially when used in conjunction with current pharmacological approaches or even as a replacement for drug therapy[9].

2. DEVELOPMENT STAGES FOR FUNCTIONAL FOODS AND NUTRACEUTICALS

The development of functional foods is an increasing challenge due to the consumer's insatiable expectancy for healthier products. In response, the food industry considers several parameters when developing functional products, such as sensory acceptance, stability, price, and chemical and functional properties[10,11].

In the realisation of functional products the following steps are typically followed: Identification of bioactive compounds from natural sources, extraction of bioactive compounds, analytical characterisation, micro or nano-encapsulation and incorporation into food matrices **(Figure 1)**.

[9] Lindsay Brown et al., "Clinical Trials Using Functional Foods Provide Unique Challenges," Journal of Functional Foods 45, no. April (2018): 233–38

[10] E. Betoret et al., "Functional Foods Development: Trends and Technologies," Trends in Food Science and Technology 22, no. 9 (2011): 498–508

[11] Peter J. Jones and Stephanie Jew, "Functional Food Development: Concept to Reality," Trends in Food Science and Technology 18, no. 7 (2007): 387–90

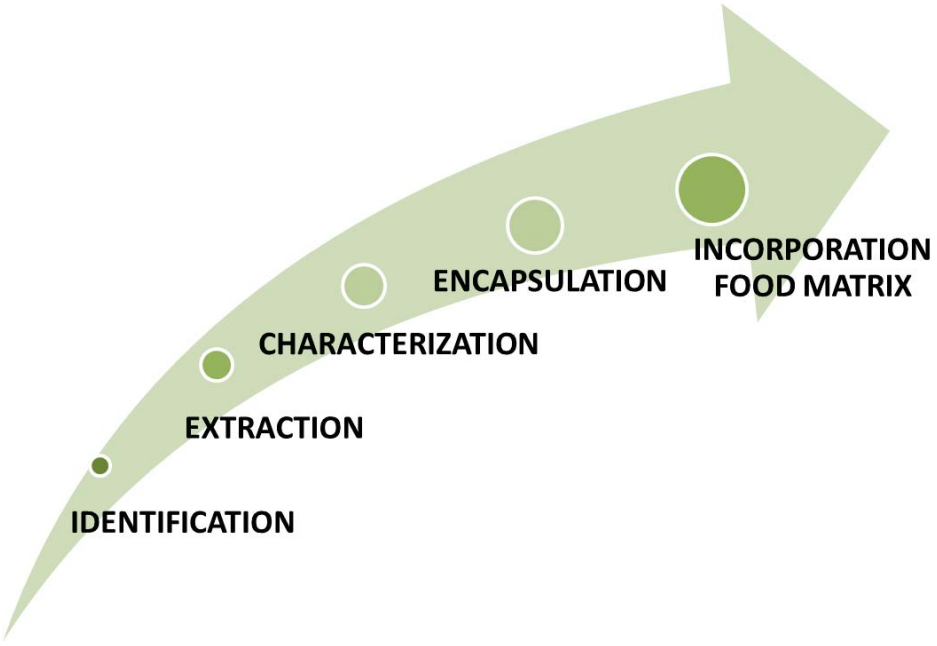


Figure 1. Stages studied in the present Thesis for the development of functional foods and nutraceuticals

2.1 Bioactive compounds from natural sources

Bioactive compounds have been defined as "*essential and non-essential compounds (e.g., vitamins or phenolic compounds) that occur in nature, are part of the food chain, and can be shown to have an effect on human health*"[12]. The most bioactive compounds are typically found in plants, although there are other sources such as microorganisms, mushrooms and in some groups of animals[13].

[12] Hans Konrad Biesalski et al., "Bioactive Compounds: Definition and Assessment of Activity," Nutrition 25, no. 11–12 (2009): 1202–5

[13] Abdelkarim Guaadaoui et al., "What Is a Bioactive Compound ? A Combined Definition for a Preliminary Consensus," International Journal of Nutrition and Food Sciences 3, no. 3 (2014): 174–79

These bioactive compounds can be classified according to different criteria, although the most accepted classification is based on their chemical structure. In this way, these compounds can be categorised as **inorganic compounds** such as minerals, or **organic compounds** such as phenolic compounds, terpenoids, lipids, proteins, oligosaccharides (prebiotics) and polysaccharides (starch) **(Figure 2)**.

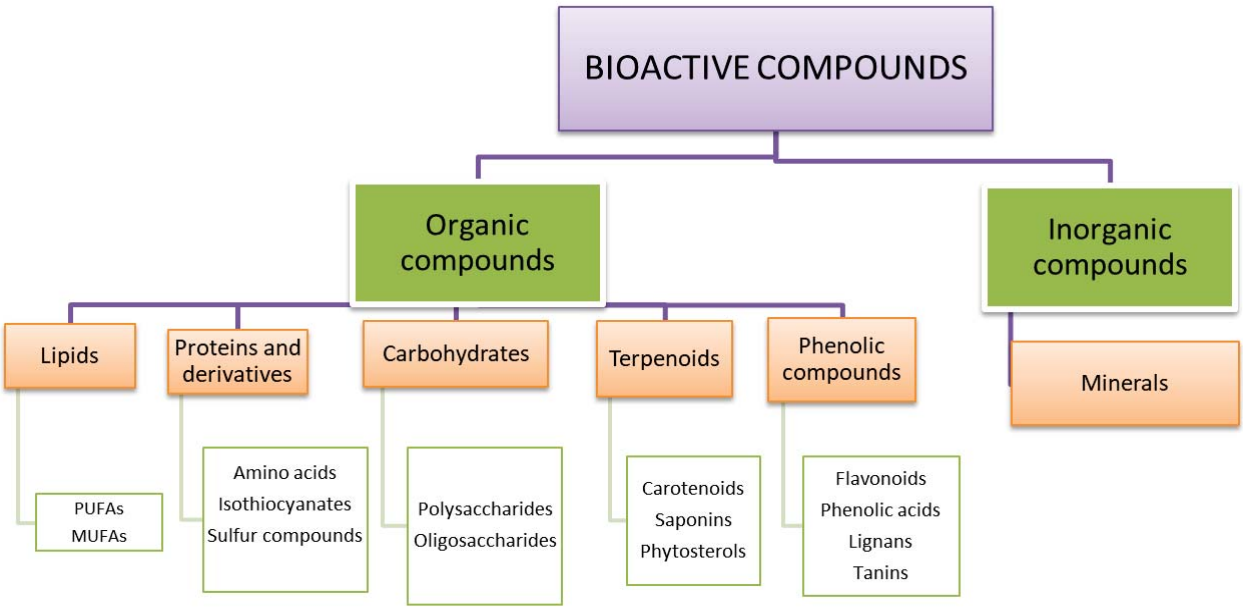


Figure 2. Classification of bioactive compounds according to their chemical structure

Bioactive compounds found in plants which have beneficial effects for humans and animals can be consumed via the leaves, stems, roots, tubers, buds, fruits, seeds and flowers of whole plants, or consumed in plant-derived foods and drinks such as chocolate, tea or coffee [14,15,16].

[14] Berit Smestad Paulsen, Highlights through the History of Plant Medicine, Bioactive Compounds in Plants - Benefits and Risks for Man and Animals, vol. 50, 2010

Selected from amongst the various classes of bioactive compounds present in plants, the current Thesis focuses on the study of **phenolic compounds** which are formed by one or more aromatic ring attached to one or more hydroxyl groups[17]. These compounds have demonstrated very interesting biological activities in recent years[18] .

2.1.1 Phenolic compounds as bioactive compounds

Phenolic compounds are an abundant class of substances with at least 10,000 different compounds having become a focus of research over the past 16 years[19]. These compounds have received tremendous attention from functional food, nutraceutical and even pharmaceutical and cosmetic industries, and consumers in general, due to their protective/preventive properties in relation to several diseases. Their **beneficial effects** are associated with their antioxidant, anti-inflammatory, anti-microbial, anti-mutagenic, anti-carcinogenic, hepatoprotective, cardioprotective neuroprotective and anti-diabetic properties, in addition to their capacity to stimulate digestive processes[20]. In addition, phenolic compounds may contribute to the bitterness, astringency, colour, flavour, odour, and oxidative stability in food processing.

[15] G. Joana Gil-Chávez et al., "Technologies for Extraction and Production of Bioactive Compounds to Be Used as Nutraceuticals and Food Ingredients: An Overview," Comprehensive Reviews in Food Science and Food Safety 12, no. 1 (2013): 5–23

[16] Anna Denny, Synthesis Report No 4 : Plant Foods and Health : Focus on Plant Bioactives, EuroFIR, 2005

[17] Ejaz Ahmed et al., "Secondary Metabolites and Their Multidimensional Prospective in Plant Life," Journal of Pharmacognosy and Phytochemistry JPP 205, no. 62 (2017): 205–14

[18] Harish Kumar et al., "Phenolic Compounds and Their Health Benefits : A Review" 2, no. 2 (2014): 46–59.

[19] Hassan Rasouli, Mohammad Hosein Farzaei, and Reza Khodarahmi, "Polyphenols and Their Benefits: A Review," International Journal of Food Properties 20, no. 2 (2017): 1700–1741

[20] Hasna El Gharras, "Polyphenols: Food Sources, Properties and Applications - A Review," International Journal of Food Science and Technology 44, no. 12 (2009): 2512–18

These compounds are not uniformly distributed in plants. Insoluble polyphenols are founds in cell walls, while soluble phenolics are compartmentalised within the plant cell vacuoles[21].

Phenolic compounds are typically classified according to their source, biological function and chemical structure. With regards to chemical structure, structures vary from simple phenols (i.e. catechols and hydrobenzoic acid derivatives) to catechol melanins $(C_6)_6$ with long chain polymers of a high molecular weight, condensed tannins $(C_6\text{-}C_3\text{-}C_6)n$ and lignins $(C_6\text{-}C_3)n$. Polyphenols can therefore be divided into different families such as phenolic acids, simple phenols, tanins, coumarins, benzoquinones, xanthones, lignans, lignins, flavonoids, etc. Of these, flavonoids are one the most important phenolic families[19].

Particular attention should be paid to **flavonoids** as they are the most common and widely distributed group of plant phenolic compounds. Daily intake of this compound normally varies between 20 mg and 500 mg and mainly comes from dietary products including tea, red wine, apples, onions and tomatoes[22]. Flavonoids have a low molecular weight which is formed by 15-carbon atoms with two aromatic rings connected by a 3-carbon bridge[23]. **Figure 3** shows the parent structure of flavonoids and their subgroups.

There are several subgroups of flavonoids. The most important groups include flavonols (e.g. quercetin, kaempferol and myricetin), flavones (e.g. apigenin and

[21] Marian Naczk and Fereidoon Shahidi, "Extraction and Analysis of Phenolics in Food," Journal of Chromatography A 1054, no. 1–2 (2004): 95–111
[22] Tian yang Wang, Qing Li, and Kai shun Bi, "Bioactive Flavonoids in Medicinal Plants: Structure, Activity and Biological Fate," Asian Journal of Pharmaceutical Sciences 13, no. 1 (2017): 12–23,
[23] A. N. Panche, A. D. Diwan, and S. R. Chandra, "Flavonoids: An Overview," Journal of Nutritional Science, 5 (2016)

luteolin), flavan-3-ols (e.g. catechin, epicatechin gallate, epigallocatechin and proanthocyanidins), isoflavones (e.g. daidzin, genistin, biochanin A and formononetin), anthocyanidins (e.g. cyaniding and delphinidin) and flavanones (e.g. hesperetin and naringenin)[24].

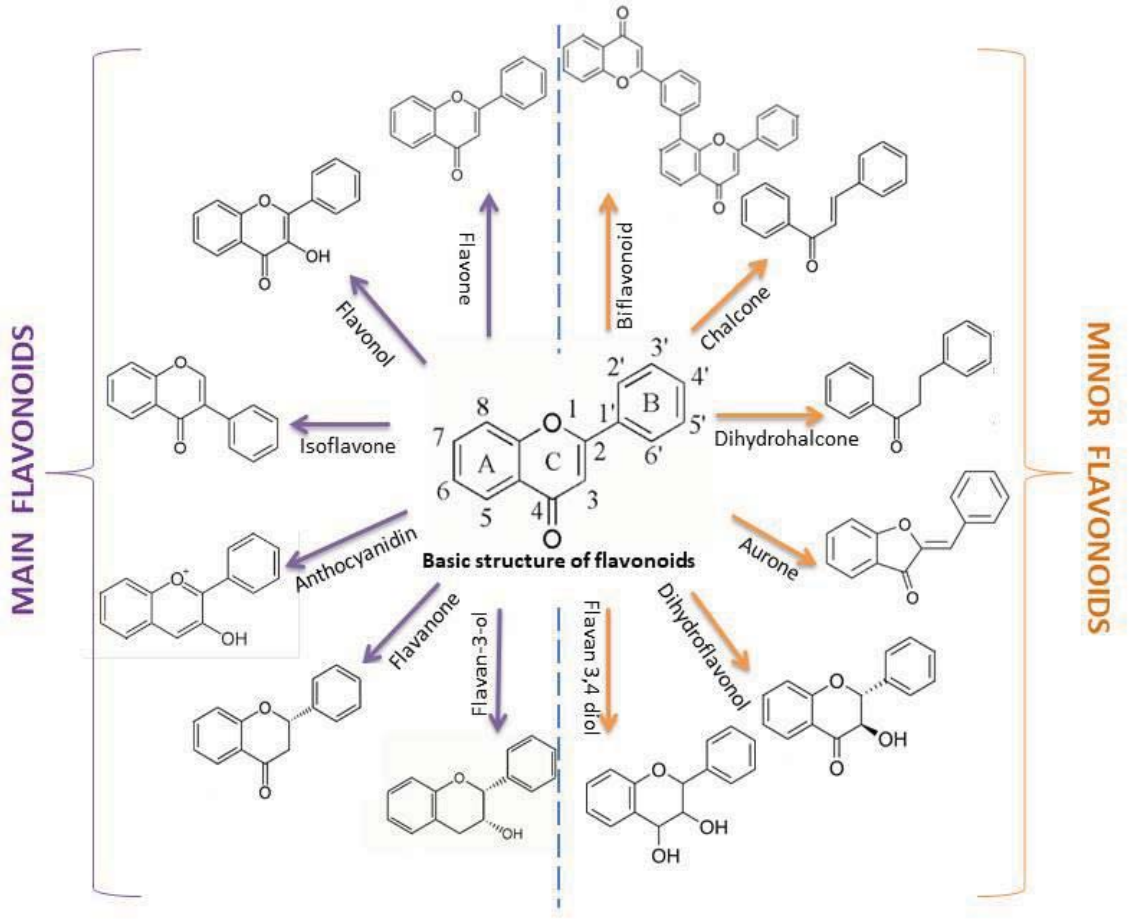


Figure 3. Classification of flavonoids

Other flavonoids occurring in nature but found in only minor quantities are biflavonoids, aurones, chalcones, flavan-3-4-diols and dihydrochalcones[22].

[24] Shashank Kumar and Abhay K Pandey, "Chemistry and Biological Activities of Flavonoids : An Overview" The Scientific World Journal, 2013 (2013).

The basic flavonoid structure varies with regards to substituents. Hydroxyl groups are usually present at the 4, 5 and 7 positions. Sugars are very common with the majority of flavonoids existing naturally as glycosides. Whereas both sugars and hydroxyl groups increase the water solubility of flavonoids, other substituents, such as methyl groups and isopentyl units, make flavonoids lipophilic[25,26].

Other phenolic compounds with dietary significance are simple phenols, phenolic acids, water-soluble tanins, coumarins, benzoquinones, acetophenones, phenylpropones, chromones, napthoquinones, xanthones, anthraquinones, lignans, lignins and stilbenes[27]. Of these, phenolic acids and stilbenes such as resveratrol are regarded as the main dietary non-flavonoids compounds.

Phenolic acids are divided in two subgroups (hydroxybenzoic acids and hydroxycinnamic acids). Although the basic structure is the same, the numbers and positions of the hydroxyl groups on the aromatic ring vary widely between these compounds[28]. With regards to bioactivity, hydroxycinnamic acids have demonstrated greater antioxidant activity than hydroxybenzoic acids. This could be because hidroxyinamic acids contain a CH=CH–COOH group which ensures higher H-donating capability and radical stabilisation than the –COOH group found in hydroxybenzoic acids[27]. **Figure 4** describes the main phenolic acids found in plants.

[25] Latifa Chebil et al., "Solubility of Flavonoids in Organic Solvents," Journal of Chemical and Engineering Data 52, no. 5 (2007): 1552–56

[26] Alan Crozier and Michael N Clifford, Terpenes, Plant Secondary Metabolites, Plant Secondary Metabolites Occurrence , Structure and Role in the Human Diet, Blackwell Publishing Ltd, 2006

[27] Nagendran Balasundram, Kalyana Sundram, and Samir Samman, "Phenolic Compounds in Plants and Agri-Industrial by-Products: Antioxidant Activity, Occurrence, and Potential Uses," Food Chemistry 99, no. 1 (2006): 191–203

[28] Rebecca J Robbins, "Phenolic Acids in Foods : An Overview of Analytical Methodology Phenolic Acids in Foods : An Overview of Analytical" Journal of Agricultural and Food Chemistry, 51, no. 10 (2003): 2866–87

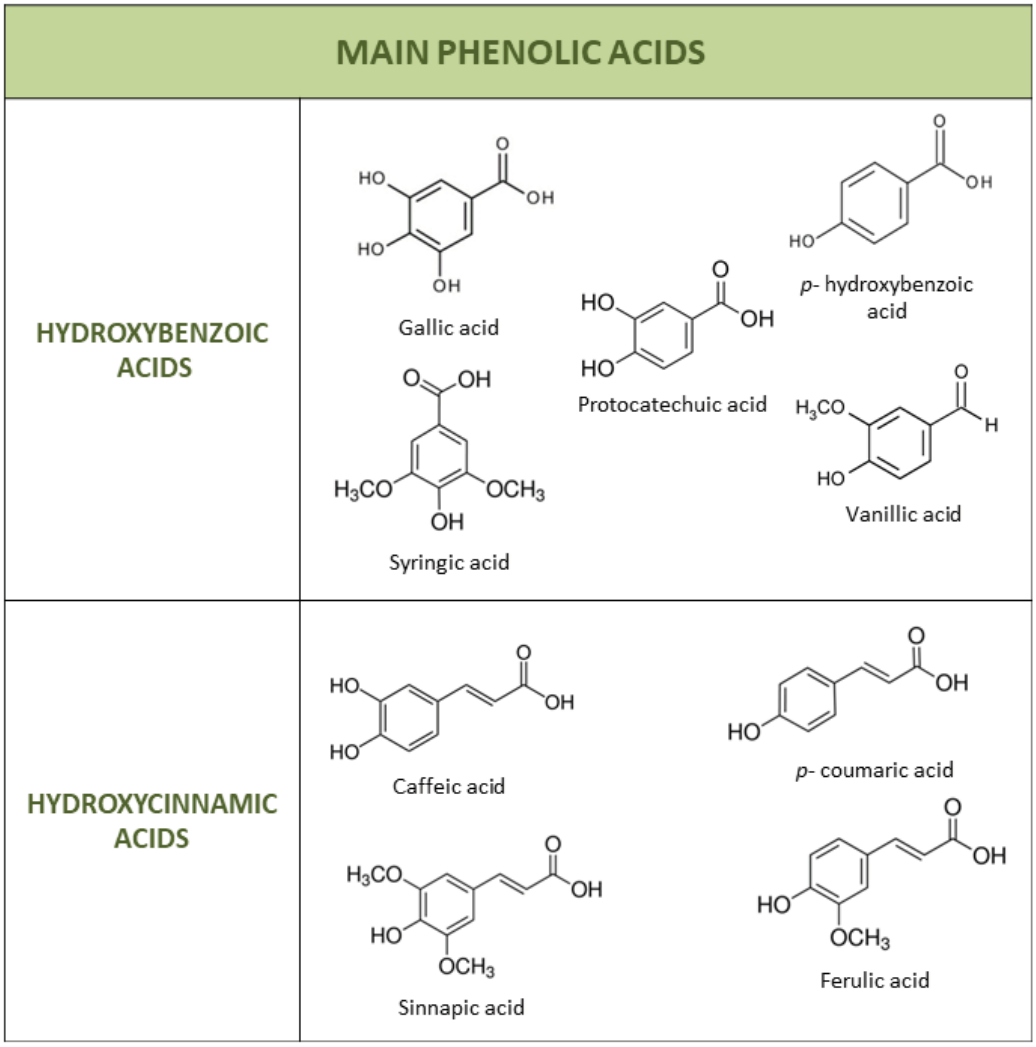


Figure 4. Classification of main phenolic acids

2.2 Extraction of phenolic compounds by green extraction technologies

Extraction involves the separation of bioactive plant compounds from its inactive components through the use of selective solvents in standard extraction procedures. This is the first step in the process required to obtain the desired chemical components for later analytical characterisation[29].

[29] S. Sasidharan et al., "Extraction, Isolation and Characterization of Bioactive Compounds from Plants' Extracts," African Journal of Traditional, Complementary and Alternative Medicines 8, no. 1 (2011): 1–10

The selection of extraction procedure depends on the chemical structure of the compounds to be isolated. The extraction method should match properties of the compound such as whether it is non-polar, polar and thermally labile. Moreover other parameters can affect the extraction process and should be considered. These include the matrix properties of the plant part, its solvent, temperature, pressure and time[30].

Different techniques can be employed to extract phenolic compounds from plant and plant-based products. Generally, extraction techniques have been classified as conventional techniques and green technologies **(Figure 5)**. The majority of conventional extraction methods rely on the extracting power of different solvents during the application of heat and/or mixing[29]. These techniques are limited due to their excessive consumption of time, energy, and polluting solvents. The last decade has resultantly seen an increase in demand for new extraction techniques which allow for automation, shorten extraction times and reduced organic solvent consumption, thus preventing pollution in analytical laboratories and reducing sample preparation costs[31]. Green extraction has been defined as a type of extraction "*based on the discovery and design of extraction processes which will reduce energy consumption, allows use of alternative solvents and renewable natural products, and ensure a safe and high quality extract/product*"[32]. In addition, these technologies should comply with

[30] J. Azmir et al., "Techniques for Extraction of Bioactive Compounds from Plant Materials: A Review," Journal of Food Engineering 117, no. 4 (2013): 426–36

[31] Marcela Bromberger Soquetta, Lisiane de Marsillac Terra, and Caroline Peixoto Bastos, "Green Technologies for the Extraction of Bioactive Compounds in Fruits and Vegetables," CyTA - Journal of Food 16, no. 1 (2018): 400–412

[32] Farid Chemat, Maryline Abert Vian, and Giancarlo Cravotto, "Green Extraction of Natural Products: Concept and Principles," International Journal of Molecular Sciences 13, no. 7 (2012): 8615–27

the Six Principles of Green Extraction as directed to establish an innovative green label[33]:

- **Principle 1**: Innovation through selection of technique and use of renewable plant resources.
- **Principle 2**: Use of alternative solvents and principally water or agro-solvents considered to be GRAS (Generally Recognised as Safe).
- **Principle 3**: Reduce energy consumption by energy recovery and use of innovative technologies.
- **Principle 4**: Production of co-products with reduced waste production.
- **Principle 5**: Reduce unit operations and safety controlled processes.
- **Principle 6**: Production of biodegradable extract with "green" values.

Green extraction technologies include ultrasound assisted extraction (UAE), microwave assisted extraction (MAE), pressurised liquid extraction (PLE) and supercritical fluid extraction (SFE). The three latter techniques were employed during the work of the present Thesis and will be discussed in the following subsections.

[33] Alper kuscu, Salih Eroglu, "Applications of Green Extraction of Phytochemicals from Fruit and Vegetables," Akademik Plataform, 2015.

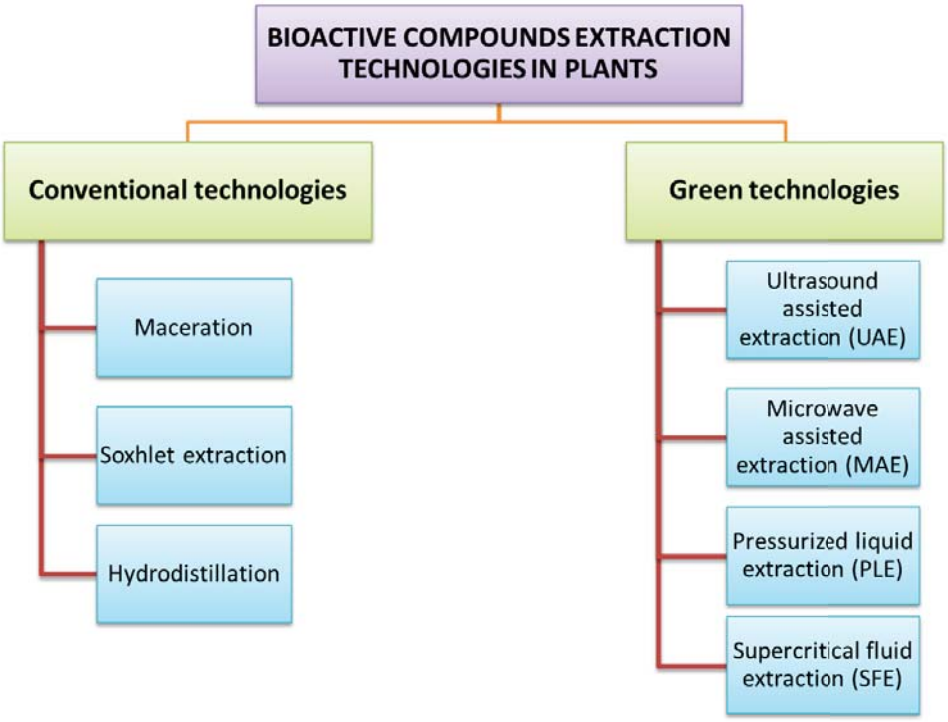


Figure 5. Types of extraction technologies used for the extraction of bioactive compounds

The extract obtained can be made ready for use as a medicinal agent in the form of fluid extracts and may be further processed to be incorporated into any dosage form such as nutraceuticals or functional foods. Thus, standardisation of extraction procedures is crucial and contributes significantly to the quality of the final herbal drug[34].

[34] Tim Dalgleish et al., "Reduced specificity of autobiographical memory and depression: the role of executive control." Journal of Experimental Psychology: General 136, no. 1 (2007): 23–42.

2.2.1 Microwave assisted extraction (MAE)

MAE is a widely used technique for extraction of phenolic components of plant material[35,36]. MAE reduces manufacturing costs by saving energy and shortening processing times, improves product uniformity and yield, and improves the properties and synthesis of new materials. Moreover, the fundamentals of MAE are different from those of conventional methods because the extraction occurs as the result of changes in the cell structure caused by electromagnetic waves[37], which consist of an electric field and magnetic field oscillating perpendicularly to each other in a frequency ranging from 0.3 to 300 GHz.

The key to the process of microwave heating is the energy transfer that takes place. During conventional heating processes, energy is transferred to the material by convection, conduction and radiation through its external surface. On the other hand, microwave energy is delivered directly to materials by molecular interactions with its electromagnetic field which convert electromagnetic energy into thermal energy[36,38]. In microwave processes, an electromagnetic wave is present that can penetrate some materials by ionic conduction and dipole rotation, interacting with polar compounds and generating heat[39]. Ionic conduction describes the electrophoretic migration of ions when an electromagnetic field is applied. Resistance within the solution to this

[35] Vanja Lovrić et al., "The Effect of Microwave-Assisted Extraction on the Phenolic Compounds and Antioxidant Capacity of Blackthorn Flowers," Food Technology and Biotechnology 55, no. 2 (2017): 243–50

[36] Marija Radojković et al., "Microwave-Assisted Extraction of Phenolic Compounds from Morus Nigra Leaves: Optimization and Characterization of the Antioxidant Activity and Phenolic Composition," Journal of Chemical Technology and Biotechnology 93, no. 6 (2018): 16, 84–93

[37] Priscilla C Veggi, Julian Martinez, and M Angela A Meireles, Microwave-Assisted Extraction for Bioactive Compounds, 2013

[38] D Acierno, A A Barba, and M D'Amore, "Heat Transfer Phenomena during Processing Materials with Microwave Energy," Heat and Mass Transfer/Waerme- Und Stoffuebertragung 40, no. 5 (2004): 413–20

[39] Chung Hung Chan et al., "Microwave-Assisted Extractions of Active Ingredients from Plants," Journal of Chromatography A 1218, no. 37 (2011): 6213–25

flow of ions produces friction and heats the solution. Dipolar rotation produces realignment of dipoles within the applied field. At 2450 MHz, which is the frequency used in commercial systems, the dipoles align and randomise 4.9×10^9 times per second. This forced molecular movement results in heating[40] **(Figure 6).**

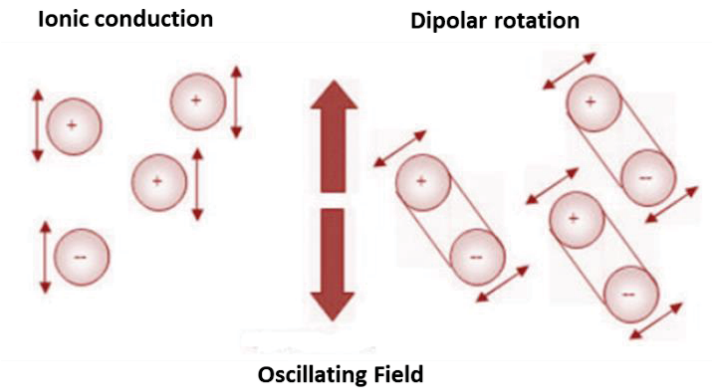


Figure 6. Mechanism of microwave heating

Extraction in closed vessels can also be accomplished at high temperatures, with increases in the mass transfer of the analyte from the sample matrix, which allows for a rapid process without any deterioration in thermally unstable compounds such as phenolic compounds[41].

Correctly choosing the appropriate solvent is fundamental in obtaining an optimal extraction process. The microwave-absorbing properties of the solvent must be considered, as must the interaction of the solvent with the matrix and its analyte solubility[42]. The ability of a solvent to absorb microwave energy and pass it on in the form of heat to other molecules will partly depend on the dissipation factor of the solvent. This is determined by the quotient between the dielectric loss (a measure of

[40] P. P. Mehta and P. Mane, "Microwave Chemistry: A Review," International Journal of Pharmacy and Technology 7, no. 1 (2015): 3210–26
[41] Ali Liazid et al., "Investigation on Phenolic Compounds Stability during Microwave-Assisted Extraction," Journal of Chromatography A 1140, no. 1–2 (2007): 29–34
[42] Cecilia Sparr Eskilsson and Erland Björklund, "Analytical-Scale Microwave-Assisted Extraction," Journal of Chromatography A 902, no. 1 (2000): 227–50

the efficiency of converting microwave energy into heat) and dielectric constant (a measure of the polarisability of a molecule in an electric field)[43]. The properties of polar and ionic solutions give them a great capacity to absorb microwave energy. This is due to the presence of a permanent dipole moment and non-polar solvents which do not heat up when exposed to microwaves. **Table 1** reports the dielectric constants and dissipation factors of commonly used solvents.

Solvent	Dielectric constant (ε)[a]	Dissipating factor (tan ɛ x 10^{-4})[b]	Boiling point (ºC)
Acetone	20.7	5555	56
Acetonitrile	37.5		82
Ethanol	24.3	2500	78
Hexane	1.89		69
Methanol	32.6	6400	65
2-Propanol	19.9	6700	82
Water	78.3	1570	100

[a]Determined at 20 ºC; [b] Determined at 101.4 kPa

Table 1. Physical constants and dissipation factors of solvents usually used in MAE

As we have seen, the efficiency of the MAE procedure is directly related to the extraction parameters selected. Factors such as solvent, extraction temperature or microwave power and extraction time should be considered[44]. To this end, numerous studies have examined the extraction parameters of phenolic extraction from plants. **Table 2** summarises some recent examples.

[43] Acierno, Barba, and D'Amore, "Heat Transfer Phenomena during Processing Materials with Microwave Energy." Heat and Mass Transfer, 40 (2004) 413-420, 2004

[44] Farid Dahmoune et al., "Optimization of Microwave-Assisted Extraction of Polyphenols from Myrtus Communis L. Leaves," Food Chemistry 166 (2015): 585–95

Plant	Objective	Optimal conditions and results	Reference
***Melastoma sanguineum* Fruit**	Optimisation of phenol yield	Optimal MAE: 31.33% ethanol, solvent/material ratio of 32.21 mL/g, 52.24 ◦C, 45 min and 500 W). The total phenolic content (TPC) was 39.02 ± 0.73 mg gallic acid equivalent (GAE)/g dry weight	45
***Vernonia cinerea* leaves**	Optimisation of phenol yield	Optimal MAE: Irradiation time, 2 min; microwave power level, 444 W; feed/solvent, 1:14 g/mL; and ethanol concentration, 47% v/v. The TPC was 85.64 ± 0.52 mg GAE / g dry weight	46
***Arbutus unedo L.* fruits**	To compare MAE, maceration and UAE on phenolic extraction	MAE, maceration and UAE were optimised. MAE demonstrated to be the most efficient technique on phenolic extraction. The optimal MAE conditions were 8.4 min and 50.1 °C	47
***Morus nigra* leaves**	Optimisation of phenol yield and to compare MAE and UAE	MAE was more efficient than UAE. Optimal condition were20 mL of ethanol:water (1:1; v/v), 120 °C, 28 min, 0.414g. The higest TPC was 19.7±2.0 mg GAE/g dry plant	35
***Cyperus esculentus L.* (Tiger nut)**	To get the highest yield and compare MAE and Soxhlet extraction	Optimal MAE: mixture petroleum ether and acetone (2:1, v/v), microwave power 420 W, temperature 75 °C, liquid to solid ratio 7.0 mL/g and time 55 min, which provided a yield of 24.12%. MAE obtained higher content of total phenolic, α-tocopherol, β-carotene, phospholipids and phytosterols compared with Soxhlet extraction	48

Table 2. MAE applications for phenolic extraction

These examples demonstrate that MAE is a suitable and green extraction technique for recovery of phenolic compounds.

2.2.2 Pressurised liquid extraction (PLE)

Pressurised liquid extraction (PLE), also known as accelerated solvent extraction (ASE), is another emergent technique in the field of phenolic compound extraction.

[45] Cai-ning Zhao et al., "Microwave-Assisted Extraction of Phenolic Compounds from Melastoma Sanguineum Fruit :," Molecules, 2018, 23, 2498

[46] Oluwaseun Ruth et al., "Industrial Crops & Products Vernonia Cinerea Leaves as the Source of Phenolic Compounds , Antioxidants , and Anti-Diabetic Activity Using Microwave-Assisted Extraction Technique," Industrial Crops & Products 122, no. December 2017 (2018): 533–44

[47] Bianca R Albuquerque et al., "Recovery of Bioactive Compounds from Arbutus Unedo L . Fruits : Comparative Optimization Study of Maceration / Microwave / Ultrasound Extraction Techniques," Food Research International 109, no. April (2018): 455–71

[48] Bin Hu et al., "Optimization of Microwave-Assisted Extraction of Oil from Tiger Nut (Cyperus Esculentus L .) and Its Quality Evaluation," Industrial Crops & Products 115, no. September 2017 (2018): 290–97

This technique employs solvents at high pressures and temperatures as extraction performance is improved relative to techniques carried out at near room temperature and atmospheric pressure[49]. In addition, this green extraction methodology has achieved shorter extraction times and better penetration of the sample even when using a smaller volume of organic solvent. The use of solvents at temperatures above their atmospheric boiling point, improves its solubility and mass transfer characteristics[50]. Temperatures above 200 °C produce a higher solubility and diffusion rate of the sample, while elevating the pressure (up to 200 bars) keeps the solvent below its boiling point. Hence, at elevated pressures and temperatures, solvents can penetrate solid samples more efficiently which reduces solvent usage and avoids degradation of the bioactive compounds[51].

PLE can follow a static or dynamic set-up. In the static set-up, the extraction process consists of one or several extraction cycles with the solvent being replaced between cycles. In contrast, when using the dynamic set-up, the extraction solvent is continuously pumped through the sample vessel[52]. During the extraction process, the sample is placed into the extractor and the solvent is then pumped into the extraction vessel using an HPLC pump. The sample placed in the extraction cell is continuously maintained at the desired temperature using an electric heating jacket, until the required pressure is attained. Once the desired combination of temperature and

[49] Perbandingan Pengekstrakan et al., "Comparison of Pressurized Liquid Extraction With Soxhlet Extraction in the Determination of Polycyclic Aromatic Hydrocarbons in Soil," Environmental Protection 13, no. 1 (2009): 141–45.
[50] Arwa Mustafa and Charlotta Turner, "Pressurized Liquid Extraction as a Green Approach in Food and Herbal Plants Extraction: A Review," Analytica Chimica Acta 703, no. 1 (2011): 8–18
[51] Joana Gil-Chávez et al., "Technologies for Extraction and Production of Bioactive Compounds to Be Used as Nutraceuticals and Food Ingredients: An Overview." Comprehensive Reviews in Food Science and Food Safety, Vol 12, 2013
[52] J. Felipe Osorio-Tobón and M. Angela A. Meireles, "Recent Applications of Pressurized Fluid Extraction: Curcuminoids Extraction with Pressurized Liquids," Food and Public Health 3, no. 6 (2013): 289–303

pressure is reached, the extraction process commences. Once the extraction process is completed, the heating system and HPLC pump are shut down. Inert gases such as nitrogen may be utilised to purge the pressurised liquid extractor and remove the residual solvent left within the extractor[53] **(Figure 7)**.

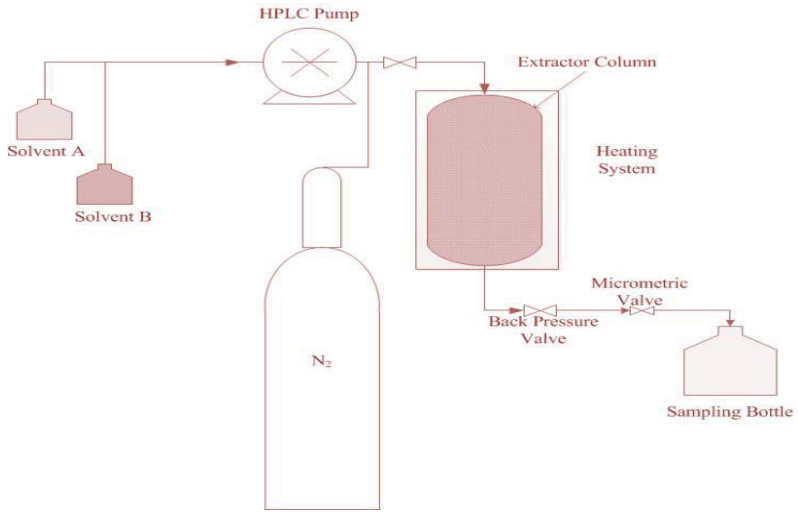


Figure 7. Basic pressurised liquid extraction diagram

It is therefore evident that the critical factors in achieving a high efficiency of extraction are temperature and pressure. Regarding extraction time, numerous investigations into phenolic compounds extraction have proposed a fixed time of 20 minutes[54,55]. In contrast, the selection of the solvent will vary to ensure solvation and release of the analyte. For phenolic compounds, ethanol is the most used solvent as it is relatively environmentally friendly and its polyphenols show high solubility. Another common solvent is an ethanol-water mixture which has been widely and successfully

[53] Kashif Ameer, Hafiz Muhammad Shahbaz, and Joong Ho Kwon, "Green Extraction Methods for Polyphenols from Plant Matrices and Their Byproducts: A Review," Comprehensive Reviews in Food Science and Food Safety 16, no. 2 (2017): 295–315

[54] Laura Jaime et al., "Pressurized Liquids as an Alternative Process to Antioxidant Carotenoids' Extraction from Haematococcus Pluvialis Microalgae," LWT - Food Science and Technology 43, no. 1 (2010): 105–12

[55] Celia Rodríguez-Pérez et al., "Optimization of Microwave-Assisted Extraction and Pressurized Liquid Extraction of Phenolic Compounds from Moringa Oleifera Leaves by Multiresponse Surface Methodology," Electrophoresis 37, no. 13 (2016): 1938–46

used for phenolic compounds extraction[53]. Numerous research studies have employed this technique for phenolic extraction of plants and have identified some of the appropriate extraction parameters to obtain a polyphenol-rich extract. **Table 3** reports some recent examples.

Plant	Objective	Optimal conditions and results	Reference
Grape marc	Optimisation of phenol yield	Optimal PLE: ethanol-water (50% w/w) at 100 °C achieved the highest TPC content (65.68 mg GAE/g dry weight	56
Goldenberry	Optimisation of antioxidant potential	Optimal PLE: 10 min and flow rate of 3 ml/min, showed higher antioxidant capacity (70.31%)	57
***Morus nigra L.* Pulp**	Optimisation of anthocyanins and phenolic content	Analysis of the model showed that the most influential factors were temperature and solvent composition. For anthocyanins, 47.2% methanol, 75.5 °C, 200 atm, purge time of 90 s, pH 3.01, and 50.2% for flushing were the best PLE condition. For the extraction of phenolics were 74.6% methanol, 99.4 °C, 100 atm, 90 s purge, pH 7, and 100% flushing.	58
Goji berry *(Lycium barbarum L.)*	Optimisation of phenolic compound extraction	Optimal PLE: 180°C and 86% ethanol obtained 65.98 mg GAE/g dry weight	59
***Lippia citriodora* leave**	To maximise recovery of phytochemicals	Optimal PLE: 200 °C, 46% ethanol and 17 min obtained a yield of 54.85%	60

Table 3. PLE applications for phenolic extraction

[56] Débora Tamires et al., "Pressurized Liquid Extraction of Bioactive Compounds from Grape Marc," Journal of Food Engineering 240, no. July 2018 (2019): 105–13.
[57] Gustavo Osmar et al., "Food and Bioproducts Processing Pressurized Liquid Extraction of Polyphenols from Goldenberry : Influence on Antioxidant Activity and Chemical Composition," Food and Bioproducts Processing 112 (2018): 63–68
[58] Estrella Espada-bellido et al., "Alternative Extraction Method of Bioactive Compounds from Mulberry (Morus Nigra L .) Pulp Using Pressurized-Liquid Extraction," 2018, 2384–95.
[59] Alejandro Cifuentes, Bienvenida Gilbert-l, and Chiara Fanali, "Elena Ib a Optimization of Pressurized Liquid Extrac- Tion by Response Surface Methodology of Goji Berry (Lycium Barbarum L .) Phenolic Bioactive Compounds," Food Analytical Methods, 2018, 1673–82
[60] Francisco Javier Leyva-jiménez et al., "Comparative Study of Conventional and Pressurized Liquid Extraction for Recovering Bioactive Compounds from Lippia Citriodora Leaves," Food Research International 109, no. April (2018): 213–22

2.2.3 Supercritical fluid extraction (SFE)

Supercritical fluid extraction (SFE) utilises the solvating characteristics of supercritical fluid (SF) by raising the pressure and temperature above the critical point (Tc- critical temperature and Pc- critical pressure)[61]. A supercritical fluid is a fluid characterised by densities similar to that of liquids, while the viscosities and diffusivities are closer to that of gases. With this technique, an efficient extraction of the compounds from the raw material is made possible by the high diffusivity and low viscosity of the supercritical fluid, which improves the penetrating power of the high mass transfer rate of the solutes into the fluid. Further, when using supercritical fluids, phenolic compounds can be extracted at low temperature, which is very important in thermo-labile compounds. Another characteristic of this technique is that SFE is environmentally friendly and the extracted material will be free of solvent residues by depressurising, allowing the supercritical fluid to return to its gas phase and evaporate the solvent residues[62]. There are a large number of solvents that can be employed in SFE (carbon dioxide, nitrous oxide, propane, ethane among others). However, carbon dioxide is the most used solvent in this extraction technique because it can easily reach supercritical conditions **(Figure 8)** (Tc = 31.1 °C, Pc = 72 bar), produces low toxicity, is inflammable and low-cost, and demonstrates high purity relative to other fluids **(Table 4)**[63]. In addition this solvent is generally recognised as safe by the FDA and EFSA (European Food Safety Authority) as it is easily removed from products and can

[61] K. M. Sharif et al., "Experimental Design of Supercritical Fluid Extraction - A Review," Journal of Food Engineering 124 (2014): 105–16

[62] Abbas K.A, et al., "A Review on Supercritical Fluid Extraction as New Analytical Method," American Journal of Biochemistry and Biotechnology 4, no. 4 (2008): 345–53

[63] M. Zougagh, M. Valcárcel, and A. Ríos, "Supercritical Fluid Extraction: A Critical Review of Its Analytical Usefulness," TrAC - Trends in Analytical Chemistry 23, no. 5 (2004): 399–405

operate at low temperatures using a non-oxidant medium which allows for the extraction of thermally labile or oxidised compounds.

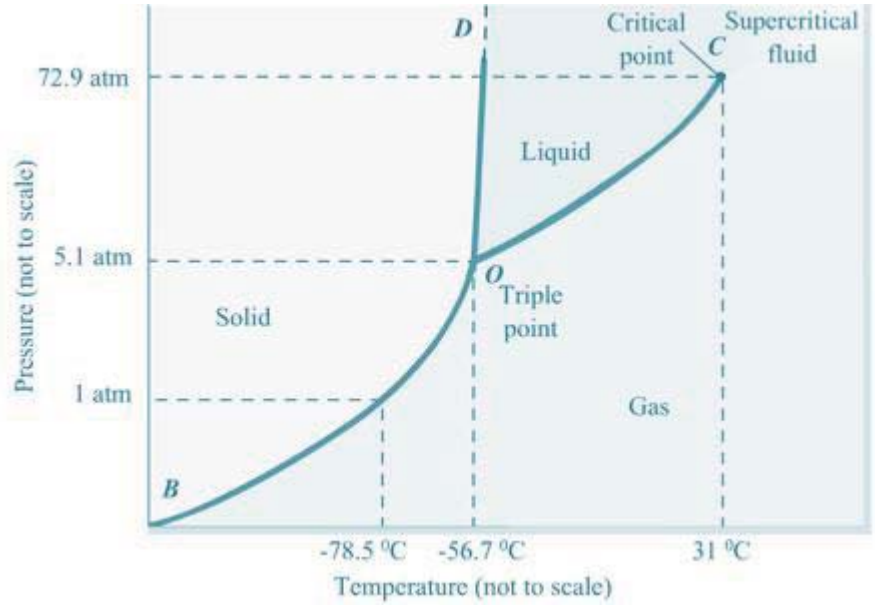


Figure 8. Phase diagram of CO_2

Solvent	Tc (ºC)	Pc (bar)
Ethylene	9.4	50.4
Ethane	32.4	48.8
Carbon dioxide	31.1	72
Dinitrogen monoxide	36.6	72.4
Ammonia	132.4	113
Isopropanol	235.3	47.3
Methanol	239.6	80.1
Toluene	318.8	41
Water	374.4	221.2

Table 4. Critical temperature and pressure of selected solvents

From the perspective of pharmaceutical, nutraceutical and food industries it is therefore a good solvent. Despite this, CO_2 is considered a non-polar solvent and is not suitable for solvating polar compounds. Hence, for the extraction of polar compounds, the addition of a small amount of a polar co-solvent such as ethanol, methanol, or water amongst others, will improve the extraction efficiency of polar targeted compounds from natural sources[64]. Varying the amount of the co-solvent can simplify the selective separation of substances with different polarities, structures or hydrogen bonds[65]. A diagram of this process is show in **Figure 9**.

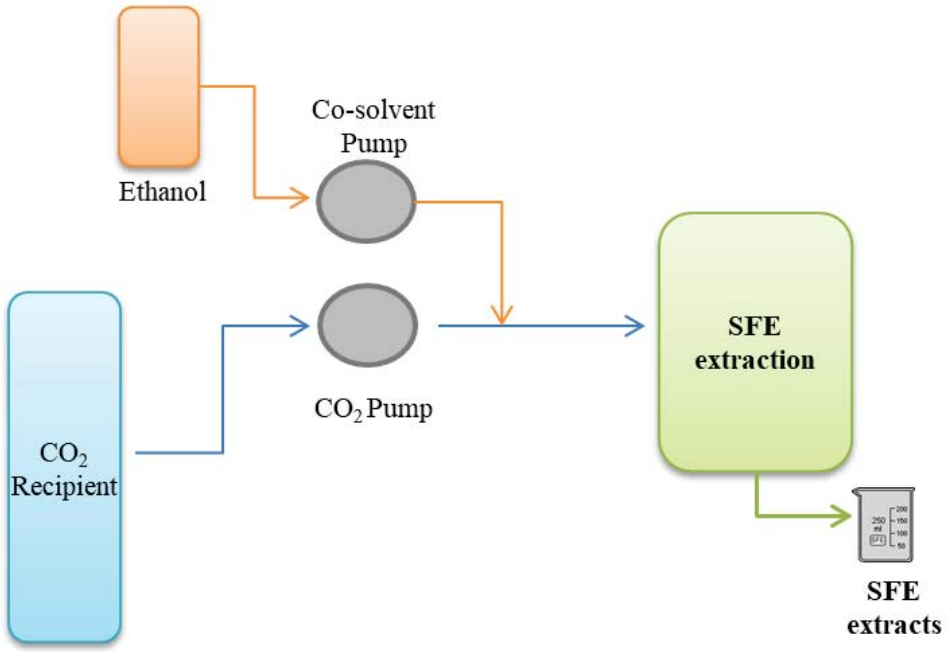


Figure 9. Process diagram of supercritical fluid extraction

[64] Milan N. Sovilj, Branislava G. Nikolovski, and Momčilo D. Spasojević, "Critical Review of Supercritical Fluid Extraction of Selected Spice Plant Materials," Macedonian Journal of Chemistry and Chemical Engineering 30, no. 2 (2011): 197–220

[65] Olga Wrona et al., "Supercritical Fluid Extraction of Bioactive Compounds from Plant Materials," Journal of AOAC International 100, no. 6 (2017): 1624–35

Extractions are conducted in a supercritical fluid extractor equipped with CO_2 and co-solvent pumps, a low-pressure heating exchange, a high pressure heating exchange, a high pressure extraction, a back pressure regulator and high pressure collection vessels where the sample is introduced.

Although SFE is less commonly employed in the extraction of polar compounds, high contents of several phenolic compounds with lower polar properties have been obtained using this technique. Some examples are summarised in **Table 5**.

Plant	Objective	Optimal conditions and results	Reference
Venezuelan Rosemary Leaves	Optimisation of carnosol and carnosic acid content	Optimal SFE: 48°C for 69 min for carnosol and 64°C for 57 min for carnosic acid	66
Mango Ginger Rhizome (*Curcuma Amada Roxb.)*	Optimisation of total phenolic content	Optimal SFE: 350 bar, 60°C and 15 hrs. The TPC obtained was 152 mg GAE/total extract	67
Hibiscus sabdariffa	Optimisation of extraction yield	Optimal SFE: 8.90 MPa, 70 °C, and 9.49 % co-solvent with predicted percentage yield of 26.73 %.	68
***Syzygium cumini* fruit pulp**	Optimisation of extraction yield of anthocyanin and phenolic compounds	The optimal conditions to obtain the maximum extraction yield of anthocyanins and TPC were 162 bar, 50 °C and co-solvent flow rate of 2.0 g/min.	69

Table 5. SFE applications for phenolic extraction

[66] Yalitza Figueroa et al., "Optimization of Supercritical Fluid Extraction of Antioxidant Compounds from Venezuelan Rosemary Leaves," International Journal of Food Engineering, no. October 2014 (2012)
[67] Krishna Murthy T P and B Manohar, "Optimization of Supercritical Carbon Dioxide Extraction of Phenolic Compounds from Mango Ginger Rhizome (Curcuma Amada Roxb .) Using Response Surface Methodology" Biomedicine and Biotechnology, 2, no. 1 (2014): 14–19
[68] Z. Idham et al., "Optimisation of Supercritical CO2 Extraction of Red Colour from Roselle (Hibiscus Sabdariffa Linn.) Calyces," Chemical Engineering Transactions 56 (2017): 871–76
[69] J Prakash Maran, B Priya, and S Manikandan, "Modeling and Optimization of Supercritical Fluid Extraction of Anthocyanin and Phenolic Compounds from Syzygium Cumini Fruit Pulp" Journal of Food Science and Technology, 51, no. September (2014): 1938–46

2.3 Characterisation and determination of phenolic compounds

A current trend in developing nutraceuticals and functional foods is to develop new analytical techniques which enable the identification and quantification of bioactive components, providing knowledge of the physical and chemical stability of these products. In this way, analytical tools can determine whether certain analytes are present or absent within a sample and, when present, estimate their concentration levels[70].

Chromatography is the most commonly used technique for separating compounds from a mixture. However, **spectrometric** techniques provide information on the structural properties of each analysed compound. In combining both technologies, the qualitative and quantitative characterisation of phenolic compounds is enabled.

The column chromatographic technique **high-performance liquid chromatography (HPLC)** is widely used to characterise polar and semi-polar compounds. For this reason, the present Thesis employed HPLC as the analytical technique to identify and quantify phenolic compounds and is discussed in the following section.

[70] Esther Trullols, Itziar Ruisánchez, and F. Xavier Rius, "Validation of Qualitative Analytical Methods," TrAC - Trends in Analytical Chemistry 23, no. 2 (2004): 137–45

2.3.1 High performance liquid chromatography (HPLC)

High performance liquid chromatography (HPLC) is a reliable, fast and efficient method, which is capable of separating very different mixtures of low and high molecular weight compounds and different polarities[71].

HPLC consists of a **stationary phase** (which contains a porous material packed into a thin metal column) and a **mobile phase** that involves an eluting solvent or mixture of solvents pumped through the column by a high pressure pump[72]. The interaction of each compound with the column components varies and this causes differences in flow rates leading to the separation of these compounds. Selection of the mobile phase is based on the target analyte, the column used and the detection system with which it is coupled. For example, the mobile phase can be modified by introducing acids or bases depending on the target compounds to be separated.

The HPLC instrumentation is presented in **Figure 10.** As is shown, it involves a pump, injector, column, detector and data acquisition system. The sample is dissolved in a small discrete volume and injected into the stream of the mobile phase where it permeates through a column composed of micrometre-sized particles. The analytes are then separated, travelling at different speeds which are dependent upon the compound chemical structure and mobile phase composition of the analyte. Each compound is eluted (rising up out of the column) one at a time, with this defined as its retention time (Rt). Following elution, all compounds are detected, generating an

[71] Lucie Nováková and Hana Vlčková, "A Review of Current Trends and Advances in Modern Bio-Analytical Methods: Chromatography and Sample Preparation," Analytica Chimica Acta 656, no. 1–2 (2009): 8–35

[72] Romdhane Karoui and Josse De Baerdemaeker, "A Review of the Analytical Methods Coupled with Chemometric Tools for the Determination of the Quality and Identity of Dairy Products," Food Chemistry 102, no. 3 (2007): 621–40

analytical signal. The intensity and duration of this signal depends on the quantity and nature of the compound[73].

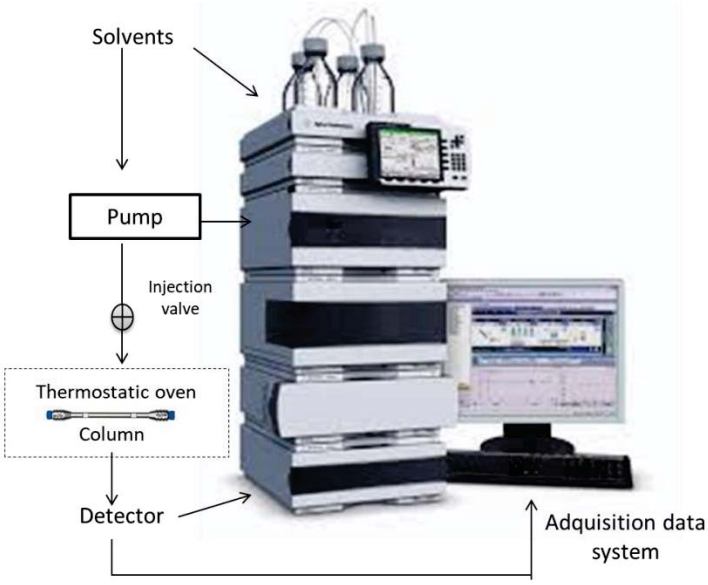


Figure 10. HPLC instrumentation

There are different types of HPLC, namely: normal phase HPLC, reverse phase HPLC, size-exclusion HPLC and ion-exchange HPLC. Of these, reverse phase HPLC (RP-HPLC) is the most widely used HPLC separation technique due to it is ability to handle compounds of diverse polarities and molecular masses. In RP-HPLC the mobile phase is polar and the stationary phase is non-polar or hydrophobic. As a result, compounds with stronger non-polar natures show greater retention[74].

In HPLC, separation efficiency is determined by the mobile phase composition and the properties of the packaging material. There is a wide variety of column types for example cyclodextrin, aryl phases, and materials containing mixed ligands or exhibiting liquid-crystalline properties[75].

[73] Mukthi Thammana, "A Review on High Performance Liquid Chromatography (HPLC)," Research & Reviews: Journal of Pharmaceutical Analysis RRJPA 5, no. 2 (2016): 22–28
[74] Kumar D Sanjay and Harish D.R Kumar, "Importance of RP-HPLC in Analytical Method Development: A Review," International Journal of Pharmaceutical Sciences and Research 3, no. 12 (2012): 4626–33.
[75] M. Ligor et al., "Study of RP HPLC Retention Behaviours in Analysis of Carotenoids," Chromatographia 77, no. 15–16 (2014): 1047–57

HPLC conditions for **phenolic compound separation** require C_{18} reverse-phased columns and a binary solvent gradient. C_{18} columns are designed specifically for the separation of basic compounds and are suitable for all samples. This type of column is filled with a packing of octadecylsilyl groups and ranges from 10 to 30 cm long, with an internal diameter of between 3.9 and 4.6 mm and a particle size of 3 to 10 µm. More recently, new types of columns (monolithic and superficially porous particles columns) ranging from 3–25 cm in length, 1–4.6 mm in internal diameter and 1.8–10 µm[76] in particles size, have been identified.

With regards to the mobile phase, an aqueous-organic solution is required. Acetonitrile and methanol are the most commonly used solutions for the mobile phases in HPLC separation of phenolic compounds. In addition, the presence of acid in the mobile phase suppresses the ionisation of these compounds, enhancing their separation within the column[77]. Thus, acetic acid, formic and phosphoric acids are widely used in acidified mobile phases[63]. In addition, different solvent gradients and flow rates are applied to improve the phenolic separation.

Between the years 2016 and 2017, more than 10000 studies have been published on the topic of HPLC for the identification and quantification of phenolic compounds (according to ScienceDirect). For this reason, this analytical technique was employed in the current Thesis.

[76] Ali Khoddami, Meredith A. Wilkes, and Thomas H. Roberts, "Techniques for Analysis of Plant Phenolic Compounds," Molecules 18, no. 2 (2013): 2328–75

[77] Lucie Řehová, Veronika Škeříkova, and Pavel Jandera, "Optimisation of Gradient HPLC Analysis of Phenolic Compounds and Flavonoids in Beer Using a CoulArray Detector," Journal of Separation Science 27, no. 15–16 (2004): 1345–59

2.3.2 Detection systems

Following separation, the next stage is **detection** of the compounds eluted from the chromatographic column. There are different types of detectors that can be coupled to chromatography and all of them demonstrate certain characteristics. The desired detector characteristics are[78]:

- High sensitivity and demonstrate a reproducible, predictable response.
- Respond to all analytes, or have predictable specificity.
- Wide linear dynamic range; a response that increases linearly with the amount of analyte.
- Response unaffected by changes in temperature and flow during the mobile phase.
- Demonstrate response that are independent of the mobile phase.
- Reliable and convenient to use.
- Non-destructive to the analyte.
- Provide qualitative and quantitative information on the detected peak.
- Fast response.

Figure 11 provides a general overview of detector systems, with ultraviolet-visible (UV-Vis) and mass spectrometry being the detectors used in the present Thesis.

[78] Michael Swartz, "HPLC Detectors: A Brief Review," Journal of Liquid Chromatography and Related Technologies 33, no. 9–12 (2010): 1130–50

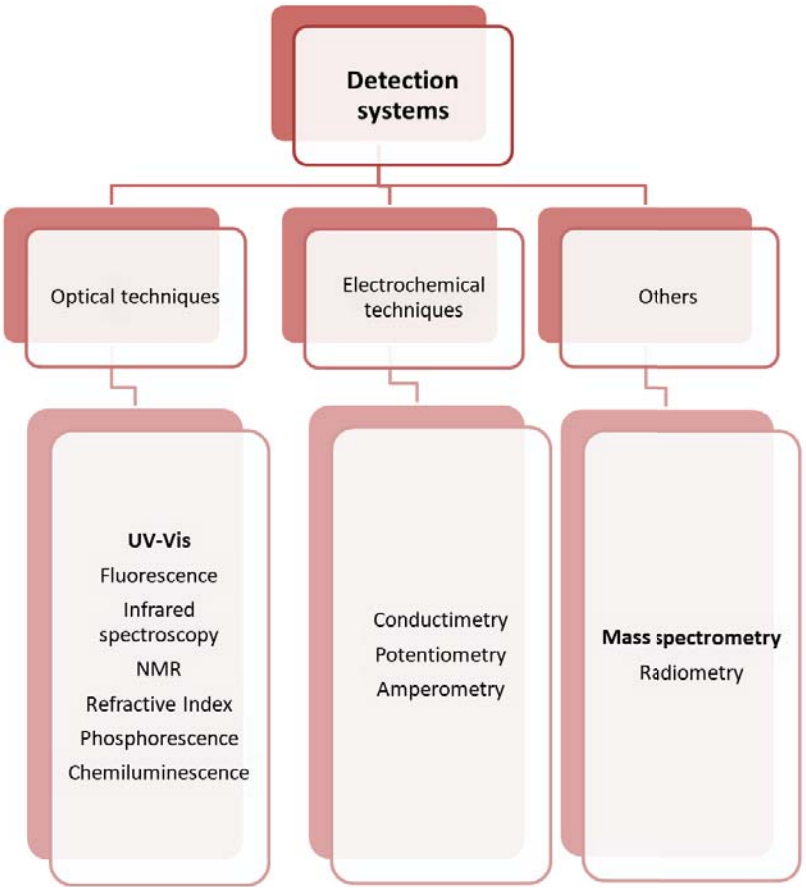


Figure 11. Overview of the most commonly used detection systems in HPLC

2.3.2.1 UV-Vis detector

HPLC coupled with UV-Vis spectroscopy is the simplest and the most popular analytical techniques used for detection. Wide availability of the instrumentation, alongside the simplicity, speed, economic-efficiency, high sensitivity and accuracy of the procedure, make the method well-suited to characterise a wide range of compounds and functional groups. In addition, UV-Vis detectors are more rigid in comparison to other detection methods[79,80].

[79] Bruno E Lendi and Veronika R Meyer, "The UV Detector for HPLC — An Ongoing Success Story," LC GC Europe 18, no. 3 (2005): 156–63.
[80] H.R.N. Bonfilio, R., De Araujo, M.B., Salgado, "Recent Applications of Analytical Techniques for Quantitative Pharmaceutical Analysis : A Review," WSEAS TRANSACTIONS on BIOLOGY and BIOMEDICINE 7, no. 4 (2010): 316–38.

Compounds that absorb within the UV or visible region (from 190-600 nm) can be detected using UV-Vis detectors. Sample concentration is related to its absorbance, which is determined by the fraction of light transmitted through the detector cell according to Beer's Law. For example, for **detection of phenolic compounds**, **Table 6** summarises the UV-Vis absorption spectra of the main families of phenolic compounds. As is shown, most benzoic acids shown high levels of absorbtion at 270-280 nm and hydroxycinamic acids at 305-325 nm. Other compounds such as anthocyanins absorb in two UV-Vis ranges (240-280 nm and 450-560 nm) depending on their chemical structure.

Phenolic compound family	UV-Vis region (nm)	
Benzoic acids	270-280	
Hydroxycinamic acids	305-325	
Cumarins	220-230	310-350
Chalcones	220-270	310-350
Dihydrochalcones	220	280
Aurones	240-270	340-390
Flavones	250-270	330-350
Flavonols	250-270	350-380
Flavonones	270-295	
Flavanols	270-280	
Anthocyanidins	240-280	450-560
Isoflavones	245-270	300-340
Proanthocyanidins	280	

Table 6. UV-Vis absorption spectra of several phenolic compound families

There are three different types of UV-Vis detector: fixed wavelength detectors, variable wavelength detectors (based on different wavelengths but with little practical use today) and **diode array detectors** (based on one or more wavelengths generated form a broad spectrum lamp). Diode array detection (DAD) is the most commonly used detector for phenolic compounds such as flavonoids because it enables the collection of on-line spectra of compounds as they pass through a measuring cell at various

wavelengths[81]. However, most applications of DAD are restricted in the selection of a narrow wavelength range and are not suitable for simultaneous determination of compounds with spectral overlapping. Further, DAD does not provide structural information. For this reason, DAD is commonly used together with mass spectrometry (HPLC-MS) or other spectroscopic methods such as infrared spectroscopy (HPLC-IR) or nuclear magnetic resonance (HPLC-NMR)[82].

2.3.2.2 Mass spectrometry

During the past decade, there has been increasing interest in the use of plants for their beneficial effects. The identification of compounds present in plants is therefore important to decipher the basis of action of therapeutic effects. To this end, mass spectrometry (MS) plays a key role in the discovery of natural products currently being recognised as a potential tool in the identification and discovery of phenolic compounds[83]. This technique is driven by the formation of gas-phase ions (positively or negatively charged) which can be isolated electrically or magnetically based on their **mass-to-charge ratio (*m/z*)**. Thus, MS analysis provides important information about the compounds such as exact mass, molecular formula and relative error therein[84]. Moreover, MS can be coupled with HPLC to enable a larger number of compounds to be analysed rapidly with high sensitivity and selectivity[85].

[81] P Mattila, J Astola, and J Kumpulainen, "Determination of Flavonoids in Plant Material by HPLC with Diode Array and Electro Array Detections," J. Agric. Food Chem. 48 (2000): 5834.
[82] P Minkiewicz and J Dziuba, "Application of High-Performance Liquid Chromatography on-Line with Ultraviolet/Visible Spectroscopy in Food Science," Polish Journal of Food, 15, no. 56 (2006): 145–53
[83] Aihua Zhang et al., "Recent Developments and Emerging Trends of Mass Spectrometry for Herbal Ingredients Analysis," TrAC - Trends in Analytical Chemistry 94 (2017): 70–76
[84] Anas El-Aneed, Aljandro Cohen, and Joseph Banoub, "Mass Spectrometry, Review of the Basics: Electrospray, MALDI, and Commonly Used Mass Analyzers," Applied Spectroscopy Reviews 44, no. 3 (2009): 210–30
[85] Aihua Zhang, Hui Sun, and Xijun Wang, "Mass Spectrometry-Driven Drug Discovery for Development of Herbal Medicine," Mass Spectrometry Reviews 37, no. 3 (2018): 307–20

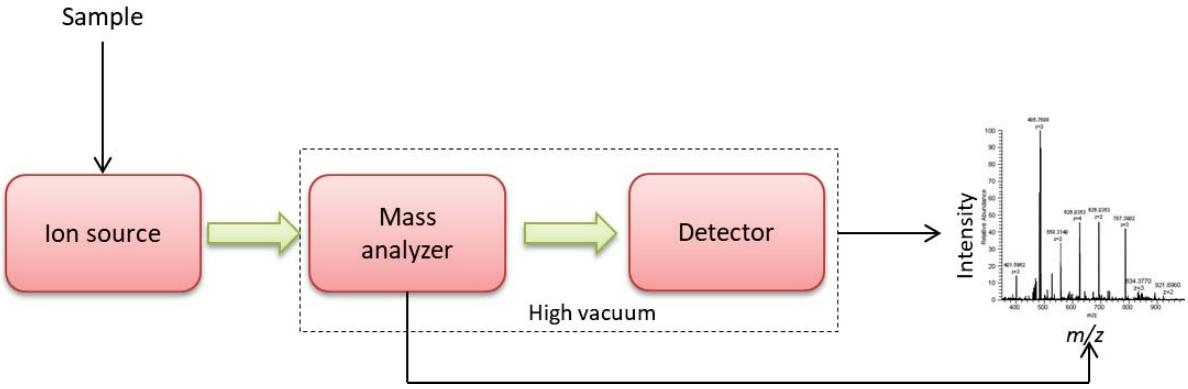


Figure 12. Main components of a mass spectrometer

The main components required to conduct MS are ion source (interface), mass analyser and detector. Molecular ions are produced in an ionisation chamber where the ion source is kept. They are then transferred to the mass analyser which separates the ions according to their *m/z* value. Finally, the isolated ions are passed into the detector to measure their concentration and the results are plotted using mass spectrum **(Figure 12)**. In addition, a large vacuum must be applied because the ions in the gas phase are very reactive and often have short half-lives[86].

Different types of ionisation sources can be coupled with HPLC for evaporation and ionisation of the molecules when they are eluted from the column. **Electrospray ionisation (ESI)** is the most commonly used interface for characterising phenolic compounds[87,88]. In ESI, the sample is nebulised when high voltage is applied. The ionisation procedure relies on the following three steps **(Figure 13)**: the production of charged droplets, the fission of the charged droplets and production of desolvated ions.

[86] Shibdas Banerjee and Shyamalava Mazumdar, "Electrospray Ionization Mass Spectrometry: A Technique to Access the Information beyond the Molecular Weight of the Analyte," International Journal of Analytical Chemistry 2012, no. May 2014 (2012): 1–40

[87] Giorgia La Barbera et al., "Liquid Chromatography-High Resolution Mass Spectrometry for the Analysis of Phytochemicals in Vegetal-Derived Food and Beverages," Food Research International 100, no. 34 (2017): 28–52

[88] Ming-Zhi Zhu et al., "Recent Development in Mass Spectrometry and Its Hyphenated Techniques for the Analysis of Medicinal Plants," Phytochemical Analysis 29, no. 4 (2018): 365–74

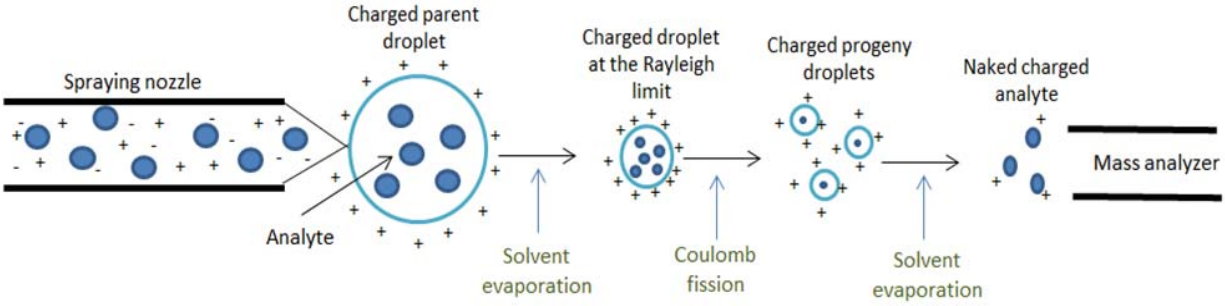


Figure 13. Schematic representation of ESI process

In the first step, a high-voltage is employed at the capillary tip where the solution is being injected in order to produce a charged droplet. The capillary tip is subjected to a neutral gas flow that collides with the droplets from the tip and causes the solvent to evaporate. In the second step, the fission of charged droplets occurs when the Coulombic repulsion between the charges is stronger than the surface tension of the liquid. The point at which this phenomenon occurs is called the Rayleigh limit. At this step in the process, the droplets decrease in size and change their state. Finally, the charged droplet undergoes solvent evaporation and a desolvated ion is produced[89]. Ionisation can take place when in a positive or a negative ion state depending on whether the capillary is set at a positive or negative potential.

Once the ionisation process has taken place, the ions are separated according to their *m/z* by a **mass analyser**. The mass analyser can be compared to a prism. A prism dissociates the component wavelengths of light for detection by an optical receptor. Currently, four main analysers are widely employed. These are called quadrupole (Q), quadrupole ion trap (QIT), time of flight (TOF) and quadrupole-time of

[89] Diogo Ribeiro, "A Short Overview of the Components in Mass Spectrometry Instrumentation for Proteomics Analyses," Tandem Mass Spectrometry - Molecular Characterization 1 (2013)

flight (QTOF) analysers. Selection of the mass analyser depends on the resolution, mass range and detection limit required for the assay being used. In the present Thesis TOF and QTOF analysers were used.

TOF analysers rely on separation of ions after initial acceleration by an electric field. They are subjected to different velocities whilst drifting in a free-field region, known as a flight tube. A detector is positioned at the end of the flight tube to measure the arrival time of charged compounds. Thus, *m/z* ratios are determined by measuring the time that ion molecules take to move through a free field region between the source and the detector. The flight time for each mass is unique and is determined by the energy with which an ion is accelerated, the distance it has to travel, and its *m/z* **(Figure 14)**. This means that ions with a larger *m/z* will travel at a lower velocity than those with a smaller *m/z*.

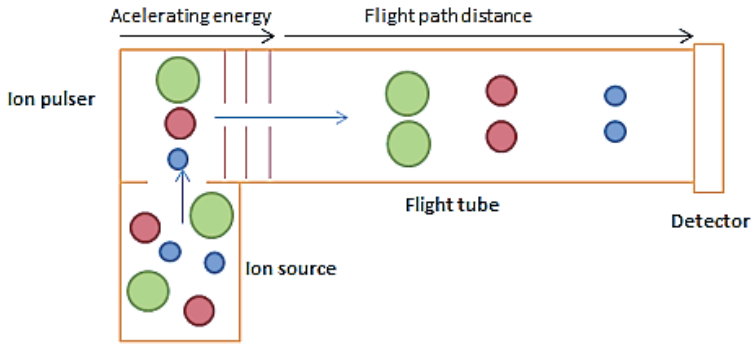


Figure 14. Ion separation in a TOF mass spectrometer

With respect to the mass resolution, this is always proportional to flight time and so the ions will demonstrate greater separation within a given time and a better mass resolution when the length of the flight tube is increased[90]. An accurate mass measurement is crucial to improve the certainty with which an analyte can be

[90] Edmond De Hoffmann and Vincent Stroobant, "Mass Spectrometry - Priniples and Applications"., Mass Spectrometry Reviews, vol. 29, 2007

accurately identified as it will decrease the number of candidate molecular formula that need to be considered. TOF instruments provide mass determinations and generally report an error lower than 2 mDa, which is a great improvement over the conventional quadrupole or ion trap instruments. However, to obtain accurate mass measurements using a TOF instrument, the instrument should be calibrated using a reference compound of a known mass. Following initial calibration, drifts occurring over the course of instrumental operation in a routine analysis are corrected by a single point online calibration[91]. **Figure 15** presents a TOF mass spectrometer.

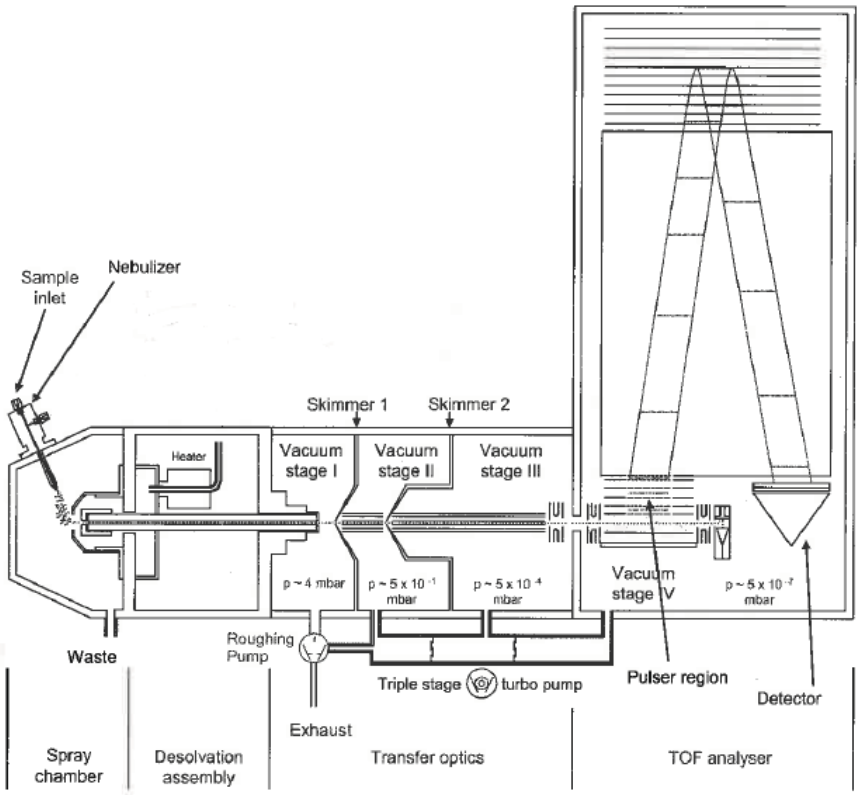


Figure 15. Schematic representation of a TOF mass spectrometer (Adapted from Bruker Daltonik Manual)

[91] M. Petrovic and D. Barceló "Application of liquid chromatography/quadrupole time-of-flight mass spectrometry (LC-QqTOF-MS) in the environmental analysis," Journal of Mass Spectrometry : JMS 41, (2006): 1259–1267

In addition to the TOF components, a QTOF mass spectrometer **(Figure 16)** also contains a collision cell and a quadrupole prior to the time-of-flight tube. In a QTOF, the ion molecules are concentrated into the quadrupole using the hexapole ion bridge. This analyser is also composed of funnels in the ion transmission area which prevent the loss of ions during ionic transmission and improve sensitivity.

QTOF is capable of both single MS and MS/MS operation modes. For MS/MS, ions are selected for collision-induced dissociation in a collision cell assisted by a collision gas (which is normally nitrogen). The fragment ions are then mass analysed in the TOF analyser. QTOF is a very attractive tool due to the combination of high sensitivity, high resolution and high mass accuracy for both precursor and fragment ions[92].

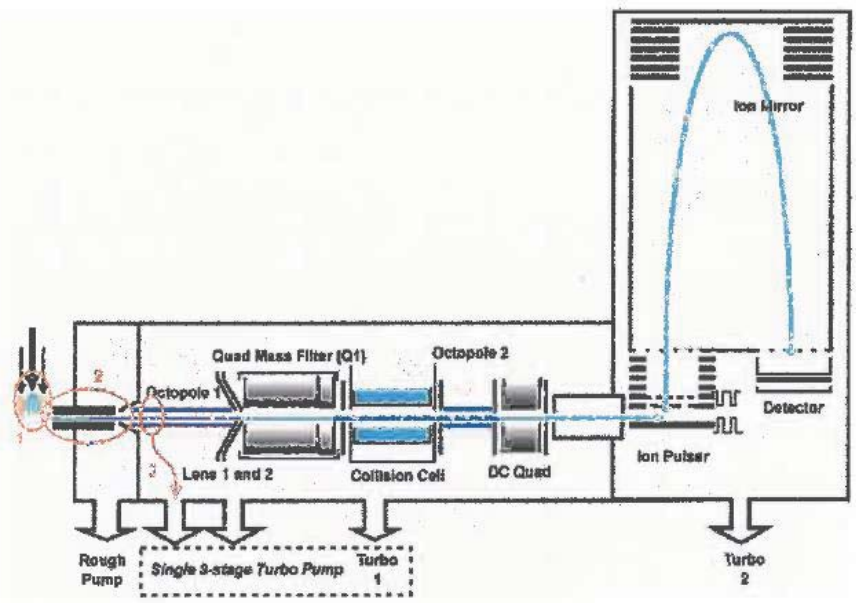


Figure 16. Schematic representation of a QTOF mass spectrometer (Adapted from Agilent QTOF manual)

[92] S. Lacorte and A. R. Fernandez-Alba "Time of flight mass spectromety applied to the lquid chromatogrphic analysis of pesticides in water and food," Mass Spectrometry Reviews 25, (2005): 866-880

In summary, HPLC coupled with DAD and TOF or QTOF, is the most employed analytical technique in the **characterisation and determination of phenolic compounds**. Numerous studies have reported on identification and quantification of these compounds using HPLC-ESI-DAD-TOF / QTOF-MS, increasing knowledge of their chemical structure. **Table 7** summarises some examples of the application of these analytical techniques.

Sample	Mobile phase	HPLC conditions	Results	Reference
Cocoa *(Theobroma cacao)*	Acetic acid 0.5% (A) and methanol (B)	Column: C_{18} (1.8 µm, 150 × 4.6 mm) Gradient: 0 min, 0% B; 5 min, 25% B; 15 min, 35% B; 20 min, 39% B; 38 min, 60% B; 40 min, 70% B; 42 min, 80% B; 44 min, 100% B; 46 min, 0% B; 48 min, 0% B Injection volume: 10 µL Flow rate: 0.3 mL/min	61 phenolic and other compounds were tentatively identified by DAD-QTOF-MS	93
Avocado peel	Acidified water (0.1% formic acid, v/v) andacetonitrile	Column: C_{18} (1.8 µm, 150 × 4.6 mm) Gradient: 0 min, 95% A; 25 min, 50% A; 33 min, 0% A; 36 min, initial conditions until 40 min Injection volume: 10 µL Flow rate: 0.5 mL/min	61 phenolic and other compounds were tentatively identified by DAD-QTOF-MS	94
***Rosmarinus Officinalis* Leaves**	0.1% formic acid (A) and acetonitrile (B)	Column: C_{18} (1.8 µm, 150 × 4.6 mm) Gradient: 0 min, 5% B; 12 min, 50% B; 17 min, 75% B; 22 min, 95% B; 25 min, 5% B. Injection volume: 5 µL Flow rate: 0.8 mL/min	34 phenolic and other compounds were tentatively identified by DAD-TOF-MS	95
25 varieties of Mexican Roselle *(Hibiscus sabdariffa)*	Water:acetonitrile (90:10, v/v) plus 1% of formic acid (A) and acetonitrile (B)	Column: C_{18} (1.8 µm, 150 × 4.6 mm) Gradient: : 0 min, 5% B; 20 min, 20% B; 25 min 40% B; 30 min 5% B; and finally a conditioning cycle of 5 min Injection volume: 10 µL Flow rate: 0.5 mL/min	37 phenolic compounds were tentatively identified by DAD-TOF-MS	96

Table 7. Application of HPLC-DAD-TOF/QTOF in phenolic compounds characterisation

[93] M. L. Cádiz-Gurrea et al., "Isolation, Comprehensive Characterization and Antioxidant Activities of Theobroma Cacao Extract," Journal of Functional Foods 10 (2014): 485–98,
[94] Jorge G Figueroa et al., "Comprehensive Identi Fi Cation of Bioactive Compounds of Avocado Peel by Liquid Chromatography Coupled to Ultra-High-de Fi Nition Accurate-Mass Q- TOF," Food Chemistry 245, no. December 2017 (2018): 707–16
[95] Isabel Borrás et al., "Rosmarinus Officinalis Leaves as a Natural Source of Bioactive Compounds," 2014, 20585–606
[96] Isabel Borrás-Linares et al., "Characterization of Phenolic Compounds, Anthocyanidin, Antioxidant and Antimicrobial Activity of 25 Varieties of Mexican Roselle (Hibiscus Sabdariffa)," Industrial Crops and Products, 69 (2015): 385-394

2.4 Micro and nano-encapsulation of phenolic compounds

It is well reported that phenolic compounds exhibit many physiological properties such as anti-allergenic, antiatherogenic, anti-inflammatory, antimicrobial, antioxidant, cardioprotective and vasodilatory effects, amongst others. Nevertheless, these bioactive properties are short-lived due to the rapid oxidation that occurs due to environmental conditions (temperature, light, oxygen etc.) or insufficient gastrointestinal resistance (resulting from enzymes, pH, low permeability or solubility within the gut etc.) and provokes a partial or even total loss meaning they are not appropriate for application in food development[97].

The effectiveness of nutraceuticals or functional foods in preventing disease depends on the preservation of the bioavailability of their phenolic compounds. In order to maintain structural integrity and increase bioavailability and physiological target, these components need to be administered alongside an additional protective factor[98, 99]. To this end, **encapsulation** is most commonly employed. Encapsulation defines a procedure to enclose one substance (bioactive compounds-phenolic compounds) within another substance (cover material)[100]. Thus, a barrier is created between bioactive ingredients and environmental conditions or between other components of a food matrix. The encapsulated substance is named the core, active or internal phase, while the substance that is encapsulating is called the coating, carrier,

[97] P. N. Ezhilarasi et al., "Nanoencapsulation Techniques for Food Bioactive Components: A Review," Food and Bioprocess Technology 6, no. 3 (2013): 628–47

[98] Sandra Pimentel-Moral et al., Nanoencapsulation Strategies Applied To Maximize Target Delivery of Intact Polyphenols, ENCAPSULATIONS Nanotechnology in the Agri-Food Industry, Volume 2 (Elsevier Inc., 2016)

[99] Matthias Baum et al., "Is the Antioxidative Effectiveness of a Bilberry Extract Influenced by Encapsulation?," Journal of the Science of Food and Agriculture 94, no. 11 (2014): 2301–7,

[100] Aude Munin and Florence Edwards-Lévy, "Encapsulation of Natural Polyphenolic Compounds; a Review", Pharmaceutics, vol. 3, 2011

capsule or membrane, amongst others[101]. The coating is generally composed of different biopolymers such as polysaccharides, proteins, sugars, gums or lipids[102, 103].

From a food industry perspective, this technology is economically advantageous as it enables the rate of delivery of the encapsulated bioactive compounds to be controlled meaning it does not have to be excessively dispensed. Encapsulation has also enabled the food industry to enhance the stability and viability of a wide of variety of products (encapsulated and protected ingredients from moisture, heat or other extreme conditions) and incorporate bioactive compounds into the development of functional foods[104]. Examples of some functions of encapsulation in the food field are: to protect an unstable ingredient from the environment; to isolate two incompatible compounds that need to coexist within the same medium; to avoid side-effects of the encapsulated compounds when consumed by the consumer; to control the delivery of the encapsulated component[105].

Different **encapsulation techniques** and coating materials have been developed to achieve these functions. These technologies can be classified according to their characteristics such as particle size. Encapsulation can therefore be categorised as microencapsulation or nanoencapsulation[106]. Further, depending on the chemical

[101] Pablo Teixeira da Silva et al., "Microencapsulation: Concepts, Mechanisms, Methods and Some Applications in Food Technology," Ciência Rural 44, no. 7 (2014): 1304–11
[102] Abdeliah El-Abbassi et al., "Recent Advances in Microencapsulation Of Bioactive Compounds," Analytical and Processing Techniques 41, no. April 2016 (2015): 129–46.
[103] Verica Đorđević et al., "Trends in Encapsulation Technologies for Delivery of Food Bioactive Compounds", Food Engineering Reviews, vol. 7, 2014
[104] David Julian McClements, "Edible Delivery Systems for Nutraceuticals: Designing Functional Foods for Improved Health," Therapeutic Delivery 3, no. 7 (2012): 801–3
[105] Claude P. Champagne and Patrick Fustier, "Microencapsulation for the Improved Delivery of Bioactive Compounds into Foods," Current Opinion in Biotechnology 18, no. 2 (2007): 184–90
[106] V. Suganya and V. Anuradha, "Microencapsulation and Nanoencapsulation: A Review," International Journal of Pharmaceutical and Clinical Research 9, no. 3 (2017): 233–39

properties of their core and coating materials, this technology can be categorised as capsules or spheres.

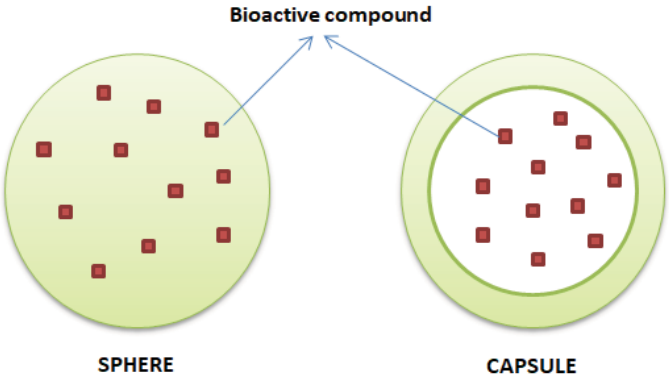


Figure 17. Sphere and capsule

Capsules use a vesicular system in which the bioactive compound is entrapped within a cavity surrounded by a coating material. Spheres use a matrix system in which the bioactive compound is uniformly dispersed **(Figure 17)**. The most commonly employed classification of encapsulation technologies relates to the intended application of the selected bioactive compound. In this regard, encapsulations can be described as chemical or mechanical[107]. Chemical methods include lipid systems, coacervation, ionic gelation, yeast encapsulation or molecular inclusion. On the other hand, mechanical methods include spray-drying, freeze-drying, spray-cooling, fluidised bed or extrusion[108].

The present Thesis uses encapsulation techniques based on **lipid based systems**. For this reason, they are discussed in depth in the following section.

[107] N. Venkata Naga Jyothi et al., "Microencapsulation Techniques, Factors Influencing Encapsulation Efficiency," Journal of Microencapsulation 27, no. 3 (2010): 187–97
[108] Disney Ribeiro Dias et al., "Encapsulation as a Tool for Bioprocessing of Functional Foods," Current Opinion in Food Science 13 (2017): 31–37

2.4.1 Lipid based systems

The application of delivery systems in the food field has increased throughout the last decade. The number of citations per year identified with the keywords "delivery system" and "food" increased from 3 in 1990 to over 10800 in 2017[109]. Also, the development of lipid-based delivery systems to encapsulate, protect and release bioactive compounds has gained traction. This is due the better thermal stability, increased oral bioavailability of compounds and better organoleptic and functional properties, offered by these systems. Further, it is important for such systems to avoid adverse effects on the desirable characteristics of the food product. In this sense, lipid based systems are composed of components (GRAS) for oral or topical administration[110].

These lipid nanocarriers consist of at least **two immiscible liquids**, with one liquid being dispersed as smaller spherical droplets than the other**.** This makes it possible to protect and control the delivery of hydrophilic and hydrophobic bioactive compounds at the same time.

Nowadays, there are a number of lipid-based encapsulation techniques such as emulsions, liposomes, or advanced lipid based nanocarries such as solid lipid nanoparticles (SLNs), nanostructured lipid carrier (NLCs) and lipid-polymer hybrid nanoparticles (LPHNs). An overview is provided in **Figure 18**.

[109] David Julian McClements, "Recent Developments in Encapsulation and Release of Functional Food Ingredients: Delivery by Design," Current Opinion in Food Science 23 (2018): 80–84
[110] David Julian McClements, "Encapsulation, Protection, and Release of Hydrophilic Active Components: Potential and Limitations of Colloidal Delivery Systems," Advances in Colloid and Interface Science 219 (2015): 27–53

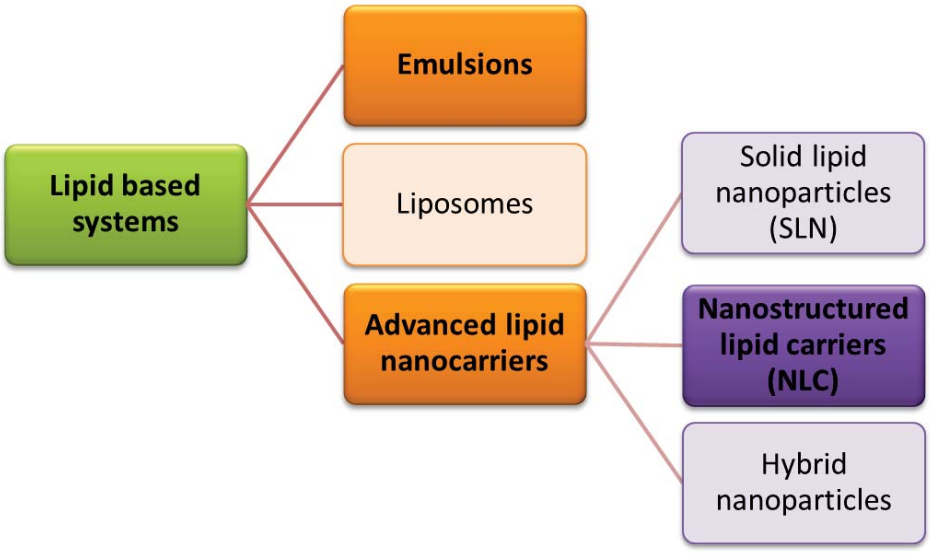


Figure 18. An overview of encapsulation techniques using lipid-based systems

The present Thesis used **emulsions** and advanced lipid nanocarriers, such as **NLCs,** as the selected encapsulation technique. This decision was based on the chemical structure of these systems, which enable them to be utilised in the near future by the food industry to encapsulate nutraceuticals and functional food components. A full review of lipid-based delivery systems is provided in the Experimental Summary of the present Thesis. Thus, in the following section only a summary of the main characteristics of emulsions and NLCs is provided.

2.4.1.1 Emulsions

In the food industry, emulsions occur in the structures of many natural and processed foods. For example, milk, cream, butter, margarine, juice, soup, sauce and ice cream are emulsion products. Indeed, this technology presents different physicochemical and organoleptic properties such as appearance, texture or flavour that allow different products to be obtained. For instance, milk is a fluid with low viscosity, yogurt is a viscoelastic gel and margarine is a semi-solid[94].

Emulsion is a technology used to encapsulate active agents within aqueous or lipid solutions. Emulsified agents can be used directly in their liquid state or can be dried to form powders via spray-drying, freeze-drying or extrusion.

This technology can be classified according to the spatial distribution of its oil and water phases. Oil droplets dispersed in an aqueous phase form an **oil-in-water (O/W)** emulsion, while water droplets dispersed in an oil phase form a **water-in-oil (W/O)** emulsion. In this way, emulsions are formed through a non-polar phase (the oil), a polar phase (the aqueous component) and an amphiphilic phase (the interfacial layer). This makes it possible to incorporate active polar, non-polar and amphiphilic ingredients within the same delivery system[111]. In addition to the simple O/W or W/O systems, there are various types of multiple emulsion systems such as **oil-in-water-in-oil (O/W/O) or water-in-oil-in-water (W/O/W)** emulsions **(Figure 19)**. In the latter, the emulsion is composed of aqueous droplets, which are dispersed inside oily drops; and these oily drops are further dispersed in an external aqueous phase. W/O/W emulsions have some advantages over conventional O/W emulsions such as**, the protection and release of hydrophilic and hydrophobic compounds**. Multiple emulsion is a technique used to produce low calorie and reduced fat foods, or to improve the sensory characteristics of foods[112].

[111] Meltem Serdaroğlu, Burcu Öztürk, and Ayşe Kara, "An Overview of Food Emulsions: Description, Classification and Recent Potential Applications," Turkish Journal of Agriculture - Food Science and Technology 3, no. 6 (2015): 430

[112] Francisco Jiménez-Colmenero, "Potential Applications of Multiple Emulsions in the Development of Healthy and Functional Foods," Food Research International 52, no. 1 (2013): 64–74

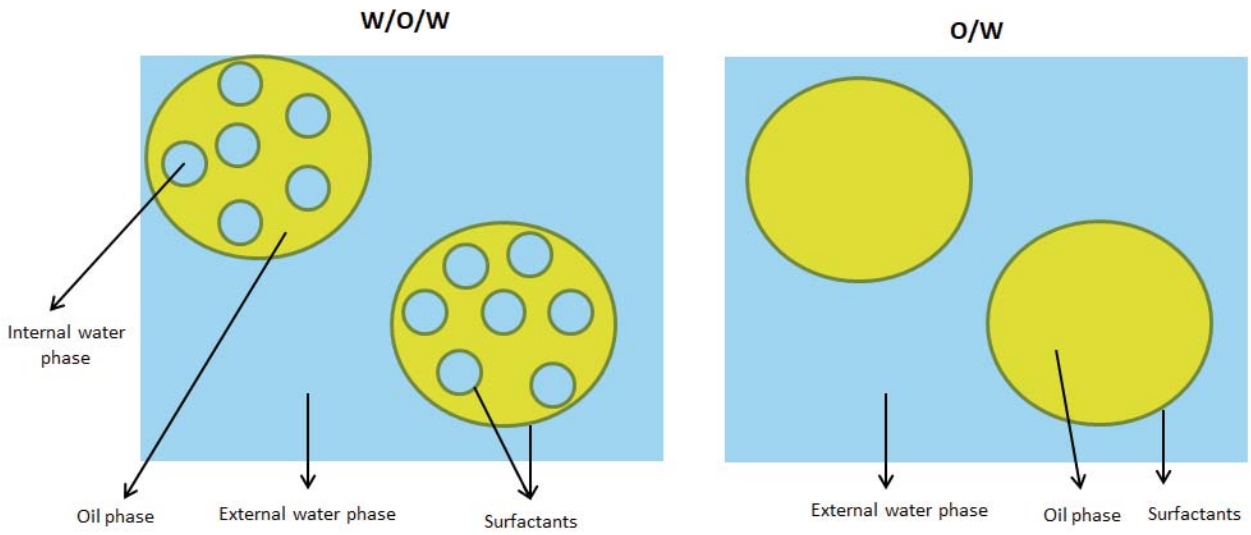


Figure 19. W/O/W and O/W emulsions

Emulsions can also be classified according to their **droplet size**: macroemulsions, microemulsions, and nanoemulsions[113]. Nanoemulsions have gained popularity over the past decade due to their properties favouring passive transport through biological membranes which enhance the bioavailability of bioactive compounds. The diameter of the dispersed droplets in a nanoemulsion is 500 nm or less[114, 115].

It is also important to note the role of **emulsifiers** for emulsion production. Surfactants are amphiphilic molecules that include both hydrophobic (non-polar, water insoluble) and hydrophilic (polar, water soluble) segments. Their properties enable them to adsorb onto the surfaces or interfaces of the system and alter to a marked degree the surface or interfacial free energies of those surfaces or interfaces. The term interface indicates a boundary between any two immiscible phases, whereas the term

[113] Marium Fatima Khan et al., "Emulsion Separation , Classification and Stability Assessment" Journal of Pharmacy and Pharmaceutical Sciences 2, no. 2 (2014).
[114] R. Ravichandran, "Nanotechnology Applications in Food and Food Processing: Innovative Green Approaches, Opportunities and Uncertainties for Global Market," International Journal of Green Nanotechnology: Physics and Chemistry 1, no. 2 (2010): 72–96
[115] MCarment Martínez-Ballesta et al., "Nanoparticles and Controlled Delivery for Bioactive Compounds: Outlining Challenges for New 'Smart-Foods' for Health," Foods 7, no. 5 (2018): 72

surface denotes an interface where one phase is a gas, usually air. Emulsifiers are routinely used in industry to lower the interfacial tension allowing a stable emulsion and avoiding breaking mechanisms such as coalescence. In an interface, surfactants are oriented with their hydrophobic groups directed towards the oil phase and their hydrophilic groups towards the water phase, decreasing the interfacial tension and delaying droplet coalescence[116]. Surfactant can be classified as anionic, non-ionic, cationic and amphoteric, depending on the nature of the hydrophilic group. Anionic and cationic surfactants possess a negative and positive charge, respectively, on their hydrophilic group. Non-ionic emulsifiers have no charge on their hydrophilic group and amphoteric surfactants can have both negative or positive charges on their hydrophilic group depending on the pH of the solution they are in. The most effective emulsifiers used to emulsify O/W or W/O are nonionic surfactants. Ionic surfactants can also be used as an emulsifier for O/W emulsions, though this system is sensitive to the presence of electrolytes. Moreover, a combination of nonionic surfactants can be more effective in emulsification and stabilisation of the emulsion. The hydrophilic–lipophilic balance (HLB) value of the emulsifiers enables the classification of non-ionic surfactants. Emulsifiers with HLB values between 3 and 8 are employed for O/W emulsions, whereas for W/O emulsions, emulsifiers with HLB values between 9 and 12 are used[117]. This parameter can decrease the number of experiments required during the formulation screening stage.

[116] Abdelhalim I.A. Mohamed et al., "Influence of Surfactant Structure on the Stability of Water-in-Oil Emulsions under High-Temperature High-Salinity Conditions," Journal of Chemistry 2017 (2017)
[117] T. Schmidts et al., "Multiple W/O/W Emulsions-Using the Required HLB for Emulsifier Evaluation," Colloids and Surfaces A: Physicochemical and Engineering Aspects 372, no. 1–3 (2010): 48–54

2.4.1.2 Advanced lipid nanocarriers: Nanostructured lipid carrier (NLC)

Despite the aforementioned advantages of emulsions, several limitations have also been which include higher particle sizes and limited physical and chemical stability during storage. To overcome these limitations, advanced and more complex lipid nanocarriers have been developed for the encapsulation of bioactive compounds and phenolic compounds.

In the field of **nanotechnology**, lipid-based systems are mainly represented by solid–lipid nanoparticles (SLNs) and nanostructured lipid carriers (NLCs) which have been commercially introduced as nanocarriers of functional compounds, largely because of their natural composition and biocompatibility[100].

The first generation of SLNs was developed at the beginning of 1990. SLNs are derived from oil-in-water nanoemulsions formed by replacing liquid oil with a solid lipid to form a crystalline undercooled matrix in which the bioactive compound is incorporated[118]. During storage time this crystallised structure could provoke expulsion of the bioactive compound. To address this, Müller, Radtke, & Wissing, (2002) developed a novel carrier namely a **nanostructure lipid carrier (NLC),** to overcome the limitations of SLN[119]. Hence, NLC methods can be described as second generation SLN, which can be produced by mixing very different lipid molecules i.e. solid (fat) and liquid (oil) at room temperature **(Figure 20)**. NLC provides better loading capacity for drug accommodation than SLN. In contrast to emulsions, these nanoparticles contain lipid droplets that are partially crystallised and have a less-ordered crystalline structure or

[118] Harjinder Singh, "Review Article Nanotechnology Applications in Functional Foods ; Opportunities and Challenges" Nanotechnology Applications in Functional Foods, 21, no. February (2016): 1–8
[119] R. H. Müller, M. Radtke, and S. A. Wissing, "Nanostructured Lipid Matrices for Improved Microencapsulation of Drugs," International Journal of Pharmaceutics 242, no. 1–2 (2002): 121–28

an amorphous solid structure, preventing the particle from coalescing by virtue of the solid matrix. In addition, NLC carry a lower risk of expulsion of the drug entrapped during storage, increase drug stability and avoid the use of toxic organic solvents which are used for liposomes formulation[120].

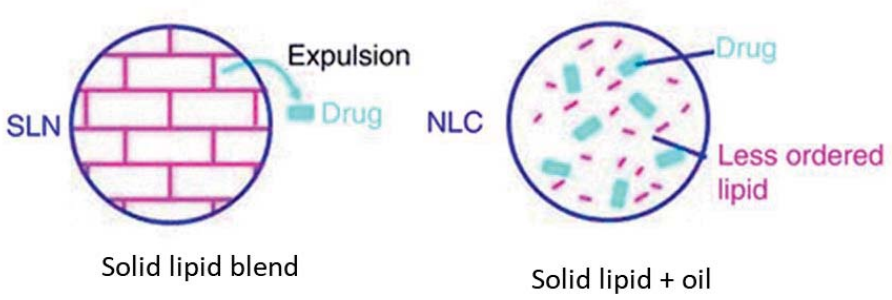


Figure 20. SLN and NLC structures

The scientific literature discusses a number of methods for producing NLC such as through high pressure homogenisation, using O/W or W/O/W emulsions, solvent emulsification-evaporation or ultrasonication[121]. The essential ingredients for NLCs include lipids, water, and surfactants. Both **solid and liquid lipids** are included in the inner cores of NLCs. The solid lipids commonly used for NLCs are glyceryl behenate (Compritol® 888 ATO), glyceril di-stearate (Vegetal BM 297 ATO) and other solid lipids which have been melted at high temperatures (e.g. > 80°C) during preparation but are introduced at room temperature. Liquid oils typically used for NLCs consist of digestible oils from natural sources (e.g. soybean oil)[122].

[120] Fardin Tamjidi et al., "Nanostructured Lipid Carriers (NLC): A Potential Delivery System for Bioactive Food Molecules," Innovative Food Science and Emerging Technologies 19 (2013): 29–43
[121] M Uner, "Preparation, Characterization and Physico-Chemical Properties of Solid Lipid Nanoparticles (SLN) and Nanostructured Lipid Carriers (NLC): Their Benefits as Colloidal Drug Carrier Systems.," Die Pharmazie 61, no. 5 (2006): 375–86.
[122] Chia-Lang Fang, Saleh A. Al-Suwayeh, and Jia-You Fang, "Nanostructured Lipid Carriers (NLCs) for Drug Delivery and Targeting," Recent Patents on Nanotechnology 7, no. 1 (2012): 41–55

NLC can be used in a wide variety of drug delivery systems such as oral drug delivery systems, transdermal drug delivery systems, injection drug delivery systems, and gene transfection. They therefore present **a promising colloidal drug carrier technology** for a number of applications.

2.4.2 Characterisation and determination of physical stability of lipid based systems

A stable lipid-based system is one in which there is a uniform distribution of the dispersed globules throughout the continuous phase. An emulsion can be formed when two immiscible liquids, water and oil, are mechanically agitated. When agitation occurs in the absence of any form of interfacial stabilisation, both liquids will form droplets that rapidly flocculate and coalesce into two separate phases upon standing. Several **breakdown procedures** can occur during storage depending on the distribution of particle sizes and density differences between the droplets and the continuous phase. Major types of physical instabilities are shown in **Figure 21** and include flocculation, creaming, sedimentation, coalescence and breaking[98,123].

[123] Tharwat F. Tadros, "Emulsion Formation,Stability, and Rheology," Emulsion Formation and Stability, 2013, 1–76

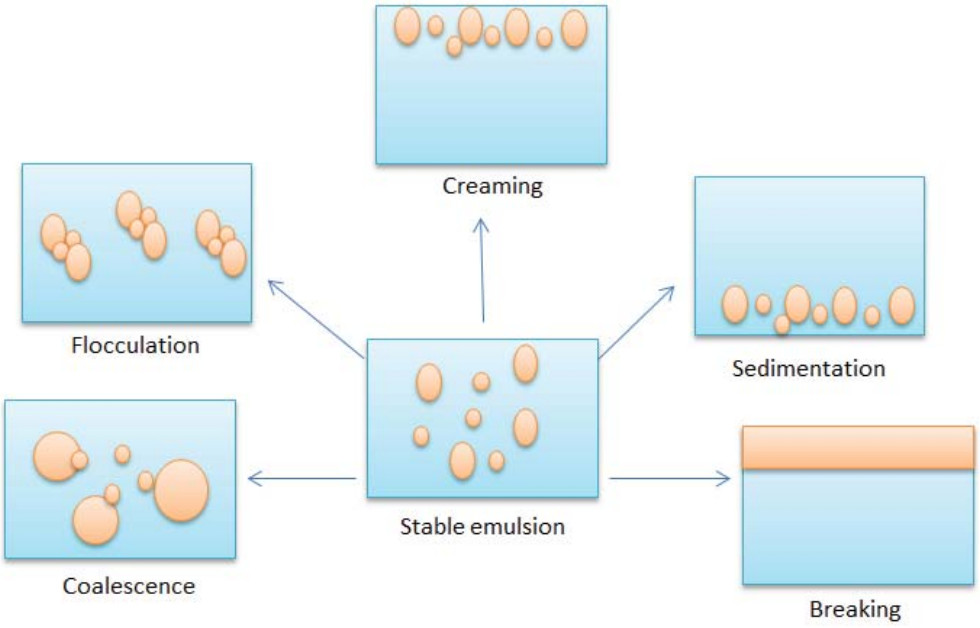


Figure 21. Physical breakdown mechanisms of lipid-based systems

Flocculation is determined by the magnitude of the attractive versus repulsive forces. This phenomenon occurs when there are not enough repulsion forces between the droplets to provoke their aggregation. Flocculation increases the rate of creaming and could be a precursor to coalescence. However, flocculation can be avoided if there is a high charge density between dispersed droplets that creates a high energy barrier and decreases the incidence of flocculation.

Creaming and sedimentation both result from external forces (gravitational or centrifugal). In the case of sedimentation, these forces force the droplets to move faster to the bottom (sedimentation) when their density is greater than that of the continuous phase. In the case of creaming, these forces force the droplets to the top when their density is lower than that of the continuous phase. These reactions are

halted when the particle size is decreased by efficient emulsification, or viscosity is increased through polymers such as polysaccharides or proteins.

Coalescence refers to the fusion of two or more droplets into larger ones through Brownian motion and van der Waals attractions[124]. Coalescence occurs when the mechanical and/or electrical barrier is not sufficient to prevent the droplets from aggregating, leading to complete phase separation (breaking). Generally, flocculation, creaming, sedimentation and coalescence occur before the breaking process.

However, all breakdown mechanisms described can be delayed by a suitable formulation. For example an efficient emulsification or the addition of emulsifiers can stabilise these lipid systems. Currently, **mixtures of biopolymers**, particularly proteins and polysaccharides, are drawing more attention in emulsion stabilisation as a potential medium for the reinforcement of oil-water and water-oil interfaces as they improve the resistance of lipid systems to environmental stresses such as pH changes, ionic strength, and temperature. In addition to this, under specific situations, proteins and polysaccharides form complexes with improved functional characteristics relative to proteins and polysaccharides alone[125]. A suitable characterisation of these lipid based systems is therefore necessary to control the quality of the final product. The **characterisation tools** selected should be sensitive to the assumptions of emulsion performance[126]. Nowadays, there are many analytical methods to determine the stability of these mediums. Indeed, complementary studies using a combination of

[124] A. J.F. Sing et al., "Interactions and Coalescence of Nanodroplets in Translucent O/W Emulsions," Colloids and Surfaces A: Physicochemical and Engineering Aspects 152, no. 1–2 (1999): 31–39
[125] Eric Dickinson, "Double Emulsions Stabilized by Food Biopolymers," Food Biophysics 6, no. 1 (2011): 1–11
[126] Wolfgang Mehnert and Karsten Mäder, "Solid Lipid Nanoparticles: Production, Characterization and Applications," Advanced Drug Delivery Reviews 64, (2012): 83–101

techniques are usually required to obtain a comprehensive view of the physicochemical properties and structures of colloidal systems.

All these mechanisms provide information on the interfacial tension, repulsion between droplets as determined by the electrical charges on the surface of the droplets, morphology, viscosity and particle size[127].

In summary, the most important key factors which directly impact stability and release kinetics are:

- Particle size
- Polydispersity index
- Zeta potential
- Degree of crystallinity and lipid structure

Another important consideration is the physical and chemical stability of the encapsulated active ingredient. Knowledge of the encapsulation efficiency informs predictions of successful emulsion performance of the entrapped bioactive compounds. In addition, the bioactivity and kinetic release of an encapsulated active compound should be determined to ensure effectiveness of the procedure. In this section, the main analytical methods used to characterise lipid encapsulation systems are briefly described.

2.4.2.1 Particle size distribution (PSD)

Droplet size distribution is an important factor to consider with regards to emulsion stability. A colloidal particle is defined as ‘‘*any particle which has some linear*

[127] T Kinyanjui, W Artz, and S Mahungu, “Organic Emulsifiers,” Encyclopedia of Food Sciences and Nutrition, 2003, 2070–77

dimension between 10^{-8} and 10^{-6} m ". Generally, droplet diameters are measured to be between 1 and 50 µm[128]. Droplet diameter is often a critical factor in the manufacture of many products. A colloid system consisting of large particles usually demonstrate a strong tendency to coalesce.

Particle size can be analysed using laser diffraction, electrical zone sensing method (or "Coulter" counter), photon correlation spectroscopy, or ultrasonic spectroscopy. In the case of laser diffraction, the electrical zone sensing method and photon correlation spectroscopy, the sample must first be diluted. In contrast, dilution is not needed in the case of ultrasonic spectroscopy but large amounts of the sample (at least 100 ml) are required[129]. Moreover, the detection limits of the equipment are important considerations when choosing the appropriate method.

Static light scattering techniques such as **laser diffraction** particle size analysers (LD) are the most broadly used to determine the size and distribution of particles. The principle of this technique is based on the Mie theory. The angular variation in intensity of light scattered as a laser beam passes through a well dispersed sample is measured. Smaller particles scatter at wide angles under relatively low light intensities, while large particles scatter more strongly at narrow angles **(Figure 22)**.

[128] D. Clausse et al., "Morphology Characterization of Emulsions by Differential Scanning Calorimetry," Advances in Colloid and Interface Science 117, no. 1–3 (2005): 59–74
[129] I. Roland et al., "Systematic Characterization of Oil-in-Water Emulsions for Formulation Design," International Journal of Pharmaceutics 263, no. 1–2 (2003): 85–94

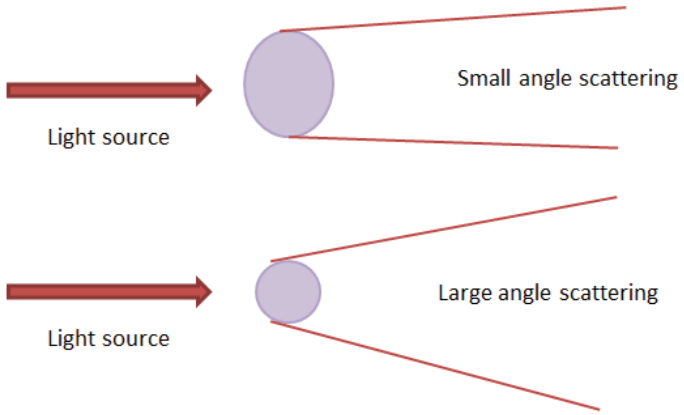


Figure 22. Scattering of light from small and large particles

An example of a LD particle size analyser is the Mastersizer 2000 (Malvern Instruments)[130]. This analyser is capable of measuring particles ranging in size from submicron to millimetre and reports particle size in terms of the diameter of a sphere that produces an equivalent light scattering pattern to the particle being measured. Thus, the refractive index of the sample and solvent must be known[131]. In order to simplify the interpretation of particle size distribution data obtained by LD analysers, a range of statistical parameters can be calculated. Means can be calculated in a number of ways. The most common methods for particle sizing are number length mean, also known as D (1,0); surface area moment mean, also known as D (3,2); and volume moment mean, also known as D (4,3). In summary, D (1,0) refers to the arithmetic mean based on the total number of particles in the sample. D (3,2) is most relevant in cases when the specific surface area is important e.g. bioavailability or dissolution. Finally D (4,3), which is also called De Brouckere Mean Diameters, relies on the size of the particles that constitute the bulk of the sample volume[132]. In addition, as mentioned above, coalescence refers to the fusion of two or more droplets into larger

[130] International Scientific, "Setting New Standards for Laser Diffraction," 2009.
[131] Malvern Instruments, "Mastersizer 2000: User Manual," 2007.
[132] Particle Properties et al., "WHITEPAPER A Basic Guide to Particle Characterization," 2014.

ones, therefore, from mean diameters it is possible to calculate the coalescence rate (*Kc*) of colloid systems at different times[133].

In order to measure particle size in the submicron region, **Dynamic Light Scattering** (DLS) is more often used. The technology behind the Photon Correlation Spectroscopy method for determining particle size is based on Brownian motion. Brownian motion describes the random motion of particles caused by collisions with surrounding solvent molecules. The main difference between DLS and LD is that in the case of the former, particle size is determined by measuring fluctuations in the intensity of scattered light, whereas in the case of the latter, particle size is determined from the measured variation in the intensity of scattered light as a function of scattering angle. Both DLS and LD base their measurements on the assumption that the particle is a perfect sphere[134] and can be used to measure nanoparticles and nano-colloidal dispersions. One example of a DLS particle size analyser **(Figure 23)** is Zetasizer Nano Series (Malvern Instruments).

Figure 23. Zetasizer equipment (Adapted from Zetasizer Nano Series Manual)

[133] E. Bosquez-Molina, I. Guerrero-Legarreta, and E. J. Vernon-Carter, "Moisture Barrier Properties and Morphology of Mesquite Gum-Candelilla Wax Based Edible Emulsion Coatings," Food Research International 36, no. 9–10 (2003): 885–93

[134] U. Teipel U. Foerter-Barth, "Characterization of Particles By Means of Laser Light Diffraction and Dynamic Light Scattering," Mineral and Material Characterization Volume 13, no. 1 (2000): Pages C1-1-C1-8

The Zetasizer analyser measures particle size from the speed of random movement whilst suspended in a liquid. It is known that small particles move quickly in a suspension, whereas large particles move slowly. By observing the movement of a particle over time it is possible to estimate its size. For example, if the particle does not change its position much over time, the particle will be large in size **(Figure 24)**. In contrast, if the particle changes is position by a large amount, then the particle of the sample will be small[135].

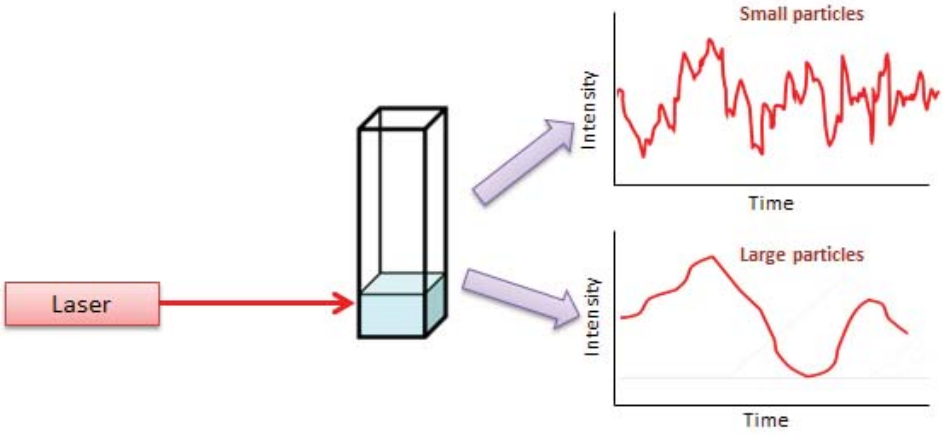


Figure 24. Intensity fluctuations for small and large particles

This technique has the following advantages with respect to LD:

- Particle size range is ideal for nano and biomaterials
- Small quantity of the sample is required
- Fast analysis and a high throughput
- Non-invasive allowing for complete sample recovery.

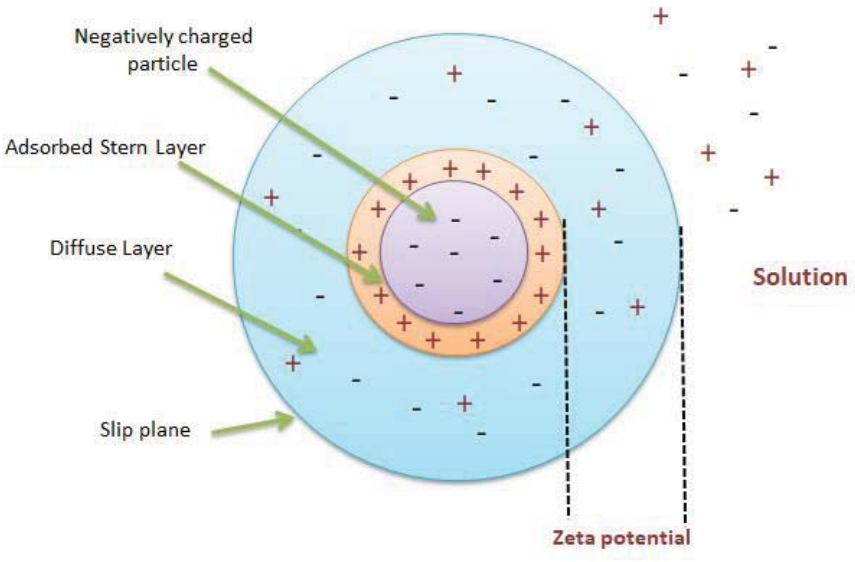
[135] Malvern instruments, "Zetasizer Nano Series User Manual," Department of Biochemistry Biophysics Facility , University of Chambridge, no. 2 (2004): 207

In addition to measuring particle size, this equipment is able to measure the zeta potential and polydispersity index of particles in suspension.

2.4.2.2 Zeta potential

Zeta potential (ZP) is defined as "*the potential at the slipping/shear plane of a colloid particle moving under electric field. This analysis determines the* ***surface charge*** *of particles in solutions. Particles have a surface charge that attracts a thin layer of ions of opposite charge to the particle surface. This double layer of ions travels with the particle as it diffuses throughout the solution*" **(Figure 25)**. Zeta potential values range from + 100 mV to – 100 mV, with values higher than + 25 mV and less than -25 mV typically showing greater stability. Suspensions with low zeta potential will be aggregated due to Van Der Waal attractions[136, 137].

Figure 25. Electrical double layer surrounding the particle



[136] Nanocomposix, "Zeta Potential Analysis of Nanoparticles," Nanocomposix Publications, 2012, 1–6.
[137] Sourav Bhattacharjee, "DLS and Zeta Potential - What They Are and What They Are Not?," Journal of Controlled Release 235 (2016): 337–51

During measurement, a **sample cell** with two gold electrodes is used and voltages up to 200 V are applied **(Figure 26)**. Movement of the particles in the electric field is detected by the equipment. Several factors can affect ZP, with the pH of the medium being the most important parameter. Other factors include ionic strength, the concentration of any additives and temperature.

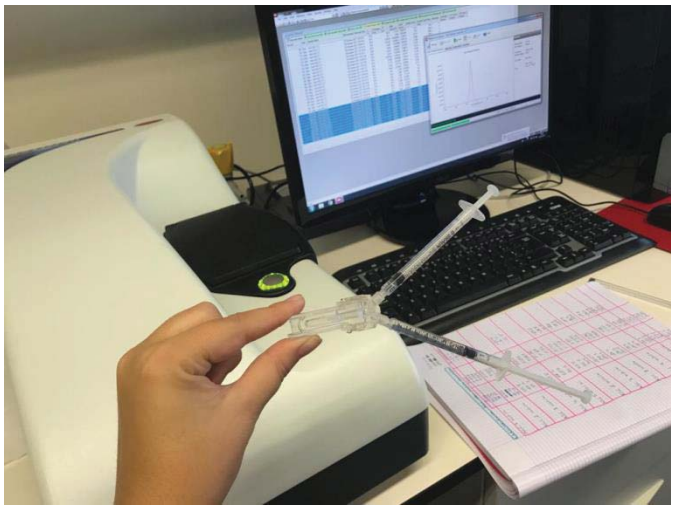


Figure 26. Sample cell for zeta potential measurement

This measure provides an important tool for understanding the stability of colloids systems.

2.4.2.3 Polydispersity index (PDI)

PDI is used to determine the **degree of non-uniformity** of a distribution of particles. This index is dimensionless and is calculated as M_w/M_n where M_w is the weight average and M_n is the average molecular weight[138]. Thus, PDI can be employed to measure the breadth of the molecular weight distribution of a polymer.

[138] Sagar S. Rane and Phillip Choi, "Polydispersity Index: How Accurately Does It Measure the Breadth of the Molecular Weight Distribution?," Chemistry of Materials 17, no. 4 (2005): 926

Values from PDI analysis typically range from 0.0 for samples with perfectly uniform particle sizes, to 1.0 for a highly polydisperse sample with a wide variety of particle sizes. For lipid encapsulation systems, a PDI value of 0.4 and lower is considered to be suitable and indicative of a homogeneous population. Alongside particle size, this parameter is an important factor for different applications (e.g., food, cosmetic, pharmaceutical, etc) and routes of bioactive administration[139].

2.4.2.4 Transmission electron microscopy (TEM)

DLS produces good measurements with regards to size, PDI and ZP of lipid systems. Nevertheless, it does not provide information about the morphology and structure of the lipid system (e.g. spherical, oval, cylindrical). In this regard, microscopic techniques give more detailed views of the **shape** of colloid systems **(Figure 27)** as well as the presence/absence of any aggregation and/or fusion[125]. Specifically, TEM has been shown to be an effective tool for the visualisation and characterisation of colloidal systems. With this technology the size of individual nanoparticles can be measured after drying the solvent and does not have to be measured in the liquid suspension. In addition, the morphology and shape of these nanoparticles can be observed with this method[140]. In the case of TEM electron microscopy, this method works by bombarding a sample with a steam of electrons and monitoring the resulting transmission. The transmission is converted into images of particle dispersion within the sample. TEM produces higher resolution images than

[139] M. Danaei et al., "Impact of Particle Size and Polydispersity Index on the Clinical Applications of Lipidic Nanocarrier Systems," Pharmaceutics 10, no. 2 (2018): 1–17

[140] A. A. Lizunova et al., "Comparison of the Results of Measurements of the Sizes of Nanoparticles in Stable Colloidal Solutions by the Methods of Acoustic Spectroscopy, Dynamic Light Scattering, and Transmission Electron Microscopy," Measurement Techniques 59, no. 11 (2017): 1–5

other electron microscopy such as Scanning Electron Microscopy (SEM), being capable of resolving images as small as 0.17 nm[141].

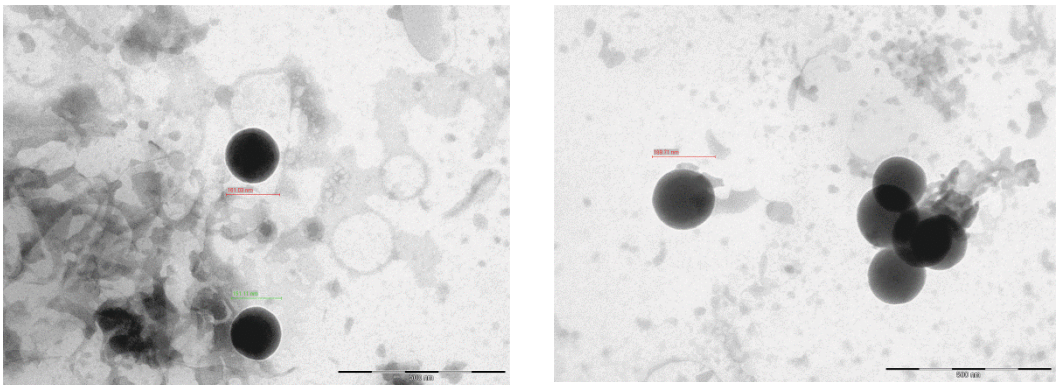


Figure 27. Nanoparticles TEM images

2.4.2.5 Centrifugation analysis of particle size distribution

Analytical centrifugation is a technique that can be used for the measurement of particle size distribution (PSD). PSD determines the state of agglomeration of the particles, in other words, the extent of the **sedimentation or creaming** process. To achieve this, a cuvette-type analytical centrifugation is used with a turbidity detector that evaluates the grouping of particulate materials when a centrifugal force is applied[142].

LUMiSizer (Figure 28) has become a commonly utilised tool which conducts PSD analysis based on analytical centrifugation and evaluates the long-term stability of nanosuspensions. This instrument measures the intensity of the light transmitted during centrifugation as a function of time and particle positioning during the duration of centrifugation. This is called STEP^{TM} – Technology (Space – and Time – resolved

[141] Shalini Charurvedi and Pragnesh N Dave, "Microscopy in Nanotechnology," Current Microscopy Contributions to Advances in Science and Technology, 2012, 14–16.
[142] Christian Ullmann et al., "Performance of Analytical Centrifugation for the Particle Size Analysis of Real-World Materials," Powder Technology 319 (2017): 261–70

Extinction Profiles). The changing transmission profiles provide information about the kinetics of the nanoparticles behaviour and inform shelf-life estimations. In this way, the instability phenomena is related to the migration of particles (sedimentation or creaming) when centrifugal acceleration is applied[143].

Figure 28. LUMiSizer equipment and cuvettes-type analytical centrifugation

2.4.2.6 Fourier-transform infrared spectroscopy (FTIR)

Fourier-transform infrared (FTIR) spectroscopy is an experimental technique used initially for qualitative and quantitative analysis of organic compounds, providing specific information on **molecular structure, chemical bonding and molecular environment**. This technology relies on infrared spectroscopy that probes the vibrations of atoms. In this sense, each infrared absorption band is associated to specific fundamental vibrations of the functional groups and in this way, it provides information about chemical composition[144]. FTIR spectroscopy is mostly used in the middle infrared (MIR) region (4000–300 cm^{-1}) in which the fundamental vibrational modes are found.

[143] A. R. Fernandes et al., "Ibuprofen Nanocrystals Developed by 2^2 factorial Design Experiment: A New Approach for Poorly Water-Soluble Drugs," Saudi Pharmaceutical Journal 25, no. 8 (2017): 1117–24
[144] Catherine Berthomieu and Rainer Hienerwadel, "Fourier Transform Infrared (FTIR) Spectroscopy," Photosynthesis Research 101, no. 2–3 (2009): 157–70

For decades, this technique has been widely used for the characterisation of colloids, analysing their lipid phase structure and organisation[145, 146]. More recently, it has become a powerful analytical tool used by the food industry in the study of edible oils and fats. Moreover, FTIR also provides the basis of the "**fingerprint technique**" as there are no two samples with the same FTIR spectra, with regards to both the number of peaks and the maximum peak intensities. As a result, with regards to lipid encapsulation, FTIR enables evaluation of the interfacial activity of aggregate structures which ensure its presence in the lipid matrix. This includes the interaction between the lipid system and the encapsulated bioactive compound[147].

2.4.3 Encapsulation efficiency (EE)

Encapsulation efficiency (EE) is defined as the proportion of bioactive ingredient incorporated into the micro or nano capsules. It refers to the total amount of core material added during the encapsulation process.

This parameter is calculated by the equation EE = (m_E / m_T) x 100, where m_E is the mass of the incorporated bioactive ingredient and m_T is the total mass of the bioactive material added. In most cases m_E value is unknown but can be calculated from the difference between the total amount of bioactive ingredient added and the amount of free or non-entrapped material, m_F ($m_E = m_T - m_F$). In a liquid delivery system, the value of m_F is obtained by centrifugation which results in the separation between m_F and m_E, with m_F being localised in the supernatant region. Hence, the first

[145] S. P. Armes et al., "Aqueous Colloidal Dispersions of Polyaniline Formed by Using Poly(Vinylpyridine)-Based Steric Stabilizers," Langmuir 6, no. 12 (1990): 1745–49
[146] Ruthven N.A.H. Lewis and Ronald N. McElhaney, "Membrane Lipid Phase Transitions and Phase Organization Studied by Fourier Transform Infrared Spectroscopy," Biochimica et Biophysica Acta - Biomembranes 1828, no. 10 (2013): 2347–58
[147] David R. Scheuing, "Fourier Transform Infrared Spectroscopy in Colloid and Interface Science," 1990, 1–21

step in the determination of encapsulation efficiency is the separation between the encapsulated drug (within the carrier) and the free drug[148]. Once the free drug is obtained, it can be quantified by UV spectroscopy under circumstances when the wavelength at which the bioactive compound absorbs is known.

2.4.4 *In vitro* release of phenolic encapsulated compounds

In recent years, interest from the food, cosmetic and pharmaceutical industries in the development of carrier systems that allow local delivery of bioactive agents has increased. **Control release** has been identified as a key step in the administration of bioactive compounds. This mechanism allows higher local concentrations to be reached which approach the effective levels demonstrated *in vitro*.

The encapsulation process has been shown potential for improving the bioavailability, solubility and shelf-life of bioactive compounds. It allows a targeted long-term release and enables the dosage and frequency of administration to be reduced. Slow release of a bioactive agent results in more positive therapeutic effects[149]. In addition, lipid-based nanostructured systems have been demonstrated to be safe and capable of achieving operative concentrations in target cells and tissues[150].

***In vitro* release testing** is employed to evaluate the release properties that reflect the combined effects of the physical (e.g. particle size) and chemical parameters (e.g. solubility) of a given dosage. The most common form of in vitro

[148] A. Laouini et al., "Preparation, Characterization and Applications of Liposomes: State of the Art," Journal of Colloid Science and Biotechnology 1, no. 2 (2012): 147–68
[149] Wei Lu, Alan L. Kelly, and Song Miao, "Emulsion-Based Encapsulation and Delivery Systems for Polyphenols," Trends in Food Science and Technology 47, no. October (2016): 1–9,
[150] Angelina Angelova et al., "Neurotrophin Delivery Using Nanotechnology," Drug Discovery Today 18, no. 23–24 (2013): 1263–71

release testing is called **Franz diffusion cell system**[151]. This technique evaluates *in vitro* drug permeability and consists of a membrane in between a donor and a receptor chamber **(Figure 29)**[152]. This membrane can be constituted of a tissue construct, a biological sample such as skin, or a synthetic membrane. The FDA advises the use of simple and porous synthetic membranes in release studies due to their characteristics of acting as a support without presenting a rate-limiting barrier[137].

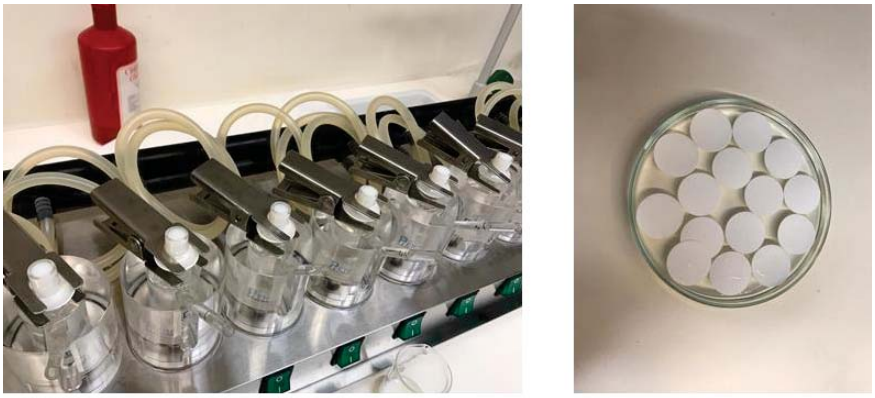


Figure 29. Franz diffusion cells and synthetic membranes

The receptor chamber is usually composed of a phosphate-buffered saline (PBS) with a pH of 7.4 and maintained at 37 ºC as these conditions simulate surface intestinal conditions[153]. An aliquot of medium is removed from the receptor compartment at predetermined time points for bioactive compound content analysis. Franz diffusion cells provide an efficient method for the evaluation of bioactive compound release.

[151] Shiow Fern Ng et al., "A Comparative Study of Transmembrane Diffusion and Permeation of Ibuprofen across Synthetic Membranes Using Franz Diffusion Cells," Pharmaceutics 2, no. 2 (2010): 209–23

[152] T. J. Franz, "Percutaneous Absorption. On the Relevance of in Vitro Data," Journal of Investigative Dermatology 64, no. 3 (1975): 190–95

[153] Erina Pretorius and Patrick J. D. Bouic, "Permeation of Four Oral Drugs Through Human Intestinal Mucosa," AAPS PharmSciTech 10, no. 1 (2009): 270–75

2.4.5 *In vitro* evaluation of oxidative stability of phenolic compounds loaded in lipid based systems

As mentioned above, bioactive compounds from natural sources exhibit many beneficial physiological effects when they are encapsulated and their functional structure is protected from oxidation. The determination of *in vitro* bioactivity of phenolic compounds loaded in colloidal delivery systems is therefore important to ensure their **bioavailability and physiological target**. To this end, the first step is **phenolic extraction** of the lipophilic phase. This procedure can be achieved by dissolving the colloidal system in lipophilic and hydrophilic solvents, which after centrifugation experience separation of both lipid and aqueous phases. The phenolic compounds in the aqueous phase are then collected and their bioactivity can be determined. The present Thesis employed *in vitro* antioxidant activity and determination of total phenolic content (TPC) to evaluate the bioactivity of the phenolic compounds loaded into the colloidal delivery systems.

The **Folin-Ciocalteu assay** is considered to be the standardised spectrophotometric assay for determination of TPC. It works by reducing a Folin-Ciocalteu reagent in the presence of antioxidant compounds in an alkaline medium such as a phenolic compound.

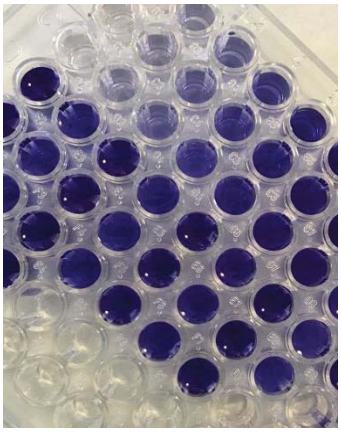


Figure 30. Folin-Ciocalteu assay

This reduction produces a blue sample with an intensity of 760 nm and containing a reduced compound content. **(Figure 30)**[154].

In order to measure antioxidant activity a number of *in vitro* methods can be employed. In fact, more than one assay is normally used to obtain more accurate information on antioxidant capacity[155, 156]. To this end, **Trolox Equivalent Antioxidant Capacity (TEAC)** and the **Ferric Reducing Antioxidant Power (FRAP)** assays have been shown to be fast-acting tools. TEAC relies on the de-colourisation of the 2.2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical ($ABTS^{\cdot+}$) which is measured at 734 nm. This radical is inhibited In the presence of antioxidant compounds this radical is inhibited and the product becomes more stable due to electron transfer or H atom transfer[157] **(Figure 31)**.

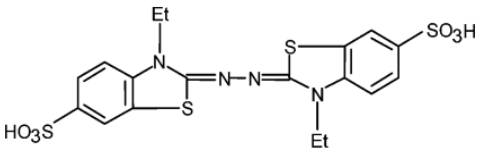


Figure 31. Structure of $ABTS^{\cdot+}$ radical

FRAP assay is based on the reduction of a colourless ferric complex with 2.4.6-trpyridyl-s-triazine (TPTZ) in its ferrous coloured form **(Figure 32)** and relies on the ability of antioxidant compounds to donate electrons. Despite the different

[154] Konstantinos Kiritsakis et al., "Olive Oil Enrichment in Phenolic Compounds during Malaxation in the Presence of Olive Leaves or Olive Mill Wastewater Extracts," European Journal of Lipid Science and Technology 119, no. 9 (2017): 1–13
[155] M. L. Cádiz-Gurrea et al., "Isolation, Comprehensive Characterization and Antioxidant Activities of Theobroma Cacao Extract," Journal of Functional Foods 10 (2014): 485–98
[156] Aránzazu Morales-Soto et al., "Antioxidant Capacity of 44 Cultivars of Fruits and Vegetables Grown in Andalusia (Spain)," Food Research International 58 (2014): 35–46
[157] Ronald L. Prior, Xianli Wu, and Karen Schaich, "Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements," Journal of Agricultural and Food Chemistry 53, no. 10 (2005): 4290–4302

mechanisms behind the reactions produced by FRAP and TEAC assays, similar compounds react to them.

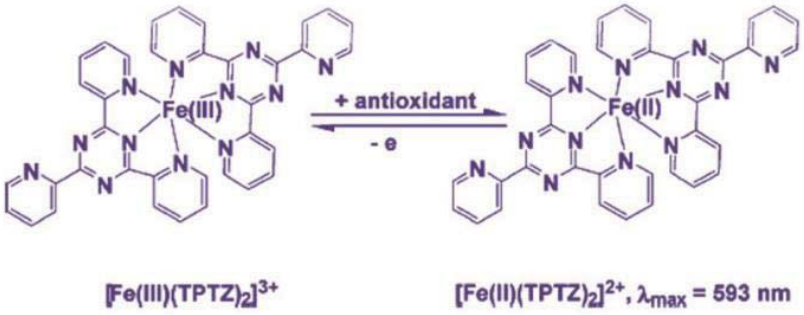


Figure 32. Reduction of Fe(III)-TPTZ complex

The FRAP method is not suitable for the determination of some phenolic compounds. This is because FRAP results depend on a relatively short time scale for analysis but some polyphenols bind ions more slowly and thus require longer reaction times for detection[143].

2.5 Incorporation of encapsulated phenolic compounds into a food matrix

2.5.1 Interaction with food components

The applications of nanotechnology in the food sector are still in their infancy but are predicted to grow rapidly in the coming years.

When food products are fortified with bioactive compounds, many technological problems can occur due to the reaction of these compounds with other food components during processing, storage and transport.

In foods there are a wide variety of interactions between molecules and colloidal particles. The repulsive and attractive forces between different food components and colloidal systems are the result of two contrasting phenomena: biopolymer incompatibility and complex formation. For example, many studies have demonstrated that **proteins** are surface-active molecules that can be used as emulsifiers and improve the stability of emulsion formulation[158]. Proteins from foods can generate repulsive interactions between oil droplets and form interfacial membranes that are resistant to rupture, stabilising the droplets against flocculation and coalescence during long-term storage.

Polysaccharides are known specifically for their water-holding characteristics. Polysaccharides make good stabilising agents because of their hydrophilic properties, high molecular weight, and gelation behaviour, which lead to the formation of a macromolecular barrier in the aqueous medium between dispersed droplets. In addition, some polysaccharides can act as emulsifiers such as gum arabic, modified

[158] David Julian McClements, "Protein-Stabilized Emulsions," Current Opinion in Colloid and Interface Science 9, no. 5 (2004): 305–13

starches and certain types of pectin[159]. Similarly, **phenolic compounds** are capable of binding with proteins and consequently decrease the astringency of foods.

2.5.2 Physical changes in food matrix

When an encapsulated bioactive compound is incorporated into a food matrix, the system must be **compatible** with this food matrix without adversely affecting the appearance, taste, flavour, texture and shelf life of the product. Indeed, encapsulation systems have been developed to improve taste, colour, flavour, texture and consistency of foodstuffs.

Texture is defined as *"the sensory and functional manifestation of the structural, mechanical and surface properties of foods detected through the senses of vision, hearing, touch and kinaesthetic"*[160]. In food products that contain colloidal systems the shape and size of particles, concentration, sample matrix and ingredient properties can impact this parameter[161].

Appearance is another important factor that should be considered when an encapsulated food ingredient is incorporated into a food matrix. This effect is determined by the directly observable structural elements of food. For example, observable fat droplets in milk would not be accepted by consumers; therefore when colloidal systems are incorporated into a dairy product a pre-defined size distribution must be maintained.

[159] Eric Dickinson, "Hydrocolloids as Emulsifiers and Emulsion Stabilizers," Food Hydrocolloids 23, no. 6 (2009): 1473–82
[160] Peter Fryer, "Food structure and functionality: a soft matter perspective," Soft Matter, 2008, 5,501
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The stability and **shelf-life** of food products containing encapsulated bioactive ingredients must be evaluated in order to ensure positive product performance of the final product within acceptable safety specifications and thresholds. Future studies should be directed towards evaluation of the stability of encapsulated bioactive components within the complex food matrix[162].

There are two other important aspects that the food industry must consider. There exists a large gap in the **regulatory framework** and many consumers are misinformed with respect to the types of encapsulation systems applied to foods. The application of a food-grade coating material would go some way to improving the confidence of consumers. However, **the cost of up-scaling** encapsulation systems is still very expensive.

From the view point of incorporation of bioactive compounds into a food matrix, the food industry will focus on advancing the development of new delivery systems for nutraceuticals and bioactive compounds.

[162] James D. Oxley, "Stability and Prediction of Shelf-Life for Microencapsulated Ingredients," Agro Food Industry Hi-Tech 23, no. 5 (2012): 60–63.

Experimental Part

Section A

Selection of natural sources of phenolic compounds: *Hibiscus sabdariffa*

The first step in developing nutraceuticals and functional foods is to identify the most appropriate sources of compounds. Bioactive compounds from plants have proven to be potentially useful candidates. A scientific search of literature on plants with high bioactive compounds content and their beneficial effects in the organism should first be performed. In the present Thesis, *Hibiscus sabdariffa* was selected due to its phenolic composition and biological effects. The main properties of this plant are summarised in the following sections.

Origin, distribution and ecology

Hibiscus sabdariffa (H. sabdariffa) is also known as "Roselle" or "Karkade" in Sudan. This plant is an important crop which grows annually in tropic and sub-tropic environments and belongs to the *Malvales* general order, the *Malvaceae* family and the *Hibiscasae* tribe. The name, *Sabdariffa*, is of Turkish origin [1]. The earliest reference to the Roselle was published by the botanist M. de L´Obel in 1576. It is believed that the crop originates from West Africa and was brought westward from India by the Mohammedans, who invaded India several centuries earlier.

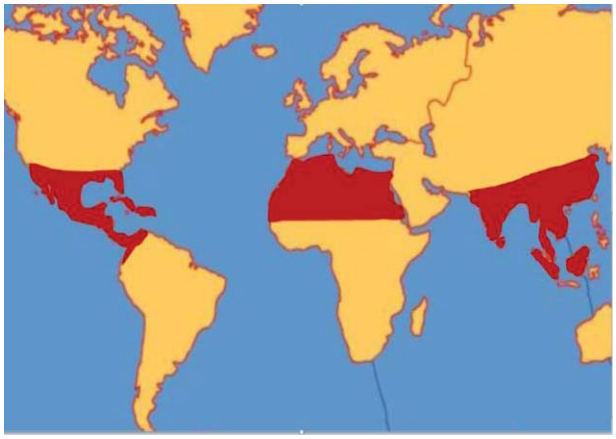


Figure 1. Regions of *H. sabdariffa* cultivation (colored red)

In 1707, this plant was discovered in America **(Figure 1)**. The first reports of its existence come from Jamaica, where Sloane described for the first time the culinary

application of the calyces. For this reason, this plant is known as “Jamaican sorrel” in Central America[2]. It is important to note that *H. Sabdariffa* is known locally in different countries by different names. Some examples are Roselle, karkade, sorrel, red sorrel, Jamaican sorrel, Indian sorrel, Guinea sorrel, sour-sour, mesta, amongst others [3] **(Table 1)**.

In the 19th century, *H. sabdariffa* was cultivated in Mexico, parts of Central America, the West Indies, and in southern Florida, Texas and California.

REGIONS	LOCAL NAMES	SOURCE
English-speaking countries	Roselle	[3]
Caribbean	sorrel	[4]
Panama	Saril	[4,5]
In North Africa and the Near East	Karkade	[6]
Spanish and Latin America	Rosa de Jamaica	[5,6]
Senegal	Bissap	[5]
Indian subcontinent	Mesta	[4,5]
Gambia	Wonjo	[4]
Namibia	Omutete	[4,5]
Nigeria	Zobo or Zoborodo	[4,5]
France	L´oiselle	[6]
Netherland	Zuring	[5]
Thailand	Krajeab	[5]
Indonesia	Rosela	[5]
Mali	Dah or Dah bleni	[4,5]
Malaysia	Asam paya, asam susur, asam	[4,5]
Florida	Karkade	[3]

Table 1. Names of *H. sabdariffa* in different regions

The plant is an erect, bushy, herbaceous sub shrub with smooth or nearly smooth, cylindrical, typically red stems [5]. It is about 3.5 m tall with a green or red coloured stalk, and a red or pale yellow calyx **(Figure 2)**. The fruit takes the form of a velvety capsule, 2-5 cm in length, which is green when immature. It has 5 valves, with each valve containing 3-4 seeds which usually contain high percentages of oil [7]. The leaves are used extensively for animal fodder and fibre, whereas the swollen calyces of the plant part are of commercial interest.

Figure 2. *Hibiscus sabdariffa*

The crop takes about 3- 4 months to reach the commercial stage of maturity before the flowers can be harvested. A tropical warm and humid climate with well-distributed rainfall of 1500 -2000 mm/year and temperatures between 18 and 35 ºC is ideal for its production [5]. Growth of the plant ceases at 14ºC and in the first months 13 hours of sunlight are needed for healthy growth. In addition, the plant thrives in well-drained humus and rich-fertile soils with a pH of 4.5 to 8.0 [4]. Propagation is achieved by seeds or by rooting shoot cuttings. The edible fleshy calyces are collected after 15-20 days of flowering. The rest of the crop is left in the field until seeds are ready for threshing. In addition, the calyces can be dried and stored in air-tight containers[3] with this process acting as an important post-harvest treatment to reduce moisture content and increase shelf life.

Nowadays, the world largest producers are Thailand and China but the greatest quality plants, as regarded by the Food and Agriculture Organisation, come from Sudan. Indeed, this plant represents Sudan's major export crop especially to western countries where it is the second most abundant crop with regards to area, after pearl millet and followed by Sesamum. Unfortunately, produce from Sudan is unpopular due

to poor packaging and distribution[3,7]. Thailand has invested heavily in *H. sabdariffa* production and now produces products of superior quality. On the other hand, China's product is less reliable and reputable due to its less stringent quality control practices. Mexico, Egypt, Senegal, Tanzania, Mali and Jamaica are also important suppliers but production is mostly for domestic use (FAO). Germany and the United States are the main countries which import this plant.

Biological effects

H. sabdariffa is cultivated for its leaves, seeds and calyces, though the **calyces** are the part most used in traditional medicines due to their healthy properties. For centuries, many cultures have employed this plant in the preparation of herbal drinks due to its antioxidant function in humans. It is now traded and used worldwide as an important ingredient in industrially produced teas and beverages.

Figure 3. *H. sabdariffa* drink

In Egypt, the fleshy calyces are used in making “cacody tea”. In Sudan and Nigeria these calyces are boiled with sugar to produce a beverage called “Karkade” or “Zorborodo” **(Figure 3)**. In Mexico this drink is known as Jamaica or “agua de Jamaica”. In India the calyces have been used as a colouring and flavouring ingredient in rum[8].

H. sabdariffa has also demonstrated antimicrobial, anti-diabetic, anti-inflammatory, hepatoprotective, anti-hypertensive, anti-obesity, cancer-preventive, antioxidant, diuretic and anti-cholesterol effects[9–12]. **Table 2** summarises the

findings of selected *in vivo* trials with regards to the main biological effects of this plant.

Biological effect	Function	Reference
Anti-hypertensive	Decreased blood pressure in 42 hypertension patients over 3 weeks of treatment	[13]
	Decreased blood pressure in 60 patients with type II diabetes with greater effects than black tea	[14]
	Decreased blood pressure and improved endothelial function in patients with metabolic syndrome	[15]
Hypoglycemic	Reduced hyperglycaemia and hyperinsulinemia in type II diabetic rats	[16]
Hypolipidemic	Reduced glucose and cholesterol levels and increased HDL level in patients with metabolic syndrome	[11]
	Decreased serum cholesterol in men and women over 1 month of treatment	[17]
Anti-inflammatory	Decreased plasma monocyte chemoattractant protein-1 in 10 healthy adults	[18]
	Inhibited ear oedema formation in xylene-induced ear oedema model mice in a dose-related manner when compared with a placebo control	[19]
Diuretic	Increased the uricosuric effect in 9 participants with no history of renal stones and 9 with a history of renal stones. This plant was provided twice daily for 15 days	[20]
Chemopreventive	Induced apoptosis of human gastric carcinoma cells in a concentration-dependent manner but was ineffective in Chang liver cells	[21]

Table 2. Biological effects of *H. sabdariffa* calyces

The functional characteristics of *H. sabdariffa* are a product of its composition which consists of different antioxidant compounds including vitamin C and phenolic compounds[22]. Its antioxidant activity results from its strong scavenging effect on reactive oxygen and free radicals. Frank et al. (2012) conducted a randomised study evaluating the impact of *H. sabdariffa* extract on systemic antioxidant status[23]. They measured antioxidant activity through the ferric reducing ability of plasma (FRAP), and showed that this extract helps to improve the systemic antioxidant potential and

decreases oxidative stress in humans. In addition, following consumption of this plant, urinary hippuric acid excretion was reported to increase due to a high microbial biotransformation of the ingested extract.

Hence, the beneficial properties of *H. sabdariffa* make it appealing to food and beverage manufacturers and pharmaceutical stakeholders who recognise the potential to exploit it as a natural food product in herbal medicines.

Nutritional value and phenolic composition

The composition of this crop make its production important. This plant has a high content of dietary fibre, vitamin C and phenolic compounds, with the latter being responsible for the previously described benefits. The specific composition of the plant, however, can differ due to differences in varieties of the crop, environment, ecology and harvesting conditions [24,25]. According to Mohamed et al. 2012, the chemical composition of red calyces is as follows: 11 % moisture, 7.88 % crude protein, 13.20 % crude fibre, 0.16 % crude fat, 57.16 % total carbohydrates, 11.00 mg/100 g ascorbic acid, 9.00 mg/100g titrable acidity, 5.00 % total soluble solids, 60 mg/100 g calcium and 25.00 mg/100g iron [2] **(Table 3).**

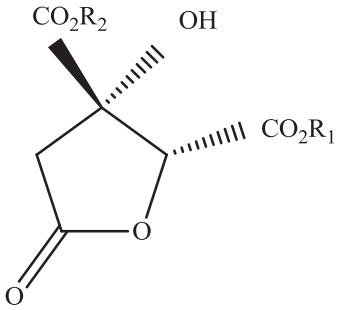
Nutritional composition of red calyces	
Moisture	11 %
Crude protein	7.88 %
Crude fibre	13.20 %
Crude fat	0.16 %
Total carbohydrates	57.16 %
Ascorbic acid	11 mg / 100 g
Titrable acidity	9 mg / 100 g
Total soluble solids	5 %
Calcium	60 mg / 100 g
Iron	25 mg / 100 g

Table 3. Nutritional composition of red calyces

The **fibre** content of calyces has been previously reported. Total dietary fibre of 33.9 ± 3.56 g/100 g dry matter has been reported, of which 4.9 ± 0.17 g/100 g dry matter corresponds to soluble dietary fibre and 29.04 ± 3.56 g/100 g dry matter refers to insoluble dietary fibre[26]. Hence, dietary fibre is a quantitatively important constituent of *H. sabdariffa.*

Regarding **vitamin C**, these calyces contain three times more vitamin C than blackcurrant (*Ribes nigrum L.*) and nine times more than citrus (*Citrus sinensis L.*) fruit[5]. Moreover, *H. sabdariffa* contains high amounts of other **organic acids** such as hibiscus acid, citric acid, malic acid and tartaric acid[27]. Hibiscus acid **(Figure 4)** is a component specific to this plant and is structurally one of the natural optical isomers of hydroxycitric acid belonging to the butyrolactone category. In addition, this organic

acid has demonstrated bioactivity as an inhibitor of starch digestion in the Caco-2 cell model system[28].



Hibiscus acid: R1=H and R2=H
Hibiscus acid hydroxyethylester: R1=OH and R2=CH_2-CH_3
Hibiscus acid dimethylester: R1=CH_3 and R2= CH_3

Figure 4. Hibiscus acid structure and its derivatives

This plant is also rich in **phenolic acids** such as, chlorogenic acid and its derivatives, caffeoylquinic acid, protocatechuic acid, gallic acid and its derivatives and *p*-coumaric acid **(Figure 5)**.

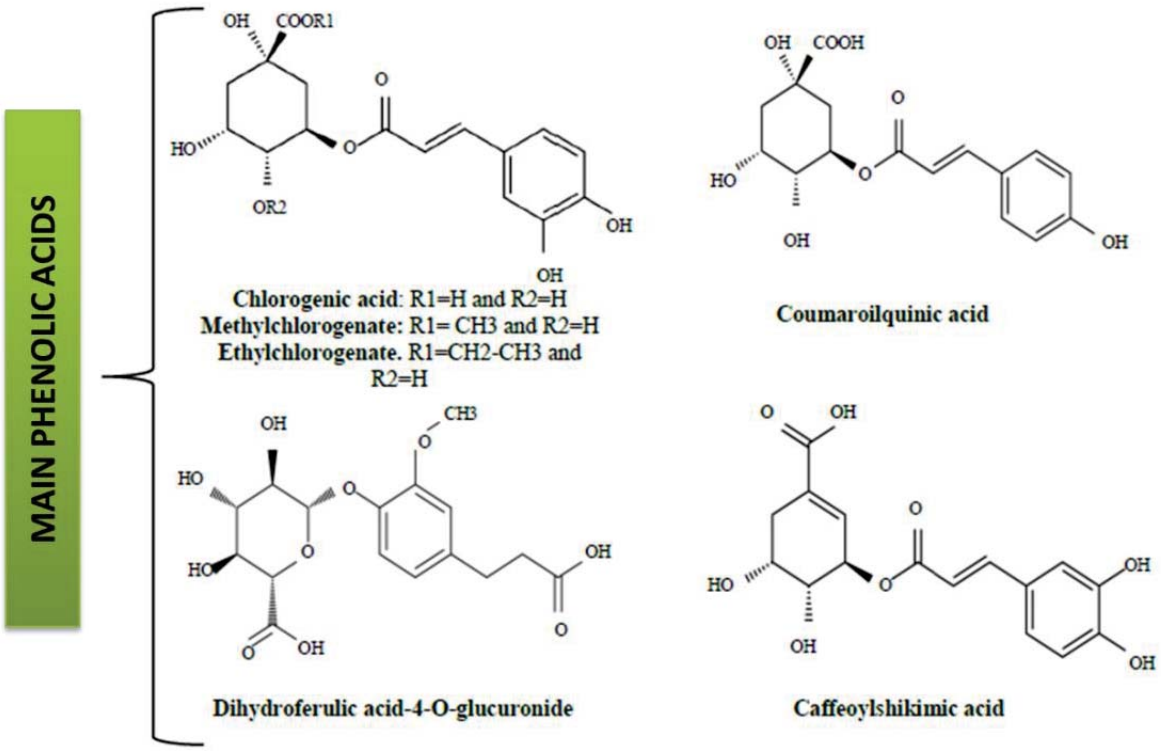


Figure 5. Main phenolic acids found in *H. sabdariffa* calyces

With regards to **flavonoids**, quercetin, kaempferol, myricetin, luteolín or gossipetin and their respective glycosides have been found in this plant **(Figure 6)**. Anthocyanidins such as cyanidin and delphinidin have been detected in large quantities in the calyces, giving them their bright red color. These anthocyanidins are normally found in their glycoside form, known as anthocyanins. Cyaniding-3-flucoside, delphinidin-3 –glucoside, cyaniding-3-samubioside and delphinidin-3-sambubioside are the most frequently occurring anthocyanins in *H. sabdariffa*[29].

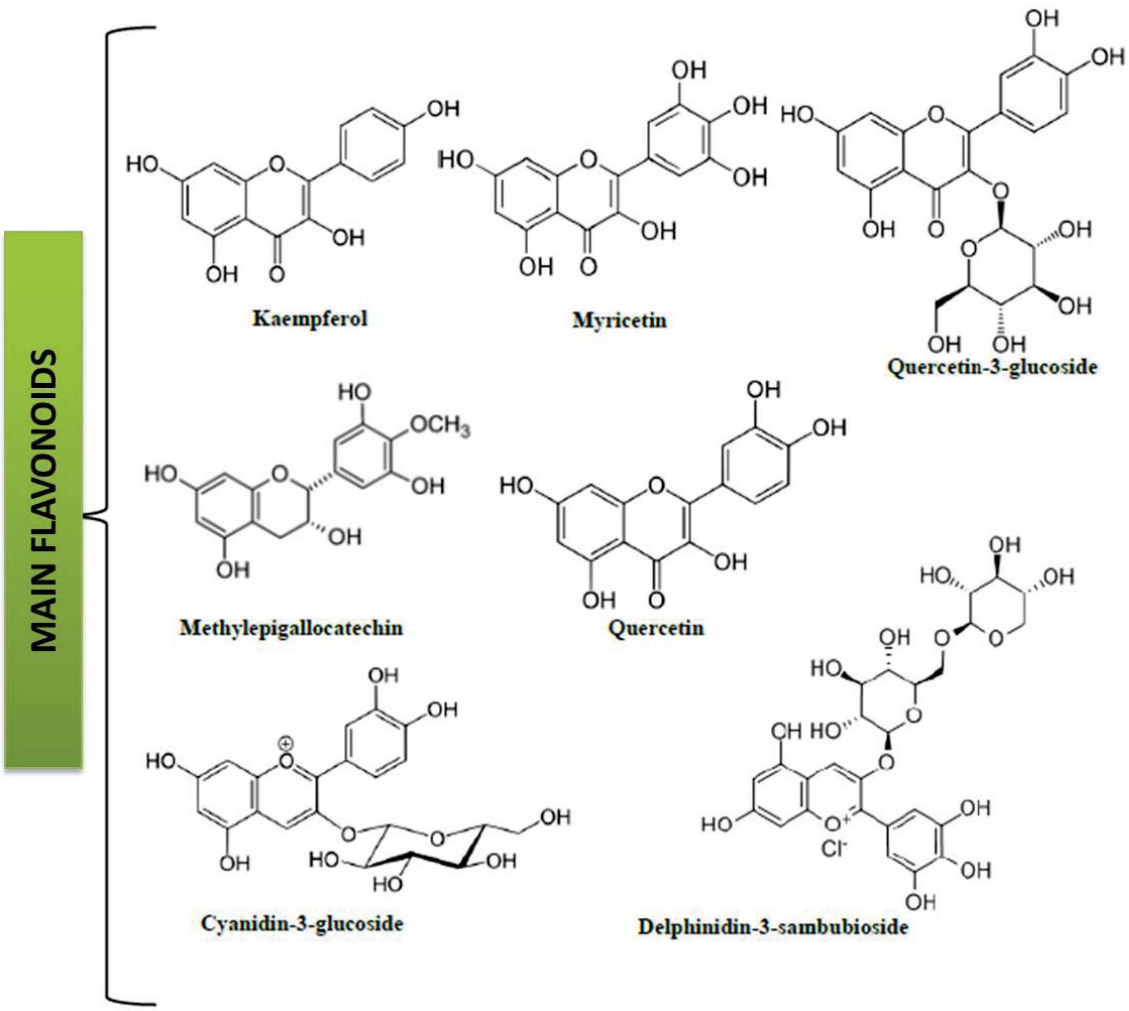


Figure 6. Main flavonoids found in *H. sabdariffa* calyces

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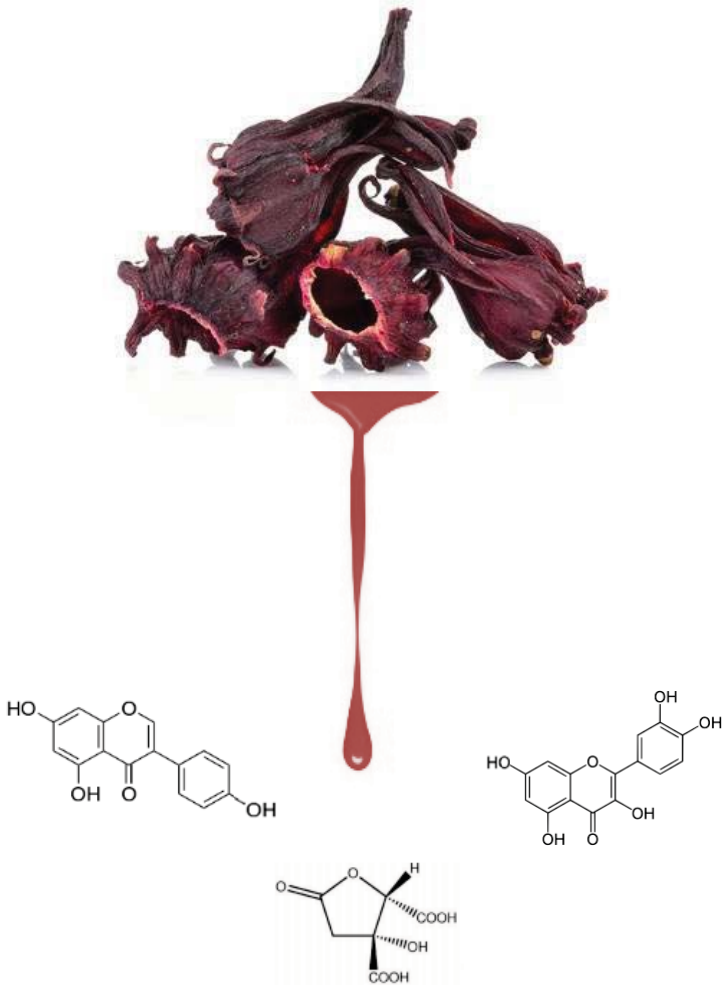
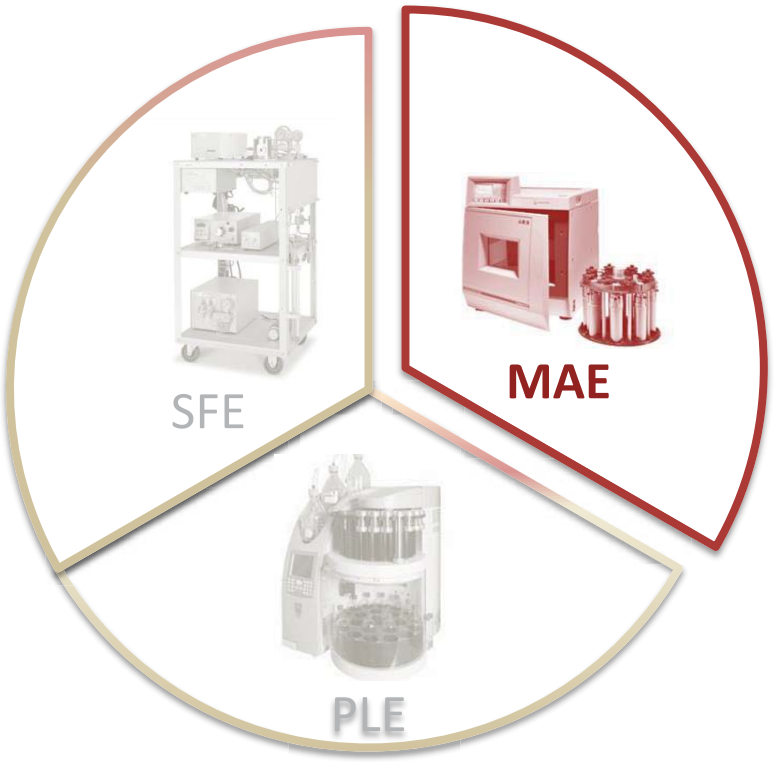
Section B

Extraction and characterization of phenolic compounds



Chapter 1

Microwave-assisted extraction for *Hibiscus sabdariffa* bioactive compounds

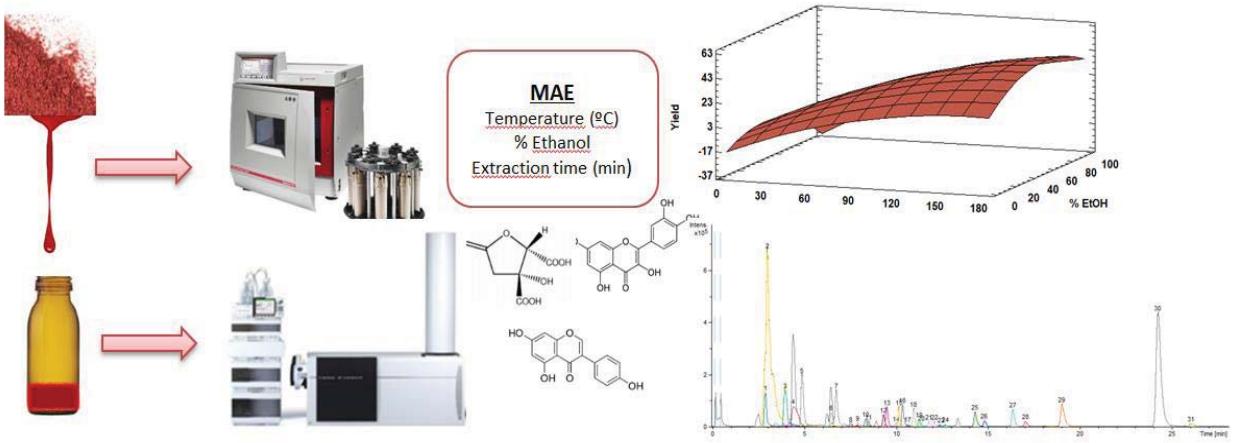


Journal of Pharmaceutical and Biomedical Analysis

Pimentel-Moral, Sandra, Borrás-Linares, Isabel, Lozano-Sánchez, Jesús, Arráez-Román, David, Martínez-Férez, Antonio, Segura-Carretero, Antonio

Microwave-assisted extraction for *Hibiscus sabdariffa* bioactive compounds

Abstract



H. sabdariffa has demonstrated positive results against chronic diseases due to the presence of phytochemicals, mainly phenolic compounds. The extraction process of bioactive compounds increases the efficient collection of extracts with high bioactivity. Microwave-Assisted Extraction (MAE) constituted a "green technology" widely employed for plant matrix. In this work, the impact of temperature (50 - 150 ºC), composition of extraction solvent (15 - 75 % EtOH) and extraction time (5 - 20 min) on the extraction yield and individual compounds concentrations were evaluated. Furthermore, the characterization of 16 extracts obtained was performed by HPLC-ESI-TOF-MS. The results showed that 164ºC, 12.5 min, 45 % ethanol, was the best extraction condition, although glycoside flavonoids were degraded. Besides that, the optimal conditions for extraction yield were 164 ºC, 60 % ethanol and 22 min.

Thus, temperature and solvent concentration have demonstrated to be potential factors in MAE for obtaining bioactive compounds from *H. sabdariffa*.

Keywords: Microwave-assisted extraction, *Hibiscus sabdariffa*, phenolic compounds, extraction yield, HPLC-ESI-TOF-MS.

Introduction

Nowadays, it is widely known that environmental stress factors could produce oxidative reactions in cells and tissues, resulting in numerous chronic diseases like diabetes, cancer or cardiovascular illnesses, among others. Therefore, antioxidants are becoming more investigated due to their ability to scavenge reactive oxygen species (ROS). These compounds are capable to accept hydrogen atoms or electrons by reactive radical to yield much lees reactive radical and non-radical species [1].

Antioxidants are widely found in plant foods. There are a wide of variety of antioxidants from plants, being the most well-known phenolic compounds, carotenoids or vitamins. Concretely, *Hibiscus sabdariffa (H. sabdariffa)* is a tropical plant commonly used for centuries in the preparation of herbal drinks, hot and cold beverages, because of its antioxidant function in humans, demonstrating positive results against chronic diseases [2,3,4] . These positive effects were associated with the presence of phenolic compounds like anthocyanins, quercetin, kaempferol, hibiscus acid or chologenic acid [5, 6].

Although the bioactivity of this plant has been demonstrated in several studies, it is possible to increase the content of the bioactive compounds present in *H. sabdariffa* by an efficient and efficacious extraction, obtaining extracts with higher bioactivity. Thus, the extraction process is an important step in order to study in depth the bioactive composition of a plant. Currently, novel green extraction techniques with efficient and rapid process have appeared in order to correct the limitations of

conventional extraction methods, such as the large quantities of solvents used, the high temperatures applied or the long extraction times needed. Those parameters, especially the high temperature and long extractions times, increase the risk of thermal degradation of thermo-labile bioactive compounds. Concretely, Microwave-Assisted Extraction (MAE) has been widely employed due to its special heating system, moderate cost and its good performance under atmospheric conditions [7]. In this extraction technique, the microwave energy is delivered through polar components interactions to generate heat by conversions of electromagnetic into thermal energies. The most important parameters involved in microwave procedure are the dielectric constant and dissipation factor, which determine the amount of the power energy that is reflected at the air–sample interface and the amount that interacts with the sample [8]. Therefore, it is important to choose a solvent with high extracting power and strong interaction with the matrix and the target bioactive compounds. Solvents like ethanol, methanol or water have the capacity to absorb microwave energy due to their high dielectric constant and dielectric loss [9]. In addition, different solvent mixtures could be combined to modify the dielectric constant.

Other important factors in MAE procedure are the temperature and extraction time. Generally, long time of exposition to microwave radiation could decreases the extraction yield because of the degradation of chemical active structures of polyphenols. Time extraction manipulation could control the exposure and improve the extract yield. Nevertheless, if longer extraction time was required, the samples could be extracted in multiple steps using consecutive extraction cycles [10]. On the other hand, at high temperatures the solvent power increases due to a decrease of

viscosity and surface tension, allowing a better solvent penetration. However, higher temperatures also decrease the extraction yield breaking down the molecular structure of bioactive compounds [9]. For these reasons, it is necessary to reach equilibrium between the applied extraction time and temperature.

In this work, response-surface methodology (RSM) was used to investigate the influence of the extraction parameters and their relationship with chemical composition of *H. sabdariffa* extracts. In addition, the impact of these factors (temperature, extraction time and dielectric constant of the solvent mixture obtained by variations in the proportion of ethanol) was evaluated on individual compound concentrations in MAE extractions by high-performance liquid chromatography couple to time-of flight mass spectrometry (HPLC-ESI-TOF-MS). Therefore, the aim of this study was to evaluate this influence achieving a quality optimal extraction conditions for each polyphenol family while improving the extract yield.

Material and Methods

Chemicals and reagents

For extractions, ultrapure water was obtained with a Milli-Q system (Millipore, Bedford, MA, USA) and absolute ethanol was purchased from Fisher Scientic (Leicestershire, UK). Regarding HPLC analysis, LC-MS grade acetronitrile was purchased from Fisher (Fisher Scientific UK, Leicesterchire, UK) and ultrapure water was obtained with a Milli-Q system describe above. For mobile phase preparation, formic acid was purchased from Sigma–Aldrich (Steinheim, Germany). Standards used for the quantification were: galic acid, chlorogenic acid, rutin, p-coumaric acid, quercetin, myricetin, quercetin-glycoside and apigenin as internal standards purchased by Sigma

– Aldrich, Steinhemin, Germany and quercitrin from Extrasynthese, Genay Cedex, France.

Sample

The calyces of *H. sabdariffa* were purchased from Monteloeder S.L (Elche, Spain). These calyces were ground using an ultra-centrifugal mill ZM 200 (Retsch GmbH, Haan, Germany). The resulting powder, with an average particle size of 2 mm, was stored avoiding light exposure and kept at room temperature until the extraction.

Extraction of phytochemicals from *H. sabdariffa* by Microwave-Assisted Extraction (MAE)

MAE was carried out in a modular microwave extraction system Multiwave 3000 (Anton Paar GmbH, Graz, Austria) equipped with equipped with two standard magnetrons of 850 W deliver up to 1500W microwave power and an autosampler (model MAS 24). Extracts were prepared by adding 3g of *H. sabdariffa* dried powder into a closed extraction vessel with 30 mL of selected solvent mixture. In this research, extraction time, temperature and water-ethanol mixtures were varied according to the experimental design, as shown in **Table 1**. After cooling, samples were centrifuged at 13000 rpm for 15 min in a centrifuge (Sorvall ST 16 R, Thermo Scientific, Leicestershire, UK) and the supernatant was collected and evaporated at 35 °C to dryness in a Savan SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK). The extract was stored at -20 °C until further use.

Run	T (ºC)	t (min)	Solvent (%)	Dielectric constant	Dissipation factor
1	50	20	75	36.43	2267.50
2	50	5	75	36.43	2267.50
3	50	20	15	63.88	1709.50
4	150	20	75	25.36	2267.50
5	100	12.5	45	42.93	1988.50
6	100	12.5	45	42.93	1988.50
7	100	12.5	6	55.23	1629.37
8	150	5	75	25.36	2267.50
9	100	22	45	42.93	1988.50
10	150	20	15	40.63	1709.50
11	164	12.5	45	30.92	1988.50
12	150	5	15	40.63	1709.50
13	50	5	15	63.88	1709.50
14	100	12.5	84	30.34	2347.63
15	100	3	45	42.93	1988.50
16	36	12.5	45	52.23	1988.50

Table 1. MAE factorial design 2^3 experimental values of tested independent variables

Characterization of MAE *H. sabdariffa* extracts by high-performance liquid chromatography coupled to electrospray ionization time-of-flight mass spectrometry (HPLC-ESI-TOF-MS)

Dried extracts of *H. sabdariffa* calyces obtained by MAE were reconstituted with the same solvent used for the extraction up to a concentration of 10 mg mL^{-1}. Prior to analysis, the solutions were filtered with single-use syringe filters (0.20 µm pore size) and injected into the HPLC system.

Analyses were performed using an Agilent 1200 RRLC system (Agilent Technologies, Palo Alto, CA, USA) of the Series Rapid Resolution equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatized column compartment and a UV–Vis detector. Compounds were separated at 25 °C using a Zorbax Eclipse Plus C_{18} analytical column (1.8 µm, 4.6×150 mm), at a flow rate of 0.5 mL/min. The mobile phases were water plus 0.1 % of formic acid as mobile phase A and acetonitrile as mobile phase B. The separation was carried out using the following multi-step gradient: 0 min, 5 % B; 8 min 22 % B; 23 min 28 % B; 27 min 95 % B, 31 min 5 % B; and finally a conditioning cycle of 5 min with the initial conditions for the next analysis. The injection volume was 10 µL.

HPLC system was coupled to a microTOF™ mass analyzer (Bruker Daltonik, Bremen, Germany) equipped with an ESI interface (model G1607A, Agilent Technologies, Palo Alto, CA, USA) operating in negative ionization mode, showing the molecular ions $[M–H]^-$. In order to ensure stable ionization conditions, the effluent from HPLC was split before being introduced into the mass analyzer. Detection was carried out considering a mass range of 50-1000 m/z. The ionization parameters were: capillary voltage, 4000 V; drying gas temperature, 210°C; drying gas flow, 9.5 L/min; nebulizing gas pressure, 2.5 Bar; and end plate offset, −500 V. The values of transfer parameters were: capillary exit, −120 V; skimmer 1, −40 V; hexapole 1, −23 V, RF hexapole, 80 V; skimmer 2, −22.5 V; the trigger time was set to 53 µs (50 µs for transfer time and 3 µs for pre-pulse storage time).

External mass spectrometer calibration was carried out with a sodium formate cluster solution (5 mM sodium hydroxide and 0.1 % formic acid in water / 2-propanol

(1:1, v/v)) in quadratic plus high-precision calibration (HPC) regression mode. The calibration solution was injected at the beginning of the run using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA) and all the spectra were calibrated before identification.

The data were processed using the software Data Analysis 4.0 (Bruker Daltonik). The identified compounds were characterized by the generation of the candidate formula with a mass accuracy limit of 5 ppm using the SmartFormula^{TM} editor and also considering their retention time (RT), mass spectrometra and the information available in literature.

Effect of MAE parameters on individual compounds concentration and extraction yield

The effect of temperature (50-150 ºC), percentage of ethanol (15-75 %) and extraction time (5 – 20 min) in the extraction by microwave was evaluated by means of a Central Composite Design 2^3 (CCD) approach. These factors were chosen for independent variables procuring a total of 16 experiments which were conducted in a randomized order **(Table 1)**. The extractions were made in triplicate. The response variable was the extraction yield. The parameters of the model (analysis of variance, ANOVA), determination of the optimum condition and plot of response surface were estimated by using Statgraphics Centurion software XVI provided by Statpoint Technologies (Warrenton, VA, USA).

The relationship between independent variables and extraction yield were analyzed by response surface plot which represents the dependent variable in function of two most influence independent variables. Optimum condition was calculated considering

the maximization of individual response variable. Therefore, independent variables were kept in ranges while response was optimized. To verify the suitability of the quadratic equation for predicting the optimum extraction yield value, the verification experiment was carried out under optimum conditions.

Results and discussion

Chemical characterization of the extracts yielded by different MAE conditions

It is commonly known that extracts yielded by different extraction conditions can be completely different in terms of chemical composition and therefore, a complete identification and quantification is necessary to evaluate the potential of each extraction parameter studied (temperature, extraction time and solvent composition). **Figure 1** shows a representative base-peak chromatogram extracted by MAE (MAE 1 condition) resulting from the HPLC-ESI-TOF-MS analysis as described above. The main compounds have been numbered according to their elution order. A maximum of 30 and a minimum of 24 compounds were identified and quantified in the studied *H. sabdariffa* MAE extracts.

The detected compounds were characterized by comparison of retention time and the MS spectra provided by the TOF mass analyzer with those of authentic standards when available. The remaining identifications were performed by interpretation of the MS spectra of the detected compounds combined with the data from the literature and data bases. The MS data of the compounds was numbered according to their elution order, together with their retention times, theoretical m/z, molecular formulas and the concentration found for each extraction condition, expressed in mg g^{-1} extract is shown for each extraction condition.

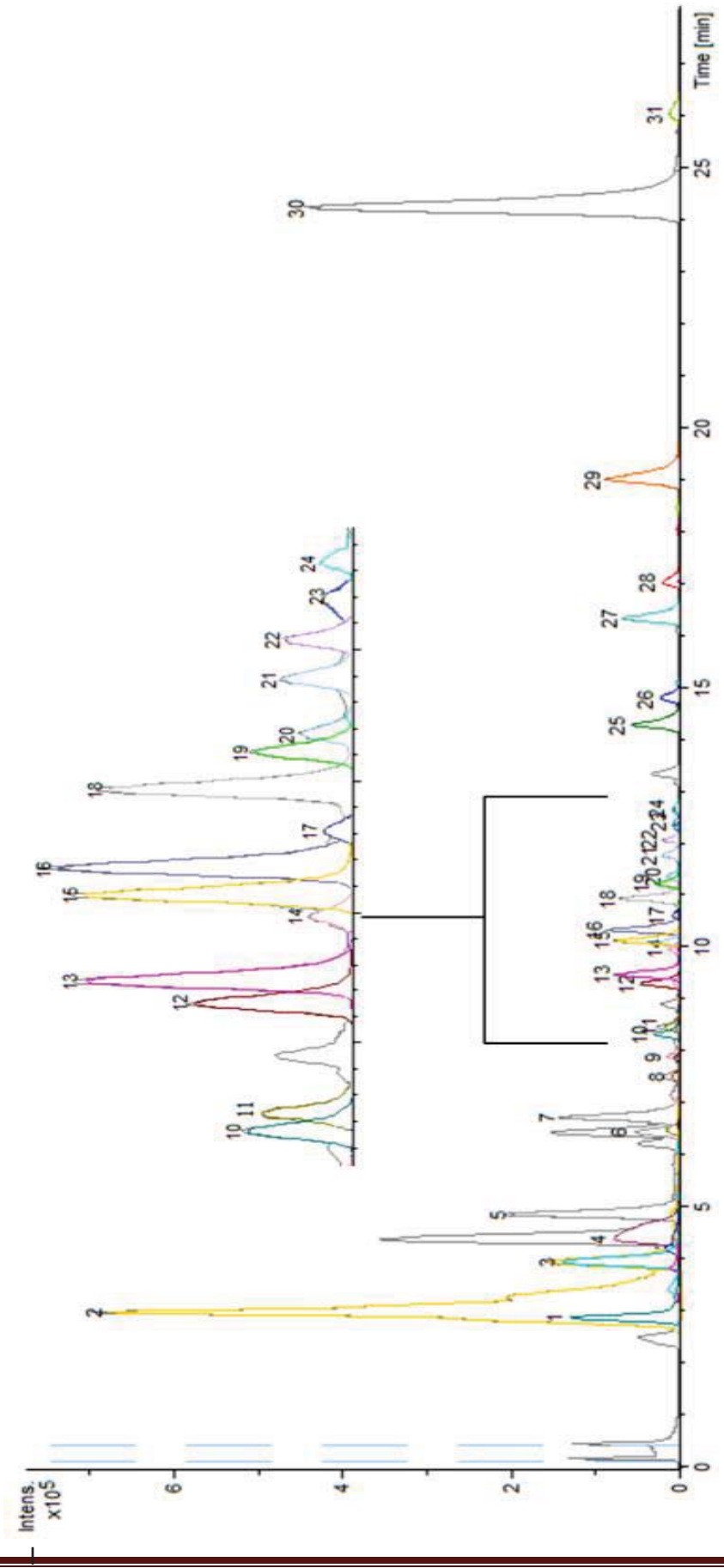


Figure 1 Base peak chromatogram (BPC) obtained from HPLC-DAD-ESI-TOF/MS analysis of the *H. sabdariffa* extract obtained under different MAE extraction conditions. Peaks have been numbered according to the elution order

In order to quantify the individual amount of each compound in the extracts, nine calibration curves with seven points were prepared using galic acid, chlorogenic acid, rutin, quercetin-glucoside, p-cumaric acid, quercetin, myricetin and quercetrin. Apigenin was used as an internal standard and the calibration points were analyzed in triplicate. The compound concentrations were determined using the corrected area of each individual compound (area standard / area internal standard) and by interpolation in the corresponding calibration curve.

Cholrogenic acid, quercetin-glucoside, quercetin and myricetin were quantified with the calibration curves of their own commercial standards. The remaining compounds were tentatively quantified on the basis of calibration curves from other compounds with structural similarities. It should be taken into account that the response of the standards can differ from that of the analytes found in the extracts, and consequently the quantification of these compounds is only an estimation of their actual concentrations although it can be considered a useful approximation. In this sense, the calibration curve of galic acid was used to quantify methyldigallate, methylepigallocatechin and N-feruloyltiramine; p-cumaric acid for hydroxicitric acid, 5-O-caffeoylshikimic acid, coumaroilquinic acid and hibiscus acids and derivatives; chlorogenic acid for derivatives of chlorogenic acid and dihydroferulic acid-4-O-glucuronide; rutin for myricetin–3–arabinogalactoside, quercetin-3-rutinoside, quercetin-3-sambubioside, prodelphinidin B3, kaempferol-3-O-sambubioside, kaempferol-3-O-rutinoside and apigenin-6,8-di-C-arabinoside; quercetrin for quercetin-3-rhamnoside and kaempferol and finally quercetin-3-glucoside for kaempferol-3-O-glucoside and kaempferol 3-(p-coumarylglucoside).

The validation of the proposed method was performed with linearity, sensitivity, and precision parameters. **Table 2** shows the limits of detection (LODs) and quantification (LOQs), calibration range, calibration equations, and regression coefficient (r^2) for all standards used. All calibration curves showed good linearity between different concentrations depending on the analytes studied. The LODs and LOQs for individual compounds in standard solutions were also calculated as S/N = 3 and S/N = 10, respectively, where S/N is the signal-to-noise ratio. The repeatability of the method was measured as the relative standard deviation (RSD %) in terms of concentration.

Analyte	LOD (µg/ml)	LOQ (µg/ml)	Calibration range (µg/ml)	Calibration equations	r^2
Galic acid	0.155	0.52	LOQ - 100	y = 0.0053 x + 0.0119	0.9939
Chlorogenic acid	0.201	0.67	LOQ - 100	y = 0.0111 x + 0.0126	0.9922
Rutin	0.037	0.12	LOQ - 100	y = 0.0152 x + 0.0311	0.9971
Quercetin-Glucoside	0.056	0.19	LOQ – 100	y = 0.0127 x + 0.0514	0.9908
p-coumaric acid	0.196	0.65	LOQ – 100	y = 0.0039 x + 0.0009	0.9964
Quercetin	0.066	0.22	LOQ – 100	y = 0.0181 x + 0.0761	0.9938
Myricetin	0.102	0.34	LOQ – 100	y = 0.0167 x + 0.0592	0.9892
Quercitrin	0.094	0.31	LOQ - 100	y = 0.0224 x + 0.0378	0.9956

Table 2 Validation of the proposed method for the quantification of phenolic compounds, performed with linearity, sensitivity, and precision parameters

The main compounds found in *H. sabdariffa* MAE extracts have been identified before [6], such as organic and phenolic acids derivatives, flavonoids and anthocyanins. Therefore, for a clearer discussion the characterization and quantification were grouped into compound families such as organic acids, phenolic acids, flavonoids, anthocyanins and others polar compounds **(Figure 2)**. Thus, organic acids and derivatives like hydroxicitric acid, hibiscus acid, hibiscus acid hydroxyethylester and hibiscus acid dimethylester were the major compounds found

in all extraction conditions (from 41.72 in MAE 10 to 156.12 mg g^{-1} extract in MAE 11). The concentration range of each individual compound was the following: hydroxicitric acid (Peak 1) from 1.31 ± 0.03 mg g^{-1} in MAE 4 to 4.28 ± 0.05 mg g^{-1} in MAE 7; hibiscus acid (Peak 2) from 29.6 ± 0.7 mg g^{-1} in MAE 4 to 69 ± 1 mg g^{-1} in MAE 11; hibiscus acid hydroxyethylester (Peak 3) from 0.63 ± 0.05 mg g^{-1} in MAE 3 to 13.1 ± 0.3 mg g^{-1} in MAE 11; hibiscus acid dimethylester (Peak 4) from 0.46 ± 0.06 mg g^{-1} in MAE 3 to 70 ± 3 mg g^{-1} in MAE 11.

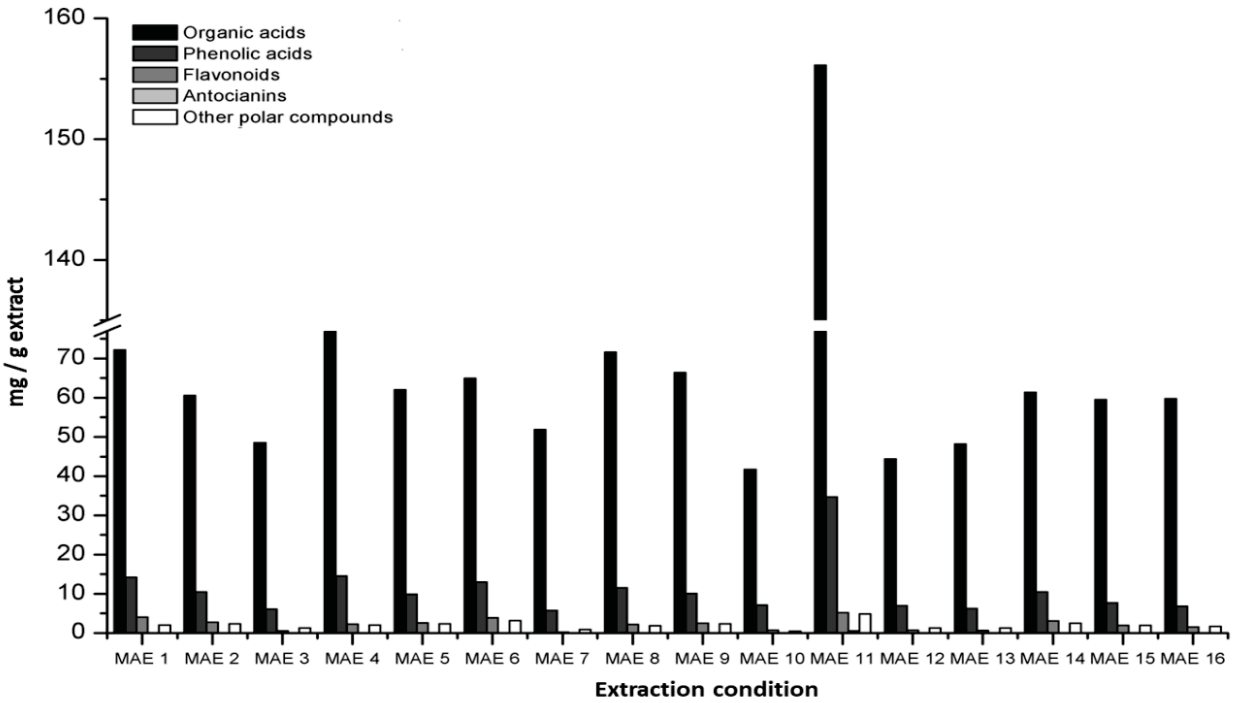


Figure 2. Concentration of the each families of compounds characterized in *H. sabdariffa* extract under different MAE conditions

With regard to the phenolic acids and derivatives, a total of 11 compounds were characterized, with a concentration range from 5.732 mg g^{-1} in MAE 7 to 34.646 mg g^{-1} in MAE 11. Respect to the isomers of ethylchlorogenate they were not

detected in MAE 3, MAE 7 and MAE 13 and were below the limit of quantification in MAE 2, MAE 12, MAE 15 and MAE 16. In addition, in all extraction conditions except MAE 1, MAE 2, MAE 6 and MAE 11 the isomers of methylchologenate were below the limit of quantification. Moreover, the content range for each phenolic acid was from 1.36 ± 0.03 mg g^{-1} in MAE 10 to 4.26 ± 0.03 mg g^{-1} in MAE 11 for neochlorogenic acid (Peak 5); from 0.271 ± 0.009 mg g^{-1} in MAE 7 to 1.92 ± 0.05 mg g^{-1} in MAE 11 for chlorogenic acid (Peak 6); from 1.17 ± 0.01 mg g^{-1} in MAE 7 to 5.0 ± 0.1 mg g^{-1} in MAE 11 for cryptocholorogenic acid (Peak 7); from 0.32 ± 0.01 mg g^{-1} in MAE 12 to 0.73 ± 0.02 mg g^{-1} in MAE 11 for methyldigallate (Peak 8); from 0.0133 ± 0.0004 mg g^{-1} in MAE 1 to 0.091 ± 0.002 mg g^{-1} in MAE 6 for methylchlorogenate (Peak 9); from 0.40 ± 0.02 mg g^{-1} in MAE 12 to 1.37 ± 0.04 mg g^{-1} in MAE 11 for coumaroilquinic acid (Peak 11); from 0.176 ± 0.005 mg g^{-1} in MAE 10 to 0.69 ± 0.01 mg g^{-1} in MAE 11 for dihydroferulic acid-4-O-glucuronide (Peak 12); from 0.04 ± 0.09 mg g^{-1} in MAE 11 to 0.089 ± 0.006 mg g^{-1} in MAE 1 for methylchlorogenate isomer II (Peak 14); from 1.45 ± 0.06 mg g^{-1} in MAE 3 to 18.1 ± 0.3 mg g^{-1} in MAE 11 for 5-O-caffeoylshikimic acid (Peak 16); from 0.066 ± 0.003 mg g^{-1} in MAE 5 to 1.318 mg g^{-1} in MAE 11 for ethylchlorogenate (Peak 20) and finally from 0.071 ± 0.001 mg g^{-1} in MAE 15 to 1.33 ± 0.05 mg g^{-1} in MAE 11 for ethylchlorogenate isomer II (Peak 21). Therefore, 5-O-caffeoylshikimic acid was the most abundant phenolic acid compound in the majority of the extraction conditions assayed.

On the other hand, in *H. sabdariffa* calyces, quercetin and kaempferol derivatives are the most important flavonoids with a high antioxidant potential in rats [11]. A total of 12 compounds belonging to this family were identified in the MAE

extracts, despite in some extraction conditions like MAE 10, only 6 flavonoids were identified and only 3 of them were above the limit of quantification. Respect to individual compounds, myricetin-3-arabinoglactoside was not detected in MAE 4, MAE 10, MAE 11 and MAE 12 and it were below the limit of quantification in the rest of extraction experiments except for MAE 1, MAE 2, MAE 5, MAE 6 and MAE 14. In addition, kaempferol and its derivatives were not identified or were below the limit of quantification in the most of the extraction conditions. Also, quercetin-3-sambubioside was not detected in MAE 10, MAE 11 and MAE 12 and it was below the limit of quantification in MAE 4 and MAE 8. Kaempferol-3-O-sambubioside (Peak 17) could not be quantified due to was not detected in MAE 4, MAE 7, MAE 8, MAE 10, MAE 11 and MAE 12 and in the other extract conditions this compound was below the limit of quantification. The higher flavonoids content was found in MAE 11 (5.230 mg g^{-1} extract) and the lower concentration in MAE 7 (0.253 mg g^{-1} extract). The concentration range of each flavonoid was: myricetin-3-arabinogalactoside (Peak 10) from 0.022 ± 0.006 mg g^{-1} in MAE 2 to 0.091 ± 0.006 mg g^{-1} in MAE 14; quercetin-3-sambubioside (Peak 13) from 0.05 ± 0.02 mg g^{-1} in MAE 1 to 0.61 ± 0.01 mg g^{-1} in MAE 6; the concentration of quercetin-3-rutinoside (Peak 15) was from 0.0854 ± 0.0003 mg g^{-1} in MAE 4 to 0.554 ± 0.006 mg g^{-1} in MAE 6 although it was below the limit of quantification in MAE 10 and MAE 12. The same applies to other kaempferol derivatives, such as kaempferol-3-O-glucoside (Peak 22) and kaempferol-3-(p-coumarylglucoside) (Peak 28), which were not detected in MAE 10 and in the other extraction conditions they were below the limit of quantification; and kaempferol (Peak 31) which was not present in MAE 3, MAE 7 and MAE 13 and could only be

quantified in MAE 11 (1.04 ± 0.04 mg g^{-1} extract). Kaempferol-3-O-rutinoside (Peak 19) was only quantified in MAE 1, MAE 6 and MAE 14 within a range from 0.041 ± 0.001 mg g^{-1} in MAE 14 to 0.10 ± 0.01 mg g^{-1} in MAE 6. Regarding to quercetin-3-glucoside (Peak 18), it was quantified in all the experiments except in MAE 7, MAE 10, and MAE 12 and its concentration range was from 0.029 ± 0.009 mg g^{-1} (MAE 13) to 0.559 ± 0.009 mg g^{-1} (MAE 6). Methylepigallocatechin (Peak 23) was not detected in MAE 3, MAE 7 and MAE 13 and its content was from 0.349 ± 0.005 mg g^{-1} in MAE 16 to 1.17 ± 0.06 mg g^{-1} in MAE 11. Myricetin (Peak 25) was detected in all extraction conditions and its concentration range was 0.055 ± 0.001 mg g^{-1} for MAE 7 to 1.17 ± 0.01 mg g^{-1} for MAE 11. Finally, the content of quercetin (Peak 29) was from 0.128 ± 0.007 mg g^{-1} in MAE 10 to 1.34 ± 0.02 mg g^{-1} in MAE 11; nevertheless in MAE 3, MAE 7 and MAE 13 this compound was below the limit of quantification.

Prodelphinidin B3 (Peak 26) was the only anthocyanin identified in these MAE extracts. However, only in MAE 1, MAE 9, MAE 11 and MAE 14 this compound was above the limit of quantification with a concentration range from 0.02 ± 0.01 mg g^{-1} in MAE 9 to 0.57 ± 0.06 mg g^{-1} in MAE 11.

Other polar compounds were also detected in *H. sabdariffa* extracts such as apigenin-6,8-di-C-arabinoside and N-feruloylyiramine. The first one was below the limit of quantification in all extraction conditions and even in MAE 16 was not detected. N-feruloyltiramine was quantified in all cases and its concentration was from 0.492 ± 0.002 mg g^{-1} in MAE 10 to 4.9 ± 0.1 mg g^{-1} in MAE 11.

Therefore, these results showed that organic acids was the family compounds most abundant in all extraction conditions **(Figure 2)**.

In addition, the quantification of individual compounds extracted by MAE has been compared with previous *H. sabdariffa* studies. In a published work, a liquid chromatography/quadrupole-time-of-flight mass spectrometry (LC-Q-TOF-MS) method was used for quantification of five phenolic compounds from a *H. sabdariffa* extract obtained using an ultrasonic cleaning bath and 70 % (v/v) methanol [12]. In this work, neochlorogenic acid, chlorogenic acid, cryptochlorogenic acid, rutin and isoquercetrin were quantified with the described methodology. However, in the present research, MAE 11 showed higher concentrations of chlorogenic acid and cryptochlogenic acid (1.92 ± 0.05 and 5.0 ± 0.1 mg g^{-1} respectively) *vs* the ultrasonicated extract (0.975 ± 0.03 and 2.3 ± 0.8 mg g^{-1}, respectively). On the contrary, lower concentration of neochlorogenic acid (4.26 ± 0.03 mg g^{-1} in MAE 11 and 5.5 ± 0.1 mg g^{-1} in ultrasonicated extract) was obtained. Furthermore, rutin and isoquercetin were not found in any MAE condition.

On the other hand, the quantification by HPLC-ESI-TOF-MS of a *H. sabdariffa* aqueous extract obtained by maceration was performed in other research [13]. It should be highlighted that in this study, only 17 compounds were identified which are in contrast with the 27 compounds tentatively proposed in the present work. Furthermore, higher concentration of hibiscus acid, chlorogenic acid, myricetin-3-arabinogalactose, quercetin-3-sambubioside, kaempferol-3-O-rutinoside, n-feruloyltiramine, kaempferol-3-(p-coumarylglucoside) and quercetin were found in the extracts obtained by MAE. Nevertheless, other compounds were not present in any MAE condition (7-hydroxycoumarin, delphinidin-3-sambubiose and cyanidin-3-sambubioside) and hydroxicitric acid was found in lower concentration respect to the

maceration. Similar results were found in other three water *H. sabdariffa* extracts, in which 15, 16 and 17 compounds were found respectively [14–16]. In one of these works [14] the phenolic composition of cold (25 ºC) and hot (90 ºC) water extractions from *H. sabdariffa* were studied. The results showed similar phenolic profile in both extracts where several compounds identified were also found in the current work by MAE. However, other compounds such as gallic acid, protocatechuic acid glucoside, delphinidin-3-sambubioside and cyanidin-3-sambubioside were not found in any MAE condition. In other study [15], an aqueous extract of *H. sabdariffa* was performed to evaluate the production of monocyte chemoattractant protein-1 in humans. The phytochemical composition of this extract was very similar to the profile obtained in MAE conditions, although only 16 compounds were tentatively characterized instead of 27 compounds proposed in the present research. In addition, this water *H. sabdariffa* extract obtained by infusion, demonstrated beneficial effects for the treatment of chronic inflammatory diseases. Therefore, the use of MAE could be interesting for obtaining *H. sabdariffa* extract with potent bioactivity against this type of diseases. Finally, other aqueous *H. sabdariffa* extract obtained by methanol-water mixture was performed to study the inhibition of hyperglycemia, hyperlipidemia and glycation-oxidative stress while improving insulin resistance [16]. This extract was composed of 17 compounds, some of them also found in MAE conditions, as well as others not found in MAE extracts such as gallic acid, 5-hydroxymethylfurfural, protocatechuic acid, feruloyl derivative, caffeic acid, galloyl ester, feruloyl quinic derivative, tiliroside, delphinidin-3-sambubiode and cyanidin-3-sambubioside. Besides that, this extract demonstrated to be a potential adjuvant for diabetic therapy.

Therefore, in the light of these results the use of novel green extraction techniques like MAE with efficient and rapid process, obtained a wider variety of bioactive compounds at higher concentration compared to conventional techniques.

Effect of temperature, percentage of ethanol and extraction time

The influence of temperature, extraction time and percentage ethanol on the quantity of each compound family and individual compounds is summarized in **Table 3**. The extraction condition with higher amount of organic acids, phenolic acids, flavonoids and anthocyanins was MAE 11 whose temperature, percentage of ethanol, extraction time, dielectric constant and dissipation factor was: 164 ºC, 12.5 minutes, 45% (v/v) of ethanol, 30.92 and 1988.50, respectively. Therefore, at elevated temperatures higher amount of phenolic compounds were obtained. In this work, the temperature limit was fixed at 176 ºC due to this value is the maximum temperature at which ethanol can be exposed to microwave energy at 18 bars without experimental problems. However, despite being the extraction with higher total concentration of phenolic compounds, in this extract 27 compounds were identified, compared to other experiments performed with temperatures below 100 ºC and percentages of ethanol between 45-84% (v/v), where 30 compounds were detected. Therefore, at higher temperatures (above 100 ºC) some compounds with more thermo-labile nature like flavonoids were degraded. Indeed, it is worth highlighting that the central points (MAE 5 and MAE 6) were the two extract conditions (100 ºC, 12.5 min, 45 % (v/v) ethanol) that obtained higher amount of some flavonoids such as myricetin-3-arabinogalactoside, quercetin derivatives (quercetin-3-sambubioside, quercetin-3-

rutinoside and quercetin-3-glucoside) and kaempferol derivatives (kaempferol-3-O-rutinoside and kaempferol-3-O-glucoside).

In addition, the effect of temperature on flavonoids content was in line with a previous research which MAE of *H. sabdariffa* seeds performed at different conditions [17]. In this study, the influence of extraction time (4-10 min), microwave power (100-300W) and solvent / solid ratio (25- 100 mL g^{-1}) was studied and the total phenolic compounds and flavonoids content (quercetin equivalents (QE)) were determined. The highest flavonoid content was achieved at 158 ºC (14.4251 mg QE g-1), but at 163 ºC lower flavonoid content (6.4524 mg QE g-1) was obtained due to the degradation of these thermo-labile compounds. However, even though in the current study applying MAE 11 extraction conditions some flavonoids were degraded and not detected by HPLC-DAD-ESI-TOF-MS analysis, this experiment obtained the highest amount of others flavonoids such as methylepigallocatechin, myricetin, quercetin and kaempferol. This could be explained because glycoside-flavonoids are more strongly degraded at high temperatures than flavonoids without glycosides [18]. In fact, it has been previously reported the thermal degradation of onion quercetin glycosides under roasting conditions. The result put into light that after a few minutes of roasting all glycoside-flavonoids showed a loss of up to 20% of the starting concentration. On the contrary, quercetin was not sensitive to degradation under heating [19].

Table 3 Tentative identification and quantification of phenolic compounds and their derivatives in *H. sabdariffa* extract obtained under different MAE conditions. ND: Compound not detected; <LOQ: Compound below the limit of quantification; Concentrations expressed in mg / g extract

Peak	Compound	RT	m/z	Molecular formula	MAE 1	MAE 2	MAE 3	MAE 4	MAE 5	MAE 6
				Organic acids						
1	Hydroxicitric acid	2.95	207.0146	$C_6H_8O_8$	4.0 ± 0.2	3.76 ± 0.03	4.1 ± 0.2	1.31 ± 0.03	3.26 ± 0.02	3.00 ± 0,06
2	Hibiscus acid	3.06	189.0041	$C_6H_6O_7$	57 ± 3	49.6 ± 0.9	43 ± 3	29.6 ± 0.7	43.9 ± 0.9	42 ± 1
3	Hibiscus acid hydroxyethylester	4.03	235.0459	$C_8H_{12}O_8$	4.9 ± 0.1	3.23 ± 0.04	0.63 ± 0.05	7.06 ± 0.08	9.0 ± 0.1	9.1 ± 0.2
4	Hibiscus acid dimethylester	4.5	217.0354	$C_8H_{10}O_7$	6.1 ± 0.2	4.0 ± 0,2	0.46 ± 0.06	40.0 ± 0.4	8.90 ± 0.08	10.8 ± 0.3
				Total	72.2	60.56	48.55	77.90	64.99	64.93
				Phenolic acids						
5	Neochlorogenic acid	4.94	353.0878	$C_{16}H_{18}O_9$	3.9 ± 0.4	2.97 ± 0.03	1.83 ± 0.07	1.48 ± 0.03	3.70 ± 0.02	3.48 ± 0.09
6	Chlorogenic acid	6.49	353.0878	$C_{16}H_{18}O_9$	0.8 ± 0.1	0.642 ± 0.002	0.34 ± 0.02	0.72 ± 0.01	0.58 ± 0.02	0.818 ± 0.007
7	Cryptochlorogenic acid	6.81	353.0878	$C_{16}H_{18}O_9$	2.8 ± 0.2	2.12 ± 0.05	1.33 ± 0.02	1.82 ± 0.01	2.85 ± 0.04	2.31 ± 0.04
8	Methyl digallate	7.56	335.0409	$C_{15}H_{12}O_9$	0.58 ± 0.04	0.495 ± 0.006	0.356 ± 0.008	0.38 ± 0.01	0.467 ± 0.007	0.604 ± 0.001
9	Methyl chlorogenate	7.96	367.1035	$C_{17}H_{20}O_9$	0.0133 ± 0.0004	0.068 ± 0.004	<LOQ	<LOQ	<LOQ	0.091 ± 0.002
11	Coumaroilquinic acid	8.54	337.0929	$C_{16}H_{18}O_8$	1.00 ± 0.04	0.74 ± 0.01	0.48 ± 0.01	0.47 ± 0.01	0.67 ± 0.01	0.858 ± 0.009
12	Dihydroferulic acid-4-O-glucuronide	9.4	371.0984	$C_{16}H_{20}O_{10}$	0.62 ± 0.03	0.44 ± 0.02	0.295 ± 0.006	0.226 ± 0.006	0.49 ± 0.02	0.508 ± 0.003
14	Methyl chlorogenate isomer II	10.02	367.1035	$C_{17}H_{20}O_9$	0.089 ± 0.006	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
16	5-O-caffeoylshikimic acid	10.38	335.0772	$C_{16}H_{16}O_8$	4.0 ± 0.1	2.94 ± 0.06	1.45 ± 0.06	8.3 ± 0.2	3.07 ± 0.04	3.77 ± 0.04
20	Ethylchlorogenate	11.4	381.1191	$C_{18}H_{22}O_9$	0.13 ± 0.02	<LOQ	ND	0.56 ± 0.02	0.066 ± 0.003	0.228 ± 0.003
21	Ethylchlorogenate isomer II	11.81	381.1191	$C_{18}H_{22}O_9$	0.228 ± 0.007	0.075 ± 0.003	ND	0.62 ± 0.03	0.325 ± 0.002	0.327 ± 0.007
				Total	14.201	10.481	6.080	14.520	12.220	12.990
				Flavonoids						
10	Myricetin-3-arabinogalactoside	8.36	611.1254	$C_{26}H_{28}O_{17}$	0.089 ± 0.009	0.022 ± 0.006	<LOQ	ND	0.148 ± 0.005	0.11 ± 0.01
13	Quercetin-3-sambubioside	9.51	595.1305	$C_{26}H_{28}O_{16}$	0.05 ± 0.02	0.365 ± 0.009	0.22 ± 0.02	<LOQ	0.62 ± 0.02	0.61 ± 0.01
15	Quercetin-3-rutinoside	10.17	489.1461	$C_{17}H_{30}O_{16}$	0.51 ± 0.03	0.340 ± 0.005	0.189 ± 0.003	0.0854 ± 0.0003	0.506 ± 0.004	0.554 ± 0.006
17	kaempferol-3-O-sambubioside	10.67	579.1355	$C_{26}H_{28}O_{15}$	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ
18	Quercetin-3-glucoside	10.97	463.0882	$C_{21}H_{20}O_{12}$	0.52 ± 0.04	0.31 ± 0.01	0.013 ± 0.009	0.23 ± 0.02	0.506 ± 0.003	0.559 ± 0.009
19	kaempferol-3-O-rutinoside	11.25	593.1512	$C_{27}H_{30}O_{15}$	0.06 ± 0.02	<LOQ	<LOQ	<LOQ	<LOQ	0.10 ± 0.01
22	kaempferol-3-O-glucoside	12.12	447.0933	$C_{21}H_{20}O_{11}$	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
23	Methylepigallocatechin	12.45	319.0823	$C_{16}H_{16}O_7$	0.466 ± 0.005	0.40 ± 0.02	ND	0.61 ± 0.01	0.495 ± 0.007	0.427 ± 0.008
25	Myricetin	14.35	317.0303	$C_{15}H_{10}O_8$	0.62 ± 0.02	0.477 ± 0.006	0.139 ± 0.001	0.45 ± 0.01	0.400 ± 0.003	0.518 ± 0.009
28	kaempferol 3-(p-coumarylglucoside)	17.17	593.1301	$C_{30}H_{26}O_{13}$	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
29	quercetin	19.15	301.0354	$C_{15}H_{10}O_7$	1.24 ± 0.07	0.84 ± 0.03	<LOQ	0.92 ± 0.04	0.9459 ± 0.0007	1.00 ± 0.02
31	kaempferol	26.24	285.0405	$C_{15}H_{10}O_6$	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ
				Total	4.043	2.750	0.559	2.2822	3.6172	3.877
				Anthocyanins						
26	Prodelphinidin B3	14.9	609.1250	$C_{30}H_{26}O_{14}$	0.09 ± 0.01	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
				Other polar compounds						
24	Apigenin-6,8-di-C-arabinoside	12.74	533.1301	$C_{25}H_{26}O_{13}$	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
27	N-feruloyltiramine	16.44	312.1241	$C_{18}H_{19}NO_4$	1.98 ± 0.06	2.38 ± 0.06	1.28 ± 0.01	1.98 ± 0.06	2.98 ± 0.06	3.19 ± 0.09
				Total	1.98	2.38	1.28	1.98	2.98	3.19

Table 3 Tentative identification and quantification of phenolic compounds and their derivatives in *H. sabdariffa* extract obtained under different MAE conditions. ND: Compound not detected; <LOQ: Compound below the limit of quantification; Concentrations expressed in mg / g extract

Compound	MAE 7	MAE 8	MAE 9	MAE 10	MAE 11	MAE 12	MAE 13	MAE 14	MAE 15	MAE 16
Organic acids										
Hydroxicitric acid	4.28 ± 0.05	1.85 ± 0.06	3.21 ± 0.06	3.23 ± 0.02	3.68 ± 0.02	2.96 ± 0.02	3.8 ± 0.2	3.34 ± 0.04	3.7 ± 0.1	4.1 ± 0.1
Hibiscus acid	44.2 ± 0.7	34 ± 3	44.4 ± 0.9	33.8 ± 0.9	69 ± 1	33 ± 1	41.8 ± 0.5	41.42 ± 0.07	44.7 ± 0.5	48.8 ± 0.8
Hibiscus acid hydroxyethylester	1.80 ± 0.03	8.0 ± 0.3	8.0 ± 0.1	2.36 ± 0.03	13.1 ± 0.3	2.61 ± 0.05	1.43 ± 0.04	8.1 ± 0.1	5.5 ± 0.1	3.21 ± 0.05
Hibiscus acid dimethylester	1.6 ± 0.9	2 ± 1	10.8 ± 0.2	2.35 ± 0.02	70 ± 3	5.82 ± 0.09	1.08 ± 0.03	8.4 ± 0.2	5.69 ± 0.07	3.63 ± 0.04
Total	51.89	71.63	66.44	41.72	156.12	44.37	48.15	61.32	59.52	59.72
Phenolic acids										
Neochlorogenic acid	1.69 ± 0.03	1.72 ± 0.06	2.63 ± 0.03	1.36 ± 0.03	4.26 ± 0.03	1.42 ± 0.03	1.85 ± 0.06	2.93 ± 0.02	2.16 ± 0.02	1.88 ± 0.02
Chlorogenic acid	0.271 ± 0.009	0.64 ± 0.02	0.59 ± 0.01	0.281 ± 0.006	1.92 ± 0.05	0.281 ± 0.007	0.333 ± 0.005	0.645 ± 0.007	0.433 ± 0.003	0.356 ± 0.004
Cryptochlorogenic acid	1.17 ± 0.01	1.572 ± 0.006	1.855 ± 0.004	1.36 ± 0.02	5.0 ± 0.1	1.20 ± 0.01	1.20 ± 0.017	1.93 ± 0.03	1.53 ± 0.05	1.31 ± 0.03
Methyl digallate	0.328 ± 0.004	0.386 ± 0.006	0.461 ± 0.003	0.334 ± 0.006	0.73 ± 0.02	0.32 ± 0.01	0.363 ± 0.007	0.492 ± 0.003	0.410 ± 0.004	0.384 ± 0.005
Methyl chlorogenate	<LOQ	<LOQ	<LOQ	<LOQ	0.027 ± 0.005	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Coumaroilquinic acid	0.431 ± 0.005	0.446 ± 0.005	0.64 ± 0.01	0.462 ± 0.006	1.37 ± 0.04	0.40 ± 0.02	0.449 ± 0.007	0.72 ± 0.02	0.527 ± 0.003	0.49 ± 0.02
Dihydroferulic acid-4-O-glucuronide	0.265 ± 0.001	0.244 ± 0.003	0.386 ± 0.004	0.176 ± 0.005	0.69 ± 0.01	0.19 ± 0.01	0.44 ± 0.01	0.450 ± 0.006	0.312 ± 0.003	0.28 ± 0.02
Methyl chlorogenate isomer II	<LOQ	<LOQ	<LOQ	<LOQ	0.04 ± 0.09	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
5-O-caffeoylshikimic acid	1.58 ± 0.01	5.92 ± 0.2	3.21 ± 0.04	2.89 ± 0.04	18.1 ± 0.3	3.22 ± 0.06	1.48 ± 0.05	3.04 ± 0.07	2.29 ± 0.02	2.09 ± 0.02
Ethylchlorogenate	ND	0.260 ± 0.003	0.108 ± 0.003	0.127 ± 0.005	1.318 ± 0.001	<LOQ	ND	0.095 ± 0.002	<LOQ	<LOQ
Ethylchlorogenate isomer II	ND	0.318 ± 0.003	0.175 ± 0.004	0.143 ± 0.005	1.33 ± 0.05	<LOQ	ND	0.157 ± 0.004	0.071 ± 0.001	<LOQ
Total	5.732	11.502	10.058	7.136	34.646	7.034	6.239	10.460	7.731	6.798
Flavonoids										
Myricetin-3-arabinogalactoside	<LOQ	<LOQ	<LOQ	ND	ND	ND	<LOQ	0.091 ± 0.006	<LOQ	<LOQ
Quercetin-3-sambubioside	0.059 ± 0.003	<LOQ	0.366 ± 0.009	ND	ND	ND	0.26 ± 0.01	0.514 ± 0.007	0.30 ± 0.03	0.26 ± 0.008
Quercetin-3-rutinoside	0.138 ± 0.006	0.168 ± 0.008	0.35 ± 0.01	<LOQ	0.26 ± 0.03	<LOQ	0.21425 ± 0.00003	0.45 ± 0.01	0.304 ± 0.006	0.21 ± 0.09
kaempferol-3-O-sambubioside	ND	ND	<LOQ	ND	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ
Quercetin-3-glucoside	<LOQ	0.35 ± 0.01	0.33 ± 0.02	<LOQ	0.25 ± 0.02	<LOQ	0.029 ± 0.009	0.38 ± 0.02	0.190 ± 0.004	0.11 ± 0.02
kaempferol-3-O-rutinoside	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ	0.041 ± 0.001	<LOQ	<LOQ
kaempferol-3-O-glucoside	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Methylepigallocatechin	ND	0.53 ± 0.01	0.40 ± 0.01	0.353 ± 0.005	1.17 ± 0.06	0.377 ± 0.005	ND	0.419 ± 0.001	0.352 ± 0.001	0.349 ± 0.005
Myricetin	0.055 ± 0.001	0.40 ± 0.01	0.401 ± 0.007	0.1985 ± 0.0003	1.17 ± 0.01	0.205 ± 0.002	0.145 ± 0.003	0.449 ± 0.001	0.342 ± 0.002	0.30 ± 0.01
kaempferol 3-(p-coumarylglucoside)	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
quercetin	<LOQ	0.70 ± 0.03	0.660 ± 0.007	0.128 ± 0.007	1.34 ± 0.02	0.135 ± 0.009	<LOQ	0.76 ± 0.02	0.45 ± 0.01	0.32 ± 0.01
kaempferol	ND	<LOQ	<LOQ	<LOQ	1.04 ± 0.04	<LOQ	ND	<LOQ	<LOQ	<LOQ
Total	0.253	2.150	2.511	0.679	5.230	0.716	0.6478	3.104	1.939	1.546
Anthocyanins										
Prodelphinidin B3	<LOQ	<LOQ	0.02 ± 0.01	<LOQ	0.57 ± 0.06	<LOQ	<LOQ	0.045 ± 0.004	<LOQ	<LOQ
Other polar compounds										
Apigenin-6,8-di-C-arabinoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	ND
N-feruloyltiramine	0.899 ± 0.008	1.83 ± 0.07	2.31 ± 0.07	0.492 ± 0.002	4.9 ± 0.1	1.248 ± 0.008	1.277 ± 0.003	2.47 ± 0.04	1.92 ± 0.01	1.72 ± 0.01
Total	0.899	1.83	2.31	0.492	4.9	1.248	1.277	2.47	1.92	1.72

On the other hand, intermediate values of percentage of ethanol and extraction time showed higher amount of these families of compounds. Ethanol and water are two food-grade solvents recognized as safe (GRAS) and they have demonstrated to be efficient for the extraction of phenolic compounds from plants [20]. Moreover different water-ethanol mixtures allowed changes on the dielectric constant which influence the irradiation time optimization [21]. Thus, for higher percentages of ethanol, the dielectric constant of the mixture decreases and although the efficiency to convert microwaves into heat is high, its capacity to absorb energy is lower. This means that the solvent itself has absorbed less microwaves and the dissipation factor is higher. In addition, although the extraction yield tends to improve with an increase of the extraction time, prolonged application of microwaves may lead to produce a degradation of target compounds [22].

RSM was employed to maximize the extraction yield. **Figure 3** shows the 3D plots of the response surface for the effect of temperature and percentage of ethanol on extraction yield during 12.5 min of extraction. Moreover, **Table 4** shows the extraction yield (mass of extract / mass of dry matter) obtained in each assayed condition. These results report that an increase of extraction time and temperature is associated with an improvement of extraction efficiency. In fact, temperatures above 100 ºC got a yield above 35%. However, at 50 ºC the highest yield obtained was 16.9 %. Thus, the optimum predicted values to maximize the extraction yield were temperature of 164 ºC, 60% (v/v) ethanol and 22 min of extraction time.

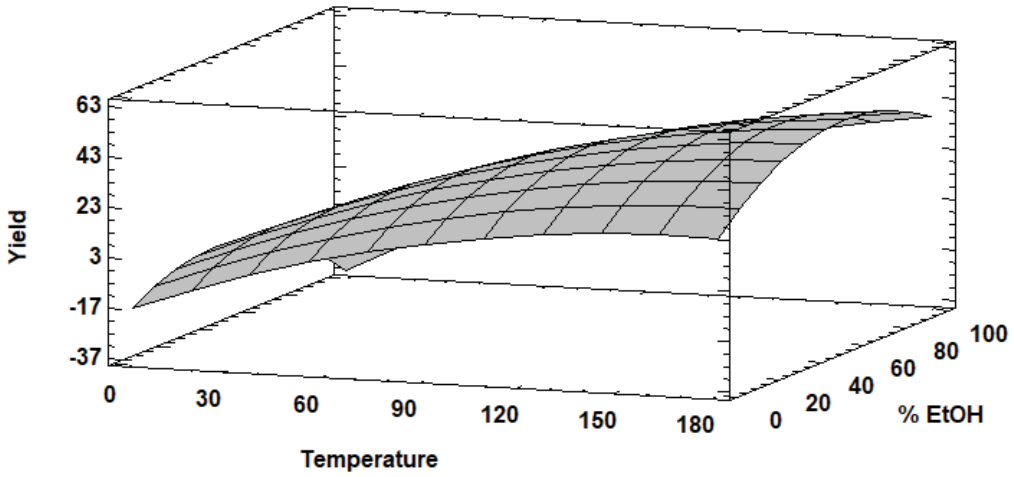


Figure 3. 3D plot of response surface for the effects of solvent (% ethanol in the mixture) and temperature at 12.5 min on the extraction yield

Generally solvents have higher capacity to solubilize components at higher temperatures improving matrix penetration due to a decrease in surface tension and solvent viscosity [9]. Additionally, high temperatures and long-time extractions could accelerate cell rupture of plant samples, which allows the destruction of sample surface and the exudation of the chemical components into the surrounding solvents achieving a higher extraction yield [23]. On the other hand, the dielectric properties of the solvent could also determine extraction yield. **Figure 3** showed that extraction yield increased while increasing the percentage of ethanol in the solvent until a maximum, and then decreased. As mentioned before, this can be explained because although ethanol-water mixture has good microwave absorbing capacity and hence heats up faster and can enhance the extraction efficiency [24], higher percentages of ethanol lead to a decrease in dielectric constant and an increase on dissipation factor, and consequently the extraction yield obtained is lower. On the other hand, an

increase of extraction time may increase the impurities or others compounds extracted like sugars.

Run	Temperature (ºC)	% EtOH	Time (min)	Yield(%)	
MAE 1	50	75	20	12,0 ± 0,1	
MAE 2	50	75	5	10,3 ± 0,1	
MAE 3	50	15	20	16,9 ± 0,3	
MAE 4	150	75	20	47,21 ± 0,02	
MAE 5	100	45	12,5	35,0 ± 0,1	
MAE 6	100	45	12,5	34,06 ± 0,06	
MAE 7	100	6	12,5	14,25 ± 0,05	
MAE 8	150	75	5	41,38 ± 0,09	
MAE 9	100	45	22	39,5 ± 0,2	
MAE 10	150	15	20	39,6 ± 0,2	
MAE 11	164	45	12,5	47,1 ± 0,1	
MAE 12	150	15	5	38,3 ± 0,3	
MAE 13	50	15	5	16,89 ± 0,04	
MAE 14	100	84	12,5	32,76 ± 0,06	
MAE 15	100	45	3	42,4 ± 0,1	
MAE 16	36	45	12,5	11,1 ± 0,2	
Optimum predicted values	164.359	59.63	22.1539	55.97	**CV = 0.65%**
Experimental verification	164.359	59.63	22.1539	55 ± 2	

Table 4. Extraction yield at different MAE conditions

Therefore, for maximize the extraction yield the optimum extraction time was higher compared to the one calculated for each compounds. These data were similar to other study carried out by Talebi, (2004) [25], who evaluated the effect of temperature and percentage of methanol on extraction yield of paclitaxel from *Taxus baccata L*. by MAE. These authors obtained an improvement in extraction efficiencies at elevated temperatures with methanol-water (90-10%, (v / v) mixture combination. In other research, different percentages of ethanol were studied for MAE of tanshinones from *Salvia miltiorrhiza bunge*. The extraction of these compounds was

increased with the increase of ethanol concentration, achieving the best result with 95% (v / v) ethanol [26].

Table 5 includes the ANOVA test of the regression model for the response variable (extraction yield). The data revealed that the value of the regression coefficient (R^2) was 95.12%. This result allowed explaining a considerable part of the variance within data. In addition to this parameter, the lack of fit was not marginally significant ($p \leq 0.10$). The obtained parameters of ANOVA enabled to confirm that this model provided a good approximation to the experimental conditions. Temperature was the variable which has shown significant effects ($p \leq 0.05$), although the percentage of ethanol was marginally significant ($p \leq 0.10$). Indeed, even though extraction time was not significant, the quadratic effect for this variable was marginally significant ($p \leq 0.10$), as well as for temperature. On the other hand, the quadratic effect of solvent mixture was significant ($p \leq 0.05$). In addition, the interaction of temperature and solvent composition was marginally significant. Fitting experimental data to a reduced model, and keeping only the significant parameters in the quadratic model, provided the model equation (Eq.1):

$$Yield = -4.02488 + 0.455724A - 0.00770215BB \quad \text{(Eq. 1)}$$

Considering the equation to explain the model for yield behaviour and understanding the influence of each independent variable, an optimization of conditions to maximize extraction were proposed.

Source	Yield				
	SS	DF	MS	F-value	p
A	2171.44	1	2171.44	94.31	**0.0091**[a]
B	46.8628	1	46.8628	2.04	**0.0616**[b]
C	2.30545	1	2.30545	0.10	0.2627
A:A	69.3888	1	69.3888	3.01	**0.0507**[b]
A:B	61.494	1	61.494	2.67	**0.0538**[b]
A:C	3.67205	1	3.67205	0.16	0.2126
B:B	263.821	1	263.821	11.46	**0.0260**[a]
B:C	4.83605	1	4.83605	0.21	0.1869
C:C	71.0378	1	71.0378	3.09	**0.0501**[b]
Lack of fit	137.707	5	27.5413	62.34	0.0946
Pure error	0.4418	1			
Total	2833.01	15			
R^2	0.9512				
Adj. R^2	0.8781				

Table 5. Analysis of variance (ANOVA) of the regression model. A = Temperature (ºC); B = % Ethanol; C = Extraction time (min); SS = sum of squares; DF = degrees of freedom; MS = mean square; R2 = Quadratic correlation coefficient; [a] Significant ($p < 0.050$); [b] Marginally significant ($p < 0.100$)

Regarding the suggested model, a great value on this response variable could be obtained under the following optimized conditions: 164 ºC, 60% of ethanol-water (v/v) and a total extraction time of 22 min. The predictable value to yield was calculated applying the obtained equation (Eq. 1) after analysis of the model and compared with experimental value when optimal conditions were applied. The theoretical and experimental values were 55.97 and 55 ± 2, respectively **(Table 4)**. Analysis of results revealed an acceptable variance (CV=0.65%) between theoretical and experimental data.

This means that the experimental validation was consistent with the mathematical description of the model, indicating the suitability of RSM in optimizing the extraction yield of *H. sabdariffa* extract by MAE.

Conclusions

The proposed MAE technology can be successfully used to optimize the extraction of phenolic compounds from *H. sabdariffa* calyces by MAE and maximize the extraction yield through RSM. The phenolic composition of the extracts was characterized by using HPLC-ESI-TOF-MS. Thus, the mixture of two GRAS solvents at 164 ºC has shown great potential to extract phenolic compounds with demonstrated bioactive properties from this plant. Nevertheless, some flavonoid glycosides were degraded at this temperature. In contrast, other non-glycoside flavonoids such as quercetin, myricetin or kaempferol were more thermo-resistant. Moreover, the composition of extraction solvent using, ethanol - water mixtures demonstrated that the dielectric constant is an important factor for the extraction of target compounds and the extraction yield. Thus, at lower dielectric factor and higher dissipation factor the extraction efficiency was lower. Indeed, values of 59.63% (v / v) of ethanol showed to be the percentage for a maximum extraction yield. In contrast, the optimum extraction time and temperature found for maximization the extraction yield were the highest applied. Therefore, to the best of our knowledge this is the first time that an experimental design is applied to maximize the extraction yield from *H. sabdariffa* calyces by MAE.

Acknowledgments

This work was supported by the Research group AGR274 “Bioactive ingredients” from the Analytical Chemistry Department of the University of Granada, the Andalusian Regional Government Council of Innovation and Science (project P11-CTS-7625) and the Spanish Ministry of Economy and Competitiveness (MINECO)

(project AGL2015-67995-C3-2). We would like to thank the Ministry of Education, Culture and Sport (MECD) for supporting the grant FPU15/01125 of SPM. The author IBL gratefully acknowledges the Spanish Ministry of Economy and Competitiveness (MINECO) in association with the European Social Fund (FSE) for the contract PTQ-13-06429 and JLS also thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for the grant IJCI-2015-26789.

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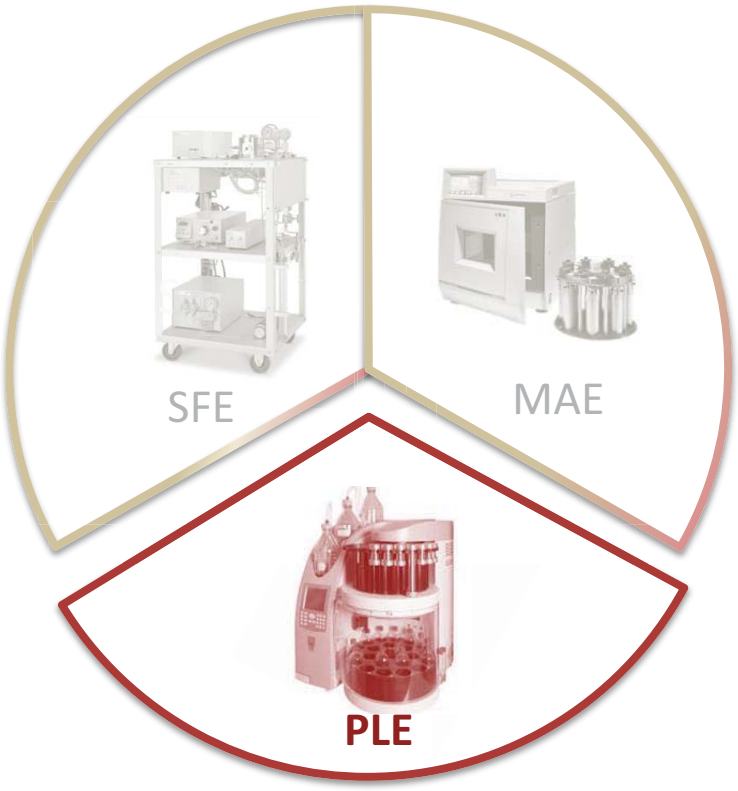
Chapter 2

Selective extraction of bioactive compounds from *Hibiscus sabdariffa* by green pressurized liquid extraction



Journal of Food and Drug Analysis

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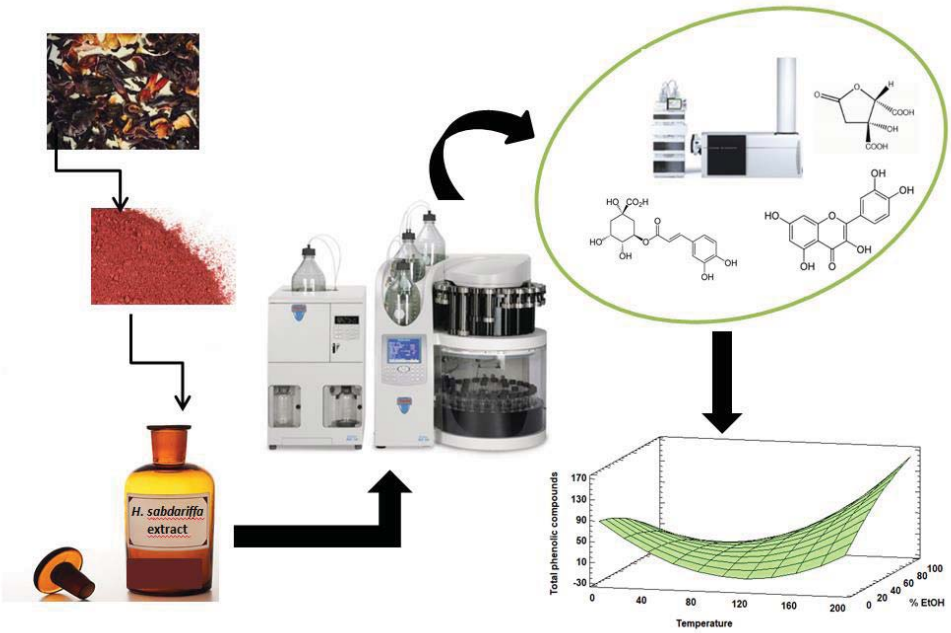
Selective extraction of bioactive compounds from *Hibiscus sabdariffa* by green pressurized liquid extraction

Abstract



Phenolic compounds from *Hibiscus sabdariffa* (Hs) have demonstrated significant health benefits against chronic diseases. Environmental friendly extraction techniques, such as Pressurized Liquid Extraction (PLE), have demonstrated a big potential for obtaining extracts with higher content in these bioactive compounds. In this work, response-surface methodology (RSM) was applied to evaluate the significance of temperature and solvent composition on the PLE efficiency of phenolic compounds. Qualitative and quantitative chemical composition was determined by high-performance liquid chromatography coupled to quadrupole time of flight mass spectrometry (HPLC-ESI-QTOF-MS). Higher concentration of organic acids, phenolic

acids, flavonoids and anthocyanins were obtained at higher temperature and ethanol percentage. However, respect to cyanidin-3-sambubioside, the temperature seemed to be an important factor, increasing its concentrations with lower extraction temperatures. Optimal extraction conditions were determined taking the maximum recovery for total phenolic compounds. The results showed that temperature and ethanol amount influenced significantly (p-value < 0.05) the extraction of total phenolic compounds. The optimum condition was obtained at 200 ºC and 100 % of ethanol. Thus, to the best of the author's knowledge, it is the first time that an experimental design of PLE has been applied to maximize the extraction of total and individual phenolic compounds from Hs calyces.

Keywords: *Hibiscus sabdariffa*; pressurized liquid extraction; phenolic compounds; response-surface 36 methodology; HPLC-ESI-QTOF-MS

Introduction

Phytochemicals have demonstrated substantial health benefits in humans. This fact has led to the isolation and purification of those bioactive compounds from different herbal matrix in order to study their bioactivities. Therefore, those compounds could be used for formulations in the food, cosmetic or pharmaceutical sectors.

As a result, the extraction process has been an extensively studied step in the required methodology in order to achieve the optimum extraction efficiency of those analytes. In this sense, Pressurized Liquid Extraction (PLE) is a green extraction technology, opposed to other conventional systems such as solid-liquid extraction

(SLE) or soxhlet extraction (SE). PLE preserves the natural environment while reduces the extraction time and the amount of used solvent [1]. Indeed, very environmentally friendly extraction solvents like ethanol and water are commonly applied [2,3].

PLE, also known as Accelerated Solvent Extraction (ASE), employs liquid solvents at elevated temperatures and pressures for extracting targeted compounds. From one hand, the elevated temperature allows the sample to become more soluble and achieve a higher diffusion rate. On the other hand, the high pressure keeps the solvent below its boiling point obtaining a deeper penetration within the sample which turn into a rise in the extraction efficiency [4]. Therefore, this technology is currently used for the extraction of bioactive compounds from herbal plants, in particular for phenolic compounds [5,6].

Despite the potential of PLE to achieve an efficient extraction of phytochemicals, different operational parameters should be controlled. Extraction temperature and solvent composition are the main variables which should be optimized. In fact, the importance of these parameters in the extraction efficiency of phenolic compounds has been highlighted in previous studies [7]. The combination of both factors together with pressure determines the dielectric constant and consequently the solute-solvent interaction. For example, water at ambient pressure and temperature has a high dielectric constant (ε) of 78, but this value decreases to 21 at 300 ºC and pressure of 3335 psi, which is similar to the value for ethanol (ε) of 24 at 25°C [8].

As mention before, the need of optimizing those parameters in the extraction process is crucial to select the best extraction condition of phytochemicals. For that

purpose, response-surface methodology (RSM) was used in this work in order to optimize the extraction of bioactive compounds from *Hibiscus sabdariffa (Hs).* This species belonging to *Malvaceae* family is a tropical plant commonly used in the preparation of herbal drinks, hot and cold beverages, because of its antioxidant function in humans. Besides that, recent studies have demonstrated that this plant provide a positive effect on prevention and treatment of chronic diseases such as diabetes mellitus, cancer, dyslipidemia and hypertension. These beneficial characteristics makes it interesting its composition study for food industry [9–11]. Moreover, it is known that this plant contains a high amount of bioactive phenolic compounds like phenolic acids, anthocyanins and flavonoids [12]. Thus, PLE optimization could be a potential technique to obtain higher content of these bioactive compounds.

In this case, solvent ratio and temperature were chosen as independent variables while the total content of phenolic compounds determined by high-performance liquid chromatography coupled to quadrupole time-of flight mass spectrometry (HPLC-ESI-QTOF-MS) was the response variable. One of the advantages is that this methodology permits the evaluation of the possible effects between variables in order to describe the behavior of a data set with the objective of making statistical predictions. Thus, in the present work, optimization by RSM was carried out to maximize the total phenolic compounds content for obtaining extracts with higher bioactivity for their potential use in cosmetic or pharmaceutical sector.

Material and methods

Chemical and Reagents

All chemicals used during this research were of analytical HPLC-MS grade. Water was obtained by purification with a Milli-Q system from Millipore (Bedford, MA, USA). Ethanol and Otawa sand for extraction was supplied by Fisher Scientific (Madrid, Spain), while cellulose filters for the preparation of extraction cells were obtained from Dionex Corp. (Sunnyvale, CA, USA). Formic acid and acetonitrile for mobile phases were purchased from Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Madrid, Spain), respectively. Finally, the standards used for the quantification were acquired from Sigma-Aldrich, (Steinhemin, Germany): gallic acid, citric acid, chlorogenic acid, rutin, p-coumaric acid, quercetin, myricetin, quercetin-glycoside and apigenin (internal standard), except quercitrin, which was supplied by Extrasynthese (Genay Cedex, France).

Plant material

All experiments were performed by using commercial dried calyces of *Hibiscus sabdariffa (Hs)* provided by Monteloeder Inc. (Elche, Alicante, Spain). The sample was grounded into fine uniform powder with a Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany) equipped with 12-tooth rotor and ring sieve with aperture size of 1 mm. After that, the sample was stored at room temperature and avoiding sun light until further experiments.

Pressurized Liquid Extraction (PLE)

The green extraction processes were carried out in a Dionex ASE 350 extractor (Dionex Corp., Sunnyvale, CA, USA), using Generally Recognized As Safe (GRAS) solvents such as ethanol and double-deionized water. Prior to use, the solvents were sonicated in an ultrasonic bath during 10 min in order to eliminate the dissolved air.

The extractions were performed in static mode at a pressure of 1500 psi during 20 min of extraction time. The sample was placed in stainless steel cells of 34 mL prepared as follows: 5g of sand at the bottom, 8 g of sample with 16 g of sand mixed homogeneously, and finally 5g of sand at the top of the cell. Moreover, disposable cellulose filters were placed into the cell´s inlet and outlet in order to prevent clogging in the metal frit of the extraction cell. After the extraction cycle, the cell was flushed with solvent (60% of the cell volume), purged with nitrogen (100 s) and the resulting extracts were collected in 200 mL amber vials and immediately cooled in ice. Afterwards, the extracts were centrifuged at 12.000 rpm for 15 min at 4 °C in a Sorvall ST 16 R centrifuge (Thermo Scientific, Leicestershire, UK). Then, the supernatants were evaporated using a Savant SC250EXP SpeedVac Concentrator (Thermo Scientific). The dried extracts were stored at −20°C and protected from light until analysis. Pior to HPLC analysis the dried extracts were reconstituted to a concentration of 4.5 mg mL^{-1}.

Chromatographic separation

To determine the chemical profile of the obtained extracts at different conditions, an analysis by HPLC-ESI-QTOF-MS was performed. The instrumentation used was an Agilent 1260 HPLC instrument (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 6540 Ultra High Definition (UHD) Accurate Mass Q-TOF equipped

with a Jet Stream dual ESI interface. Chromatographic separation was performed with a reversed-phase C18 analytical column (Agilent Zorbax Eclipse Plus, 1.8 µm, 4.6×150 mm).

The compounds were eluted with two polar mobile phases consisted of phase A (water-acetonitrile, 90:10 v/v plus 0.1 % of formic acid) and phase B (acetonitrile). The chromatographic parameters were optimized for the best resolution. Thus, the elution program was a multi-step linear gradient at a flow of 0.3 mL min^{-1}, beginning at 0 min with 5% of mobile phase B, followed by 20 % phase B at 34 min, at 45 min increasing until 95 % phase B, at 55 min back to 5 % of phase B. Finally, the initial conditions were maintained for 5 min. The sample injection volume was 10 µl, whereas the column and auto-sampler compartments temperatures were set at 25 ºC and 4 ºC, respectively.

ESI-QTOF-MS conditions

The MS detection were performed in negative ionization mode with a mass range of 100-1700 m/z, the detection window was set to 100 ppm and data acquisition (2.5 Hz) was performed in centroid mode. The capillary voltage was set +4000V, nebulizer pressure 20 psi, fragmentor 130 V, nozzle voltage 500 V, skimmer 45 V and octopole 1 RF Vpp 750 V. Ultrahigh pure nitrogen was used as drying and nebulizer gas at temperatures of 325 and 350 ºC and flows of 10 and 12 L min^{-1}, respectively.

With the intention of recalibrate each single mass spectra acquired during the analysis providing accurate mass measurement typically better than 2 ppm, a continuous infusion of two reference masses were performed: trifluoroacetate anion

(m/z 112.985587) and hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine or HP-921 (m/z 1033.988109).

The MS data were processed through the software Qualitative Analysis of MassHunter workstation version B.06.00 (Agilent Technologies, Palo Alto, CA, USA).

Standards for individual compound quantification

The commercial standards used for quantifying the main identified compounds in PLE extracts, were myricetin, quercitrín, quercetin-3-glucoside, quercetin, rutin, gallic acid, chlorogenic acid, p-coumaric acid and citric acid. Therefore, nine calibration curves were performed using these standards. The linear range were set at seven concentration levels and analyzed in triplicate. In addition, apigenin was the internal standard used in this HPLC analysis at a concentration of 20 mg L^{-1}.

To determine the compound concentration the corrected area of each compound was calculated as area standard / area internal standard and the result was interpolated in the corresponding calibration curve. However, although for myricetin, quercetin-glucoside, quercetin and chlorogenic acid, the calibration curves of their own commercial standards were used for their quantification, for the quantification of the other compounds identified, which had no commercially available standards, were tentatively quantified with calibration curves from other compounds with similar structure. Hence, the calibration curve of gallic acid was used for the quantification of protocatechuic acid glucoside, protocatechuic acid, methyldigallate and N-feruloyltiramine. The regression data of citric acid was applied for the quantification of

its ester with gluconic acid, quinic acid, hydroxycitric acid, hibiscus acid, hibiscus acid hydroxyethylesther, hibiscus acid dimethylesther and hibiscus acid hydroxyethyldimethylesther. Chlorogenic acid curve was used for chlorogenic acid and derivatives such as neochlorogenic acid, methylchlorogenate and ethylchlorogenate, and also for 2-O-trans-caffeoyl-hydroxycitric acid, laricin, kinsenoside, furanosil derivative, dihydroferulic acid glucuronide and verbascoside. P-coumaric acid was used for the estimation of phenolic acids like caffeoylshikimic acid, sinapic acid, coumaroilquinic acid and the benzopyran derivative; flavonoids such as quercetin-3-sambubioside, myricetin-3-arabinogalatoside, quercetin di-O-glucoside, quercetin-3-rutinoside, eriocitrin, kaempferol-3-O-sambubioside, kaempferol-3-O-rutinoside, prodelphinidin B3 and cyanidin-3-sambubioside were quantified by rutin curve. On the other hand, quercetin-3-glucoside was used for quantification of myricetin 3-glucoside, kaempferol-3-O-glucoside, cordifolioside B and kaempferol 3-(p-coumarylglucoside). Finally, the quantification of kaempferol was performed with quercetin standard curve parameters.

Response-Surface Methodology

Response-surface methodology (RSM) by central composite rotatable design (CCRD) was performed to determine the influence of independent variables, (temperature and solvent composition, ethanol and water) on total phenolic compounds concentration. It was also important to note that the experimental design cover the entire operational range that the equipment allows to the independent variables (from 40 to 200 ºC and from 0 to 100% v/v ethanol).

A total of 10 experimental runs were carried out at different experimental conditions, 4 to the full factorial design, 4 start points and 2 center points **(Table 1)**. As is known, the center points ensure the reproducibility of this experimental design decreasing the number of experiments. In addition, these assays were carried out randomly in order to minimize errors. The experimental data obtained was processed with the program Statgraphics Centurion software XVI provided by Statpoint Technologies (Warrenton, VA, USA) in order to determine the optimum condition to obtain higher phenolic compounds content.

Run	T (ºC)	Solvent Composition (% Ethanol)	Dielectric constant (Ɛ)
1	40	50	49
2	63	85	31.4
3	63	15	60.9
4	120	100	19
5	120	50	34.7
6	120	0	50.5
7	176	85	21.8
8	176	15	34.8
9	200	50	26.8
10	120	50	34.7

Table 1. Central composite rotatable design (CCRD) independent variables.

Results and discussion

Characterization of phytochemicals from *Hs* extracts by different PLE conditions

As mentioned before, extraction is an important step for recovering bioactive compounds from plant. In this sense, PLE is considered a green technology which increase the extraction yield compared with traditional extraction methods. Hence, a complete identification and quantification of the compounds extracted by PLE from *Hs* calyces was carried out by HPLC-ESI-QTOF-MS. Compound identifications were performed by comparison with standards when available or by interpretation of the MS spectra obtained by the QTOF mass analyzer in combination with the data recovered from data bases and literature. **Table 2** describes the peak number (numbered according to their elution order), retention time, theoretical m/z, error (ppm), score and molecular formula of each tentatively characterized compound, as well as the bibliographic reference where was previously described.

The objective was to study the effect of temperature and composition of extraction solvent on chemical profiles of *Hs* extracts obtained by PLE at different experimental conditions. Thus, **Figure 1** showed a representative PLE extract base-peak chromatogram obtained at 200 °C with 50 % EtOH (PLE condition 9). A total of 52 compounds were tentatively identified and quantified among all extraction experiments.

Peak	Tentative Identification	Molecular formula	*Rt* (min)	theoretical m/z	Error (ppm)	Score	References
1	Gluconic acid ester with citric acid	$C_{12}H_{18}O_{13}$	4.642	370.0747	-4.06	79.67	(Bankova, et al., 2000)
2	Quinic acid	$C_7H_{12}O_6$	4.648	192.0634	-4.57	99.21	(Ramirez-Rodrigues, et al, 2011)
3	Hydroxycitric acid	$C_6H_8O_8$	4.769	208.0219	-3.12	96.43	(Borrás-Linares et al., 2015)
4	Hibiscus acid	$C_6H_6O_7$	4.991	190.0114	2.39	98.32	(Borrás-Linares et al., 2015)
5	Quercetin sambubioside	$C_{26}H_{28}O_{16}$	6.612	596.1377	2.86	60.96	(Rodríguez-Medina et al., 2009)
6	Hibiscus acid hydroxyethylester	$C_8H_{12}O_8$	6.8	236.0532	0.31	67.36	(Borrás-Linares et al., 2015)
7	Protocatechuic acid glucoside	$C_{13}H_{16}O_9$	6.989	316.0806	3.8	65.85	(Da-Costa-Rocha, et al, 2014)
8	Cyanidin-3-sambubioside	$C_{26}H_{28}O_{15}$	7.843	580.1428	0.28	86.37	(Da-Costa-Rocha et al., 2014)
9	Neochlorogenic acid	$C_{16}H_{18}O_9$	8.075	354.0951	-3.72	84.79	(Zhen et al., 2016)
10	Hibiscus acid dimethylester	$C_8H_{10}O_7$	8.213	218.0427	-0.63	73.7	(Herranz-López et al., 2012)
11	Protocatechuic acid	$C_7H_6O_4$	9.742	154.0266	0.56	79.53	(Da-Costa-Rocha, et al, 2014)
12	Chlorogenic acid	$C_{16}H_{18}O_9$	11.061	354.0951	-7.01	63.94	(Borrás-Linares et al., 2015)
13	Chlorogenic acid isomer II	$C_{16}H_{18}O_9$	11.668	354.0951	-7.21	77.29	(Borrás-Linares et al., 2015)
14	Hibiscus acid hydroxyethyldimethylester	$C_{10}H_{16}0_8$	12.066	264.0846	0.31	97.94	(Borrás-Linares et al., 2015)
15	Methylchlorogenate	$C_{17}H_{20}O_9$	12.286	368.1107	-8.13	67.74	(Borrás-Linares et al., 2015)
16	2-O-trans-caffeoyl-hydroxycitric acid	$C_{15}H_{14}O_{11}$	12.728	370.0536	-8.27	63.53	(Borrás-Linares et al., 2015)
17	Methyl digallate	$C_{15}H_{12}O_9$	12.927	336.0481	-7.67	67.4	(Herranz-López et al., 2012)
18	Chlorogenic acid isomer III	$C_{16}H_{18}O_9$	14.627	354.0951	0.62	80.06	(Borrás-Linares et al., 2015)
19	Laricin	$C_{16}H_{22}O_8$	15.261	342.1315	1.32	93.2	(Borrás-Linares et al., 2015)
20	Myricetin 3-arabinogalactoside	$C_{26}H_{28}O_{17}$	15.835	612.1326	-1.48	76.5	(Da-Costa-Rocha, et al, 2014)
21	Coumaroylquinic acid	$C_{16}H_{18}O_8$	16.216	338.1002	-1.29	76.43	(Borrás-Linares et al., 2015)
22	Kinsenoside	$C_{10}H_{16}O_8$	16.63	264.0845	-2.64	96.7	(Borrás-Linares et al., 2015)
23	Feruloyl derivative	$C_{21}H_{30}O_{12}$	17.646	474.1737	1.29	92.15	(Da-Costa-Rocha, et al, 2014)
24	Methylchlorogenate isomer II	$C_{17}H_{20}O_9$	18.034	368.1107	0.65	89.63	(Borrás-Linares et al., 2015)
25	Quercetin 3 7-di-O-glucoside	$C_{27}H_{30}O_{17}$	18.336	626.1483	-1.3	78.05	(Ismailov, et al., 1994)
26	Dihydroferulic acid-4-O-	$C_{16}H_{20}O_{10}$	19.23	372.1056	-0.85	95.58	(Contreras, et al., 2015)

	glucuronide						
27	Myricetin 3-glucoside	$C_{21}H_{20}O_{13}$	19.908	480.0904	-2.17	82.7	(Herranz-López et al., 2012)
28	Quercetin sambubioside isomer II	$C_{26}H_{28}O_{16}$	20.184	596.1377	-0.37	95.03	(Rodríguez-Medina et al., 2009)
29	Ethylchlorogenate	$C_{18}H_{22}O_9$	21.432	382.1264	-0.06	98.71	(Borrás-Linares et al., 2015)
30	Caffeoylshikimic acid isomer I	$C_{16}H_{16}O_8$	21.47	336.0845	-1.51	75.3	(Da-Costa-Rocha, et al, 2014)
31	Caffeoylshikimic acid isomer II	$C_{16}H_{16}O_8$	21.978	336.0845	-2.12	80.33	(Da-Costa-Rocha, et al, 2014)
32	Quercetin-3-rutinoside	$C_{27}H_{30}O_{16}$	23.209	610.1534	-0.74	98.32	(Da-Costa-Rocha, et al, 2014)
33	Eriocitrin	$C_{27}H_{32}O_{15}$	24.325	596.8504	-0.85	97.65	(Wang et al., 2016)
34	Kaempferol-3-O-sambubioside	$C_{26}H_{28}O_{15}$	25.041	580.1428	-3.16	80.03	(Da-Costa-Rocha, et al, 2014)
35	Verbascoside	$C_{29}H_{36}O_{15}$	25.312	624.2054	2.27	85.28	(Oyourou, et al., 2013)
36	Quercetin 3-glucoside	$C_{21}H_{20}O_{11}$	25.836	464.0955	-0.45	99.78	(Borrás-Linares et al., 2015)
37	Sinapic acid	$C_{11}H_{12}O_5$	26.118	224.0685	5.1	73.48	(Min Zhang, 2011)
38	Ethylchlorogenate isomer II	$C_{18}H_{22}O_9$	26.548	382.1264	-0.38	88.9	(Borrás-Linares et al., 2015)
39	Kaempferol-3-O-rutinoside	$C_{27}H_{30}O_{15}$	28.138	594.1585	-1.3	97.46	(Herranz-López et al., 2012
40	Ethylchlorogenate isomer III	$C_{18}H_{22}O_9$	28.69	382.1264	-0.76	86.5	(Borrás-Linares et al., 2015)
41	Methylepigallocatechin	$C_{16}H_{16}O_7$	29.126	320.0896	-2.09	78.86	(Herranz-López et al., 2012)
42	Kaempferol-3-O-glucoside	$C_{21}H_{20}O_{11}$	30.925	448.1006	-5.72	75.56	(Da-Costa-Rocha, et al, 2014)
43	Cordifolioside B	$C_{22}H_{32}O_{13}$	32.355	504.1843	1.18	94.34	(Shen et al., 2012)
44	Myricetin	$C_{15}H_{10}O_8$	37.427	318.0376	-2.48	96.03	(Borrás-Linares et al., 2015)
45	4H-1-Benzopyran-4-one derivative	$C_{35}H_{34}O_{18}$	37.995	742.1745	-0.62	99.06	(Alluis, et al., 2000)
46	N-feruloylyiramine	$C_{18}H_{19}NO_4$	39.497	313.1314	7.51	68.2	(Borrás-Linares et al., 2015)
47	Prodelphinidin B3	$C_{30}H_{26}O_{14}$	42.4	610.1323	-0.41	93.45	(Herranz-López et al., 2012)
48	N-feruloyltyramine Isomer II	$C_{18}H_{19}NO_4$	43.57	313.1314	-0.43	98.95	(Borrás-Linares et al., 2015)
49	Kaempferol 3-(p-coumarylglucoside)	$C_{30}H_{26}O_{13}$	44.508	594.1373	-3.25	90.84	(Da-Costa-Rocha, et al, 2014)
50	Quercetin	$C_{15}H_{10}O_7$	45.148	302.0427	-0.84	97.12	(Borrás-Linares et al., 2015)
51	Apigenin (IS)	$C_{15}H_{10}O_5$	46.484	270.0528	-1.92	94.52	IS
52	Kaempferol	$C_{15}H_{10}O_6$	46.799	286.0477	-0.4	89.49	(Zhen et al., 2016)

Table 2. HPLC-ESI-QTOF-MS data of the identified compounds in *Hs* calyces extracted by PLE 9 condition.

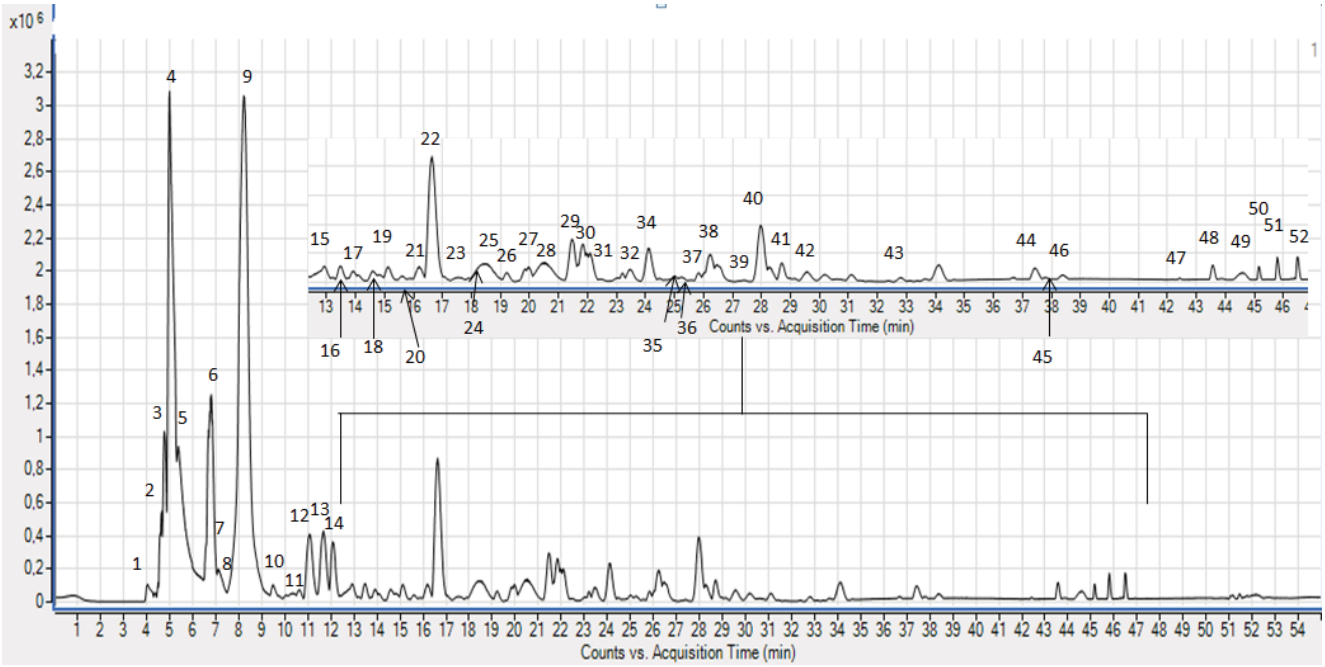


Figure 1. Base peak chromatogram (BPC) obtained from HPLC-ESI-QTOF-MS analysis of the *Hs* extract obtained under different PLE extraction conditions. Peaks have been numbered according to the elution order.

In addition, the sensitivity and validation of this assay was evaluated based on the limits of detection (LODs) whose signal / noise ratio was 3 and limit of quantification (LOQs) whose signal / noise ratio was 10 for each compound in standard solutions. All respective data including regression equation, linearity range and correlation coefficients are described in **Table 3**. Moreover, the repeatability was deduced by relative standard deviations (RSDs) for multiple injections of the same sample and intermediate precision was determined based on intra- and inter- day variances. Thus, *Hs* extract obtained with the PLE condition 9 was injected several times (n = 6) on the same day (intraday precision) and 3 times on 2 consecutive days (interday precision, n=12). The intraday repeatability of the developed method for all analytes ranged from 0.81 to 7.87%, whereas the interday repeatability ranged from 1.03 to 9.60%.

Standard	Calibration range (mg/L)	LOD (mg/L)	LOQ (mg/L)	Calibration equation	R^2
p-coumaric acid	LOQ-100	0.0006 ± 0.0002	0.0020 ± 0.0006	y = 0.0859 x + 0.1541	0.987
Gallic acid	LOQ-100	0.0028 ± 0.0010	0.009 ± 0.003	y = 0.0468 x + 0.1761	0.960
Citric acid	LOQ-100	0.005 ± 0.002	0.015 ± 0.005	y = 0.0408 x + 0.6539	0.995
Chlorogenic acid	LOQ-100	0.010 ± 0.002	0.033 ± 0.006	y = 0.0333x + 1.1761	0.983
Quercetin	LOQ-100	0.010 ± 0.003	0.03 ± 0.01	y = 0.0973x + 0.3613	0.951
Myricetin	LOQ-100	0.003 ± 0.001	0.010 ± 0.003	y = 0.1129x + 0.2238	0.980
Quercitrin	LOQ-100	0.06 ± 0.01	0.19 ± 0.04	y = 0.0205x + 1.6884	0.956
Quercetin-3-glucoside	LOQ-100	0.0040 ± 0.0007	0.013 ± 0.002	y = 0.0262x + 2.1191	0.960
Rutin	LOQ-100	0.004 ± 0.001	0.012 ± 0.003	y = 0.0852x + 0.2182	0.963

Table 3. Analytical parameters of the proposed method for the quantification of phenolic compounds

The identified compounds could be classified into four families: organic acids and derivatives, phenolic acids and derivatives, other polar compounds and flavonoids, which were divided in two groups due to different chemical structure and different demonstrated thermo-stability. The total content of each family and the concentration of individual compound in all extraction conditions expressed in mg g^{-1} are recovered in **Table 4** whose significant figures were rounded depending on its standard deviation. For a clearer explanation, the results will be explained separately depend on the families in the following sections.

Peak	Compound	PLE 1	PLE 2	PLE 3	PLE 4	PLE 5	PLE 6	PLE 7	PLE 8	PLE 9	PLE 10
					Organic acids						
1	Gluconic acid ester with citric acid	6.4 ± 0.5	6.4 ± 0.3	4.61 ± 0.06	2.9 ± 0.1	3.5 ± 0.3	3.6 ± 0.1	7.1 ± 0.5	1.7 ± 0.1	18.9 ± 0.9	3.8 ± 0.1
2	Quinic acid	<LOQ	<LOQ	<LOQ	0.60 ± 0.07	0.31 ± 0.03	0.053 ± 0.007	5.4 ± 0.5	0.57 ± 0.03	13.1 ± 0.8	0.5 ± 0.1
3	Hydroxicitric acid	2.0 ± 0.7	4.4 ± 0.6	5.07 ± 0.08	4.0 ± 0.3	7.8 ± 0.4	4.9 ± 0.4	261 ± 9	75.9 ± 4	22 ± 3	6.5 ± 0.4
4	Hibiscus acid	20 ± 2	14.7 ± 0.7	21 ± 1	20 ± 1	20 ± 2	29.2 ± 0.8	205 ± 1	52 ± 6	131 ± 7	21 ± 1
6	Hibiscus acid hydroxyethylester	5.1 ± 0.4	14.9 ± 0.2	<LOQ	26 ± 1	21.2 ± 0.5	<LOQ	28 ± 2	1.9 ± 0.5	90 ± 2	20 ± 1
10	Hibiscus acid dimethylester	10.0 ± 0.5	21 ± 2	3.14 ± 0.08	72 ± 7	60 ± 1	0.7 ± 0.04	44.3 ± 0.8	71 ± 3	480.0 ± 0.1	60.5 ± 0.9
14	Hibiscus acid hydroxyethyldimethylester	<LOQ	1.4 ± 0.1	<LOQ	22.0 ± 0.2	4.63 ± 0.2	<LOQ	67 ± 1	0.85 ± 0.03	19 ± 1	3.5 ± 0.4
16	2-O-trans-caffeoyl-hydroxycitric acid	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Total	**43.5**	**62.8**	**33..82**	**147.5**	**117.44**	**38.453**	**617.8**	**203.92**	**774.0**	**115.8**
					Phenolic acids						
7	Protocatechuic acid glucoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	1.09 ± 0.02	<LOQ
9	Neochlorogenic acid	2.9 ± 0.06	1.6 ± 0.1	2.33 ± 0.09	1.6 ± 0.1	3.1 ± 0.3	2.3 ± 0.1	5.2 ± 0.3	1.8 ± 0.1	23 ± 1	3.8 ± 0.4
11	Protocatechuic acid	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.54 ± 0.02	<LOQ	0.9 ± 0.1	<LOQ
12	Chlorogenic acid	3.10 ± 0.04	1.7 ± 0.4	<LOQ	1.6 ± 0.1	4.6 ± 0.3	<LOQ	8.9 ± 0.6	3.5 ± 0.2	43 ± 3	5.5 ± 0.7
13	Chlorogenic acid isomer II	2.8 ± 0.1	1.2 ± 0.1	1.88 ± 0.03	0.8 ± 0.2	4.2 ± 0.2	1.8 ± 0.1	6 ± 1	3.2 ± 0.2	19 ± 3	3.8 ± 0.2
15	Methylchlorogenate	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
17	Methyl digallate	1.07 ± 0.01	0.04 ± 0.02	0.376 ± 0.007	0.45 ± 0.06	1.8 ± 0.2	0.06 ± 0.05	1.1 ± 0.1	<LOQ	12 ± 2	1.9 ± 0.1
18	Chlorogenic acid isomer III	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
21	Coumaroylquinic acid	0.03 ± 0.01	0.022 ± 0.004	<LOQ	0.02 ± 0.01	0.23 ± 0.04	<LOQ	1.3 ± 0.1	0.13 ± 0.02	2.9 ± 0.1	0.3 ± 0.1
24	Methyl chlorogenate isomer II	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.11 ± 0.07	<LOQ	0.58 ± 0.04	<LOQ
26	Dihydroferulic acid-4-O-glucuronide	0.024 ± 0.005	0.08 ± 0.02	<LOQ	0.08 ± 0.03	0.20 ± 0.03	<LOQ	1.31 ± 0.05	<LOQ	1.8 ± 0.2	0.35 ± 0.02
29	Ethylchlorogenate	<LOQ	0.88 ± 0.01	<LOQ	0.86 ± 0.07	0.57 ± 0.06	<LOQ	10.8 ± 0.3	0.02 ± 0.01	2.2 ± 0.1	0.32 ± 0.01
30	caffeoylshikimic acid	0.47 ± 0.03	0.73 ± 0.02	<LOQ	1.28 ± 0.06	1.9 ± 0.1	<LOQ	12 ± 1	0.97 ± 0.06	11 ± 2	2.6 ± 0.2
31	caffeoylshikimic acid isomer II	<LOQ	<LOQ	<LOQ	<LOQ	0.46 ± 0.04	<LOQ	2.0 ± 0.5	0.62 ± 0.07	4.4 ± 0.9	0.23 ± 0.03
35	Verbascoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
37	Sinapic acid	<LOQ	ND	<LOQ	<LOQ	<LOQ	<LOQ	ND	ND	0.436 ± 0.001	<LOQ
38	Ethylchlorogenate isomer II	<LOQ	0.341 ± 0.004	<LOQ	2.67 ± 0.09	0.37 ± 0.05	<LOQ	19 ± 1	0.052 ± 0.004	3.1 ± 0.4	0.21 ± 0.06
40	Ethylchlorogenate isomer III	<LOQ	0.67 ± 0.06	<LOQ	3.4 ± 0.3	0.52 ± 0.08	<LOQ	21.0 ± 0.7	0.42 ± 0.04	3.9 ± 0.9	0.85 ± 0.07
	Total	**10.394**	**7.263**	**9.172**	**12.76**	**17.95**	**8.32**	**89.26**	**10.712**	**129.306**	**19.86**

Table 4 (Continued)

Flavonoids; Flavonols and derivatives, catechins and flavanones											
5	Quercetin sambubioside	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ
20	Myricetin 3-arabinogalactoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.31 ± 0.06	<LOQ
25	Quercetin 3 7-di-O-glucoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
27	Myricetin 3-glucoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
28	Quercetin sambubioside isomer II	0.20 ± 0.01	0.30 ± 0.04	<LOQ	0.08 ± 0.04	0.07 ±0.05	<LOQ	0.26 ± 0.07	<LOQ	1.1 ± 0.1	0.36 ± 0.03
32	Quercetin-3-rutinoside	0.04 ± 0.02	0.25 ± 0.04	<LOQ	0.42 ± 0.07	0.28 ± 0.01	<LOQ	0.62 ± 0.06	<LOQ	0.9 ± 0.1	0.28 ± 0.02
33	Eriocitrin	ND	<LOQ	ND	ND	ND	ND	ND	ND	ND	ND
34	kaempferol-3-O-sambubioside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ	<LOQ
36	Quercetin-3-glucoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.44 ± 0.07	<LOQ	0.85 ± 0.04	<LOQ
39	kaempferol-3-O-rutinoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
41	Methylepigallocatechin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.051 ± 0.006	<LOQ	<LOQ	<LOQ
42	Kaempferol -3-O-glucoside	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ
44	Myricetin	0.059 ± 0.004	0.29 ± 0.01	<LOQ	0.47 ± 0.06	0.50 ± 0.03	<LOQ	2.7 ± 0.1	<LOQ	2.41 ± 0.09	0.30 ± 0.01
49	kaempferol 3-(p-coumarylglucoside)	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
50	quercetin	0.028 ± 0.005	0.49 ± 0.02	<LOQ	0.37 ± 0.08	0.5 ± 0.1	<LOQ	1.332 ± 0.009	<LOQ	0.48 ± 0.08	0.75 ± 0.03
52	kaempferol	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Total	**0.327**	**1.33**	**0**	**1.34**	**1.35**	**0**	**5.403**	**0**	**6.05**	**2.09**
Flavonoids: Anthocyanins and proanthocyanins											
8	Cyanidin -3- sambubioside	0.33 ± 0.01	<LOQ	<LOQ	ND	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ
47	Prodelphinidin B3	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.24 ± 0.05	<LOQ	0.36 ± 0.01	<LOQ
	Total	**0.33**	**0**	**0**	**0**	**0**	**0**	**0.24**	**0**	**0.36**	**0**
Other polar compounds											
19	Laricin	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
22	Kinsenoside	0.03 ± 0.01	4.5 ± 0.2	<LOQ	83 ± 1	21.6 ± 0.8	<LOQ	170 ± 5	1.67 ± 0.07	69 ± 4	22.3 ± 0.5
23	Furanosil derivative	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
43	Cordifolioside B	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
45	4H-1-Benzopyran-4-one derivative	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.28 ± 0.02	<LOQ
46	N-feruloyltyramine	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	0.20 ± 0.06	<LOQ
48	N-feruloyltyramine isomer II	0.20 ± 0.02	0.40 ± 0.03	<LOQ	0.53 ± 0.04	0.32 ± 0.02	<LOQ	2.18 ± 0.02	<LOQ	2.4 ± 0.1	0.23 ± 0.02
	Total	**0.23**	**4.9**	**0**	**83.53**	**22.92**	**0**	**172.18**	**1.67**	**71.88**	**22.53**

Table 4. Concentrations expressed in mg g^{-1} extract of phenolic compounds and their derivatives in *Hs* extracts obtained under different PLE conditions. ND: Compound not detected; <LOQ: Compound below the limit of quantification.

Organic acids and derivatives

These compounds showed to be the major family in all extraction conditions **(Figure 2a)**. This family comprised the compounds gluconic acid esther with citric acid, quinic acid, hydroxicitric acid, hibiscus acid, hibiscus acid hydroxyethylesther, hibiscus acid hydroxyethyldimethylesther and 2-O-trans-caffeoyl-hydroxycitric acid. These organic acids were described in previous *Hs* studies [12–14], except gluconic acid derivative, which has been described in propolis [15].

Respect to their total contents quantified in the different extracts, the lowest total concentration was found in PLE 3 (whose extract condition was 63 ºC and 15% of ethanol) with 33.82 mg g^{-1} extract, whereas the highest value (774.0 mg g^{-1} extract) was detected in PLE 9 performed at 200 ºC and 50% of ethanol **(Figure 2).**

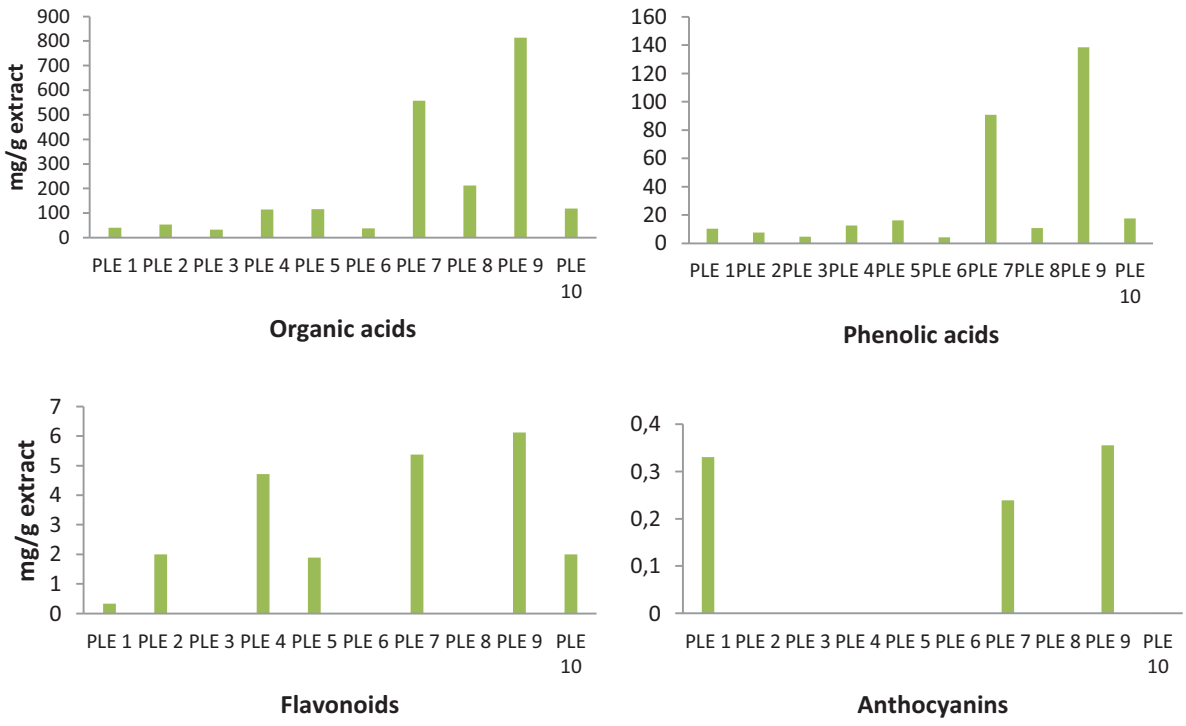


Figure 2. Concentration of each group of compounds characterized in *Hs* extract under different PLE conditions. a) organic acids, b) phenolic acids, c) flavonoids, d) anthocyanins.

Regarding to individual concentrations, gluconic acid esther with citric acid (Peak 1), quinic acid (Peak 2), hibiscus acid hydroxyethylester (Peak 6) and hibiscus acid dimethylester (Peak 10) presented their higher concentration also in PLE 9. For these compounds, the concentration ranges were as follows: for gluconic acid esther with citric acid from 1.7 ± 0.1 in PLE 8 to 18.9 ± 0.9 mg g^{-1} extract; from 0.053 ± 0.007 in PLE 6 to 13.1 ± 0.8 mg g^{-1} extract for quinic acid; for hibiscus acid hydroxyethylester was from 1.9 ± 0.5 in PLE 8 to 90 ± 2 mg g^{-1} extract; and finally from 0.70 ± 0.04 in PLE 6 to 480.0 ± 0.1 mg g^{-1} extract for hibiscus acid dimethylester. Nevertheless, some of these compounds were below the limit of quantification in some cases. In PLE 1, PLE2 and PLE 3 quinic acid was present in amounts below the limit of quantification; moreover, hibiscus acid hydroxyethylester was below the limit of quantification in PLE 3 and PLE 6.

As it can be observed, for this kind of compounds a large amount was obtained in PLE 9 compared to other extraction conditions. However, hydroxicitric acid (Peak 3), hibiscus acid (Peak 4) and hibiscus acid hydroxyethyldimethylester (Peak 14) were obtained in higher concentration in PLE 7 (performed at 176 ºC and 85 % of ethanol) compared to the other extractions. In this case, the concentration ranges were from 2.0 ± 2 in PLE 1 to 261 ± 9 mg g^{-1} extract, from 14.7 ± 0.7 in PLE 2 to 205 ± 1 mg g^{-1} extract and from 0.85 ± 0.03 in PLE 8 to 67 ± 1 mg g^{-1} extract, respectively. Despite that, hibiscus acid hydroxyethyldimethylester was below the limit of quantification in PLE 1, PLE 3 and PLE 6. In addition, 2-O-trans-caffeoyl-hydroxicitric acid (Peak 16) was also below the limit of quantification in all extraction conditions.

The results highlight that PLE performed at high temperature and high percentage of ethanol seemed to increase the organic acids concentration. These results were in accordance to a previous study [16], in which the effect of temperature and solvent composition on organic acids content from radish sprouts was studied. To this end, microwave – assisted and ultrasound – assisted extraction techniques were applied and the results showed that the optimal extraction condition was achieved with the first extraction technique using the highest applied temperature. Hence, higher temperatures and higher ethanol concentration seemed to have a positive effect on the collection of this family group. Moreover, in the present study, most of the organic acids found resulted to be ester derivatives, so it could be presume that higher temperatures and percentages of ethanol would favor the collection of those derivatives. Nevertheless, according the author's knowledge, it is the first time that the effect of temperature and solvent concentration on organic acids concentration in *Hs* has been evaluated by PLE.

Phenolic acids and derivatives

A total of 18 compounds belonging to this family group were found in some of the extraction conditions, named as protocatechuic acid and its glucoside form, four isomers of chlorogenic acid, two isomers of methylchlorogenate and three of ethylchlorogenate, coumaroylquinic acid, two isomers of caffeoylshikimic acid and sinapic acid. All of these compounds have been found in previous *Hs* researches [12,14,17,18]. Moreover, dihydroferulic acid-4-O-glucuronide and verbascoside, also characterized in the studied extracts were previously found in cranberry syrups and

Lippia citriodora leaves respectively (Contreras, Arráez-Román, Fernández-Gutiérrez, & Segura-Carretero, 2015; Quirantes-piné, Herranz-lópez, Funes, & Borrás-linares, 2013).

In this family, the highest total concentration was obtained also for PLE 9 (129.306 mg g^{-1} extract); whereas the lowest total content was found in PLE 2 (7.263 mg g^{-1} extract), followed by PLE 6 (8.32 mg g^{-1} extract) and PLE 3 (9.172 mg g^{-1} extract). These extract conditions corresponding to the lowest temperature (63 ºC) in PLE 2 and PLE 3 and the lowest ethanol concentration in the case of PLE 6 (0%).

With regard to the individual compound quantification, methylchlorogenate (Peak 15), chlorogenic acid isomer III (Peak 18) and verbascoside (Peak 35) were below the limit of quantification in all extraction conditions. Moreover, protocatechuic acid glucoside (Peak 7) and sinapic acid (Peak 37) could only be quantified in PLE 9 (1.09 ± 0.02 mg g^{-1} extract and 0.436 ± 0.001 mg g^{-1} extract, respectively). On the other hand, protocatechuic acid (Peak 11) and methylchlorogenate isomer II (Peak 24) were above the limit of quantification only in PLE 9 (0.9 ± 0.1 mg g^{-1} extract and 0.58 ± 0.04 mg g^{-1} extract, respectively) and PLE 7 (0.54 ± 0.02 mg g^{-1} extract and 0.11 ± 0.07 mg g^{-1} extract, respectively). For the other phenolic acids, PLE 9 was the extract condition with higher concentration **(Figure 2)**, except for ethychlorogenate and their derivatives (Peak 29, 38 and 40), where PLE 7 obtained a higher amount of these compounds. Thus, the concentration range of each individual compound was the following: for neochlorogenic acid (Peak 9) from 1.6 ± 0.1 in PLE 2 and PLE 4 to 23 ± 1 mg g^{-1} extract; chlorogenic acid (Peak 12) was found from 1.6 ± 0.1 in PLE 4 to 43 ± 3 mg g^{-1} extract, despite this compound was below the limit of quantification in PLE 3 and PLE 6.

Chlorogenic acid isomer II (Peak 13) was present from 0.8 ± 0.2 in PLE 4 to 19 ± 3 mg g^{-1} extract; the concentration range of methyldigallate (Peak 17) was from 0.04 ± 0.02 in PLE 2 to 12 ± 2 3 mg g^{-1} extract, being below the limit of quantification in PLE 8. For coumaroylquinic acid (Peak 21), the highest concentration was 2.9 ± 0.1 mg g^{-1} extract and the lowest was found in PLE 4 (0.02 ± 0.01 mg g^{-1} extract). In addition, this compound could not be quantified in PLE 3 and PLE 6. Respect to dihydroferulic acid-4-O-glucuronide (Peak 26), it was present from 0.024 ± 0.005 in PLE 1 to 1.8 ± 0.2 mg g^{-1} extract, being below the limit of quantification in PLE 3, PLE 6 and PLE 8. The first isomer of caffeoylshikimic acid (Peak 30) was also more abundant in PLE 9 (11 ± 2 mg g^{-1} extract) versus to PLE 1 (0.47 ± 0.03 mg g^{-1} extract); the second isomer (peak 31) was from 0.23 ± 0.03 in PLE 10 to 4.4 ± 0.9 mg g^{-1} extract. By the same token than other compounds, in PLE 3 and PLE 6 these compounds were below the limit of quantification; as well as in PLE 1, PLE 2, and PLE 4 in the case of compound 31. Finally, ethylchlorogenate and their isomers (Peak 29, 38, 40) were below the limit of quantification in PLE 1, PLE 3 and PLE 6. However, in PLE 7 the concentrations were the highest (10.8 ± 0.3, 19 ± 1 and 21.0 ± 0.7 mg g^{-1} extract, respectively) and the lowest concentration was found in PLE 8 (0.02 ± 0.01, 0.052 ± 0.004 and 0.42 ± 0.04 mg g^{-1} extract, respectively).

Therefore, for phenolic acids and derivatives, PLE 3 and PLE 6 were also the extraction conditions with the lowest phenolic acids concentrations, which coincide with the lowest percentage of ethanol and temperature. However, PLE 7 and PLE 9,

whose dielectric constant was lower, were performed at higher temperatures and reported higher concentration of these compounds.

These results could be explained due to most phenolic acid derivatives present in plant matrixes are stored in vacuoles, and consequently, the use of high temperature in combination with alcoholic or organic solvents favored their extraction [21].

Flavonoids

This family was divided in two groups. On one hand, flavonols as quercetin, kaempferol and myricetin and their derivatives were the major flavonoids found in these extraction conditions. On the other hand, anthocyanins and proanthocyanins were evaluated separately respect to the other flavonoids due to these compounds possess different chemical structure and demonstrated lower thermos-stability [22] .

a) Flavonoids: Flavonols and derivatives, catechins and flavonones

16 peaks were identified of which 14 were flavonols and derivatives (two isomers of quercetin-sambubioside, myricetin 3-arabinogalactoside, quercetin-3-rutinoside, kaempferol-3-O-sambubioside, quercetin-3-glucoside, kaempferol-3-O-rutinoside, kaempferol-3-O-glucoside, myricetin, kaempferol 3-(p-coumaryglucoside), quercetin and kaempferol), one catechin (methylepigallocatechin) and one flavonones (eriocitrin). All of these flavonoids have been found in earlier *Hs* studies [12,14,17,23], althogh quercetin 3 7-di-glucoside and eriocitrin that were present in cotton and Chinese plants (Ismailov, Karimdzhanov, Yu, & Rakhimkhanov, 1994;Wang et al., 2016).

In term of quantification, as well as phenolic acids, PLE 9 and PLE 7 were the two extraction conditions that extracted higher concentration of this type of flavonoids (6.05 mg g^{-1} extract and 5.403 mg g^{-1} extract respectively). On the contrary, PLE 3, PLE 6 and PLE 8 with an ethanol concentration lower than 15 % did not obtain quantitatively amounts.

Indeed, only six compounds were above the limit of quantification in some extraction condition. Quercetin 3 7-di-O-glucoside (Peak 25), myricetin 3-glucoside (Peak 27), kaempferol-3-O-rutinoside (Peak 39), kaempferol 3-(p-coumarylglucoside) (Peak 49) and kaempferol (Peak 52) were below the limit of quantification in all extraction conditions. Besides, quercetin-sambubioside (Peak 5) was not detected in PLE 4 and PLE 7, and kaempferol- 3-O-sambubioside (Peak 34) and kaempferol – 3-glucoside (Peak 42) were below the limit of quantification in all extraction conditions and not detected in PLE 6. On the other hand, eriocitrin (Peak 33) was the only flavonoid that was detected in PLE 2.

The compounds belonging to this group found above the limit of quantification were: myricetin 3-arabinogalactoside (Peak 20) that only could be quantified in PLE 9 (0.31 ± 0.06 mg g^{-1} extract) and methylepigallocatechin (Peak 41) only in PLE 7 (0.051 ± 0.006 mg g^{-1} extract). Myricetin and quercetin (Peaks 44 and 50, respectively) were obtained in higher amount in PLE 7, with a concentration range of 0.059 ± 0.004 in PLE1 to 2.7 ± 01 mg g^{-1} extract for myricetin and from 0.028 ± 0.005 to 1.332 ± 0.009 mg g^{-1} extract for quercetin. Nevertheless, both compounds were below the limit of quantification in PLE 3, PLE 6 and PLE 8. Other compounds as quercetin-sambubioside

isomer II (Peak 28), quercetin-3-rutinoside (Peak 32) and quercetin 3-glucoside (Peak 36) were extracted in higher concentration in PLE 9 showing ranges from 0.07 ± 0.05 in PLE 4 to 1.1 ± 0.1 mg g^{-1} extract, from 0.04 ± 0.02 in PLE 1 to 0.9 ± 0.1 mg g^{-1} extract and from 0.44 ± 0.07 in PLE 7 to 0.85 ± 0.04 mg g^{-1} extract, respectively. In addition, it is important to note that quercetin 3-glucoside was only above the limit of quantification in PLE 7 and PLE 9; while quercetin-sambubioside isomer II and quercetin-3-rutinoside were below the limit of quantification in PLE 3, PLE 6 and PLE 8.

Therefore, all compounds identified in PLE 3, PLE 6 and PLE 8, whose extraction parameters were the lower ethanol amount (from 0 to 15 %, v/v) and higher dielectric constant, were below the limit of quantification **(Figure 2)**. Conversely, higher percentage of ethanol and lower dielectric constant exerts a positive effect on the flavonoids content. Similar results were observed in a previous study [26], in which the effect of ethanol concentration and temperature using PLE on total flavonoid content in *Momordica charantia* was determined. In this work, total flavonoid content was measured using the Dowd method and expressed by g quercetin equivalents (QE) per 100 g dried powder. The results showed that high ethanol concentration and high temperature obtained the maximum amount of flavonoids (1.48 g QE per 100 g at 160 ºC and 80 % (v/v) ethanol). In contrast, the minimum was found at 61 ºC and 20 % ethanol (0.07 g QE per100 g). The same results have been observed for this type of *Hs* flavonoids extracted by PLE.

b) Anthocyanins and proanthocyanins

Two compounds were identified belonging to this phenolic family in these *Hs* extracts. Cyanidin-3-sambubioside (Peak 8) and prodelphinidin B3 (Peak 47) have been described in other *Hs* researches [14,17].

This kind of compounds could only be quantified in PLE 1, PLE7 and PLE 9 with concentration values of 0.33 ± 0.01, 0.24 ± 0.05 and 0.36 ± 0.01 mg g^{-1} extract respectively. However, in the rest of extraction conditions these two compounds were below the limit of quantification and in PLE 4 and PLE 8 cyanidin-3-sambubioside was not detected.

Besides, cyanidin-3-sambubioside was only above the limit of quantification in PLE 1 (0.33 ± 0.01 mg g^{-1} extract), extract obtained with the lowest temperature applied (40 ºC). These findings indicate that temperature has a negative effect on the extraction of this kind of compounds. Indeed, the thermal kinetic degradation of anthocyanins in *Hs* infusion was already studied by Aurelio, Edgardo, & Navarro-Galindo, (2008). On the contrary, the opposite effect occurs in the case of prodelphinidin B3, which was extracted in larger quantities in PLE 7 and PLE 9 (0.24 ± 0.05 and 0.36 ± 0.01 mg g^{-1} extract, respectively). As mentioned before, these extract conditions present higher temperatures (176 ºC in PLE 7 and 200 ºC in PLE 9) (Figure 2). This difference observed for both compounds could be related to chemical structure, indeed, proanthocyanidins unlike anthocyanins, are composed by two, three, four or more flavan-3-ol molecules that formed stable composition. Moreover, proanthocyanidins are mainly located in cell vacuoles while anthocyanins like cyanidin-3-sambubioside are located in the upper cellular layers [27]. These characteristics

suggest that prodelphinidin B3 is more temperature – stable than cyanidin-3-sambubioside.

Other polar compounds

In addition to the previously mentioned phenolic compounds, it was possible to identify other seven polar compounds present in all extraction conditions characterized by HPLC-ESI-QTOF-MS, such as laricin, kinsenoside, a furanosil derivative and n-feruloyltyramine which have been described in previous *Hs* studies [12,17]. Additionally, cordifolioside B and a benzopyran derivative have been characterized in the Hs extracts and have been described in other plants [28,29].

Regarding to individual quantification, laricin (Peak 19), the furanosil derivative (Peak 23) and cordifolioside B (Peak 43) were below the limit of quantification in all extractions, whereas the benzopyran derivative (Peak 45) and n-feruloyltyramine (Peak 46) could only be quantified in PLE 9 with concentrations of 0.28 ± 0.02 and 0.20 ± 0.06 mg g^{-1} extract, respectively. The major compound belonging to this family was kinsenoside (Peak 22), whose concentration range was from 0.03 ± 0.01 in PLE 1 to 170 ± 5 mg g^{-1} extract in PLE 7, followed by PLE 4 (83 ± 1 mg g^{-1} extract). Besides, this compound was below the limit of quantification in PLE 3 and PLE 6 as well as all compounds of this family group.

Therefore, PLE 7 was the experimental run that higher concentration of these polar compounds obtained (172.18 mg g^{-1} extract). This extraction was carried out using high percentage of ethanol and high temperature (85 % and 176 ºC) although not

reaching the extremes (200 ºC in PLE 9 and 100 % v/v in PLE 4). Therefore, it seems that for these compounds the results are in concordance with the ones founds for the rest of groups.

To sum up, a total of 51 compounds were identified in conditions PLE 1, PLE 2, PLE 3, PLE 5, PLE 9 and PLE 10 (PLE 5 and PLE 10 represent the two central points). However, in PLE 3 only 7 compounds were above the limit of quantification while in PLE 6, from the 50 identified compounds only 8 were above the limit of quantification. In contrast, 51 and 48 compounds were identified in PLE 9 and PLE 7, of which 33 and 29 compounds were quantified. Also, it is important to note that PLE 3 and PLE 6 correspond to the lowest ethanol amount in the solvent mixture (15 and 0 % v/v of ethanol, respectively) whereas PLE 9 and PLE 7 were performed with higher temperature and higher percentage of ethanol. Thus, the higher concentration of phenolic compounds was found with PLE 9 extract condition **(Figure 3)**. These finding are in line with the outcome of previous investigation, in which the antioxidant capacity and anthocyanin contents were measured in *Hs* extract when they were heated and stored at different temperatures [30]. To this end, several antioxidant assays were performed and all of these assays showed an increase in the percentage of phenolic compounds at higher temperature, except for anthocyanins (DEL assay) that decreased from about 80 % to about 50 %.

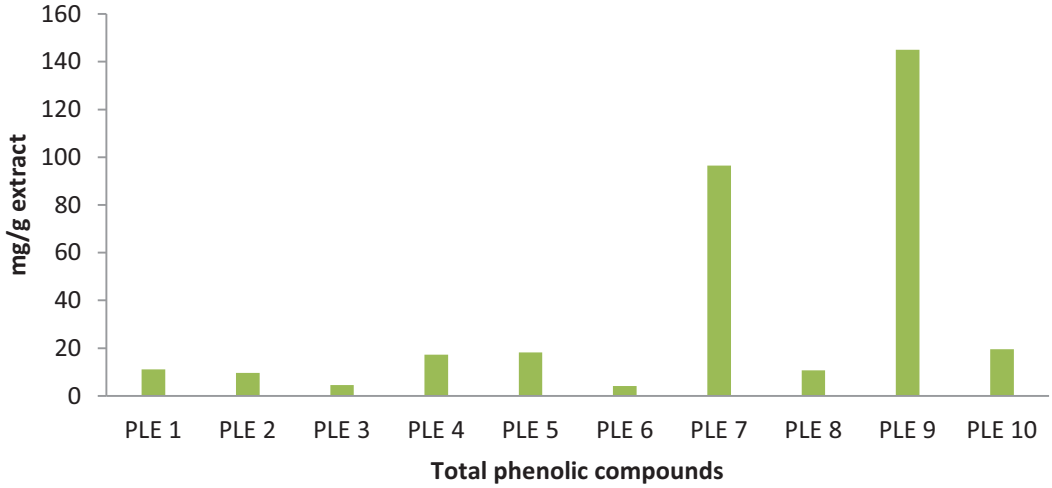


Figure 3. Concentration of total phenolic compounds characterized in *Hs* extract under different PLE conditions.

These results highlight that the majority of phenolic compounds are better extracted from *Hs* calyces by PLE with high temperatures and percentages of ethanol in the aqueous extraction mixtures, except for anthocyanins, compounds that are very sensitive to high temperatures.

As previously mentioned, the extraction conditions are strongly important to obtain higher amount of bioactive compounds. Moreover, it is necessary to evaluate the impact of temperature and solvent mixture on the recovery of phytochemicals. As a conclusion, the results obtained in this work showed that PLE 9 was the best extraction condition for phenolic acids and flavonoids such as flavonols and derivatives and proanthocyanins, the main bioactive compounds present in *Hs*. The experiment PLE 9 was carried out at the maximum temperature allowed by the equipment (200 ºC) and using 50 % of ethanol as solvent. However, similar results were obtained in PLE 7 with a temperature and percentage of ethanol of 176 ºC and 85 %. Therefore, these

results highlighted that high temperatures and high ethanol amount increased the concentration of phenolic compounds **(Figure 4)**.

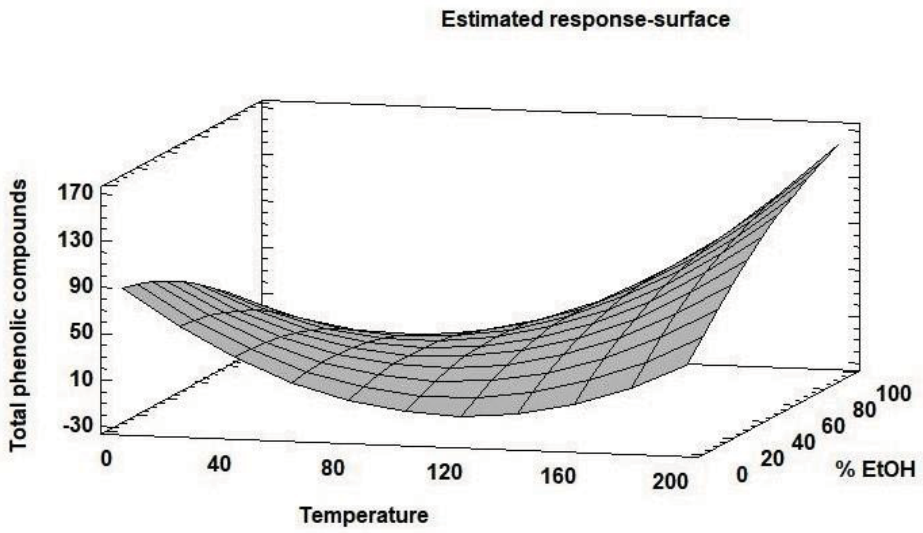


Figure 4. 3D plot of response surface for the effects of solvent and temperature on the extraction of total phenolic compounds.

Optimization of total phenolic compounds extraction by RSM

RSM was applied to maximize phenolic compound recovery. **Table 5** summarizes the statistical parameters of the experimental design. According to the results, the model presented a great correlation coefficient (R^2=0.89) indicating a slight variance of data and a good prediction of the model to extract these compounds. The independent variables, temperature and ethanol, exert effect on the total polar compounds (*p*-value < 0.05). With regards to quadratic effects, temperature interactions were also significant. Hence, keeping only the significant parameters the model equation provided was (Eq.1):

Phenolic compounds = 109,836 - 1,96004A - 0,952867B + 0,00839877AA + 0,0107168AB (Eq. 1)

In addition, the lack of fit was not significant (p-value > 0.05), which indicates that this model is suitable to the data observed at 95% confidence level. Regarding the theoretical and suitable model, the optimized conditions to maximize the phenolic compounds concentrations by PLE were 200 ºC and 100 % of ethanol. In the current work, the optimum extraction conditions were established taking into account the total phenolic compounds calculated as a sum of individual phenolic concentrations determined by HPLC-ESI-TOF MS. To the best author´s knowledge, there are no previous studies about PLE from *Hs*, although, similar results in other plants were found in previous studies where RSM has been used [31–34].

Source	Total phenolic compounds content				
	SS	DF	MS	F-value	p
A	12248.9	1	12248.9	3488.47	**0.0108**[a]
B	633.524	1	633.524	180.43	**0.0473**[a]
A:A	2530.54	1	2530.54	720.69	**0.0237**[a]
A:B	1796.49	1	1796.49	511.64	**0.0281**[a]
B:B	260.509	1	260.509	74.19	0.0736
Lack of fit	1940.88	3	646.96	184.25	0.0534
Pure error	3.51125	1	3.51125		
Total	17799.2	9			
R^2	0.89076				
Adj. R^2	0.754209				

Table 5. Analysis of variance (ANOVA) of the regression model. A = Temperature (ºC); B = % Ethanol; SS = sum of squares; DF = degrees of freedom; MS = mean square; R^2 = Quadratic correlation coefficient; [a] Significant ($p < 0.050$)

In one study the influence of solvent composition (32 – 88 % v/v methanol) and temperature (66 - 129 ºC) on antioxidant activity and total polyphenol content measured spectrophotometrically in sage (*Salvia officinalis* L.), basil (*Ocimum basilicum* L.) and thyme (*Thymus vulgaris* L.) were evaluated. For all these spices, the highest

temperature assayed (129 ºC) was the optimum temperature while different methanol concentration was found optimum depending on the plant (58 % v/v for sage, 60% v/v for basil and 33% v/v for thyme), being both variables highly significant for total phenol content and antioxidant activity [33]. In other work, temperature and ethanol concentration was also optimized by RSM for PLE from apple pomace considering antioxidant activity measured by DPPH, total phenol content measured by Folin-Ciocalteu assay and three polyphenol groups determined by HPL-DAD as response variables. The highest antioxidant activity was also achieved for the extracts obtained at maximum temperature (200 ºC), [31]. However, in both studies, spectrophotometric techniques were used, and although, these methods are widely used for quantification of phenolic compounds, on the one hand it only provides a general estimation of this group of bioactive compounds whereas other classes of compounds could act as interferences in the determination. For this reason, it should be noted the importance of the quantification of each individual compound, since the extraction performance depends on the chemical structure. Thus, in the current work, it is the first time for our knowledge that phytochemicals extracted by PLE from *Hs* have been individually quantified. Therefore, the extraction parameters have been evaluated for the recovery of each detected compound.

Conclusions

The current work has pointed out that PLE is a suitable technique to maximize the extraction of phenolic compounds from *Hs* calyces. The individual compound concentrations determined by HPLC-ESI-QTOF-MS showed that for the majority of

compounds, higher temperatures and ethanol percentages and for instance lower dielectric constant had a positive effect on their recovery from *Hs* calyces. In contrast, cyanidin-3-sambubioside could only be quantified in the extraction condition performed at the lowest temperature (40 ºC). This result was expected due to the thermolabile nature of anthocyanins. In addition, to maximize the total phenolic compounds content, the predicted optimal extraction conditions by RSM were 200 ºC and 100 % (v/v) of ethanol. Furthermore, temperature and solvent composition and the dielectric constant which depends on the applied previous-mentioned parameters, demonstrated to be significant factors for the extraction of target compounds. To our knowledge, it is the first time that an experimental design of PLE has been applied to maximize the extraction of total and individual phenolic compounds from *Hs* calyces.

Acknowledgements

This work was supported by the projects co-financed by Andalusian Regional Government Council of Innovation and Science (Project P11-CTS-7625), the Spanish Ministry of Economy and Competitiveness (MINECO) (project AGL2015-67995-C3-2) and the Research group AGR274 “Bioactive ingredients” from the Analytical Chemistry Department of the University of Granada. We would like to thank the Ministry of Education, Culture and Sport (MECD) for supporting the grant FPU15/01125 of SPM. The author IBL gratefully acknowledges the Spanish Ministry of Economy, and Competitiveness (MINECO) in association with the European Social Fund (FSE) for the contract PTQ-13-06429. JLS also thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for the grant IJCI-2015-26789.

Author Contributions:

SPM performed the statistical analysis and prepared the manuscript. IBL conducted the extraction experiment and the analysis by HPLC-ESI-QTOF-MS, realized the analytical data treatment and revised the manuscript. JLS performed the extraction experimental design, supported the experimental work and revised the data. DAR and ASC revised the manuscript.

Conflicts of Interest:

The authors declare no conflict of interest.

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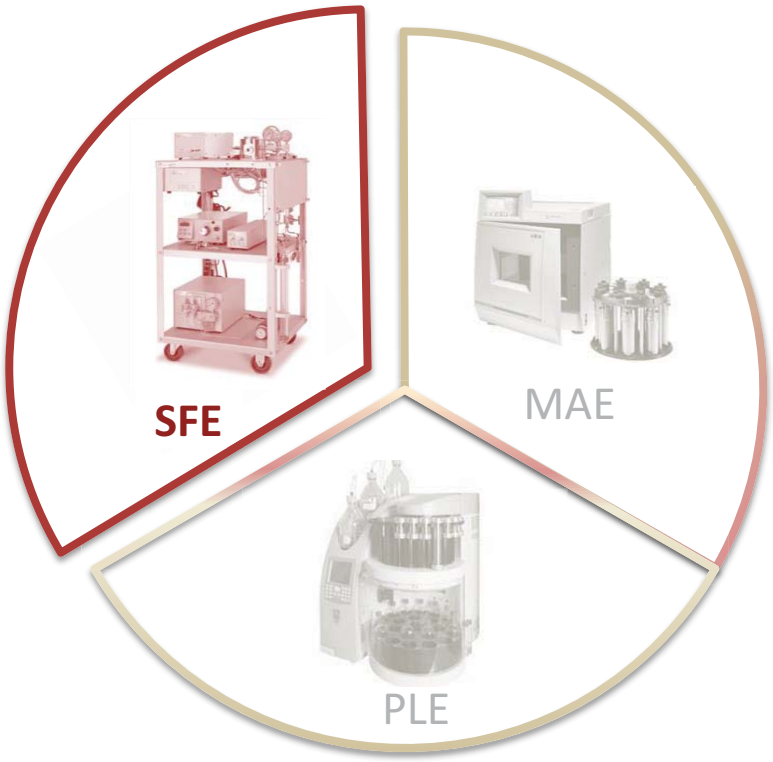
Chapter 3

Supercritical CO_2 extraction of bioactive compounds from *Hibiscus sabdariffa*

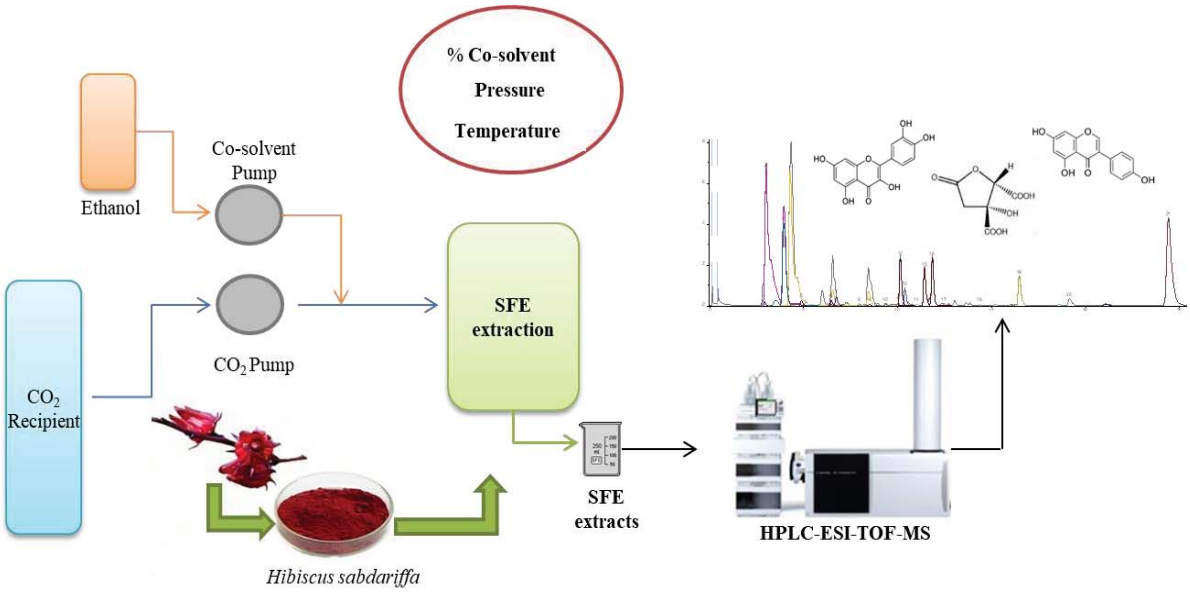


Journal of Supercritical Fluids

Pimentel-Moral, Sandra, Borrás-Linares, Isabel, Lozano-Sánchez, Jesús, Arráez-Román, David, Martínez-Férez, Antonio, Segura-Carretero, Antonio

Supercritical CO_2 extraction of bioactive compounds from *Hibiscus sabdariffa*

Abstract



Hibiscus sabdariffa is used as potent phytochemical agent due to its phenolic composition. On the other hand, Supercritical Fluid Extraction (SFE) is considered a green extraction technique that provides a selective extraction using a supercritical solvent. In this work, extraction parameters such as temperature, pressure of CO_2 and percentage of co-solvent were evaluated on the chemical composition of *Hibiscus sabdariffa* by Response Surface Methodology (RSM). After that, individual compound concentration was determined by HPLC-ESI-TOF-MS. This design, allowed found the optimal conditions for total phenolic and organic acids content. Each chemical group showed different behavior against SFE. Despite organic acids exhibited an irregular trend; the quadratic model was fitted for total phenolic compounds. Therefore, SFE has demonstrated to be a suitable and selective technique to maximize the extraction of several phytochemical compounds from *H. sabdariffa* calyces.

Keywords: supercritical fluid extraction, *Hibiscus sabdariffa,* phenolic compounds, response surface methodology, HPLC-ESI-TOF-MS.

Introduction

The increasing evidence correlating diet and chronic diseases such as diabetes, obesity, hypertension, cancer or other illnesses, made the incorporation of natural food additives one of the most important trends in the food industry. Hence, phenolic compounds are a widely studied natural family of compounds, which have demonstrated several bioactive applications, such as anti-inflammatory, antitumor, antimicrobial, anti-obesity and antioxidant functions.

In this sense, the collection of polyphenol-enriched extracts generates a great interest and attracts strong demand today. To this end, extraction process is an important step for separation and recovery of bioactive compounds from plants. In this way, supercritical fluid extraction (SFE) is considered a green extraction technique that provides attractive features overcoming many of the limitations of conventional extractions. The main advantages of SFE are the use of solvents generally recognized as safe (GRAS), lower extraction times and higher efficiency. Moreover, this technique is selective, does not require further cleaning and can be performed on small amounts of sample. SFE is the most technologically advanced extraction technique with application in environmental, chemical, food, agriculture, pharmaceutical and nutraceutical industries [1–3].

One of the main characteristic of SFE is the use of supercritical solvents. This type of solvents enhances the extraction rate due to their low viscosity and high

diffusivity coefficient, which allow rapid penetration of the solvent into the matrix. In addition, in this technique it is possible to modify the density of the fluid by changing its pressure and / or temperature [4]. Regarding extraction fluid, carbon dioxide is widely used in SFE because of its innocuous nature to human health and environment, as well as its moderate critical temperature (31.2 ºC), which permit the preservation of thermo-labile bioactive compounds. Also, this fluid prevents reactions of oxidation by avoiding the contact with air and it is readily available and reusable gas [5]. Nevertheless, the polarity of CO_2 is low and therefore, is less effective in extracting more polar phytochemical embedded in the cell wall of vegetable samples. For this reason, for the extraction of polar compounds, such as many phenolic compounds, there are polar solvent used as modifiers or co-solvents (ethanol, methanol or water) that, added in small amounts, can induce substantial changes of the solvent properties of CO_2 [2]. Among them, ethanol has been reported to be the better co-solvent due to its lower toxicity and improved extraction of phenolic compounds [6].

Besides that, operating parameters such as pressure and temperature can control the SFE process. Thus, an increase in pressure results in an increase in fluid density and enhanced solubility of the solute [5]. In addition, the combination of pressure and temperature influence on the physical properties of SFE solvent (density, viscosity and diffusivity) by mean that selectivity in extraction of target bioactive compounds can be gleaned exclusively by SFE. Generally a temperature range of 40 to 60 ºC and a pressure range of 200 to 400 bar are employed in SFE for the extraction of bioactive compounds [4]. Therefore, the use of SFE represent an alternative to the extraction of natural compounds from plant origin [1,6,7].

On the other hand, *Hibiscus sabdariffa (H. sabdariffa)* is a tropical plant belonging to the *Malvaceae* family which is used as potent phytochemical agent in the therapeutic treatment of hypertension, hyperlipidemia, diabetes, obesity, inflammatory diseases among others [8–12]. These beneficial effects are based on its composition, which is rich in bioactive compounds such as hibiscus acid, phenolic acids, anthocyanins and flavonoids. Therefore, the extraction and recovery of phenolic compounds from *H. sabdariffa* have showed great interest from food industries to be incorporated in many functional foods or nutraceuticals.

Hence, the objective of this work was the evaluation of the extraction parameters (temperature, pressure and percentage of co-solvent) and their relationship with chemical composition of *H. sabdariffa* extracts. For that purpose, individual compound concentration was measured by high-performance liquid chromatography coupled to time-of-flight mass spectrometry (HPLC-ESI-TOF-MS). For SFE optimization, a response surface methodology (RSM) based on central composite design (CCD) 2^3 model with 16 experiments including center and axial points was performed.

Materials and methods

Chemicals

All chemicals were of analytical reagent grade. Absolute ethanol and Otawa sand were supplied from Fisher Scientific (Leicestershire, UK). Glass wool was purchased from Sigma – Aldrich, (Steinhemin, Germany). For HPLC-ESI-TOF-MS analysis, formic acid and acetonitrile for mobile phases were purchased from Sigma-Aldrich (Steinheim, Germany) and Fisher Scientific (Madrid, Spain), respectively.

Standards used for the quantification (gallic acid, chlorogenic acid, rutin, p-coumaric acid, quercetin, myricetin, quercetin-glycoside and apigenin) were purchased from Sigma – Aldrich, (Steinhemin, Germany) while quercitrin was obtained from Extrasynthese, (Genay Cedex, France).

Sample

Dried calyces of *H. sabdariffa* were purchased from Monteloeder Inc. (Elche, Alicante, Spain). The % moisture of the raw material was determined by the loss of mass by desiccation in an oven during 72 h. The % of moisture of the calyces was 7.78 ± 0.1 %, determined in triplicate. This sample was homogenized using an Ultra Centrifugal Mill ZM 200 (Retsch GmbH, Haan, Germany) equipped with 12-tooth rotor and ring sieve with aperture size of 2 mm, which guaranties to obtain a uniform powder with this particle size. The homogenized samples were stored at -20 ºC avoiding environment conditions until extraction procedure. The bed density and bed porosity were 601 ± 25 kg / m^3 and 0.17, respectively. The and a real density of plant, bed density and bed porosity were calculated according to Minozo et al. 2012 [13] whose results were 1890 ± 52 kg/m3, 601 ± 25 kg / m3 and 0.17, respectively.

Supercritical CO_2 extraction (SFE) of bioactive compounds from *H. sabdariffa*

Extractions were carried out in a supercritical fluid extractor (Waters Prep Supercritical Fluid Extraction systems, SFE-100) equipped with CO_2 P-50 and co-solvent P-50 pumps, a low-pressure heating exchange, a high pressure heating exchange, a high pressure extraction cell, a back pressure regulator, a high pressure collection vessels and a chiller (Thermo Fisher Scientific™ , Leicestershire, UK). The samples were introduced into the high-pressure extraction vessel whose volume is 100 mL in a 3

layer sandwich structure: 5g sea sand + 30 g *H. sabdariffa* calyces + 5g sea sand. Glass wool was packed at both ends of the extraction vessel to prevent entrainment of the sample. All SFE extractions were performed in a dynamic mode at a constant total flow (25g / min) and extraction time (90 min). Temperature (from 40 to 60 ºC), pressure (from 150 to 350 bars) and different solvent combinations (CO_2 plus ethanol from 7 to 15 %) were varied according to the experimental design with the conditions showed in **Table 1**. After extraction, the collected extracts were evaporated using a Savant SC250EXP SpeedVac Concentrator (Thermo Scientific) and stored at −20°C protected from light until HPLC-ESI-TOF analysis.

Experimental design

Response-surface methodology (RSM) by Central Composite Design 2^3 (CCD) with axial points was applied to optimize the extraction of phytochemical compounds from *H. sabdariffa*. Temperature (40 , 50, 60 ºC), pressure (150, 250, 350 bar) and co-solvent composition (ethanol 7, 11 and 15 %) were selected as independent variables and individual phytochemical concentrations were dependent variables procuring a total of 16 experiments **(Table 1)** which were conducted in a randomized order. The response variables were the chemical composition of the extracts determined by HPLC-ESI-TOF-MS.

The experimental data obtained was processed with the program Statgraphics Centurion software XVI provided by Statpoint Technologies (Warrenton, VA, USA) in order to determine the optimum extraction conditions to obtain higher phenolic compounds content. The suitability of the model obtained was checked by evaluating

coefficient of determination (R^2) and the lack of fit. Significant values for independent variables and their interactions were considered according to the *p value* (< 0.05).

Code	Temperature (ºC)	Pressure (bar)	% Co-solvent
SFE 1	60	350	15
SFE 2	60	150	7
SFE 3	40	350	15
SFE 4	50	250	11
SFE 5	64	250	11
SFE 6	50	250	5.3
SFE 7	50	250	11
SFE 8	36	250	11
SFE 9	40	150	7
SFE 10	40	150	15
SFE 11	40	350	7
SFE 12	60	150	15
SFE 13	60	350	7
SFE 14	50	391	11
SFE 15	50	109	11
SFE 16	50	250	16.7

Table 1. SFE experimental conditions

Characterization of SFE *H. sabdariffa* extracts by HPLC-ESI-TOF-MS

Dried extracts of *H. sabdariffa* calyces obtained by SFE were reconstituted with ethanol used for their extraction up to a concentration of 10 mg / mL. In addition, these samples were filtered with single-use syringe filters (0.20 µm pore size) prior to inject into the HPLC system.

Chemical composition of these extracts was analyzed by HPLC-ESI-TOF-MS using an Agilent 1200 RRLC system (Agilent Technologies, Palo Alto, CA, USA) of the Series Rapid Resolution equipped with a binary pump, vacuum degasser, an autosampler, a thermostatized column compartment and a UV-Vis detector. Chromatographic separation was carried out in a Zorbax Eclipse Plus C_{18} analytical column (1.8 µm, 4.6×150 mm). The separation method was performed according to

previous work [14]. Briefly, the flow rate applied was 0.5 mL min^{-1}, the injection volume was 10 µL, the column temperature was 25 °C and the mobile phase was water plus 0.1 % of formic acid as eluent A and acetonitrile as eluent B. The elution gradient was 0 min, 5 % B; 8 min 22 % B; 23 min, 28 % B; 27 min, 95 % B, 31 min, 5 % B; and finally a conditioning cycle of 5 min with the initial conditions before the next analysis.

ESI-TOF-MS analyses

The HPLC system was coupled to the time of flight mass analyzer (TOF-MS) using an ESI interface operating in negative ionization mode. The ionization parameters were capillary voltage, 4000 V, gas heater temperature, 210°C, drying gas flow, 9.5 L min^{-1}; nebulizing gas pressure, 2.5 bar and end plate offset, −500 V. To obtain the mass accuracy necessary to identify the compounds, an external calibration was performed. To this end, a sodium formate cluster solution (5mM sodium hydroxide and 0.1 % formic acid in water / 2-propanol (1:1, v/v)) was used. This calibrant was injected at the beginning of the rum using a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, Illinois, USA). Thus, all spectra were calibrated before identification.

The data were processed using the software Data Analysis 4.0 (Bruker Daltonik). The identified compounds were characterized by the generation of the candidate formula with a mass accuracy limit of 5 ppm using the SmartFormula™ editor and also considering their retention time (RT), mass spectra and the information available in literature.

For the quantification of each compound present in the extracts, calibration curves with seven concentration levels were prepared using standards such as

chlorogenic acid, *p*-cumaric acid, gallic acid, quercetin, quercetin-glucoside, rutin, myricetin and quercetrin. Moreover, apigenin was used as internal standard and included in all the extracts and calibration level samples at a concentration of 20 µg/mL. All calibration curves showed good linearity over the study range (R^2=0.9971) [14]. All the individual samples were analyzed in triplicate. The concentration of each compound was calculated using the corrected area of each individual compound calculated as "compound area / internal standard area" and interpolating in the corresponding calibration curve.

Thus, chlorogenic acid, quercetin-glucoside, quercetin and myricetin could be quantified with the calibration curves of their own commercial standards. Nevertheless, the other compounds, which had no commercially available standards, were tentatively quantified from calibration curves of standards with similar structure. Therefore, the quantification of these compounds is only an estimation of their actual concentrations, although it can be considered a useful approximation. Hence, the calibration curve of gallic acid was used to quantify methylepigallocatechin and N-feruloyltiramine; *p*-cumaric acid standard equation was utilized for hydroxicitric acid, 5-O-caffeoylshikimic acid, coumaroilquinic acid and hibiscus acids derivatives; chlorogenic acid for its derivatives and dihydroferulic acid-4-O-glucuronide; and quercetrin was expressed as kaempferol.

Results and discussion

Identification and quantification of the *H. sabdariffa* SFE extracts

In order to evaluate the effect of temperature, pressure and co-solvent percentage on chemical composition of *H. sabdariffa* SFE extracts, an exhaustive

identification and quantification of phytochemicals was performed. To that end, a complete analysis using HPLC-ESI-TOF-MS has been carried out in this research. **Figure 1** showed a representative base-peak chromatogram from the *H. sabdariffa* SFE extract obtained at 60 ºC, 350 bars and 15 % co-solvent flow rate (condition SFE 1). The compounds have been numbered according to their elution order. A total of 21 compounds were tentatively identified and quantified by interpretation of their MS spectra and the data from the literature and open-access mass-spectra databases. **Table 2** summarizes the proposed compounds together with their peak number (numbered according to their elution order), retention time, mass, measured m/z, error (ppm) and molecular formulas.

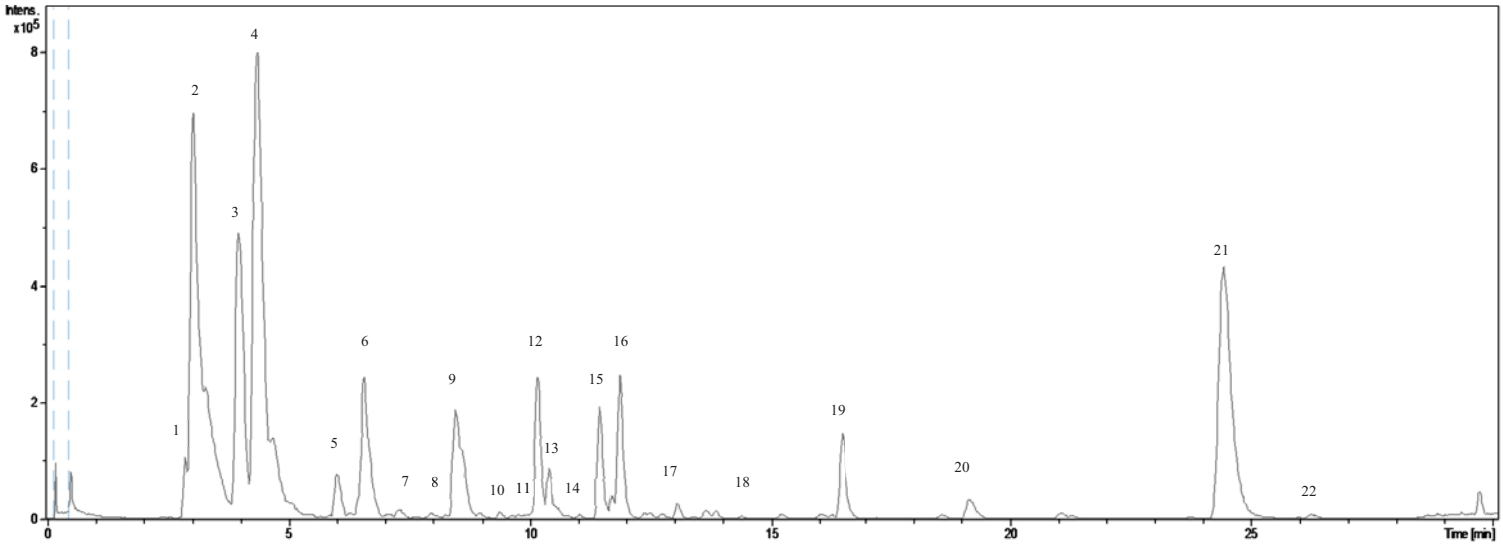


Figure 1. Base peak chromatogram (BPC) obtained from HPLC-DAD-ESI-TOF/MS analysis of the *H. sabdariffa* extract SFE 1. Peaks have been numbered according to the elution order

Peak	Proposed Compound	Molecular formula	Rt (min)	Mass	Measured m/z	Error (ppm)	Reference
1	Hydroxycitric acid	$C_6H_8O_8$	2.97	208.0219	207.0144	1.1	[22]
2	Hibiscus acid	$C_6H_6O_7$	3.04	190.0114	190.004	0.2	[22]
3	Hibiscus acid hydroxyethylester	$C_8H_{12}O_8$	3.98	236.0532	236.0459	0.2	[22]
4	Hibiscus acid dimethylester	$C_8H_{10}O_7$	4.33	218.0427	217.0355	0.9	[22]
5	Neochlorogenic acid	$C_{16}H_{18}O_9$	5.92	354.0951	353.0872	1.1	[23]
6	Chlorogenic acid	$C_{16}H_{18}O_9$	6.5	354.0951	353.0895	1.1	[22]
7	Cryptochlorogenic acid	$C_{16}H_{18}O_9$	7.37	354.3110	353.0887	2.4	[22]
8	Methylchlorogenate isomer I	$C_{17}H_{20}O_9$	7.97	368.1107	367.1035	0.9	[22]
9	Coumaroylquinic acid	$C_{16}H_{18}O_8$	8.51	338.1002	337.0941	1.5	[22]
10	Dihydroferulic acid-4-O-glucuronide	$C_{16}H_{20}O_{10}$	9.38	372.1056	371.1002	1.8	[24]
11	Methylchlorogenate isomer II	$C_{17}H_{20}O_9$	10.06	368.1107	367.1053	1.1	[22]
12	5-O-caffeoylshikimic acid	$C_{16}H_{16}O_8$	10.4	336.0845	335.0779	1.1	[25]
13	Quercetin-3-glucoside	$C_{21}H_{20}O_{12}$	11.02	464.0955	463.0907	0.9	[22]
14	Ethylchlorogenate isomer I	$C_{18}H_{22}O_9$	11.44	382.1264	381.1221	8	[22]
15	Ethylchlorogenate isomer II	$C_{18}H_{22}O_9$	11.44	382.1264	381.1221	8	[22]
16	Ethylchlorogenate isomer III	$C_{18}H_{22}O_9$	11.86	382.1264	381.1221	7.7	[22]
17	Methylepigallocatechin	$C_{16}H_{16}O_7$	12.48	320.0896	319.0831	2.6	[26]
18	Myricetin	$C_{15}H_{10}O_8$	14.38	318.0376	317.0316	4.1	[22]
19	N-feruloyltyramine	$C_{18}H_{19}NO_4$	16.47	313.1314	312.1257	4.9	[22]
20	Quercetin	$C_{15}H_{10}O_7$	19.14	302.0427	301.0362	2.7	[22]
21	Apigenin (IS)	$C_{15}H_{10}O_5$	24.39	269.0455	269.0462	2.6	
22	Kaempferol	$C_{15}H_{10}O_6$	26.21	286.0477	285.0407	1	[23]

Table 2 HPLC-ESI-QTOF-MS data of the identified compounds in *Hs* calyces extracted by SFE

Table 3 descripts the individual compound concentration for the assayed extraction conditions. For a better understanding, the compounds were separated in four groups: organic acids, phenolic acids, flavonoids and other polar compounds. **Figure 2** shows the total phytochemical amount obtained in each extraction condition.

Peak	Compound	SFE 1	SFE 2	SFE 3	SFE 4	SFE 5	SFE 6	SFE 7	SFE 8
					Organic acids				
1	Hydroxycitric acid	0.4631 ± 0.0008	0.47 ± 0.02	0.43 ± 0.03	0.62 ± 0.02	0.38 ± 0.02	ND	0.51 ± 0.02	0.44 ± 0.02
2	Hibiscus acid	37.0 ± 0.2	34 ± 1	35.3 ± 0.4	38.6 ± 0.6	31 ± 1	15.0 ± 0.3	36.9 ± 0.6	33.4 ± 0.8
3	Hibiscus acid hydroxyethylester	16.1 ± 0.1	34 ± 2	12.62 ± 0.09	15.4 ± 0.3	11.4 ± 0.4	2.00 ± 0.04	14.7 ± 0.3	13.1 ± 0.2
4	Hibiscus acid dimethylester	32.6 ± 0.8	26.3 ± 0.5	30.3 ± 0.4	34 ± 1	27.2 ± 0.7	3.2 ± 0.2	31.21 ± 0.07	28.6 ± 0.7
	Total	**86 ± 1**	**95 ± 3**	**78.6 ± 0.9**	**88 ± 2**	**70 ± 2**	**20.2 ± 0.4**	**83 ± 1**	**76 ± 3**
					Phenolic acids				
5	Neochlorogenic acid	0.176 ± 0.004	ND	<LOQ	<LOQ	<LOQ	ND	0.037 ± 0.002	0.102 ± 0.002
6	Chlorogenic acid	0.11 ± 0.01	ND	<LOQ	<LOQ	<LOQ	ND	0.07 ± 0.07	0.061 ± 0.006
7	Cryptochlorogenic acid	0.38 ± 0.02	ND	0.177 ± 0.001	0.146 ± 0.003	<LOQ	ND	0.15 ± 0.01	0.234 ± 0.007
8	Methyl chlorogenate	<LOQ	ND	ND	<LOQ	ND	ND	<LOQ	<LOQ
9	Coumaroylquinic acid	0.206 ± 0.004	ND	0.198 ± 0.001	0.151 ± 0.009	ND	ND	0.153 ± 0.003	0.172 ± 0.004
10	Dihydroferulic acid-4-O-glucuronide	<LOQ	ND	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ
11	Methyl chlorogenate isomer II	<LOQ	ND	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ
12	Ethylchlorogenate	2.04 ± 0.03	<LOQ	0.76 ± 0.05	1.03 ± 0.04	0.54 ± 0.03	ND	1.11 ± 0.03	1.35 ± 0.03
13	5-O-caffeoylshikimic acid	2.35 ± 0.05	0.29 ± 0.02	1.580 ± 0.009	1.44 ± 0.02	0.80 ± 0.03	0.140 ± 0.008	1.49 ± 0.07	1.80 ± 0.04
15	Ethylchlorogenate isomer II	1.65 ± 0.02	<LOQ	0.63 ± 0.01	0.80 ± 0.01	0.38 ± 0.01	ND	0.85 ± 0.02	1.03 ± 0.01
16	Ethylchlorogenate isomer III	2.15 ± 0.03	<LOQ	0.780 ± 0.008	1.12 ±0.02	0.60 ± 0.02	ND	1.19 ± 0.02	1.28 ± 0.02
	Total	**9.1 ± 0.1**	**0.29 ± 0.01**	**4.12 ± 0.06**	**4.69 ±0.09**	**2.32 ± 0.09**	**0.140 ± 0.008**	**5.04 ± 0.08**	**6.03 ± 0.08**
					Flavonoids				
14	Quercetin-3-glucoside	<LOQ	ND	ND	ND	ND	ND	ND	<LOQ
17	Methylepigallocatechin	0.47 ± 0.01	ND	0.45 ± 0.02	0.408 ± 0.007	0.340 ± 0.003	ND	0.360 ± 0.006	0.40 ± 0.03
18	Myricetin	0.057 ± 0.002	ND	ND	ND	ND	ND	ND	ND
20	Quercetin	<LOQ	ND	<LOQ	<LOQ	<LOQ	ND	<LOQ	<LOQ
22	Kaempferol	<LOQ	ND	ND	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	Total	**0.53 ± 0.01**	**0.00**	**0.45 ± 0.02**	**0.408 ± 0.007**	**0.340 ± 0.003**	**0.00**	**0.360 ± 0.006**	**0.40 ± 0.03**
					Other polar compounds				
19	N-feruloyltyramine	3.92 ± 0.09	1.45 ± 0.04	3.12 ± 0.06	3.75 ± 0.03	2.35 ± 0.02	1.18 ± 0.03	3.6 ± 0.1	3.19 ± 0.07
	Total	**3.92 ± 0.09**	**1.45 ± 0.04**	**3.12 ± 0.06**	**3.75 ± 0.03**	**2.35 ± 0.02**	**1.18 ± 0.03**	**3.6 ± 0.1**	**3.19 ± 0.07**
	Total phytochemicals	**99.6 ± 0.9**	**96 ± 3**	**86 ± 1**	**96 ± 1**	**75 ± 2**	**21.5 ± 0.4**	**92 ± 2**	**85 ± 3**
	Extraction yield (%)	**5.75**	**1.02**	**1.15**	**3.09**	**3.76**	**1.39**	**3.17**	**3.16**

Table 3 (Continued)

Compound	SFE 9	SFE 10	SFE 11	SFE 12	SFE 13	SFE 14	SFE 15	SFE 16
Organic acids								
Hydroxycitric acid	0.45 ± 0.02	1.21 ± 0.08	0.72 ± 0.01	1.35 ± 0.06	1.26 ± 0.05	1.42 ± 0.07	1.27 ± 0.01	1.33 ± 0.03
Hibiscus acid	33.5 ± 0.3	41.7 ± 0.6	34.1 ± 0.1	44 ± 3	39.47 ± 0.02	41 ± 3	39.1 ± 0.2	42 ± 1
Hibiscus acid hydroxyethylester	10.6 ± 0.2	15.9 ± 0.6	10.5 ± 0.2	14.9 ± 0.6	12.3 ± 0.1	15.6 ± 0.8	10.9 ± 0.7	17.3 ± 0.5
Hibiscus acid dimethylester	27.5 ± 0.2	38.0 ± 0.7	26 ± 2	38 ± 1	32.5 ± 0.6	38 ± 1	40 ± 1	38.8 ± 0.7
Total	**72.0 ± 0.3**	**97 ± 1**	**72 ± 1**	**86.2 ± 0.4**	**86 ± 2**	**95 ± 4**	**91 ± 2**	**99 ± 5**
Phenolic acids								
Neochlorogenic acid	ND	0.09 ± 0.02	ND	<LOQ	<LOQ	<LOQ	<LOQ	0.19 ± 0.01
Chlorogenic acid	ND	ND	ND	0.2 ± 0.1	ND	ND	ND	<LOQ
Cryptochlorogenic acid	<LOQ	0.29 ± 0.01	ND	0.15 ± 0.02	ND	0.162 ± 0.005	<LOQ	0.40 ± 0.02
Methyl chlorogenate	ND	<LOQ	ND	ND	ND	<LOQ	ND	<LOQ
Coumaroylquinic acid	ND	ND	ND	ND	ND	ND	ND	0.20 ± 0.02
Dihydroferulic acid-4-O-glucuronide	<LOQ	ND	ND	<LOQ	ND	ND	ND	<LOQ
Methyl chlorogenate isomer II	<LOQ	ND	ND	ND	ND	ND	ND	<LOQ
Ethylchlorogenate	0.203 ± 0.001	1.54 ± 0.03	0.1456 ± 0.0006	0.82 ± 0.02	0.17 ± 0.05	1.24 ± 0.04	0.80 ± 0.02	2.39 ± 0.04
5-O-caffeoylshikimic acid	0.626 ± 0.008	1.88 ± 0.07	0.67 ± 0.03	1.77 ± 0.08	0.8 ± 0.2	1.74 ± 0.07	0.97 ± 0.02	2.77 ± 0.07
Ethylchlorogenate isomer II	0.114 ± 0.005	1.22 ± 0.04	0.076 ± 0.002	0.61 ± 0.02	0.10 ± 0.02	0.94 ± 0.05	0.586 ± 0.001	1.84 ± 0.04
Ethylchlorogenate isomer III	0.192 ± 0.008	1.4 ± 0.1	0.1457 ± 0.006	0.77 ± 0.04	0.21 ±0.01	1.28 ± 0.06	0.75 ± 0.03	2.18 ± 0.04
Total	**1.134 ± 0.003**	**6.4 ± 0.2**	**1.03 ± 0.03**	**4.3 ± 0.3**	**1.3 ± 0.3**	**5.4 ± 0.2**	**3.10 ± 0.06**	**10 ± 1**
Flavonoids								
Quercetin-3-glucoside	ND	ND	ND	ND	ND	ND	ND	ND
Methylepigallocatechin	ND	0.435 ± 0.006	ND	0.46 ± 0.02	ND	ND	ND	0.53 ± 0.01
Myricetin	ND	ND	ND	ND	ND	ND	ND	0.083 ± 0.003
Quercetin	ND	<LOQ	ND	ND	ND	<LOQ	<LOQ	<LOQ
Kaempferol	ND	<LOQ	ND	ND	ND	<LOQ	ND	<LOQ
Total	**0.00**	**0.435 ± 0.006**	**0.00**	**0.46 ± 0.02**	**0.00**	**0.00**	**0.00**	**0.61 ± 0.01**
Other polar compounds								
N-feruloyltyramine	2.40 ± 0.06	3.32 ± 0.05	1.99 ± 0.03	2.48 ± 0.07	2.7 ± 0.4	3.4 ± 0.2	2.39 ± 0.07	3.6 ± 0.1
Total	**2.40 ± 0.06**	**3.32 ± 0.05**	**1.99 ± 0.03**	**2.48 ± 0.07**	**2.7 ± 0.4**	**3.4 ± 0.2**	**2.39 ± 0.07**	**3.6 ± 0.1**
Total phytochemicals	**75.5 ± 0.3**	**107 ± 2**	**75 ± 1**	**93.4 ± 0.3**	**90 ± 1**	**103 ± 4**	**96 ± 2**	**113 ± 2**
Extraction yield (%)	**1.95**	**3.60**	**1.80**	**3.16**	**1.95**	**3.43**	**3.12**	**7.40**

Table 3 Quantification and extraction yield of phenolic compounds and their derivatives in *H. sabdariffa* SFE extracts and yield obtained under different extraction conditions. ND: Compound not detected; <LOQ: Compound below the limit of quantification; Concentrations expressed in mg / g extract (x ± s).

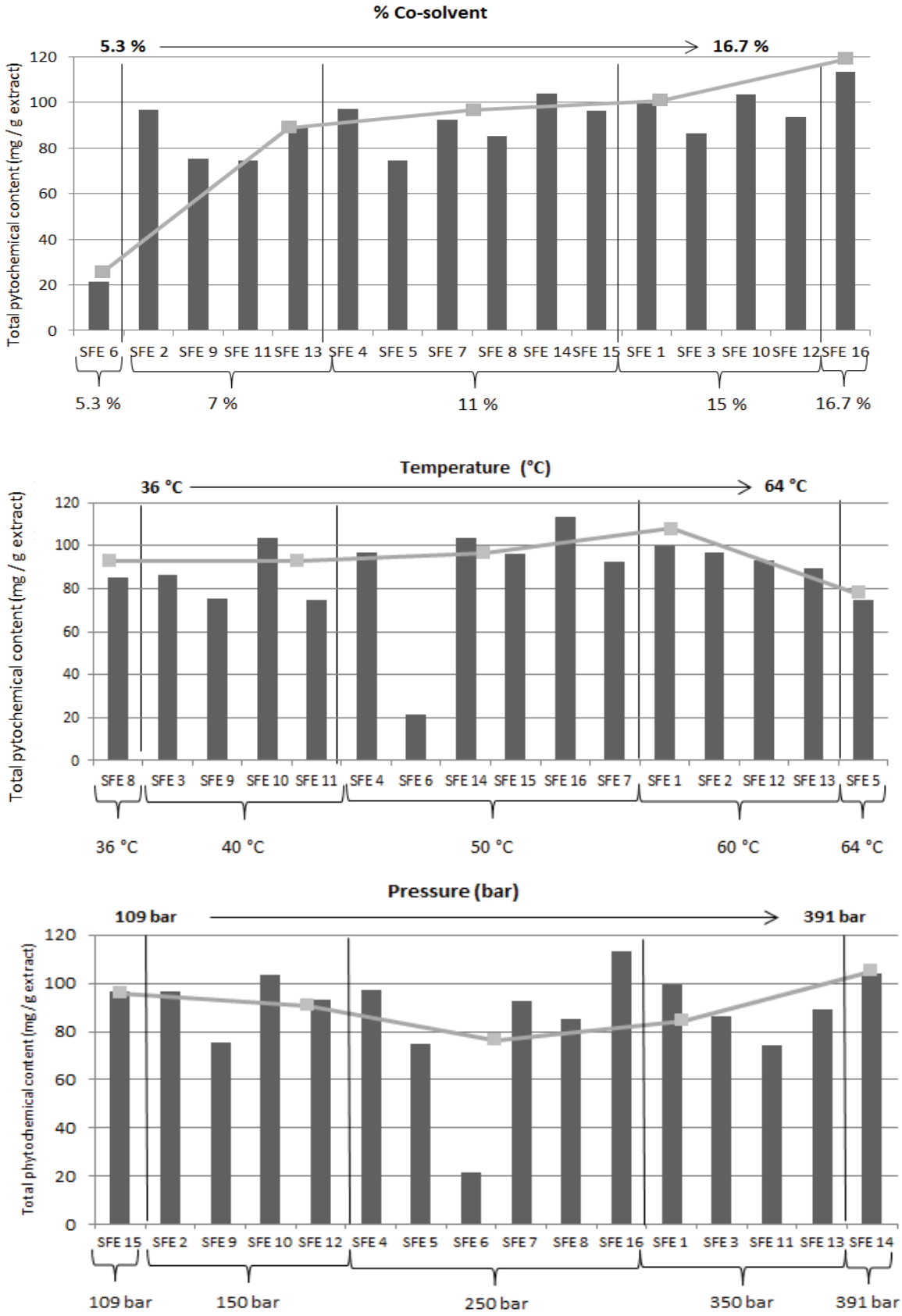


Figure 2 Concentration of total phytochemicals characterized in *H. sabdariffa* extract under different SFE conditions.

Therefore, organic acids and derivatives like hydroxycitric and hibiscus acids and its derivatives (hibiscus acid hydroxyethylester and hibiscus acid dimethylester) were found in all extraction conditions with a total concentration range from 20.2 ± 0.4 mg / g extract in SFE 6 (50 ºC, 250 bar and 5.3 % co-solvent) to 99 ± 1 mg / g extract in SFE 16 (50 ºC, 250 bar, 16.7 % co-solvent). Therefore, these first results demonstrated that co-solvent amount is the main factor for SFE of organic acids from *H. sabdariffa.* Respect to individual organic acids concentration, hydroxycitric acid (Peak 1) was found at 0.38 ± 0.02 mg / g extract in SFE 5 (64 ºC, 250 bar and 11 % co-solvent) to 1.42 ± 0.07 mg / g extract in SFE 14 (50 ºC, 391 bar and 11 % co-solvent) and this compound was not detected at 50 ºC, 250 bar and 5.3 % co-solvent. Thus, high pressure provide higher amount of this compound. In contrast for hibiscus acid (Peak 2) lower pressure and higher co-solvent amount obtained higher hibiscus acid content. The concentration range was from 15.0 ± 0.3 mg / g extract at 50 ºC, 250 bar and 5.3 % co-solvent to 44 ± 3 mg / g extract in SFE 12 (60 ºC, 150 bar and 15 % co-solvent). In addition, for hibiscus acid derivatives also lower pressures demonstrated higher content: hibiscus acid hydroxyethylester (Peak 3) was obtained from 2.00 ± 0.04 mg / g extract at 50 ºC, 250 bar and 5.3 % co-solvent, to 34 ± 2 mg /g extract at 60 ºC, 150 bar and 7 % co-solvent; and finally for hibiscus acid dimethylester (Peak 4) was from 3.2 ± 0.2 mg / g extract at 50 ºC, 250 bar and 5.3 % co-solvent to 40 ± 1 mg / g extract at 50 ºC, 109 bar and 11 % co-solvent.

It is important to note that in this study, higher amount of hibiscus acid and derivatives have been obtained in comparison to other extraction methods [14, 15]. Compared to other *H. sabdariffa* extracts obtained using conventional techniques, the levels of these compounds were higher in the SFE extracts obtained in the current

work. An example of that could be observed in the work carried out by Fernandez-Arroyo, (2011) [15] where the phenolic fraction of a *H. sabdariffa* aqueous extract obtained by conventional maceration was individually quantified. The hibiscus acid content in this extract was a third of the quantity obtained in SFE (31 ± 11 mg / g extract from literature *vs* 97 ± 5 in SFE 16). Besides that, other green extraction technology such as microwave-assisted extraction has been previously used to obtain bioactive *H. sabdariffa* extracts [14]. In these extracts lower amounts of hibiscus acid derivatives (around two-thirds) were also obtained in most extraction conditions assayed (from 38 to 76 mg / g extract) compared to the levels obtained in the current SFE extracts. The high content of hibiscus acid derivatives obtained by SFE compared with other extraction techniques could be explained due to the non-polar chains that present these compounds in their structure. Therefore, the use of non-polar solvents as CO_2 in SFE allows obtaining high amount of these less polar compounds.

The importance of these compounds reside in the fact that hibiscus acid and its methylester derivatives have demonstrated beneficial healthy effects as α-amylase inhibitor of starch digestion in the caco-2 cell model [16]. The suppression of the activity of digestive enzymes would result in a decrease of glucose absorption and consequently the reduction of postprandial blood glucose level elevation. Therefore, SFE could be consider as a selective and interesting technique that provides high contents of *H. sabdariffa* bioactives with demonstrated potential against diabetes and other glucose -related diseases, like metabolic syndrome.

Regarding to phenolic acids, 11 compounds were detected in the obtained SFE extracts. The concentration range of this family group was from 0.140 ± 0.008 mg /g

extract at 50 ºC, 250 bar and 5.3 % co-solvent to 10.1 ± 0.1 mg /g extract at 50 ºC, 250 bar and 16.7 % co-solvent. Hence, co-solvent amount plays an important role in the phenolic acids extraction. However, some phenolic acids were not detected or were below the limit of quantification (LOQ) in some extraction conditions although, 5-O-caffeoylshikimic acid (Peak 13) was quantified in all extraction conditions, being the phenolic acid more abundant in all SFE extracts.

On the other hand, flavonoids were the minor family of compounds obtained with this green extraction technique. Indeed, in all extraction conditions with lower values of 11 % co-solvent not a single flavonoid was detected. Therefore, a polar co-solvent like ethanol is necessary for flavonoid extraction. This can be explained due to flavonoids are compounds with high content in hydroxyl groups, which provide a higher polar property than other phenolic compounds, and therefore, high co-solvent content should be used for their extraction [17]. Respect to individual compounds, only methylepigallocatechin (Peak 17) was quantified in the most extraction conditions with high co-solvent amount.

Finally, N-feruloyltyramine (Peak 19) was quantified in all the extraction experiments with a concentration range from 1.18 ± 0.03 mg / g extract in SFE 6 to 3.92 ± 0.09 mg / g extract in SFE 1.

Therefore, to summarize these quantification results, the major compounds found in all extraction conditions were hibiscus acid and its derivatives. On the contrary, flavonoids were the family with lower concentration in all extractions. Hence, individual compound quantification using HPLC-ESI-TOF-MS provide an exhaustive information analysis to know the best extract condition for each compound or family of compounds.

In previous works [18, 19] extractions of *H. sabdariffa* calyces using supercritical CO_2 were also performed. The main objective of the first study [19] was the optimization of the extraction conditions to obtain the red color appearance. Thus, the best operating conditions to obtain the highest red color appearance was 8.90 MPa, 70 ºC and 9.49 % of ethanol. Respect to the other work [18], the focus was the maximization of the total phenolic compounds (TPC) measured using the spectrophotometric assay Folin-Ciocalteu method. However, although this determination is widely used for quantification the total phenolic compounds content, it is commonly known that it possess some disadvantages. On the one hand, this assay only provides a general estimation of this group of bioactive compounds, whereas other classes of compounds could act as interferences in the determination, like sugars, which are present in *H. sabdariffa* composition. For this reason, it should be noted the importance of the present research which carried out a complete quantification of each individual compound, since the extraction performance depends on the chemical structure [20]. Thus, for our knowledge, the current work is the first available report in which the effect of extraction conditions has been studied for individual phytochemicals extracted by SFE from *H. sabdariffa*. Therefore, the extraction parameters have been evaluated for the recovery of each detected compound.

Analysis of SFE extraction conditions

The influence of temperature, pressure and co-solvent percentage on the quantity of each individual phenolic compound were evaluated and optimized. For this purpose, a response surface methodology based on a CCD 2^3 with two axial points was assayed in order to maximize the response variables. For that, an analysis of variance

(ANOVA) for each response was performed in order to fit and optimize the statistical model.

Thereafter, 16 different SFE extracting conditions were performed and an exhaustive evaluation of the phenolic composition was carried out by HPLC-ESI-TOF-MS. Hence, the results are showed in **Figure 2** where it can be observed that the highest total phytochemical content was obtained in SFE 16 (113 ± 2 mg / g extract). This extraction was performed at 50 ºC, 250 bar and the maximum value of % of ethanol (16.7 %). In contrast, the assayed extraction that obtained the lowest phytochemicals content was SFE 6 (21.5 ± 0.4 mg / g extract) whose temperature, pressure and percentage of co-solvent were 50 ºC, 250 bar and the minimum value of % of ethanol (5.4 %). This behavior could be explained because supercritical CO_2 is a solvent with low polarity and, consequently, less effective in the extraction of polar compounds from plant origin, such as polyphenols. Thus, the use of supercritical CO_2 combined with percentages around 15 % of polar co-solvent like ethanol provides a selective technique to obtain bioactive compounds from natural sources with polar nature. Indeed, as is shown in **Figure 2**, the percentage of co-solvent is the most significant parameter, providing an increase in total phytochemical content when the amount of co-solvent is higher. In contrast, the modification of temperature and pressure did not produce significant changes in total phytochemical content.

On the other hand, in this study different effects of extraction parameters in the extraction efficiency of organic acids and phenolic compounds content were found by RSM analysis. For a clearer explanation, in the following sections the results will be explained separately.

Organic acids

Table 4 describes the ANOVA results for the responses variables organic acids content (Y_1), total phenolic compounds (Y_2) and total phenolic acids content (Y_3).

Source	Y_1					Y_2				
	SS	DF	MS	F-value	*p*-value	SS	DF	MS	F-value	*p*-value
A	94.3719	1	94.3719	7.01	0.2299	1.18707	1	1.18707	24.23	0.1276
B	74.3898	1	74.3898	5.52	0.2561	3.85405	1	3.85405	78.68	**0.0715**[b]
C	2033.87	1	2033.87	151.01	**0.0517**[b]	114.816	1	114.816	2343.93	**0.0131**[a]
A:A	41.7212	1	41.7212	3.10	0.3289	2.14009	1	2.14009	43.69	**0.0956**[b]
A:B	1.01531	1	1.01531	0.08	0.8294	8.28326	1	8.28326	169.10	**0.0489**[a]
A:C	95.151	1	95.151	7.06	0.2291	1.59811	1	1.59811	32.62	0.1103
B:B	225.277	1	225.277	16.73	0.1527	3.22354	1	3.22354	65.81	**0.0781**[b]
B:C	38.5003	1	38.5003	2.86	0.3400	0.36911	1	0.36911	7.54	0.2224
C:C	426.996	1	426.996	31.70	0.1119	0.34262	1	0.34262	6.99	0.2301
Lack of fit	1860.52	5	372.104	27.63	0.1415	14.9192	5	2.98383	60.91	0.0957
Pure error	13.468	1	13.468			0.04899	1	0.04899		
Total	5351.94	15				149.099	15			
R^2	65 %					90%				

Table 4 Analysis of variance (ANOVA) of the regression model for organic acids (Y_1) and phenolic compounds (Y_2); A = Temperature (ºC); B = % Ethanol; SS = sum of squares; DF = degrees of freedom; MS = mean square; R^2 = Quadratic correlation coefficient; [a] Significant (p < 0.050); [b] Marginally significant (p < 0.100).

Regarding the first one, the statistical treatment showed that the CCD was not fitted for this response variable. Despite that, the lack of fit was not significant and the value of R^2 revealed that the experimental design was not suitable (64 %). In this sense, any variable which have a significant effect on the response provided a *p*-value lower than 0.05. In this case, the *p*-value of temperature and pressure were 0.2299 and 0.2561 and therefore, did not show significant effects on organic acids content. However, the *p*-value of co-solvent amount was 0.0517, which showed a marginally significance (p-value < 0.100). In spite of that lack of adequacy, it could be observed that lower pressure seemed to positively impact the extraction of organic acids from *H.*

sabdariffa calyces **(Figure 3)**. On the other hand, temperatures higher than 47 ºC decreased the organic acids content. In contrast, high co-solvent amount provided higher organic acids content due to the polar characteristic of this type of compounds.

The corresponding regression model, knowing that not provide a good adequacy was (Eq. 1) representing temperature, pressure and % co-solvent as A, B, C, respectively.

$$Y_1 = -97.0277 + 3.4225A - 0.19176B + 18.0678C - 0.0212216AA - 0.00035625AB - 0.0862187AC + 0.000493124BB - 0.00548437BC - 0.424314CC \quad \text{(Eq. 1)}$$

In addition, the optimum predicted extraction condition proposed by CCD for these compounds were 47 ºC, 15.9 % of co-solvent and 81 bar.

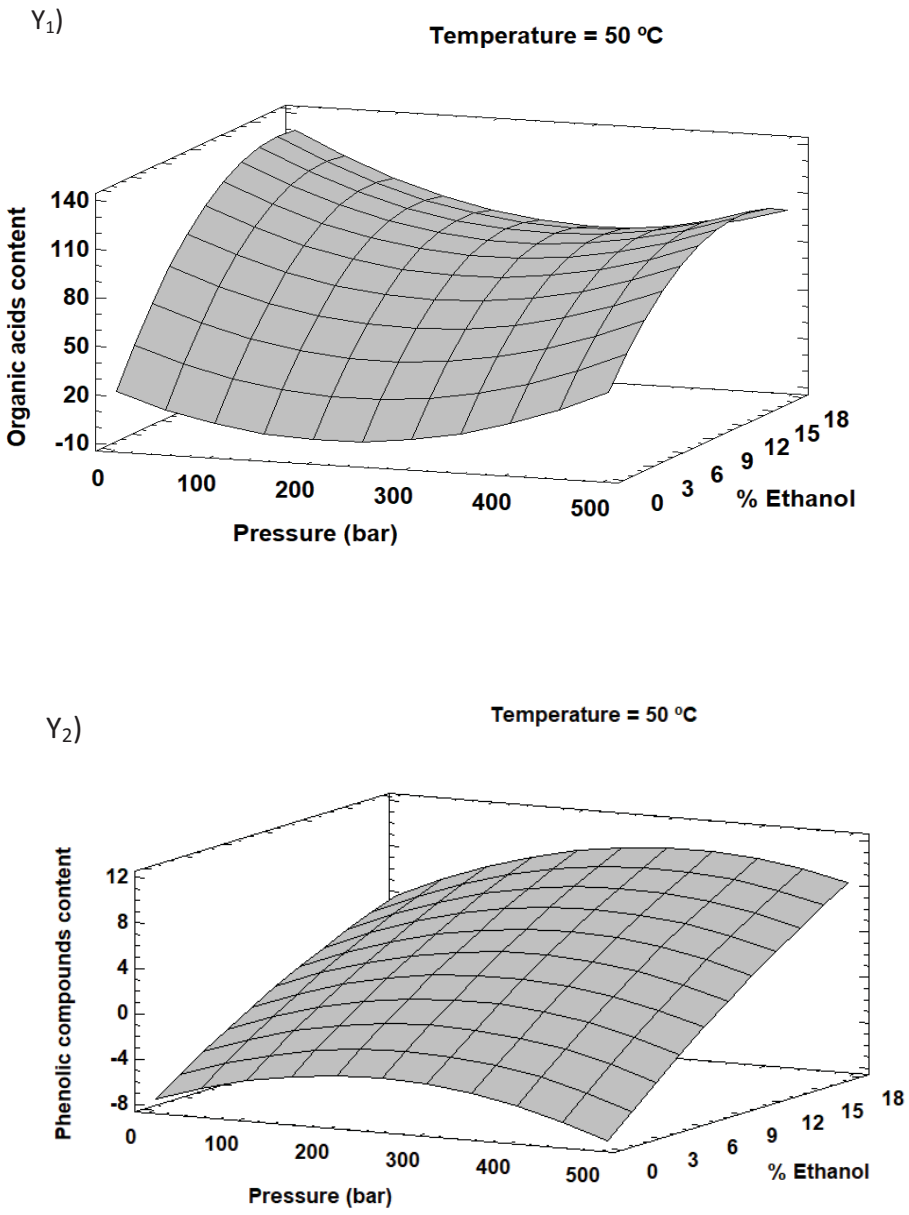


Figure 3 3D plot of response surface for the effects of SFE parameters on the organic acids content (Y_1) and total phenolic compound content (Y_2)

Phenolic compounds

Regarding the statistical analysis represented in **Table 4** for total phenolic compounds (Y_2), the regression model showed a great correlation coefficient (R^2 = 90%) and the lack of fit was not significant. Therefore, this CCD was suitable for this

response variable. The extraction parameters which exert a significant effect on total phenolic compounds ($p < 0.05$) were % co-solvent and the interaction between temperature and pressure; whereas pressure and its quadratic interaction and the quadratic effect of temperature were marginally significant ($p < 0.10$). These results were summarized in the following equation keeping only the significant parameters to predict this response (Eq. 2):

$$Y_2 = 0.699023 + 0.0738529A - 0.0219782B + 0.296369C - 0.00480634AA + 0.00101755AB - 0.0000589881BB \quad \text{(Eq. 2)}$$

Figure 3 shows the effect of pressure and % co-solvent on the phenolic compounds content from *H. sabdariffa*. As it could be observed, higher co-solvent amounts, temperature and pressure increased the extraction of these compounds. Therefore, respect to the optimal conditions proposed by this experimental model, it was found that the ones of the axial point (64 ºC, 391 bar and 16.7 % of ethanol) was the best conditions.

These results are in accordance to a previous research [18] where phenolic compounds from *H. sabdariffa* were extracted using supercritical CO_2 and measured by Folin- Ciocalteu method. Although in this work the effect of temperature and pressure on each individual compound concentration and co-solvent percentage were not studied, the results showed that the highest temperature and pressure assayed (240 bar and 70 ºC) were the best conditions to obtain the highest total phenolic content (8.63 mg GAE / g dried). In the current research, the extraction with higher phytochemical content was performed at 50 ºC, 250 bar and 16.7 % of ethanol which provide an estimated total phenolic content of 113 ± 2 mg / g extract. Besides that,

other authors have reported the extraction of bioactive compounds from *H. sabdariffa* by conventional and advanced technologies. In a study, a conventional extraction with different solvents (water, ethanol, methanol and acetone) was performed to evaluate the effect of each solvent on polyphenolic content and antioxidant activity of *H. sabdariffa*. The results showed that ethanol and methanol were better solvents than water or acetone for phenolic extraction [21]. For this reason, in the current work, higher ethanol amount provide higher phytochemical content. On the other hand, comparing SFE with other extraction techniques in terms of yield, the value obtained with this SFE methodology is lower than MAE or conventional extraction techniques [14, 21]. The highest yield was obtained with MAE and conventional method, with values of 47.1 % and 12.8 % respectively, while the best result of SFE provided a value of 7.4 % **(Table 3)**. Despite that, this methodology has demonstrated its ability for obtaining higher content of less polar compounds present in this plant. Thus, hibiscus acid derivatives which contain non-polar chains and have demonstrated high bioactivity were obtained in higher content by SFE compared to conventional and MAE methods.

Conclusions

The individual compound concentrations determined by HPLC-ESI-TOF-MS showed that SFE improved the collection of hibiscus acid and derivatives (which have demonstrated beneficial effects on glucose absorption) compared to other conventional and green extraction technologies. In addition, temperature, pressure and percentage of co-solvent were evaluated, demonstrating that at higher temperatures, pressure and co-solvent amount employed higher concentration of

phenolic compounds were obtained. Thus, to the best of our knowledge, this is the first time that SFE of *H. sabdariffa* was implemented to optimizing the individual compound extraction found in their calyces. The statistical analysis showed that the quadratic model was fitted for total phenolic compounds. However, for organic acids, this experimental design was not fitted. Therefore, SFE has demonstrated to be a suitable and selective technique to maximize the extraction of several phytochemical compounds from *H. sabdariffa* calyces such as hibiscus acid and derivatives.

Acknowledgments

This work was supported by the Research group AGR274 "Bioactive ingredients" from the Analytical Chemistry Department of the University of Granada, the Andalusian Regional Government Council of Innovation and Science (project P11-CTS-7625) and the Spanish Ministry of Economy and Competitiveness (MINECO) (project AGL2015-67995-C3-2). We would like to thank the Ministry of Education, Culture and Sport (MECD) for supporting the grant FPU15/01125 of SPM. The author IBL gratefully acknowledges the Spanish Ministry of Economy and Competitiveness (MINECO) in association with the European Social Fund (FSE) for the contract PTQ-13-06429 and JLS also thanks the Spanish Ministry of Economy and Competitiveness (MINECO) for the grant IJCI-2015-26789.

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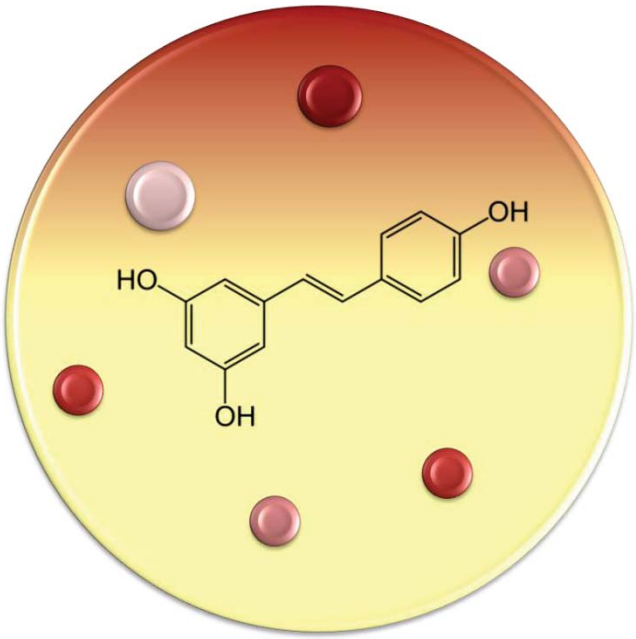
Section C

Micro and nano-encapsulation and incorporation into a food matrix

Chapter 4

Lipid nanocarriers for the loading of polyphenols



Advances in colloids and interface science

Pimentel-Moral, Sandra, Teixeira, Maria du Ceu, Fernandes, Ana Rita, Arráez-Román, David, Martínez-Férez, Antonio, Segura-Carretero, Antonio, Souto, Eliana

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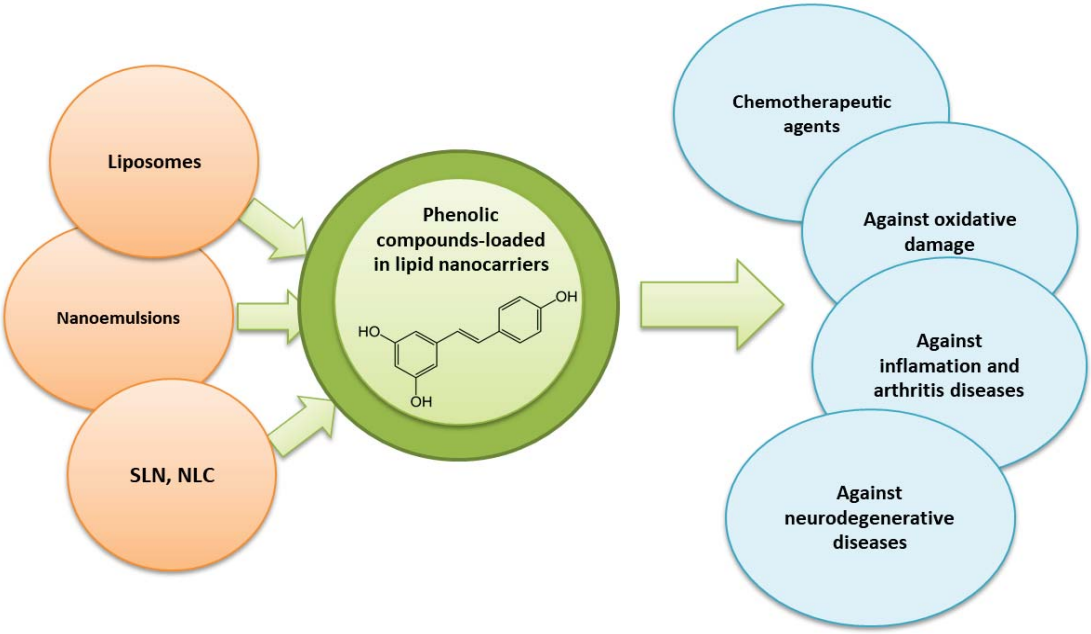
UNIVERSIDADE DE COIMBRA

Lipid nanocarriers for the loading of polyphenols – a comprehensive review

Abstract



Polyphenols are secondary metabolites found in all vascular plants and constitute a large group of at least 10,000 unique compounds. Particular attention is currently being paid to polyphenols attributed to their beneficial effects in the protection and prevention of several diseases. While their use in food, pharmaceutical and cosmetic industries is largely documented, several environmental conditions (e.g. light, temperature or oxygen) may affect the physicochemical stability of polyphenols, compromising their bioactivity *in vivo*. To overcome these limitations, the loading of polyphenols into nanoparticles has been proposed aiming at both increasing their bioavailability and reducing eventual side effects. Lipid nanoparticles offer several advantages, namely their biodegradability and low toxicity, with the additional capacity to modify the release profile of loaded drugs.

This paper is a review of the recent advances of lipid nanocarriers commonly used for the encapsulation of polyphenols, highlighting their added value to increase

bioavailability and bioactivity of this group of compounds as well as their application in several diseases.

Keywords: Polyphenols; Lipids; Nanoencapsulation; Bioavailability; Neurodegenerative diseases; antioxidant

Introduction

Polyphenols are secondary metabolites found in all vascular plants and constitute a large group of at least 10,000 unique compounds that contain one or more aromatic rings with one or more hydroxyl groups attached to them [1]. Thus, the majority of vegetables, fruits, whole grains or beverages such as fruit juices, coffee, wine, chocolate or tea from plant extract are rich sources of polyphenols.

These compounds have been classified by their source, biological function, and chemical structure. According to chemical structure, polyphenols are classified as flavonoids (flavonols, flavones, flavanones, anthocyanidins, catechins, isofavones, chalcones), phenolic acids (hydroxybenzoic acid and derivates and hydroxycinnamic acids and derivates), stilbenes, lignans, curcuminoids and tannins [2].

Nowadays, polyphenols have received tremendous attention among food, pharmaceutical and cosmetic industries and the general consumers due to their potential towards the protection/prevention of several diseases, given the amount of health promoting properties that have been studied and described. Examples of their beneficial effects are associated with their antioxidant, anti-inflammatory, anti-microbial, anti-mutagenic, and anti-carcinogenic properties, as well as capacity to stimulate the digestive process [3]. Polyphenols have therefore been widely studied as nutraceuticals in the prevention of several diseases such as diabetes, obesity, cardiovascular diseases, cancer, inflammatory diseases or microbial diseases [1,4,5].

However, numerous factors may affect the content of polyphenols in daily food or plant extracts **(Figure 1)**. Environmental conditions such as light, temperature or oxygen, food processing and storage (e.g. heating, acidification, light and oxygen) or the digestive tract reactions (acidic pH, enzymes, presence of other nutrients) are the main parameters that could produce a decrease in the concentration of polyphenols and provoking a partial or even total loss bioactivity. These factors limit their availability for human oral administration, posing serious challenges for the loading of these compounds in nutraceutical products.

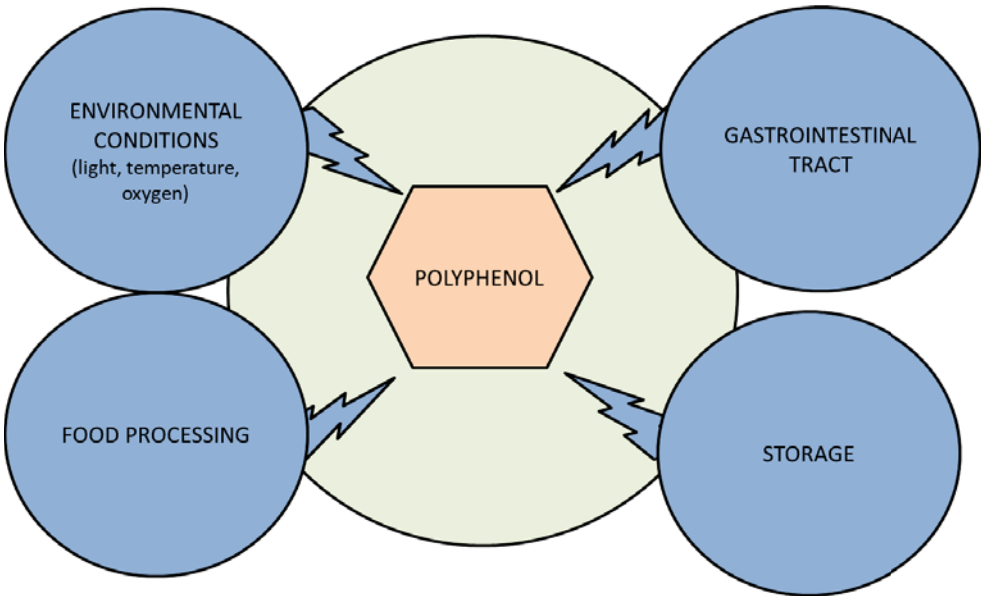


Figure 1. Factor that affect the polyphenol content and their bioavailability

Moreover, their incorporation into food related products may cause quality issues such as astringent taste and increased haze in beverages and act as substrates for browning reactions resulting in undesirable color changes and flavors in food products; furthermore, complexation with food proteins may jeopardize nutritional value, since proteins become unavailable for absorption and digestion [6]. Therefore,

oral administration of polyphenols needs an additional protection to keep their structural integrity and increase their bioavailability and physiological targeting.

Nanoencapsulation of bioactive compounds such as polyphenols within appropriately structured carriers may overcome these problems without adverse effect on sensory characteristics and appearance of final product. In general terms, encapsulation may be defined as a process to load a bioactive compound within another substance called cover material, protecting them from gastrointestinal conditions (pass through esophagus, acid pH in the stomach, pass through the small intestine and digestive juices) as well as environment conditions such as light, oxygen, temperature among others. In this scenario, a correct choice of carrier formulations, able not only to increase polyphenol concentration by colloidal solubilization but also to improve absorption and stability and to provide targeted delivery, is of paramount importance to achieve the best safety to effect ratios.

Nanoparticulate carriers (NPs) have been largely described to this aim. There are a wide variety of delivery systems for the encapsulations of active ingredients such as lipid-based nanoparticles, coacervation, spray-drying, fluid bed, extrusion, ionic gelation or yeast encapsulation [7]. Among them, lipid-based nanosystems offer several advantages, namely the improvement in design and function of some materials or systems at a nanoscale level, the possibility of improving the stability of the incorporated compounds and their interaction with the environment in which the system/material is included as well as enabling a modulated release action. Among the array of characteristics that can be improved, e.g. better water solubility, thermal stability, increased oral bioavailability of compounds (particularly lipophilic molecules, e.g. labile drug molecules can be protected from external environment during storage)

and the digestive process protection (following oral administration) when the compounds are not resistant to the passage through digestive tract (from the oral cavity to the intestine where they are absorbed) and the digestive juices and enzymes secreted. On the other hand, these systems may also improve the organoleptic and functional properties. In addition, these lipid nanocarriers include, in general, compounds that hold a Generally Recognized as Safe (GRAS) status for oral and topical administration [8]. Lipid nanocarriers are the most commonly used technique for hydrophobic polyphenols, such as curcumin [9,10] or resveratrol [11] attributed to their capacity for protection and control release of both hydrophilic and hydrophobic drugs. However, although lipid nanocarriers are more commonly used for the loading of hydrophobic compounds, there are also several examples of many hydrophilic polyphenols successfully loaded in lipid nanocarriers [12].

Thus, this review will primarily focus on lipid nanocarriers that can be used for encapsulation of polyphenols such as emulsions, liposomes, phytosomes, solid lipid nanoparticles (SLNs), nanostructured lipid carrier (NLCs) and lipid-polymer hybrid nanoparticles (LPHNs) and the biological effects of polyphenols loaded trough these lipid nanocarriers.

Interactions between polyphenols and lipids

Polyphenols are highly reactive molecules and interactions between polyphenols and lipids (or lipid metabolites) have been described. Considering oral delivery, while passing through the gastrointestinal tract (GIT), phenolic molecules can interact with lipid peroxidation products, resultants of the digestives process. This interaction can cause damaging effects which could potentially decrease polyphenol activity [13]. As previously referred, the main advantage of nanoencapsulation of

polyphenols is the protection conferred to the molecule by the nanocarrier. Therefore, while protected by a nanocarrier, this kind of reactions can be avoided, having a positive effect on the bioavailability and activity of the polyphenolic molecules in lower regions of the GIT, and also promoting an increased absorption to circulation.

However, the knowledge of other interactions between polyphenols and lipids can be useful for the rational engineering of suitable polyphenol lipid-based nanocarriers. Interactions between lipids and phenolic compounds that are considered to form these nanosystems were subject of several reviews [4,14,15]. As example, we highlight several properties and interactions studies that can be considered as influence in both formulation process and behaviour. The property that we consider as one of the most important is the solubility of phenolic molecules in lipid matrixes. For instance, solubility studies on resveratrol, fisetin or quercetin showed an increased solubility of these in oily phases [16,17]. However, formulation studies of quercetin and fisetin lipid nanocarriers, it appears that these distribute, rather, at the oil/tensioactive interface. Thus, these compounds exhibit an amphiphilic behaviour, necessitating hydrophobic interactions, as well as hydrogen bond interactions with the aqueous medium to be encapsulated [16,18]. Moreover, other studies have shown that lipid nanocarrier can allow the encapsulation of hydrophilic polyphenols, such as epigallocatechin gallate (EGCG) [19,20]. These findings show that, despite the versatility, it is important to screen the solubility of the polyphenol in several lipids, in order to choose the most suitable ones to build the nanocarrier and increase encapsulation efficiency.

For a more specific example, in the case of liposomes, the interaction of the phenolic compounds with the lipid bilayer is also an important factor. These

interactions are considered to be: (i) the affinity or partition of more non-polar compounds within the hydrophobic portion of the membrane; and (ii) the formation of hydrogen bonds between the most hydrophilic polyphenols and the polar head groups of lipids and within the membrane interface [21]. The lipophilic character of a phenolic compound, that is, the capacity of the polyphenol to dissolves in each of two immiscible solvent phases, is determined by its partition coefficient. This concept can be defined as the ratio of concentration of a polyphenol in a mixture of the two immiscible phases at equilibrium. This ratio is therefore a measure of the difference in solubility of the phenolic compound in these two phases. These interactions will also influence both the encapsulation efficiency and the release rate of polyphenol.

Mostly, the preparation methods of lipid nanocarriers rely on emulsification processes that result in a wide range of emulsions with different properties [22]. Any molecule which might act on emulsion properties can potentially change and affect these properties. The molecules partitioning within the oil droplet, the water phase that surrounds the oil droplet or the interfacial region is also important in this matter. As referred, lipophilic molecules can be found in the lipid phase, hydrophilic molecules in the aqueous phase and amphiphilic molecules at the interface of these two regions. Studies on the influence of polyphenols on the emulsification process *in vitro* have given evidence that, from their positions, these molecules can influence the surface, the size and the shape of the oil droplet. For instance, Shishikura, Khokhar, & Murray, (2006) studied the influence of tea polyphenols on the properties of a model emulsion from olive oil, phosphatidylcholine, and bile salt [23]. It was found that green and black tea polyphenols affected emulsions by increasing droplet size and decreasing specific surface area by interacting phosphatidylcholine at the exterior of the emulsion droplet.

Tea polyphenols are more hydrophilic having many hydroxyl groups. They may interact with the polar head of the phosphatidylcholine creating complexes and even act as a linker between complexes leading to aggregation, thus increasing droplet size.

We have earlier pointed out the bioactive characteristics of polyphenols, namely their protective antioxidizing effect in aqueous media. Several studies, for instance with liposome formulations of EGCG [24] and resveratrol [25] have also showed the same protective antioxidizing effect on lipids. However, the same studies could not clarify if this effect was due to a particular positioning of the polyphenol at the surface or within the lipidic compartment of the liposomes. Despite this fact, protective behaviour in lipid matrices against oxidation and degradation, contributes to increase the formulation stability.

Lipid nanocarriers for polyphenols encapsulation

As previously referred, lipid nanocarriers are usually used as a vehicle for delivery of bioactives compounds such as phenolic compounds. The lipids in these systems are normally used in the building block to form the nanoparticles [26]. In this section, we review the most recent research efforts made on this topic, focusing on specific nanocarriers such as nanoemulsions, liposomes and the most advanced nanoplatforms like solid lipid nanoparticles (SLNs), nanostructured lipid carriers (NLCs) and lipid-polymer hybrid nanoparticles (LPHNs).

Nanoemulsions

Oil-in-oil emulsions are, normally described as, a binary system which consists in dispersed droplets that contain smaller droplets inside. These droplets present similar polarity and immiscible properties between them [27]. The structural

properties of these emulsions have been shown their ability to control the release of the compound that is in the inner phase [28]. Oil-in-oil emulsions are a good option in several cases, once, offers the possibility to former a reservoir. This characteristic is very useful, for example, to achieve controlled release of the bioactive molecule [29]. Thus, all these properties allow that this kind of formulation can be used in several applications in some fields, such as pharmacy, medicine, separation processes among others.

A special type of emulsions are the nanoemulsions which are characterized by the size of the oil droplets that is below 100 mm. Nanoemulsions can be oil-in-water, water-in-oil, bi-continuous, liquid-in-liquid or liquid-in-solid [30]. Due to size, some nanoemulsions can be not stable thermodynamically and have high stability kinetic. There are generally two methods to produce nanoemulsions, the high shear and high energy homogenization or, using the low energy methods, the spontaneous self-emulsification methods [31]. The advantages of nanoemulsions are their greater surface area which leads to a greater absorption, the improvement of their physical stability (to gravitational separation, flocculation and coalescence) and the increase of the bioavailability of the drug. Nanoemulsions allow reaching a faster release of the encapsulated compounds due to their characteristics, such as the small size of the droplets. Their small size of the particles and, consequently, a large surface area permits to quick digestion and the faster release of the encapsulated substances. Thus, the particles with a small size can be absorbed through the lymphatic vessels by the mucous layer present in the intestine or can be absorbed via paracellular and endocytosis [22]. All these advantages improve the therapeutic efficacy of the drugs and therefore the adverse effects are minimized as well as the toxic reactions that are

undesirable [32]. However, some nanoemulsions limitations have been described, namely, the instability, is usually due to intrinsic factors, that can include some characteristic events, such as, creaming, flocculation, and coalescence. Other external factors that can influence the stability of the emulsions are the temperature and the pH [33]. The use of a large amount of the surfactants and/or co-surfactants to stabilize the nanoemulsions is one of the disadvantages of these particles, once high concentrations of surfactant compromise the solubility of substances that a have high melting point. In addition, it is also known that the encapsulation of the natural extracts provide a substantial antioxidant activity which helps in the maintenance of the lag phase in the process of oxidation of nanoemulsions.

On the other hand, nanoemulsions have been used to increase the bioavailability and stability of phenolic compounds, for example, the EGCG, a polyphenol that is present in the green tea as the most significant substance [34]. In a research was described that the nanoencapsulation of EGCG increased significantly the *in vitro* activity and the *in vitro* bioavailability [35]. It was possible to observe that the *in vitro* bioavailability of the EGCG when it was encapsulated in a nanoemulsion was doubled when compared to the free EGCG. Hence, the nanoemulsion had the ability to improve the systemic absorption of the EGCG, when administered orally, which may be a consequence of the small size of the droplets that lead to an increase of the penetration through the blood-brain barrier. Besides that, several studies have demonstrated that emulsions improve the stability of these bioactive compounds over time. Thus, in a work, the catechins from decaffeinated green tea were encapsulated in nanoemulsions using palm oil and sunflower oil demonstrating a physical and chemical stability for 14 days [36]. Other example was the incorporation of a

hydrophilic *Hibiscus sabdariffa* extract in emulsions. This extract contains phenolic acids, flavonoids and anthocyanins which have demonstrated a potential benefit for human diseases. In this study, this extract was encapsulated in emulsions using olive oil and sunflower oil and the physicochemical profile was evaluated for 30 days. The results showed that the chemical composition of *Hibiscus sabdariffa* extract was stable over time when it was incorporate in emulsion [37]. In addition, this lipid nanocarrier has been used to enhance the intestinal permeability. In this way, catechins encapsulated in nanoemulsion improved the intestinal permeability of these compounds [38]. It was evaluated using Caco-2 cell as an *in vitro* model and could be caused by soy protein used in the formulation of the nanoemulsions to increase the stability. Thus, the smaller droplets size of the particles and the increase in the liquid interface area of droplets provide a major physical stabilization to the gravitational separation and to the aggregation [39].

Liposomes and phytosomes

Nanoliposomes are small spheres whose composition is characterized by a phospholipid bilayer membrane. These particles are liquid crystals formed by an aqueous core caged by one or multiple lipid bilayers. Structures with a single layer are unilamellar and with various layers are multi-lamellar liposomes. Liposomes composition insert phospholipids inside an aqueous medium and, consequently, their association with each other results in the formation of a bilayer sheet arrangement to protect their hydrophobic sections from the water molecules whereas avoiding the contact with the aqueous phase through the hydrophilic groups [40]. Due to their lamellarity, the liposomes can be categorized as unilamellar, oligolamellar and multilamellar vesicles [41,42]. The advantages of liposomes and nanoliposomes are

their ability to release hydrophilic and lipophilic bioactive substances, as well as, the possibility of large-scale production, the targetability, [42,43] and the possibility of production using safe natural compounds [44]. Another advantage is that these particles are their similarity to natural cell membranes, their biocompatibility and their small size, [30,45].

The preparation method (hydration of thin lipid film) of liposomes was described in 1965 by Bangham, Standish, & Watkins [46]. However, there is another method of post-formation process that is known and normally are used to decrease the number of bilayers and consequently, the size of the liposomes obtained [47]. Other methods are the sonication, freeze-thaw, the hydration and dehydration [48–50]. On the other hand, there still have non-mechanical methods that may include the reverse-phase evaporation, the removal of detergents, as well as the solvent injection and another heating methods [40]. Despite their multiple advantages, liposome technology has been limited in the food sector, mainly due to poor stability inside complex environments and high production costs. One way to increase the stability of liposomes is using cholesterol and saturated phospholipids with long-chain [51]. Another solution is the substitution of cholesterol by phytosterols due to their properties and benefits for human health, such as the decrease of the risk of heart disease, once decrease the blood cholesterol levels [52]. In addition, there are in the literature some satisfactory results that showed that is possible to improve the stability of liposomes through the modification of liposomes with some substances, for example, polysorbate 80, dextran derivatives, chitosan, among other [53–57].

In case of food application, liposomes must be designed using, for example, free fatty acids, monoacylglycerols, diacylglycerols, phospholipids and polar

amphiphilic products of lipid oxidation. All these substances normally self-aggregate when on contact to colloids [58]. Bryła, Lewandowicz, & Juzwa, (2015) compared the suitability of different lecithins for nanoencapsulation of elderberry extract, which is rich in phenolic compounds such as anthocyanins and proanthocyanins and has demonstrated beneficial effects in inflammatory diseases [60]. The results of this study showed that liposomes that have in their constitution egg yolk lecithin are larger and more complex when compared to the liposomes formed with soybean lecithin that are smaller and more uniform [59]. In other research, polyphenol-rich grape seed extract was encapsulated in chitosan-coated liposomes showing good stability for 98 days of storage. In addition, the cationic chitosan coating improved the oxidative stability demonstrating high antioxidant activity [54]. De Pace *et al.,* (2013) described the effect of EGCG encapsulated chitosan-coated nanoliposomes and concluded that the stability of EGCG the capacity to sustained release and the intracellular EGCG content in MCF7 breast cancer cells was expressively improved when compared to EGCG free and empty chitosan-coated nanoliposomes [61]. Moreover, in lower concentrations, these particles demonstrated their antiproliferative and proapoptotic activity while the natural EGCG did not show any advantageous effects. All these results showed an enormous potential of EGCG encapsulated chitosan-coated nanoliposomes in the treatment of breast cancer, as well as in the prevention. Another author evaluated the encapsulation of catechin and EGCG of green tea in soy lecithin liposomes and concluded that high encapsulation efficiency was achieved from the incorporation of catechin or EGCG inside the liposome structure [62]. Thus, the utilisation of entrapped polyphenols can increase the bioavailability and the half-life of the drug *in vivo* and *in vitro*.

On the other hand, another specific type of nanocarriers that use phospholipids and polyphenols as building blocks are phytosomes. ''Phyto'' means plant and ''some'' signify cell-like. To produce phytosomes the polyphenol mixture reacts chemically with a phospholipid, mainly phosphatidylcholine (the phospholipid of living tissues). However, is necessary to distinguish the phytosomes from the liposomes **(Figure 2)**. The phytosomes is a molecular association between two molecules, one of phosphatidylcholine and another is the phenolic compound [63]. The liposome is composed of lots of phospholipid aggregates into a spherule where the drugs are inside but do not present a specific bond. Nevertheless, in some cases, the liposomes still an unproven concept as a reservoir for oral delivery of drugs and the phytosome is already described to dramatically increase the oral delivery [64]. Phytosomes are hybrid molecules that have properties like the phosphatidylcholine that is being significantly lipid-soluble and water-soluble.

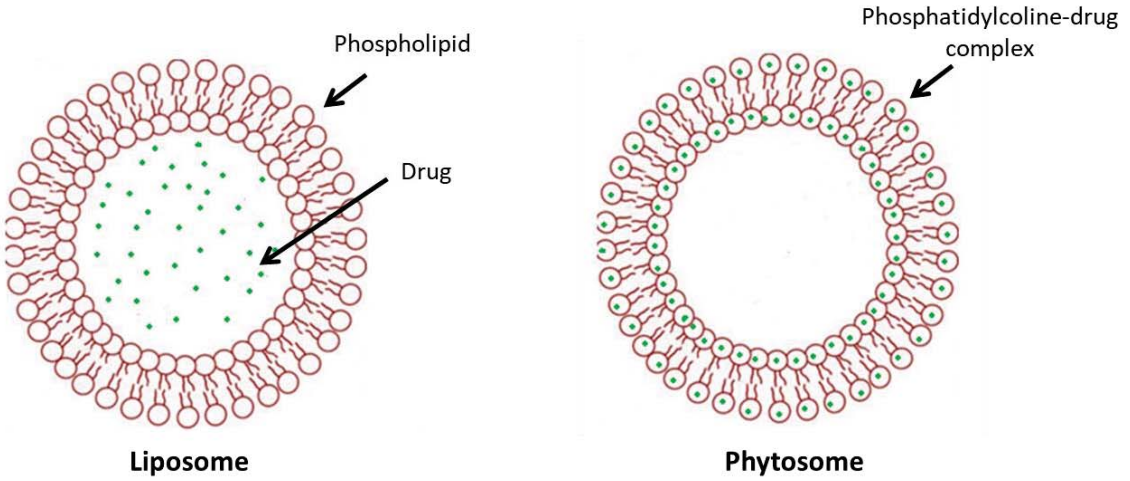


Figure 2. Structure difference between liposome and phytosome

Phytosome as a hybrid molecule when goes through the intestinal tract simplify the transition between the water-soluble environment of the lumen and the lipid-

soluble environment of the cell membranes [65]. Indeed, in some studies, phytosome preparations showed that when administered orally, the phytosome complexation increases dramatically the level of polyphenol in the blood almost 2-6 times [66,67]. Then, the phytosomes have proven that are a revolution in the clinical applicability of herbal/botanical polyphenols once increase the bioavailability and consequently improve the drug efficacy. Some advantages of phytosome complexes are the improved intestinal absorption and the targeted tissue delivery of polyphenols, the enhance of efficacy when compared to the correspondent phytosome form and are safe. Several studies have demonstrated these beneficts. For example, phytosomes of curcumin and naringenin showed higher antioxidant activity than pure curcumin in all dose levels tested [68]. In other work, phytosomes of gallic acid improved the antioxidant potential of this phenolic acid on carbon tetrachloride (CCl4)-induced oxidative damage in rat liver [69]. Also, chlorogenic acid phytosome was developed and evaluated against oxidative stress produced in the rat skin due to UVA exposure. Compared to the conventional formulation, the complex showed better protection when UVA irradiation was performed after 4 h of topical application [70]. Finally, these phytosomes have also been formulated with other polymers like chitosan to improve the stability. Thus, Zhang, Tang, Xu, & Li, (2013) described a novel polyphenol delivery system by curcumin phytosome loaded chitosan microspheres (Cur-PSCMs) by combining polymer and lipid nanocarrier [71]. The results showed that this combination demonstrated better effects of oral absorption and prolonged the retention time of curcumin. All these results have been summarized in **Table 1**.

Lipid nanocarrier	Phenolic compound encapsulated	Objetive	Reference
Nanoemulsion	EGCG	Increased the *in vitro* bioavailability	[35]
	Catechins	Increased the physical and chemical stability	[36]
	Hibiscus sabdariffa extract	Increased the chemical stability	[37]
	Catechins	Enhanced the intestinal permeability	[38]
Liposomes	Elderberry extract	Compared 3 different lecithins to improve the stability	[59]
	Grape seed extract	Improved the oxidative stability for 98 days	[54]
	EGCG	Improved the stability of EGCG the capacity to sustained release and the intracellular EGCG content in MCF7 breast cancer cells	[61]
Phytosomes	Catechins and EGCG	Achieved high encapsulation efficiency within a low fat hand cheese	[62]
	Curcumin and naringenin	Increased the antioxidant activity	[68]
	Gallic acid	Improved the antioxidant potential on CCl-4-induced oxidative damage in rat liver	[69]
	Chlorogenic acid	Enhanced the protection after 4 h of UVA exposure in the rat skin	[70]
	Curcumin	Improved the oral absorption and prolonged the retention time of curcumin	[71]

Table 1. Studies on phenolic compounds encapsulated by nanoemulsions and liposomes

Advanced lipid based nanocarriers

Despite the described advantages for the previous lipid nanocarriers (nanoemulsions and liposomes), several limitations have also been noted **(Table 2)**. To overcome these limitations, the engineering of advanced and more complex lipid nanocarriers, such as nanoparticles composed of solid lipids (solid lipid nanoparticles – SLN - and nanostructured lipid carriers - NLC) and hybrid nanoparticles composed of a blend of lipids and polymers, has been studied also for the encapsulation of bioactive molecules [72].

As previously reported, SLN and NLC, are an advanced type of lipid nanocarriers which structures are formed basically by solid lipids formulated to improve drug stability, bioavailability and release rate modulation. SLN are characterized by the

presence of a mixture of one or more solid lipids and the drug is entrapped within the solid lipid matrix. NLC composition consists in a mixture of solid lipid and liquid lipid (oil) in order to enhance their drug-loading capacity [73]. The production methods for both SLN and NLC, consists in a top-down approach based on the incorporation of the drug in the melted lipid that is then mixed with the aqueous surfactant solution. After this primary mixing high energy (high-pressure homogenization (HPH), ultrasound techniques and supercritical fluid technologies) or low energy processes (solvent emulsification evaporation, coacervation, microemulsion technique and phase-inversion temperature method), can be used followed by immediate formulation cooling that results in the lipid recrystallization and nanoparticles formation [8,74]. Both SLN and NLC are recognize of limited acute and chronic toxicity [75,76]. Despite the many offered improvements of these nanosystems, the fact that the loading capacity is directly proportional to the crystalline structure of the solid lipid leads to an increase in particle size, flocculation, aggregation, and compound release during storage time [77].

To overcome the possible limitations, nanoparticles composed of a blend of both lipid and polymeric materials (LPHNs) have been proposed as a new nanoplatform for drug delivery, offering several therapeutic advantages by altering pharmacokinetics, biodistribution, and toxicity profile of drug payload compared to free drug solutions, thus enhancing physical stability and biocompatibility. The hybrid classification is used because these systems combine the characteristics of both lipid and polymeric particles as LPNHs consisting of building blocks of lipids and polymers. The combinations of both approaches are also a result of the combination of the production methods for both nanoparticle, in a one-step or two-step procedures [78].

Recently, the use these advanced nanoplatforms for polyphenols encapsulation have been also extensively studied. Due to their versatile engineering, they allow the encapsulation of hydrophobic and hydrophilic phenolic compounds, or even the co-encapsulation of combinations of both.

Nanoemulsion and liposomes	Advances lipid nanocarriers: SLN, NLC
• **Possible cytotoxic effect**	• SLN are 10–100 fold less cytotoxic
• **Residual contamination from the production process, for example by organic solvents, large polymer aggregates and toxic degradation products**	• Most of the lipids and surfactants used for these lipid nanocarriers are recognized like GRAS (Generally Recognized As Save) due to their low toxicity
• **Limited physical and chemical stability during storage**	• Chemical protection of labile incorporated compounds, controlling the release over several weeks and enhancing the bioavailability of entrapped drug
• **Difficulty to produce on a large industrial scale**	• Production by high pressure homogenization is suitable for scaling-up
• **Expensive production methods**	• Excellent reproducibility with a low cost
• **Higher particle sizes**	• Smaller particle sizes with a wide potential application spectrum such as intravenous, dermal, per oral, topical

Table 2. Limitations of nanoemulsions and liposomes covered by advances lipid nanocarriers

Recent studies which use these advanced lipid nanocarriers are showed in **Table 3**. Starting with resveratrol, a hydrophobic polyphenol with low and variable oral bioavailability, Pandita, Kumar, Poonia, & Lather, (2014) explored SLNs as a suitable nanocarrier for the improvement of the oral delivery of this compound [79]. Resveratrol was loaded into stearic acid-based SLNs coated with poloxamer 188 produced by solvent diffusion-solvent evaporation method with an average diameter of 134 ± 7.6 nm and a zeta potential of −34.3 ± 2.5 mV. The encapsulation efficiency of

resveratrol in SLNs was of 88.9 ± 3.1 %. *In vitro* release studies showed prolonged drug release up to 120 h following Higuchi kinetics. Pharmacokinetic parameters after oral administration of SLNs and a resveratrol control were investigated in Wistar male rats. These results showed that the SLNs produced a significant 8.035-fold improvement in the oral bioavailability of resveratrol as compared to the free drug suspension.

Hajj Ali *et al.,* (2016) developed shea butter SLNs (SB-SLNs) for curcumin encapsulation [80]. Curcumin is a yellow hydrophobic polyphenol extracted from the rhizome of turmeric (*Curcuma longa*). SB-SLNs were prepared by the sonication method using nonionic surfactants and the experimental design methodology enabled to control particles size from 50 nm to more than 1 mm according to the mixture composition. Then, curcumin, a natural polyphenol, has been encapsulated in nanoparticles with a wide range of diameters (50-230 nm) and the encapsulation efficiency has been related to the particles sizes. The bigger the nanoparticles, the lower the encapsulation efficiency. The lipid structure at non-dispersed state and under SLN form has been studied by differential scanning calorimetry and X-ray diffraction. From the obtained results, encapsulated curcumin did not affect the lipid properties at the SLN state unlike at non-dispersed state. The localization of the curcumin in the outer shell of the lipid nanoparticles explained the evolution of the encapsulation efficiency according to the nanoparticles size.

As referred earlier, the engineering of advanced lipid-based nanosystems also allow the co-encapsulation of different types of bioactive compounds. Within this premise, studies on the development of lipid-core nanocapsules (LCN), containing the combination of resveratrol and curcumin, aiming to improve the antioxidant effects of both polyphenols, have been made. Coradini *et al.,* (2014) evaluated the

physicochemical characteristics of the co-encapsulation systems against formulations containing each polyphenol individually [81]. Co-encapsulation did not influence nanotechnological characteristics, and all formulations presented mean diameter around 200 nm, low polydispersity index, and encapsulation efficiency close to 100 %. This approach also increased the photostability of both resveratrol and curcumin. This work also evaluated the *in vitro* antioxidant activity of polyphenols against radicals which was proven to be was enhanced by nanoencapsulation, and an even better effect was observed after co-encapsulation. Taking into account a more specific application, Friedrich *et al.,* (2015) explored the effect of co-delivery of resveratrol and curcumin by LNC upon topical application on excised human skin [82]. Compared to the single polyphenol formulation, resveratrol penetrated into deeper skin layers when co-encapsulated with curcumin. Also, results on the evaluation of the interaction between LNC and primary human skin cells revealed a cellular uptake within 24 h suggesting intracellular effects of the polyphenols.

Other hydrophobic polyphenol of interest in the formulation of new food and nutraceutical products is rosmarinic acid (RA). The research group of Campos, Madureira, Gomes, Sarmento, & Pintado, (2014) and Madureira *et al.,* (2015) studied the potential use of SLNs for the encapsulation of RA. The first system to by developed, where Witepsol H15 was used as lipid and Polysorbate 80 (Tween 80) as surfactant, resulted in initial mean diameters between 270 and 1000 nm with a slight increase over controlled storage time occurred, but without occurrence of aggregation and with low polyphenol releases from SLNs throughout storage time [83]. The aim of the second work was to produce SLNs using carnauba wax as lipidic matrix. Physical properties, surface morphology and association efficiencies were studied at time of

production and after 28 days at refrigerated storage. The particles produced has a size range between 35 and 927 nm, showing high stability, with no aggregation verified during the stability study. All of SLN initial properties were maintained through the 28-day refrigerated storage period, and no RA particles release occurred. These results indicate a better compatibility between RA and this lipid matrix [84].

Lipid nanoparticles have also been tested for sustained release and specific targeting of EGCG, a polyphenol with hydrophilic nature associated with the pleiotropic health benefits of green and white tea [85]. However, pure EGCG's oral delivery has been impaired due poor stability and intestinal permeability. To improve these limitations, Granja *et al.,* (2017) produced EGCG-loaded NLC and functionalized with folic acid. Size, zeta potential and encapsulation efficiency (EE) of the produced spherical nanoparticles were evaluated and an *in vitro* release study in simulated gastric and intestinal fluids was preformed, as well as an evaluation on the storage stability of the nanoparticles over an 8-week period. The optimized NLC presented a size of approximately 350 nm and negative zeta potential around 30 mV points. The encapsulation efficiency (EE) of EGCG was higher than 80%. In simulated gastrointestinal conditions, this nanosystem allowed the controlled release of EGCG with an initial burst release in the first hours of only 10% of compound in gastric conditions. Also, the EGCG-NLC presented good stability up to 8 weeks in storage [86].

Anthocyanins, another hydrosoluble molecles are the main polyphenol components from red cabbage (*Brassica oleracea L. Var. Capitata f. Rubra*), and another red colorful plants and vegetables extracts with inherent antioxidant activities. Anthocyanins undergoing simulated pancreatic digestion, the total phenol recovery is of approximately 25%. With the aim of anthocyanins protection against harsh

environmental conditions (e.g., pH and temperature), Ravanfar, Tamaddon, Niakousari, & Moein, (2016) prepared SLNs by the dilution of water in oil (w/o) microemulsions containing anthocyanins in aqueous media. The formulations were optimized and characterized for particle size and encapsulation efficiency. Anthocyanins loaded SLNs mean particle size was of 455 ± 2 nm with a calculated entrapment efficiency of 89.2 ± 0.3 % [87].

Polyphenol	Nanosystem	Description	Reference
Resveratrol	SLN	Nanoencapsulation for oral delivery improvement	[79]
Curcumin	SLN	Shea butter SLNs physicochemical parameters; Pre-formulation studies	[80]
Resveratrol + Curcumin	Lipid core nanocapsules (LPHN)	Co-encapsulation of resveratrol and curcumin; Studies on the effect on skin permeation	[81] [82]
Rosmarinic acid	SLN	SLNs with different lipid matrixes and their influence on physicochemical properties and storage stability	[83] [84]
EGCG	NLC	Nanoencapsulation for oral delivery improvement	[86]
Anthocyanins	SLN	Nanoencapsulation for oral delivery improvement	[87]

Table 3. Recent studies on advanced lipid nanocarriers for polyphenols encapsulation and delivery

Biological effects of polyphenols incorporated in lipid nanocarriers

As is already known, the most important encapsulation goal is to keep the structural integrity and increase the bioavailability and physiological target of bioactive compounds. Although there are a wide of variety of encapsulation techniques that could achieve this purpose, the previous reviewed reports have clearly indicated that the lipid nanocarriers improve the bioavailability and efficacy of polyphenols. The

majority of investigations in literature that applied this technique focus on lipid-soluble polyphenols such as resveratrol and curcumin.

Resveratrol has been successfully encapsulated by lipid nanocarriers, whose effect on oxidative stress and tumor cell has been tested. One example was the study of resveratrol liposomes on cerebromicrovascular endothelial cells to evaluate the attenuation of oxidative stress. Resveratrol was encapsulated in fusogenic liposomes and the effect on transcriptional activity of Nrf2 in aged cerebromicrovascular endothelial cells was investigated. The results showed a significant increase in luciferase activity over the vector control assessed by a flow cytometry; therefore, this formulation activated Nrf2-driven antioxidant defense mechanisms [88]. In other study, resveratrol and 5-fluorouracil (phytochemical and chemotherapeutic agent) were co-encapsulated using PEGylated nanoliposomes. This formulation was evaluated *in vitro* on a head and neck cancer cell line NT8e demonstrating a GI_{50} similar to that of free 5-fluorouracil. However, the combination of resveratrol and 5-fluorouracil showed different effects on different genes that may influence the net antagonistic effect. Thus, this lipid nanocarrier demonstrated less cytotoxicity than free combination of resveratrol and 5-fluororacil [89]. In addition, resveratrol is a neuroprotective compound but after intravenous injection, it is rapidly metabolized without being able to exercise its functional action. For this reason, resveratrol-loaded SLN were functionalized with apolipoprotein E which can be recognized by the LDL receptors overexpressed on the blood-brain barrier. These nanoparticles were assessed in human cerebral microvascular endothelial cells (hCMEC/D3) demonstrating a significant permeability increase for resveratrol-loaded SLN with apolipoprotein E when compared to non-functionalized ones [90].

Curcumin has been shown to suppress inflammatory processes and induce apoptotic cell death of several tumor cells [91]. In a research, the biological effects of a liposomal curcumin formulation on human synovial fibroblast (SW982) and mouse macrophages (RAW 264) were evaluated. Regarding toxicity, liposomal curcumin formulation was less toxic in both types of cells than free curcumin. In addition, this formulation decreased pro-inflammatory cytokine/chemokine expression in synovial fibroblasts and in macrophages without affecting cell viability [92]. In other study, the anti-inflammatory potential for the treatment of osteoarthritis of curcumin and bisdemethoxycurcumin liposomes was studied. Liposomes were prepared using thin-film hydration method and particle size was stable for 2 weeks. Moreover, both types of liposome were less toxic than free curcuminoid and inhibited macrophage inflammation. Regarding 7F2 osteoblast cells both curcuminoids liposomes decreased osteoclast activity and maintained osteoblast functions due to these liposomes downregulated the expression of inflammatory markers on osteoblasts and show a high osteoprotegerin (OPG)/receptor activator of nuclear factor κB ligand (RANKL) ratio to prevent osteoclastogenesis [93]. On the other hand, curcumin oil-water nanoemulsions (CM-Ns) were carried out to be administered through intravenous injection on adjuvant-induced arthritis in rats. These nanoemulsions were formulated by high-pressure homogenizing technique, achieving an encapsulation efficiency of 90% and a particle size of 150 nm without degradation in simulated gastrointestinal conditions. In addition, similar decreases in the levels of TNF-α and interleukin-1β in both synovial fluid and blood serum were obtained from oral administration of CM-Ns and intravenous injection [94]. Therefore, liposomal and nanoemulsions curcumin might be a potential tool in inflammatory diseases.

On the other hand, there are also several studies that evaluated the biological effect of hydrosoluble polyphenols. Thus, polyphenols from *Arnica montana L*. were encapsulated in lipid vesicles using a hydration method followed by sonication and extrusion. This formulation was tested to know its anti-inflammatory effect in fibroblast cell culture by pro-inflammatory cytokines production. The results showed higher anti-inflammatory effect than free forms and better protection of cell membrane against oxidative damage [95].

Also, EGCG was encapsulated into nanolipidic particles to improve the bioavailability and α-secretase inducing ability of this hydrosoluble polyphenol for the treatment of Alzheimer's disease and HIV-associated dementia. Thus, in previous study, EGCG has been shown to modulate amyloid precursor protein cleavage and reduce cerebral amyloidosis in Alzheimer transgenic mice [96]. However, due to poor bioavailability and inefficient system delivery, the application in human clinical trials has presented difficulties. Therefore, encapsulation of this phenolic compound could improve its bioavailability. Indeed, EGCG loaded nanolipidic particles were developed in a research improving the neuronal α-secretase ability *in vitro* by up to 91% and the oral bioavailability *in vivo* by more than two-fold over free EGCG [35]. Other phenolic compound with a significant antioxidant activity in Alzheimer´s disease is ferulic acid. In a work, this compound was entrapped into SLN demonstrating a higher protective activity than free ferulic acid against oxidative stress on human neuroblastoma cells (LAN 5) [97]. Similar results were found in SLN of quercetin [98]. These nanoparticles were tested in Wistar rats and evaluated their permeation across the blood-brain barrier. The results showed markedly better memory-retention and pure quercetin-treated rats having significant therapeutic potential to treat Alzheimer´s disease.

Moreover, in other work, this flavonoid was encapsulated in NLC and SLN for improve the brain permeability. These SLN and NLC were substantially uptaken by Caco-2 cells and enhanced the relative bioavailability and they were able to deliver the quercetin to brain [99].

Hence, all these reports **(Table 4)** are a demonstration that lipid nanocarriers are also a potential technique for polyphenol encapsulation in order to improve their bioavailability and interactions with physiological targets.

Phenolic compounds	Lipid nanocarrier	Biological effect	Reference
Resveratrol	Liposomes	Actived antioxidant defense mechanisms in cerebromicrovascular endotelial cells	[88]
Resveratrol and 5-fluoracil	Liposomes	Demonstrated to be chemotherapeutic agent with less cytotoxicity than free combination of resveratrol and 5-fluororacil in head and neck cancer cell line NT8e	[89]
Resveratrol and apolipoprotein E	SLN	Permeability increase in human cerebral microvascular endothelial cells (hCMEC/D3) for the treatment of Alzheimer's disease	[90]
Curcumin	Liposomes	Decreased pro-inflamatory cytokine/chemokine expression in synovial fibroblast and in macrophages without affecting cell viability	[92]
Curcumin and bisdemethoxycurcumin	Liposomes	Inhibited macrophage inflammation and decreased osteoclast activity	[93]
Curcumin	Nanoemulsion	Decreased the levels of TNF-α and interleukin-1β in both synovial fluid and blood serum in induced arthritis rats	[94]
Arnica montana L.	Liposomes	Higher anti-inflammatory effect than free forms and better protection of cell membrane against oxidative damage	[95]
EGCG	Nanoemulsion	Improved the bioavailability and α-secretase ability *in vitro* by up to 91% and the oral bioavailability *in vivo* by more than two-fold over free EGCG for the treatment of Alzheimer's disease and HIV-associated dementia	[35]
Ferulic acid	SLN	Higher protective activity than free ferulic acid against oxidative stress on human neuroblastoma cells	[97]
Quercetin	SLN	Better memory-retention and pure quercetin-treated rats	[98]
Quercetin	NLC and SLN	These SLN and NLC were substantially uptaken by Caco-2 cells and enhanced the relative bioavailability	[99]

Table 4. Biological effects of phenolic compound loaded in lipid nanocarriers

Concluding Remarks

Phenolic compounds have become very important functional compounds due to their ability to scavenge reactive oxygen species (ROS). In addition, they are showed physiological properties such as anti-atherogenic, anti-inflammatory, cardioprotective, antioxidant and antimicrobial effects, wakening a great interest in health, food and cosmetics industries.

However, the oxidation of these compounds occurs very quickly, and they are very unstable under harsh environmental conditions, GTI digestive process, food processing and storage. Thus, the scientific community has focused in establishing new strategies for protection and improves their bioavailability. As show in this review, several works have demonstrated that lipid nanocarriers are a potential technology for polyphenol encapsulation to improve their bioavailability at their biophysiological target.

Also, *in vitro* and *in vivo* research has been carried out as a proof-of-concept for the produced nanocarriers. Although these results have not been yet translated to the clinic, it should be taken into account that most researchers found were published within the last five years and there are new lipid-based strategies like SLNs, NLCs or LPHNs that are demonstrating to be great delivery systems and could lead to potentially efficacious clinical and nutraceutical products. Therefore, it is hope that these lipid-based formulations will contribute in the prevention and improvement of human health.

Acknowledgments

This work was supported by the Research group AGR274 “Bioactive ingredients” from the Analytical Chemistry Department of the University of Granada,

and “REQUIMTE/LAQV” group of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra. Also, the authors would like to thank the financial support received from the Spanish Ministry of Economy and Competitiveness (MINECO) (project AGL2015-67995-C3-2), from Portuguese Science and Technology Foundation (FCT/MCT) and from European Funds (PRODER/COMPETE) under the project references M-ERA-NET/0004/2015-PAIRED and UID/QUI/50006/2013, co-financed by FEDER, under the Partnership Agreement PT2020. We would like to thank the Ministry of Education, Culture and Sport (MECD) for supporting the grant FPU15/01125 of SPM and the stay in the University of Coimbra supported by FPU short – term stay. MCT wishes to acknowledge FCT and Dendropharma - Investigação E Serviços De Intervenção Farmacêutica, Sociedade Unipessoal Lda. for the individual fellowship (PD/BDE/135086/2017).

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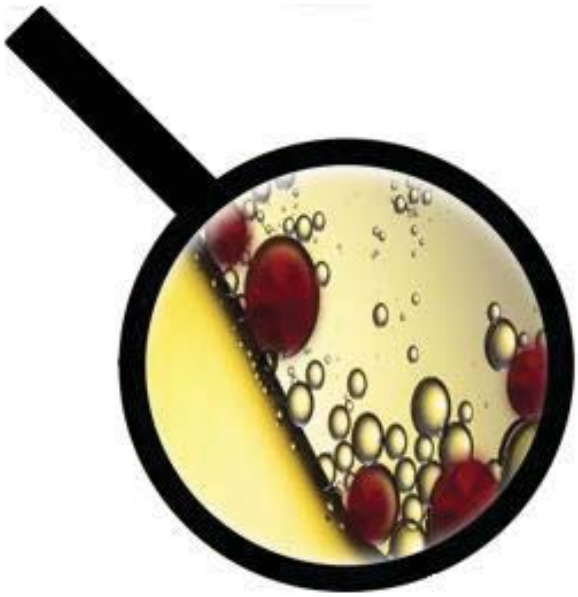
Chapter 5

Development and stability evaluation of water-in-edible oils emulsions formulated with the incorporation of hydrophilic *Hibiscus sabdariffa* extract

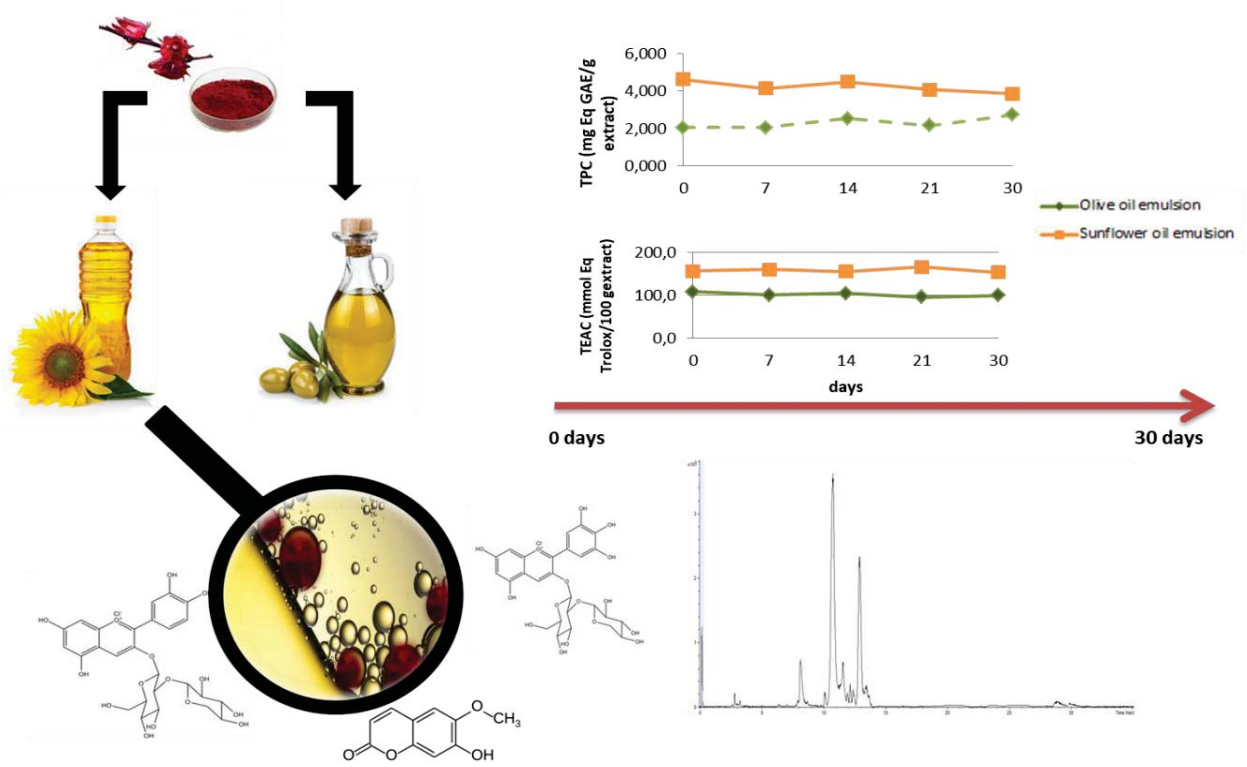


Food Chemistry

Pimentel-Moral, Sandra, Rodríguez-Pérez, Celia, Segura-Carretero, Antonio, Martínez-Férez, Antonio

Development and stability evaluation of water-in-edible oils emulsions formulated with the incorporation of hydrophilic *Hibiscus sabdariffa* extract

Abstract



New functional oils (extra virgin olive oil, EVOO and sunflower oil, SO) containing antioxidants from *Hibiscus sabdariffa* extract were developed by W/O emulsion. Their physical and chemical stability were measured over time. The lowest coalescence rate was obtained with 8 wt% and 12 wt% surfactant amount for EVOO and SO emulsions, respectively. Before the evaluation of the oxidative stability, an optimization of phenolic compounds extraction from emulsions by multi-response surface methodology (RSM) was performed. EVOO emulsions were chemically more stable over time than SO emulsions in terms of total phenolic content (TPC),

antioxidant activity and chemical composition measured by HPLC-ESI.TOF-MS. TPC significantly increased (from 2.024 ± 0.074 to 2.714 ± 0.062 mg Eq GAE/g extract) and the antioxidant activity obtained by TEAC remained constant during 30 days. Thus, W/O emulsion technology has proven to be a potential method to vehiculize and stabilize bioactive compounds from *H. sabdariffa* into edible oils.

Keywords: Emulsion; Stability; Olive oil, Sunflower oil, Antioxidant activity; *Hibiscus sabdariffa*; Functional oils

Introduction

Functional foods represent one of the most intensively investigated and widely promoted areas in the food industry and nutrition sciences today (Siró, Kápolna, Kápolna, & Lugasi, 2008). The significant increase of interest of consumers on healthy eating and the development of new technologies to extend the quality and the shelf-life of foods is moving to design longer-lasting products that are able to reduce the risk of disease as well. In this context, *Hibiscus sabdariffa* (*H. sabdariffa*), has been traditionally used for preparing herbal drinks, both, hot and cold beverages. Currently, *H. sabdariffa* is one of the most promising emerging natural sources to be incorporated in many functional foods due to its healthy properties. In this regard, *H. sabdariffa* has demonstrated to have positive effects as anti-obesity, hypotensive, antioxidant, hypocholesterolemic, antidiabetic, anti-cancer, immunomodulatory, hepatoprotective and diuretic (Gurrola-Díaz et al., 2010; Jiménez-Ferrer et al., 2012; Kao, Yang, Hung, Huang, & Wang, 2016; Lin et al., 2012; Micucci et al., 2015; Moyano et al., 2016; Rosemary, Rosidah, & Haro, 2014; Usoh et al., 2012; Celia Rodríguez-Pérez, Segura-Carretero, & del Mar Contreras, 2017). These effects have been

attributed to its chemical composition, mainly based on phenolic compounds i.e. hibiscus acid, chlorogenic acid, anthocyanins, quercetin or kaempferol. For example, Segura-Carretero et al., (2008) demonstrated that the anthocyanins delphinidin-3-sambubioside and cyaniding-3-sambubioside were the main components in the aqueous extract of *H. sabdariffa* using solid phase extraction-capillary electrophoresis-mass spectrometry. Also, Alarcón-Alonso et al., (2012) reported that 1 g of *H. sabdariffa* water extract contains 56.5 mg delphinidin-3-O-sambubioside, 20.8 mg/g cyanidin-3-O-sambubioside, 3.2 mg/g quercetin, 2.7 mg/g chlorogenic acid and 2.1 mg/g rutin. Numerous studies have defined the anthocyanins as an important group of water-soluble plant pigments, belonging to the flavonoid family, with a nutritional interest due to their protective mechanism against environmental stress factors (De Pascual-Teresa & Sanchez-Ballesta, 2008).

Therefore, the incorporation of *H. sabdariffa* into oils, will give rise to functional oils of interest for, both, food industry and consumers. However, it should not be forgotten that phenolic compounds from *H. sabdariffa* are unstable and especially sensitive to changes in pH, temperature or atmospheric composition. This chemical instability characteristic makes necessary to preserve them from agents such as light, oxygen, temperature, or even gastric residence time. Therefore, in order to establish a vehicle for incorporating *H. sabdariffa* antioxidants in a food matrix is necessary to reduce or prevent degradation of flavonoids. To achieve it, the development of products able to protect flavonoids and other phenolic compounds from any chemical modification, thus, ensuring their bioactivity is crucial. Consequently, functional oils formulation appears as an available and scalable

technique to carry out this challenge that could allow the prolongation of the shelf life antioxidants extracted from *H. sabdariffa*. For functional oils formulation, both, the choice of the best oils and the use of emulsions are important factors to be considered. For its purpose, and despite they are more susceptible to lipid oxidation due to unsaturated fatty acids (Friberg, Larsson, & Sjoblom, 2003), vegetable oils such as sunflower and olive oil have been the most widely employed because of their sensory and technological properties. Functional food formulation using water in oil (W/O) emulsions has already been used to protect antioxidants from natural plant e.g. rosemary, thyme, green tea or lotus extract achieving better stability of these compounds (Gallego, Hakkarainen, & Almajano, 2017; Mahmood, Akhtar, & Manickam, 2014). In this regard, Sahat, Zaidel, Muhamad, & Alam, (2014), studied the stability of water-in-oil emulsions containing anthocyanins from *Red cabbage*. The parameters studied were the storage and stability characteristics of the emulsion such as, temperature or solid fat content from oil phase. That study demonstrated that anthocyanins affected positively the stability of the oil phase at the same time that these anthocyanins were more stable into the oil phase. On the other hand, *H. sabdariffa* extract has demonstrated to be a potential antioxidant when added to fat products due to its chemical composition. In a recently study, *H. sabdariffa* extracts were incorporated in emulsions containing whey proteins to study the antioxidant effect on soybean oil, sunflower oil and palm oil (Gagaoua et al., 2017). By Rancimat test, a positive effect on oxidative stability of the emulsions was observed.

Therefore, to get an adequate structure and stability over time of W/O emulsions incorporating plant extracts, numerous factors must be controlled. Thus,

the aims of this study were 1) to incorporate a hydrophilic *H. sabdariffa* extract containing 10% of anthocyanidins into extra virgin olive (EVOO) and sunflower (SO) oils in order to develop functional oils in the form of W/O emulsions; 2) to study the physical stability of the emulsions i.e. droplet size distribution, concentration and composition of dispersed phase and emulsifier concentration; 3) to optimize a method for the extraction of phenolic compounds from *H. sabdariffa* emulsions by multi-response surface methodology (RSM) and 4) to monitor the oxidative stability of the emulsions i.e. antioxidant capacity, total phenolic compounds and chemical composition measured by HPLC-ESI-TOF-MS.

Materials and methods

Materials

A commercial hydrophilic *H. sabdariffa* extract containing 10% of anthocyanidins was vehiculized into the inner phase of W/O emulsion. The dried calyces of *H. sabdariffa* were provided by Monteloeder Inc. (Elche, Alicante, Spain). The oil phase of the emulsions (O) was made up commercially sunflower oil (SO) or extra virgin olive oil (EVOO). To get an adequate hydrophilic-lipophilic balance (HLB), polyethylene glycol sorbitan monolaurate (Tween 20), and sorbitane monooleate (Span 80) were employed as hydrophilic and hydrophobic surfactants respectively (both from Merck, Hohenbrunn, Germany). The adequate HLB was obtained in a previous study (data not published). Acetonitrile, methanol, hexane, ethanol, gallic acid, Folin–Ciocalteu reagent, 2,2 -azinobis (3-ethylbenzothiazoline- 6-sulfonate(ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman- 2-carboxylic acid (Trolox), 1,3,5-triphenyltetrazolium chloride (TPTZ) , fluorescein, potassium persulfate ferric sulfate

and potassium persulfate were purchased from Sigma–Aldrich (Steinheim, Germany). Ultrapure water with a resistivity value of 18.2 M was from a Milli-Q system (Millipore, Bedford, MA, USA). Dehydrated sodium phosphate, trihydrated sodium acetate, sodium acetate, ferric chloride, hydrochloric acid, and acetic acid were purchased from Panreac (Barcelona, Spain).

Methods

Development of water-in-oil (W/O) emulsions

W/O emulsions were formulated with different total surfactant concentrations (8 wt %, 10 wt % and 12 wt %) and different HLB (8, 9.5, 10.5, and 11.5). The dispersed phase consisted of 10 wt % or 20 wt % of *H. sabdariffa* extract. The dispersed phase mass fraction (φ) was 0.3. Tween 20 and Span 80 were used to get the adequate HLB for stabilizing sunflower or extra virgin olive oil continuous phase. All emulsions were carried out using the rotor stator system (Ultra-Turrax IKA T25) at 12000 rpm for 5 minutes (20ºC).

Physical stability: Particle size distribution and rate of coalescence

To determine the physical stability of the emulsions, the particle size distribution was measured after its preparation and during storage. The droplet sizes of the emulsions were determined by Malvern Mastersizer 2000S (Malvern Instruments Ltd., U.K.). Each measurement of droplet size of the emulsions was determined over time in triplicate and the results were expressed as volume diameter ($D_{4.3}$). The optical parameters selected were a dispersed phase refractive index of 1.333 (water refractive index) and a continuous phase refractive index of 1.467 and 1.470 for extra virgin olive oil and sunflower oil respectively. Also, the rate of

coalescence (Kc) was determined according to Pimentel-Moral, Ochando-Pulido, Segura-Carretero, & Martinez-Ferez, (2018). Both methods were evaluated for 30 days to evaluate the emulsion behavior in the long term.

Extraction of phenolic compounds

The extraction procedure was as follows: 0.5 g W/O emulsion was diluted with hexane (5 mL) and stirred in vortex for 1 min to remove the lipophilic phase from the oils. Then, 5 mL of different mixtures of methanol:water (range of methanol 0% to 50%) were added and stirred again in vortex for 2 minutes and 30 seconds. Ultrasound-assisted extraction was carried out in a sonicator Branson B3510 for 10 min at room temperature and finally samples were centrifuged at different revolution/minute (range of rpm was 5000 rpm to 10000 rpm). The supernatant was collected and the solvent was evaporated using a rotary evaporator under vacuum at 35ºC.

The extraction was optimized by response surface methodology (RSM), which is a compilation of mathematical an statistical techniques based on the fit of a polynomial equation to the experimental data, in order to describe the behavior of a data set with the aim of making statistical predictions (Yolmeh & Jafari, 2017). The independent variables considered were the proportion of methanol in the solvent mixture (0-50% methanol) and the speed of centrifuge (5000-10000 rpm) while the factor responses were recovery, total phenolic compounds and antioxidant activity. To study the influence of percentage of solvents and the speed of the centrifuge on the abovementioned responses, a full factorial experimental design at three levels (3^2) with 3 central points which included 12 experimental points was employed. Optimal extraction conditions were estimated by multiple linear regressions using Statgraphics

Centurion XVI software (Statpoint Technologies, Warrenton, Virginia, USA). To verify the suitability of the quadratic equations for predicting optimal responses values, the verification experiments for each extraction were made in triplicate under optimal conditions.

Oxidative stability of *Hibiscus sabdariffa* extract as W/O emulsion

a) Antioxidant activity assays

The oxidative stability of W/O emulsions for 30 days was determined. To this end, the emulsions were maintained at room temperature and the antioxidant activity trough TEAC and FRAP assays described by Morales-Soto et al., (2014) were carried out. Briefly, in the trolox equivalent antioxidant capacity (TEAC) assay, the ABTS radical cation ($ABTS^{+}$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and keeping the mixture in darkness at room temperature for 12 to 24 h before use. The $ABTS^{+}$ solution was diluted with water until reaching an absorbance value of 0.70 (±0.02) at 734 nm. For the spectrophotometric assay, 300 µL of the $ABTS^{+}$ solution and 30 µL of the extract were mixed and measured immediately using a Synergy Mx Monochromator – Based Multi – Mode Microplate reader by Bio-Tek (Winooski, VT, USA). The result of each sample was then compared with a standard curve made from the corresponding readings of trolox (0.5–30 µM in the microplate wells). The results were expressed as mmol Eq Trolox / 100 g extract. On its behalf, in the ferric ion reducing antioxidant power (FRAP) assay, 40 µL the extract was mixed on a 96-well plate with 250 µL of FRAP reagent. Samples were incubated at 37ºC for 10 min. The absorbance at 593 nm was recorded for 4 min on the microplate reader. Finally the final absorbance of each sample was compared with

those from the standard curve made from $FeSO_4 \cdot 7H_2O$ (12.5–200 µM in the microplate wells). The results were expressed as mmol Eq $FeSO_4$ / 100 g extract.

b) Total phenolic content

The total phenolic content (TPC) was measured at time 0, 7, 14, 21 and 30 days, using the Folin-Ciocalteu assay reported by Kiritsakis, Rodríguez-Pérez, Gerasopoulos, & Segura- Carretero, (2017). In brief, the total volume of the reaction mixture was reduced to 1 mL. Next, 10 µL of each sample were mixed in 50 µL of Folin-Ciocalteu reagent. After 10 min, 150 µL of 2% (w/v) Na_2CO_3 were added and the volume was made up to 1.0 mL with water. The mixture was incubated for 2 h at room temperature in darkness and, then, 200 µL of the mixture was transferred into a well of a microplate. The absorbance was measured at 760 nm and compared to a gallic acid calibration curve (25-500 µg/mL) elaborated in the same manner. The total phenolic content was calculated as mean ± SD (n = 3) and expressed as mg of gallic acid per g of oil.

c) Characterization of phenolic compounds from *H. sabdariffa* emulsions by HPLC-ESI-TOF-MS

The individual phenolic compounds extracted from *H. sabdariffa* emulsions at different times were tentatively characterized. To that end, HPLC analyses were made with an Agilent 1200 series rapid resolution LC system (Agilent Technologies, Palo Alto, CA, USA) equipped with a binary pump, an autosampler and a diode-array detector (DAD). Separation was carried out with a Zorbax Eclipse Plus C_{18} analytical column (150 mm × 4.6 mm, 1.8 m particle size). Water–formic acid (90:10, v/v) and acetonitrile were used as mobile phases A and B, respectively. The HPLC system was coupled to a microTOF-Q II mass spectrometer (Bruker Daltonik GmbH, Bremen, Germany)

equipped with electrospray ionization (ESI) operating in positive mode. Flow rate was set at 0.5 mL/min using the following gradient: 0–13 min, linear gradient from 0% B to 20% B; 13–20 min, linear gradient from 20% B to 30% B; 20–25 min, linear gradient from 30% B to 80% B; 25–30 min, linear gradient from 80% B to 0% B; and 30–35 min, isocratic of 0% B described by Iswaldi et al., (2012). The injection volume in the HPLC system was 10 µL.

Statistical analysis

Quantitative data are represented as mean ± SD. To evaluate the differences at 95% confidence level ($p \leq 0.05$), a one-way analysis of variance (ANOVA) followed by Tukey's test was performed using Origin (version Origin Pro 8 SR0, Northampton, MA, USA).

Results and discussion

Physical stability of W/O emulsions

Figure 1 depicts the evolution of $D_{4,3}$ for (W/O) emulsions as a function of type of oil used. It can be seen that oil type, amount emulsifiers and HLB influenced considerably on particle size of W/O emulsions. This was already reported by Diamante & Lan, (2014); Friberg et al., (2003).

Regarding the oil type, EVOO emulsions showed smaller droplet sizes than SO emulsions. EVOO emulsions showed droplet sizes around 0.68 µm while values around 0.93 µm were obtained for SO emulsions droplet sizes after 30 days at room temperature. In the case of SO emulsions, we have found an improvement of the physical stability (lower droplet size) at higher surfactant concentration and HLB values for both *H. sabdariffa* extract concentrations studied. On the contrary, the amount of

emulsifiers affected the physical stability of EVOO emulsions only with 10 % wt extract concentration. Therefore, oil type and surfactants amount demonstrated to be two important factors in the emulsion stability.

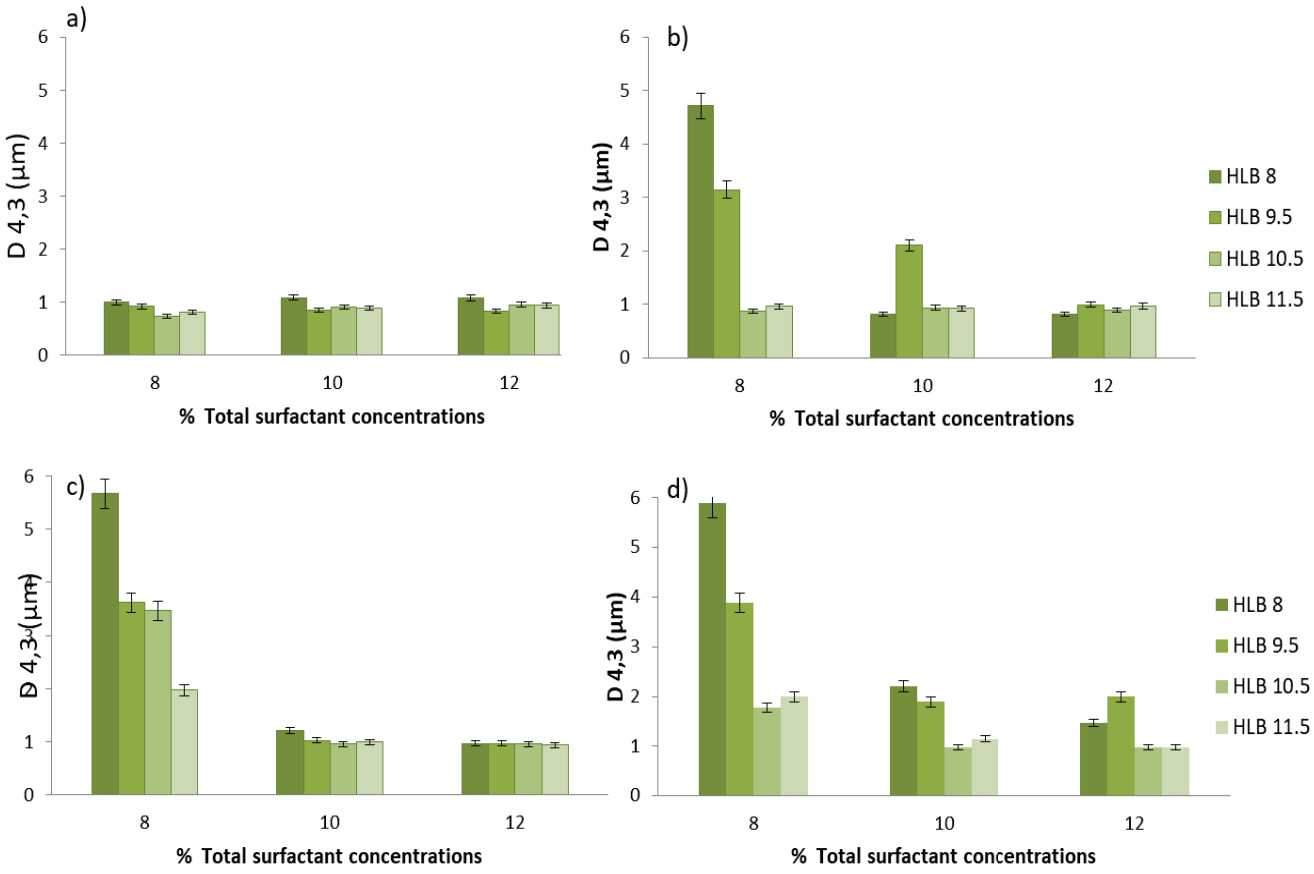


Figure 1. Evolution of $D_{4,3}$ for (W/O) emulsions as a function of type of oil used. a) (W/O) 20% extract concentration in EVOO emulsions, b) (W/O) 10% extract concentration in EVOO emulsions, c) (W/O) 20% extract concentration in SO emulsions, d) (W/O) 10% extract concentration in SO emulsions.

This behaviour could be explained by the different viscosities of the different vegetable oils. In fact, olive oil showed higher viscosity as compared to sunflower oil (0.592 vs 0.0488 Pa·s) (Diamante & Lan, 2014). In addition, this difference of viscosity could explain the smaller droplet size in EVOO emulsions and the different optimal surfactant amount among oils. Indeed, the size of the droplets after emulsification

depends on the ratio and amount of emulsifier which must be enough present to cover the droplet surfaces (Friberg et al., 2003). These results are in agreement with the study carried out by Kowalska, Zbikowska, Wozniak, & Kucharczyk, (2017) in which the long-term stability of rose oil emulsion with six different emulsifiers amount was studied. They confirmed that greater amounts of emulsifier lead to a stable particle size distribution.

Other important factor related to physical stability was the extract concentration. Emulsions with 20% of extract concentration showed smaller droplet sizes than those with 10% extract concentration. This could be explained by the *H. sabdariffa* extract acidity i.e. pH 2.7 and 3.2 were obtained for 20 % and 10 % wt extract concentration, respectively. The pH of the solution determines the ionization of surface groups and therefore the final surface charge density. For that reason a decrease of pH induces a higher repulsion between droplets (surfaces negatively charged), showing as a consequence smaller droplet size distribution (Israelachvili, 2011). Previous studies have determined the impact of pH on physical stability of emulsions. Recently, the effect of pH on curcumin emulsions was investigated (Kharat, Du, Zhang, & McClements, 2017). The authors found that more than 85 % of curcumin was present after one month at 37 ºC when acidic conditions were employed. However, emulsions at pH 7.0, 7.4 and 8.0 contained only 62, 60, and 53% of the initial curcumin respectively, demonstrating worse stability. On the other hand, the impact of pH on the stability of emulsions stabilized by pectin-zein complexes has also been studied (Juttulapa, Piriyaprasarth, & Sriamornsak, 2013). They found a greater cross-linking polymer network at pH 4 than pH 7, providing thus a smaller droplet size

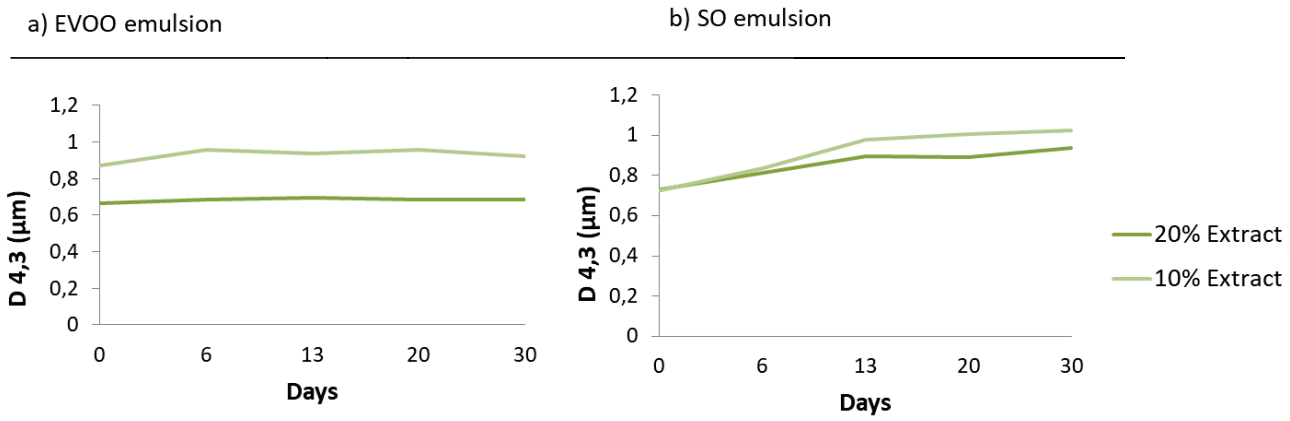


Figure 2. Volume mean diameter ($D_{4,3}$) over time at room temperature as a function of extract concentration. a) EVOO emulsion, b) SO emulsion.

Figure 2 shows the evolution of $D_{4,3}$ for (W/O) EVOO (a) and SO (b) emulsions over time. It can be seen that SO and EVOO emulsions showed stable droplet sizes after 30 days of storage at room temperature. These results were confirmed with the coalescence rate **(Table 1)**. The lowest *Kc* value for sunflower oil emulsions was achieved with 20% extract concentration, 12% wt total surfactants concentration and 10.5 HLB. In contrast, for olive oil emulsions, the lowest *Kc* value was obtained with 20% wt extract concentration, 8% wt total surfactants concentration and 10.5 HLB.

E.V. Olive oil emulsions			
Surfactant concentration	**HLB**	**Kc (W/O) (s-1) (20% extract concentration)**	**Kc (W/O) (s-1) (10% extract concentration)**
8%	8	4.91615E-11	2.95795E-09
	9.5	3.06323E-11	2.76667E-10
	10.5	7.12E-12	2.6095E-11
	11.5	2.21973E-11	4.01623E-11
10%	8	4.87282E-11	4.97137E-10
	9.5	3.79982E-11	2.08997E-10
	10.5	2.27605E-11	1.67916E-11
	11.5	1.32626E-11	4.36756E-11
12%	8	3.60981E-11	7.85222E-11
	9.5	2.23664E-11	2.70558E-10
	10.5	1.08935E-11	4.17886E-11
	11.5	1.26799E-11	3.4675E-11
Sunflower oil emulsions			
Surfactant concentration	**HLB**	**Kc (W1/O) (s-1) (20% extract concentration)**	**Kc (W1/O) (s-1) (10% extract concentration)**
8%	8	2.54121E-11	1.8494E-09
	9.5	1.7524E-11	4.27467E-10
	10.5	1.00081E-11	8.33183E-11
	11.5	2.63235E-11	1.40699E-11
10%	8	2.44348E-11	2.76459E-11
	9.5	3.42648E-11	4.84179E-10
	10.5	3.53715E-11	3.31253E-11
	11.5	2.15639E-11	4.6571E-11
12%	8	5.75725E-11	3.84442E-10
	9.5	2.15919E-11	5.85638E-11
	10.5	8.64906E-12	2.92929E-11
	11.5	1.36906E-11	2.69632E-11

Table 1. Coalescence rates of the W/O emulsions

Oxidative stability

Optimization of extraction procedure by multi-response surfaces (RSM)

The extraction of phenolic compounds from *H. sabdariffa* in form of emulsions was carried out by RSM, which provided the optimal conditions to maximize extract yield, TPC and antioxidant activity. A ratio of water:methanol in the solvent mixture and speed of centrifuge were the independent variables.

The optimal conditions for EVOO emulsions were as follows: 1.22 % methanol and 8023 rpm. These values were determined on the basis of the desirability function, which was 0.609. However, for SO emulsions, the optimal conditions were 50 % methanol and 7036 rpm. In this case, the optimization desirability was 0.689. **Figure S1** (supplementary material) represents the 3D plots of the response surface for solvent percentage and speed of centrifuge in phenolic extraction of olive oil emulsions (S1.a) and sunflower oil emulsions (S1.b).

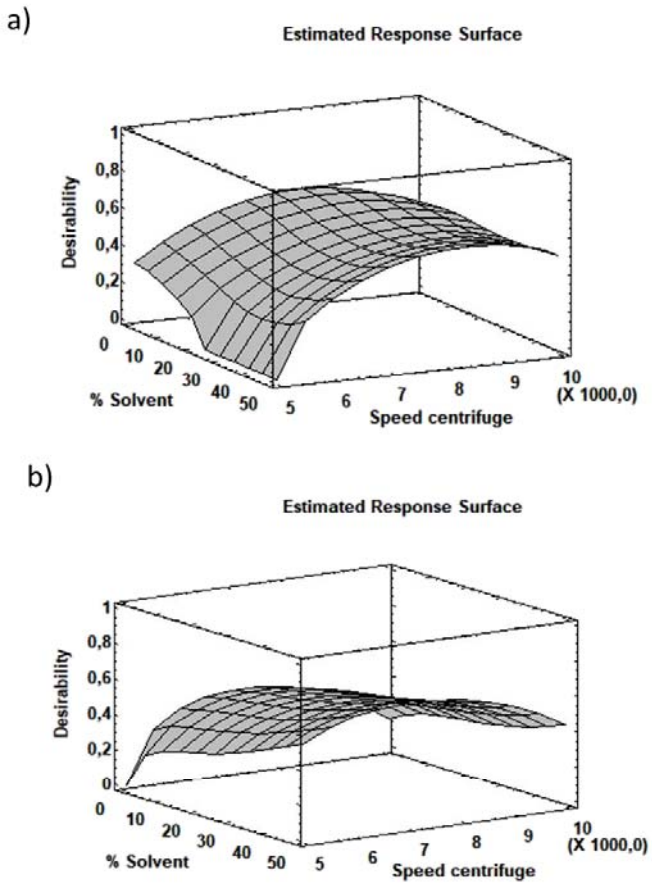


Figure.S1. 3D plots of response surface for the effects of solvent (% methanol in the mixture) and speed of centrifuge (rpm) on the overall desirability in olive oil emulsions (a) and sunflower oil emulsions (b).

To verify the experimental process with theoretical mathematical model, the optimum conditions were carried out in triplicate and it was possible to observe that the results were closer to the predicted one. According the author's knowledge, it is the first time that an optimization method for antioxidant extraction from emulsions has been carried out. In addition, this method has demonstrated that speed of centrifuge and methanol amount are two important factors for the antioxidant extraction from emulsions.

	Independent variables			Investigated responses		
Run	Solvent (%)	Speed centrifuge (rpm)	Yield (%)	TPC (mg Eq GAE/g extract)	FRAP (mmol Eq FeSO4/100 g extract)	TEAC (mmol Eq Trolox/100 g extract)
1	25	7500	138.00	3.22	87.41	93.86
2	50	10000	123.00	3.17	88.63	117.18
3	0	5000	128.33	3.72	70.65	106.83
4	25	7500	134.33	3.50	80.01	91.57
5	25	10000	120.67	3.39	83.64	104.99
6	0	7500	144.00	4.28	68.10	110.72
7	50	5000	129.00	2.82	88.08	75.66
8	25	7500	134.33	3.14	82.44	93.79
9	0	10000	150.33	3.22	63.54	114.14
10	25	5000	102.67	2.66	69.81	82.15
11	50	7500	103.00	2.98	92.06	115.00
12	25	7500	126.67	3.21	85.18	89.60

Response	**Predicted**	**Observed**
Yield (%)	146.286	135.30 ± 5.13
TPC (mg Eq GAE/g extract)	3.735	2.02 ± 0.07
FRAP (mmol Eq $FeSO_4$/Kg)	7.089	78.41 ± 0.33
TEAC (mmol Eq Trolox/100 g extract)	112.224	108.24 ± 1.29

Optimized desirability: 0.609 Extraction conditions: 8023.79 rpm; 1.2153 % methanol

Table S4. Three – level factorial design (3^2) of the two-level and four-variable and observed responses under different experimental conditions. Predicted and observed values of each individual reponse for EVOO oil emulsions.

Independent variables			Investigated responses			
Run	Solvent (%)	Speed centrifuge (rpm)	Yield (%)	TPC (mg Eq GAE/g extract)	FRAP (mmol Eq FeSO4/100 g extract)	TEAC (mmol Eq Trolox/100 g extract)
1	25	7500	123.00	4.26	116.27	145.44
2	50	10000	112.67	4.27	109.72	133.87
3	0	5000	125.67	2.83	95.14	148.49
4	25	7500	123.33	3.82	118.21	145.98
5	25	10000	114.33	3.79	103.99	147.18
6	0	7500	129.00	4.74	91.31	138.44
7	50	5000	107.67	3.77	119.22	146.67
8	25	7500	123.33	3.64	105.07	126.64
9	0	10000	114.33	4.42	96.43	122.99
10	25	5000	120.67	3.48	103.51	159.95
11	50	7500	115.67	4.12	139.07	152.05
12	25	7500	126.67	3.58	110.13	137.02

Response	Predicted	Observed
Yield (%)	122.581	120.00 ± 3.46
TPC (mg Eq GAE/g extract)	4.2779	4.61 ± 0.15
FRAP (mmol Eq $FeSO_4$/Kg)	12.9331	107.56 ± 0.17
TEAC (mmol Eq Trolox/100 g extract)	142.798	155.85 ± 5.66

Optimized desirability: 0.689. Extraction conditions: 7036.29 rpm; 50% methanol

Table S5. Three – level factorial design (3^2) of the two-level and four-variable and observed responses under different experimental conditions. Predicted and observed values of each individual reponse for sunflower oil emulsions

Table S4 and S5 (Supplementary material) show the responses obtained for each experimental condition. In this context, there were differences between both types of emulsions.

The yield was higher in EVOO emulsions (from 102.67 to 138.00 % *vs* 107.67 to 129.00 % corresponding to SO emulsion). However, the total phenolic content and antioxidant activity, measured by the TEAC and FRAP spectrophotometric methods, were higher in SO emulsions. Specifically, EVOO and SO emulsions showed a TPC

ranging from 2.655 to 4.276 and from 2.832 to 4.747 mg Eq GAE / g extract, respectively. TEAC assay showed values from 75.66 to 117.18 in EVOO emulsion *vs* values from 122.99 to 159.95 mmol Eq Trolox / 100 g extract in SO emulsion. Finally, FRAP assay values ranged from 3.54 to 92.06 in EVOO emulsions *vs* values from 91.31 to 139.07 mmol Eq $FeSO_4$ / 100 g extract in SO emulsions. This different behavior can be explained by the different oxygen solubility in olive oil and sunflower oil. In this regard, our results are in agreement with Chaix, Guillaume, & Guillard, (2014) who investigated the oxygen solubility and diffusivity in solid food matrices showing that the oxygen solubility in olive oil was 5.5 E-08 whereas in sunflower oil was 8.6 E-09 $mol \cdot kg^{-1} \cdot Pa^{-1}$. These results demonstrated higher oxygen solubility in olive oil which could explain the higher TPC and antioxidant activity in SO than in EVOO emulsions.

Evolution of antioxidant activity and total phenolic content over time of *Hibiscus sabdariffa* extracted from olive oil and sunflower oil emulsions

Despite FRAP and TEAC may be comparable in terms of antioxidant activity capacity, FRAP results can vary tremendously depending on the time scale of analysis and some polyphenols binds the iron more slowly and require longer reaction times for detection (Prior, Wu, & Schaich, 2005). For that reason, to evaluate the chemical stability of *H. sabdariffa* as W/O emulsion over time, TEAC assay was chosen. This methods represents one of the most employed assays for determining antioxidant activity from foods, plants and plant-based products, allowing further comparisons between samples and laboratories (Qualities, 2003; Zhen et al., 2016). In addition, previous researches revealed a good ability of the major compounds present in *H. sabdariffa* i.e. cyanidin, delphinidin and their glycosylated derivatives to scavenge the

radical cations (Christian, Nair, & Jackson, 2006). Furthermore, the TPC measured by Folin-Ciocalteu assay was employed. **Figure 3** and **Table S6** (Supplementary material) show the evolution of these factors at 0, 7, 14, 21 and 30 days.

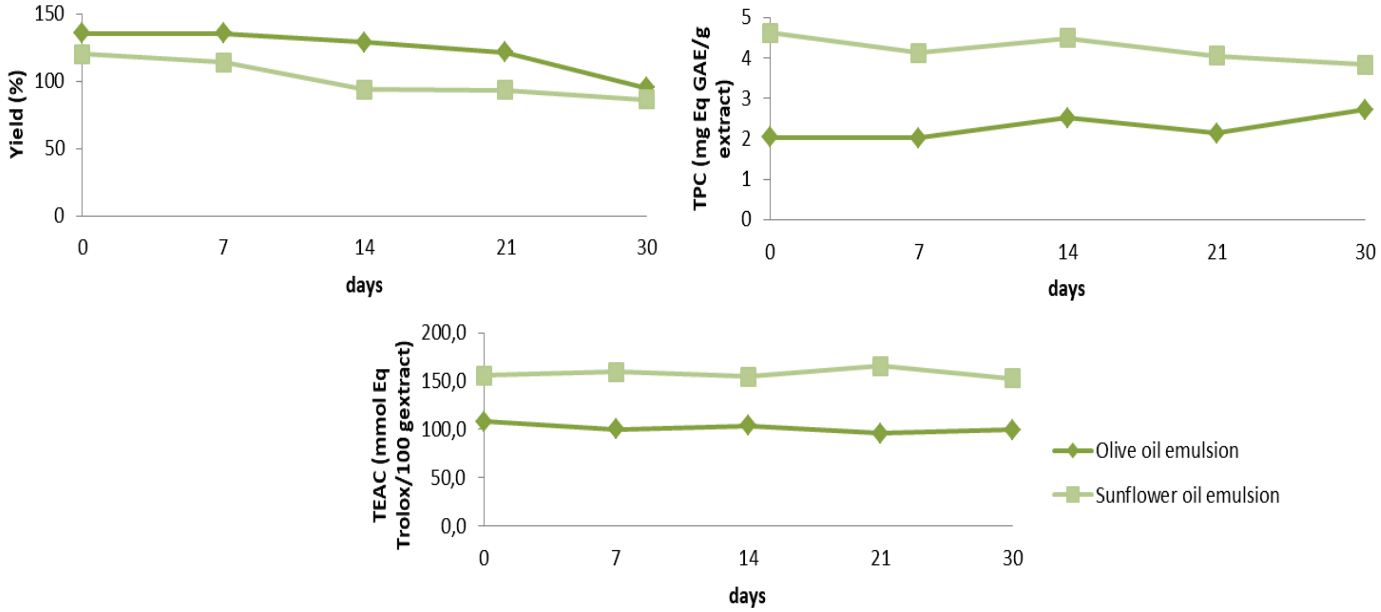


Figure 3. Evolution of oxidative stability over time.

It can be seen that the yield significant decreased over time in both types of emulsions (from 135.30 % ± 3.54 to 95.00 % ± 5.30 in EVOO emulsion and from 120.00 % ± 2.31 to 86.21 % ± 4.23 in SO emulsions). However, the total phenolic content obtained by Folin-Ciocalteu and the antioxidant activity obtained by TEAC kept relatively constant over time during storage at room temperature for EVOO and SO emulsions. Specifically, EVOO emulsions showed a significant increment in TPC (from 2.024 ± 0.074 to 2.714 ± 0.062 mg Eq GAE / g extract). In contrast, SO emulsions significantly decreased the TPC over time (from 4.61 ± 0.15 to 3.83 ± 0.09 mg Eq GAE / g extract). No significant differences in antioxidant activity measured by TEAC between

time 0 and time 30 were found for both types of emulsions (from 108.24 ± 1.30 to 99.60 ± 4.46 mmol Eq Trolox / 100 g extract and from 155.85 ± 5.66 to 152.52 ± 9.52 mmol Eq Trolox / 100 g extract for EVOO and SO emulsion, respectively).

EVOO emulsions			
Time (days)	Yield (%)	TPC (mg Eq GAE/g extract)	TEAC (mmol Eq Trolox/100g extract)
0	135.30 ± 3.54 [a]	2.02 ± 0.07 [a,c]	108.24 ± 1.30 [a]
7	135.60 ± 3.21 [a]	2.02 ± 0.09 [a,c]	100.12 ± 7.40 [a]
14	128.96 ± 4.32 [b]	2.52 ± 0.24 [b]	103.90 ± 4.95 [a]
21	121.30 ± 4.98 [b]	2.14 ± 0.08 [a,c]	95.50 ± 7.20 [a]
30	95.00 ± 5.30 [c]	2.71 ± 0.06 [c,d]	99.60 ± 4.46 [a]
SO emulsions			
Time (days)	Yield (%)	TPC (mg Eq GAE/g extract)	TEAC (mmol Eq Trolox/100g extract)
0	120.00 ± 2.31 [a]	4.61 ± 0.15 [a]	155.85 ± 5.66 [a]
7	114.00 ± 4.60 [a]	4.13 ± 0.11 [b,c]	159.25 ± 8.55 [a]
14	94.00 ± 4.78 [b]	4.49 ± 0.11 [d]	154.50 ± 2.31 [a]
21	93.33 ± 3.72 [b]	4.05 ± 0.19 [b,c,e]	165.71 ± 14.32 [a]
30	86.21 ± 4.23 [b]	3.83 ± 0.09 [f]	152.52 ± 9.52 [a]

Table S6. Evolution of each individual response at different times for 30 days

To the best of our knowledge, this is the first time that a W/O emulsion for formulating functional oils enriched in bioactive compounds from *H. sabdariffa* has been developed, thus, hampering comparisons with other studies in terms of oxidative stability. However, similar studies have reported the use of this technology to encapsulate bioactive food ingredients. In this regards, a research studied the antioxidant stability of resveratrol into multilayer emulsions during four weeks. The results showed lower antioxidant stability in resveratrol-enriched corn oil, *versus* resveratrol emulsions (Acevedo-Fani, Silva, Soliva-Fortuny, Martín-Belloso, & Vicente, 2017). In other study, the antioxidant activity degradation of *Equisetum arvense* extract nanoemulsions was evaluated (Hernández-Jaimes, Fouconnier, Pérez-Alonso, Munguía-Guillén, & Vernon-Carter, 2013). The method used was DPPH radical

scavenging activity and the measurements were made each week for 12 weeks. The antioxidant loss rate for nanoemulsions was eleven times slower and the time required decreasing their antioxidant activity by half was larger than for the free *Equisetum arvense.*

Chemical characterization of *Hibiscus sabdariffa* extract as W/O emulsion by HPLC-ESI-TOF-MS

Although, spectrophotometric assays such as TPC are considered to be a good option for quantifying phenolic compounds and useful for screening purposes and for acquiring a general estimate of some groups of bioactive compounds in different matrices, it is important to complement these results with the analysis of each individual compound, since the behavior depend on the chemical structure (C. Rodríguez-Pérez et al., 2015). Therefore, the phenolic composition of the *H. sabdariffa* free extract and the functional oils at 0, 7, 14, 21 and 30 days were tentatively characterized to evaluate the potential effect of storage time on the chemical composition.

Figure S2 (Supplementary material) shows the base-peak chromatogram (BPC) for the free extract (A) and the extract from W/O emulsions at time 0 (B) resulting from the HPLC-ESI-TOF-MS method previously described (Iswaldi et al., 2012). Each peak was numbered according to its elution order. Moreover, **Table S7** (Supplementary material) and **Table 2** show a list of the compounds tentatively characterized including their retention time (RT), experimental m/z, molecular formula and mass error of free extract (Table S7, Supplementary material) and extract from W/O EVOO emulsions and SO emulsions (Table 2) at time 0 respectively.

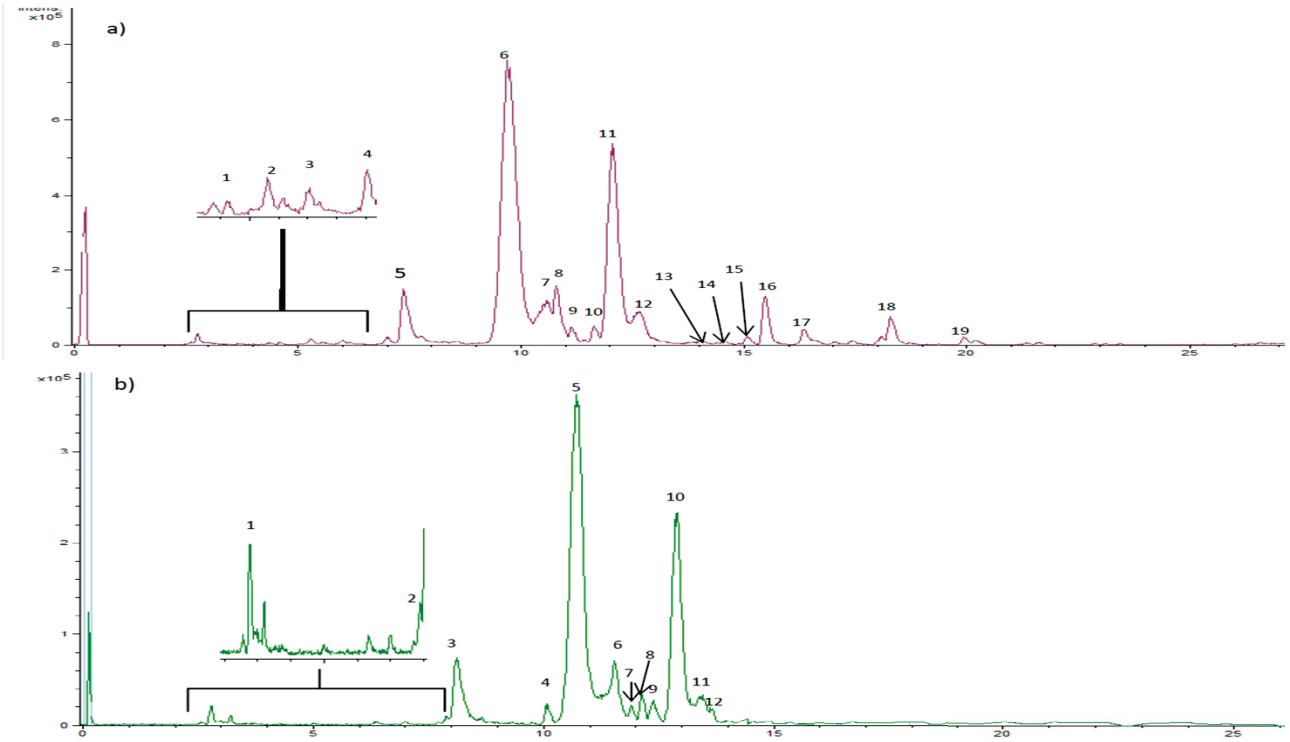


Figure S2. Base-Peak Chromatogram (BPC) of free H. sabdariffa extract (a) and extracted from W/O EVOO emulsion at time 0 (b) analyzed by HPLC-ESI-TOF-MS in positive ion mode

Peak	RT	$[M+H]^+$	Molecular formula	Error (ppm)	mSigma	Compound
1	4.4	252.0757	$C_9H_9N_5O_4$	-1.8	15.5	Aspartic acid, N-purin-6-yl-
2	4.68	259.0408	$C_{10}H_{10}O_8$	7.4	12.7	Aceglatone
3	5.6	195.0288	$C_9H_6O_5$	3.2	9.5	Trihydroxycoumarin
4	7.04	136.0618	$C_5H_5N_5$	0.7	3.5	Adenine
5	7.41	163.0390	$C_9H_6O_3$	-1.5	6.2	Hydroxycoumarin isomer I
6	9.57	597.1450	$C_{26}H_{28}O_{16}$	-2.8	16.6	Delphinidin 3-sambubioside
7	10.57	303.0499	$C_{15}H_{10}O_7$	4	16.7	Quercetin isomer I
8	11.17	193.0495	$C_{10}H_8O_4$	3.0	6.0	Scopoletin
9	10.82	163.0390	$C_9H_6O_3$	0.7	1.1	Hydroxycoumarin isomer II
10	11.72	163.0390	$C_9H_6O_3$	2.2	3.7	Hydroxycoumarin isomer III
11	12.06	581.1501	$C_{26}H_{28}O_{15}$	-1.4	8.2	Cyanidin 3-sambubinoside
12	12.71	287.0550	$C_{15}H_{10}O_6$	3	3	Kaempferol isomer I
13	14.05	319.0448	$C_{15}H_{10}O_8$	7.7	7.1	Gossypetin isomer I
14	15.47	319.0448	$C_{15}H_{10}O_8$	0	3.9	Gossypetin isomer II
15	17.04	303.0499	$C_{15}H_{10}O_7$	-3.6	2.4	Quercetin isomer II
16	18.09	163.0390	$C_9H_6O_3$	6.6	16.2	Hydroxycoumarin isomer IIII
17	18.26	303.0499	$C_{15}H_{10}O_7$	5.4	3.5	Quercetin isomer III
18	19.95	287.0550	$C_{15}H_{10}O_6$	3.1	1.6	Kaempferol isomer II
19	20.3	303.0499	$C_{15}H_{10}O_7$	-5	23.2	Quercetin isomer IIII

Table S7. HPLC-ESI-TOF-MS data of the compounds identified in free *H. sabdariffa* extract

A total of 19 compounds were tentatively characterized in the free extract including anthocyanidins (delphinidin 3-sambubioside and cyanidin 3-sambubinoside), flavonoids (quercetin, kaempferol and gossypetin), and cumarins (hydroxycoumarin and scopoletin). In addition, the compound scopoletin and two isomers of gossypetin have been tentatively characterized for the first time in *H. sabdariffa.* As expected, the main compounds identified were the anthocyanidins delphinidin 3-sambubioside and cyanidin 3-sambubinoside.

Peak	RT	$[M+H]^+$	Molecular formula	Compound	EVOO emulsion	SO emulsion
1	7.71	163.0390	$C_9H_6O_3$	Hydroxycoumarin isomer I	+	+
2	7.95	136.0618	$C_5H_5N_5$	Adenine	+	+
3	8.15	163.0390	$C_9H_6O_3$	Hydroxycoumarin isomer II	+	+
4	8.68	195.0288	$C_9H_6O_5$	Trihydroxycoumarin	+	+
5	10.54	597.1450	$C_{26}H_{28}O_{16}$	Delphinidin 3-sambubioside	+	+
6	10.79	303.0499	$C_{15}H_{10}O_7$	Quercetin	+	+
7	11.56	163.0390	$C_9H_6O_3$	Hydroxycoumarin isomer III	+	+
8	11.93	193.0495	$C_{10}H_8O_4$	Scopoletin	+	-
9	12.43	163.0390	$C_9H_6O_3$	Hydroxycoumarin isomer IIII	+	+
10	12.78	581.1501	$C_{26}H_{28}O_{15}$	Cyanidin 3-sambubinoside	+	+
11	12.96	287.0550	$C_{15}H_{10}O_6$	Kaempferol isomer I	+	+
12	13.68	287.0550	$C_{15}H_{10}O_6$	Kaempferol isomer II	+	-

Table 2. HPLC-ESI-TOF-MS data of the compounds identified in H. sabdariffa extracted from W/O EVOO emulsions and SO emulsions

From these 19 compounds, 12 were found in EVOO emulsions whereas a total of 10 compounds were tentatively characterized in SO emulsions (Table 2). Among them, it is possible to observe that the compounds described as bioactive compounds in *H. sabdariffa* extract, have been successfully incorporated into the W/O emulsions. However, not all compounds previously characterized in *H. sabdariffa* extract i.e. anthocyanidins, cumarins or flavonoids were found in the emulsions. The absence of

some compounds i.e. gossypetin, some isomers of kaempferol or quercetin in the emulsions could be due to the formulation of emulsions by ultra-turrax which could have a negative impact on some compounds due to shear force that is produced by this mechanism. When comparing between extract from EVOO emulsions and SO emulsions (**Figure S3** Supplementary material) chemical composition, it is possible to observe slight differences. There were two compounds (scopoletin and kaempferol isomer II) present in extract from olive oil emulsion and in extract from sunflower oil not. This could be explained through a major viscosity of olive oil (Diamante & Lan, 2014) compared to sunflower oil that (0.592 vs 0.0488 Pa·s) could confer a greater protection against homogenization process to develop W/O emulsions is obtained in EVOO emulsions.

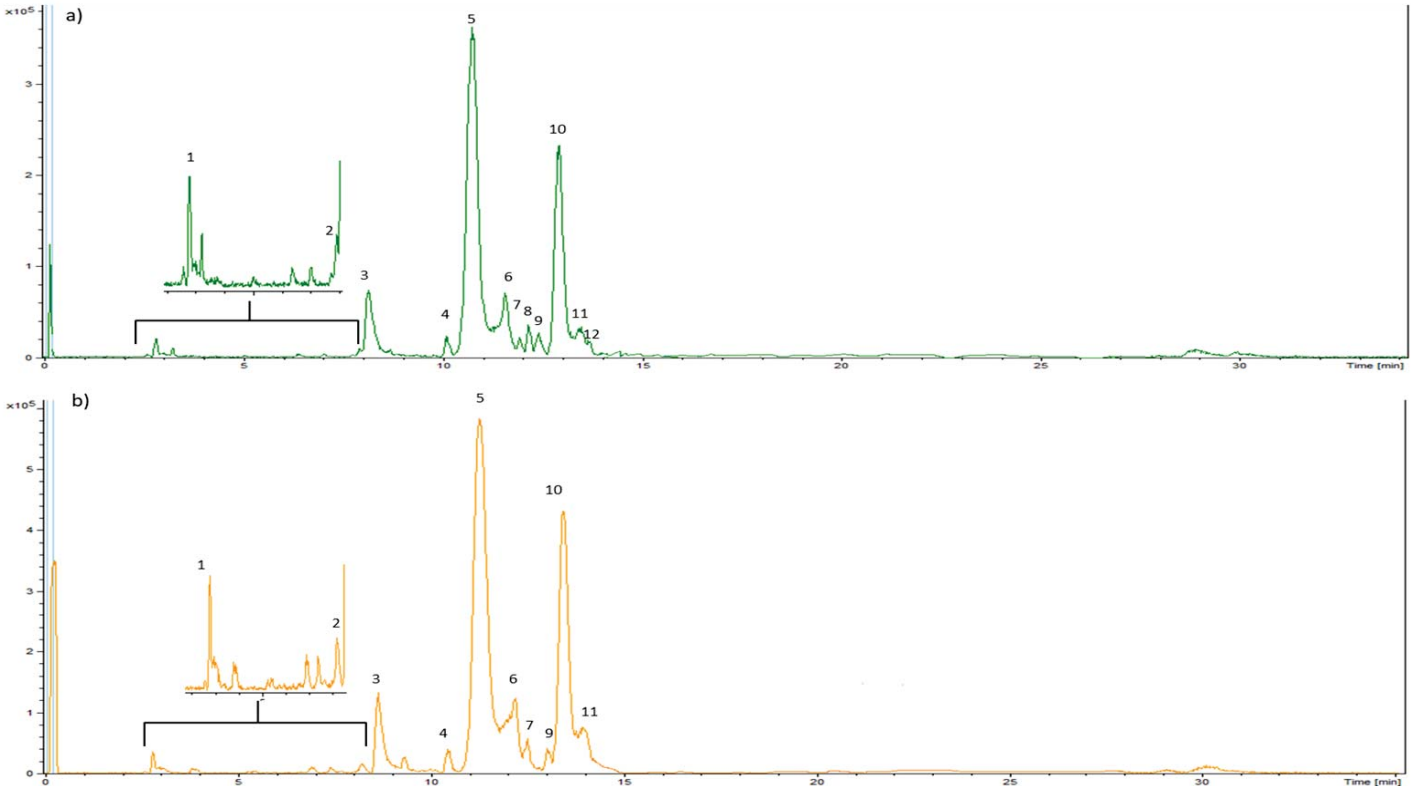


Figure S3. Base-Peak Chromatogram (BPC) of *H. sabdariffa* extracted from W/O EVOO emulsion at time 0 (a) and extracted from W/O SO emulsion at time 0 (b) analyzed by HPLC-ESI-TOF-MS in positive ion mode.

Interestingly and contrarily to the spectrophotometric methods, the chemical composition for 30 days for each type of emulsions was stable when it was compared with the initial emulsion (time 0). By analyzing the chromatograms (**Figure 4**), could be detect slight qualitative and quantitative differences between EVOO (a) and SO (b) emulsions. The normalized areas showed that the anthocyanidins delphinidin 3-sambubioside and cyanidin 3-sambubinoside were the main compounds presenting a normalized area 58.15 and 26.07% respectively in EVOO emulsions and 57.99 and 29.44% respectively in SO emulsions. 7-Hydroxycoumarin was also representative with a normalized area of 7.96% in EVOO emulsion and 7.16% in SO emulsion (Table S8, Supplementary material). These results showed not important differences between EVOO emulsion and SO emulsion. In addition, the normalized area measured at different times showed a good stability for both types of emulsions. For example, the normalized area for delphinidin 3-sambubioside in SO emulsion at time 0 was 57.99 and after 30 days was 58.25. On the other hand, in EVOO emulsion at time 0 was 58.15 and after 30 days 57.95. Thus, all these data corroborate the results from antioxidant assays carried out. Therefore, emulsions technology as a potential technique to maintain a good chemical stability over time.

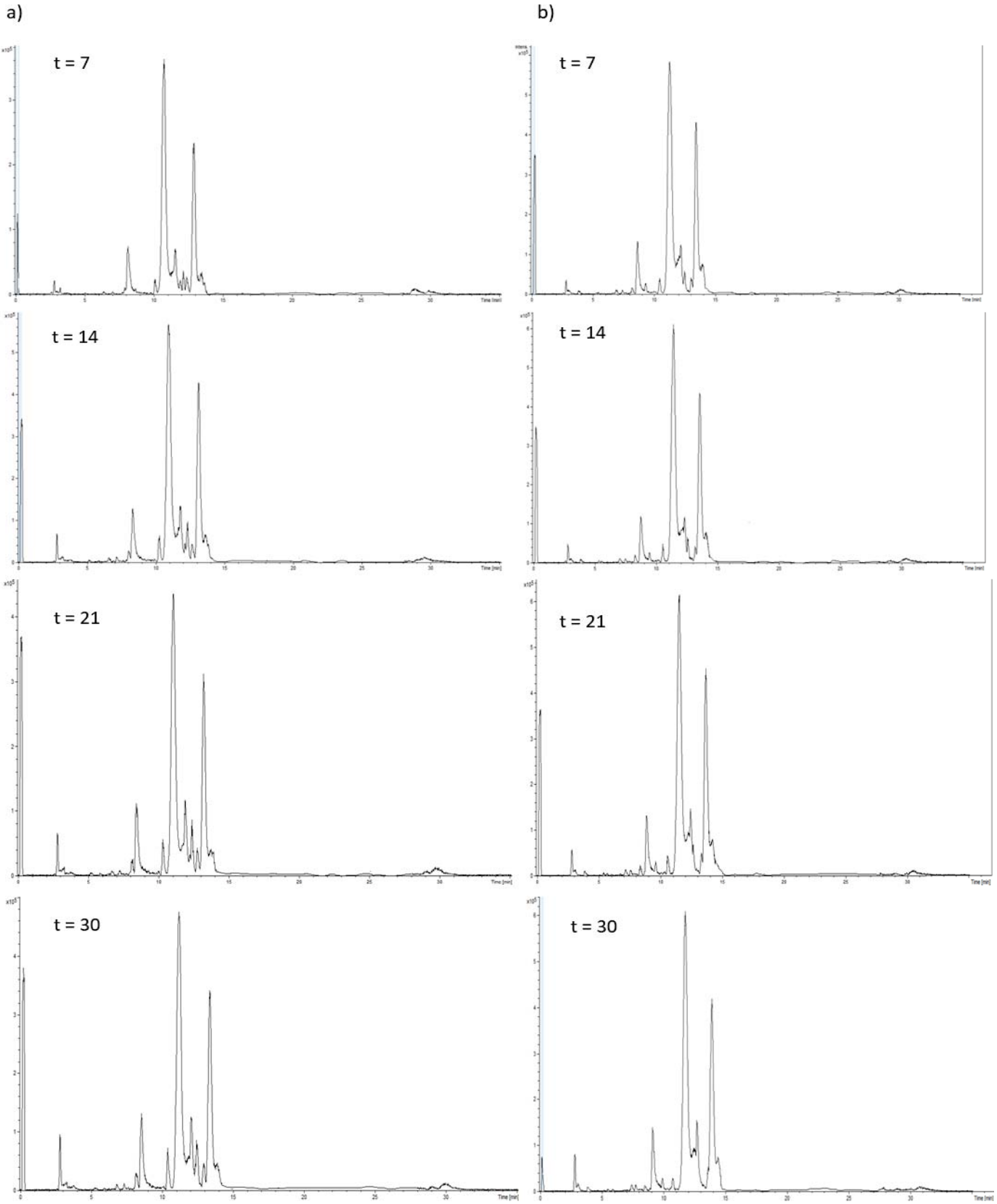


Figure 4. Base-Peak Chromatogram (BPC) of *H. sabdariffa* extracted from W/O EVOO emulsion at different times (a) and extracted from W/O sunflower oil emulsion at different times (b) analyzed by HPLC-ESI-TOF-MS in positive ion mode.

			Normalized area (%) EVOO emulsion					Normalized area (%) SO emulsion				
Compound	**Rt (min)**	**m/z**	**0 days**	**7 days**	**14 days**	**21 days**	**30 days**	**0 days**	**7 days**	**14 days**	**21 days**	**30 days**
Hydroxycoumarin isomer I	7.71	163.0390	0.05	0.03	0.04	0.02	0.06	0.04	0.01	0.01	0.03	0.03
Adenine	7.95	136.0618	0.09	0.51	0.59	0.61	1.43	0.54	0.61	0.40	0.53	0.36
Hydroxycoumarin isomer II	8.15	163.0390	7.96	8.73	4.96	8.05	7.26	7.16	6.66	5.55	5.91	7.07
Trihydroxycoumarin	8.68	195.0288	0.03	0.08	0.07	0.04	0.04	0.57	0.59	0.66	0.65	0.71
Delphinidin 3-sambubioside	10.54	597.1450	58.15	53.56	55.18	54.71	57.95	57.99	54.9	59.38	57.53	58.25
Quercetin	10.79	303.0499	0.03	0.06	0.03	0.07	0.02	0.04	0.02	0.14	0.24	0.15
Hydroxycoumarin isomer III	11.56	163.0390	1.25	4.37	1.71	2.94	3.66	0.60	1.00	1.51	1.79	2.22
Scopoletin	11.93	193,0495	0.36	0.31	0.19	0.16	2.41	-	-	-	-	-
Hydroxycoumarin isomer IV	12.43	163.0390	0.71	1.34	0.43	0.57	1.68	0.46	0.56	0.43	0.52	0.27
Cyanidin 3-sambubinoside	12.78	581.1501	26.02	24.25	30.62	25.46	26.00	29.44	31.40	27.68	28.93	27.39
Kaempferol isomer I	12.96	287.0550	0.05	0.10	0.04	0.03	0.03	0.06	0.46	0.28	0.21	0.02
Kaempferol isomer II	13.68	287.0550	0.84	0.51	0.13	0.22	0.11	-	-	-	-	-

Table S8. Normalized areas of compounds of EVOO and SO emulsions

Conclusions

New functional oils containing hydrophilic antioxidants from *H. sabdariffa* extract have been developed and their physical and oxidative stability has been evaluated. It is the first time that functional oils using bioactive compounds from *H. sabdariffa* have been formulated. The results demonstrated a successful incorporation of bioactive compounds from *H. sabdariffa* into the emulsions and a good oxidative stability during one month of storage. Therefore, emulsion technology has proven to be a powerful method to vehiculize bioactive compounds from plants into edible oils. This study represents a new insight into the design of new generation of functional oils for health promotion. The presented promising results are the beginning for further research focused on optimizing functional oils formulation.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

The emulsification work was supported by the Research group TEP025 “Technologies for Chemical and Biochemical Processes” from the Chemical Engineering Department of the University of Granada. On the other hand, the analytical work was supported by the Research group AGR274 “Bioactive ingredients” from the Analytical Chemistry Departament of the University of Granada. We would like to thank the Applied Physics Department (especially Prof. Angel Delgado) of the University of Granada for making possible to use the Malvern Mastersizer. The Ministry of Education, Culture and Sport (MECD) is deeply grateful for supporting the grant

FPU15/01125 of Ms. Pimentel-Moral. The Andalusian Regional Government Council of Innovation and Science (grant number P11-CTS-7625) and the Spanish Ministry of Economy and Competitiveness (MINECO) (grant number AGL2015-67995-C3-2) are also acknowledged. The authors are grateful to the University of Granada for a "Contrato Puente" postdoctoral contract (C. Rodríguez-Pérez).

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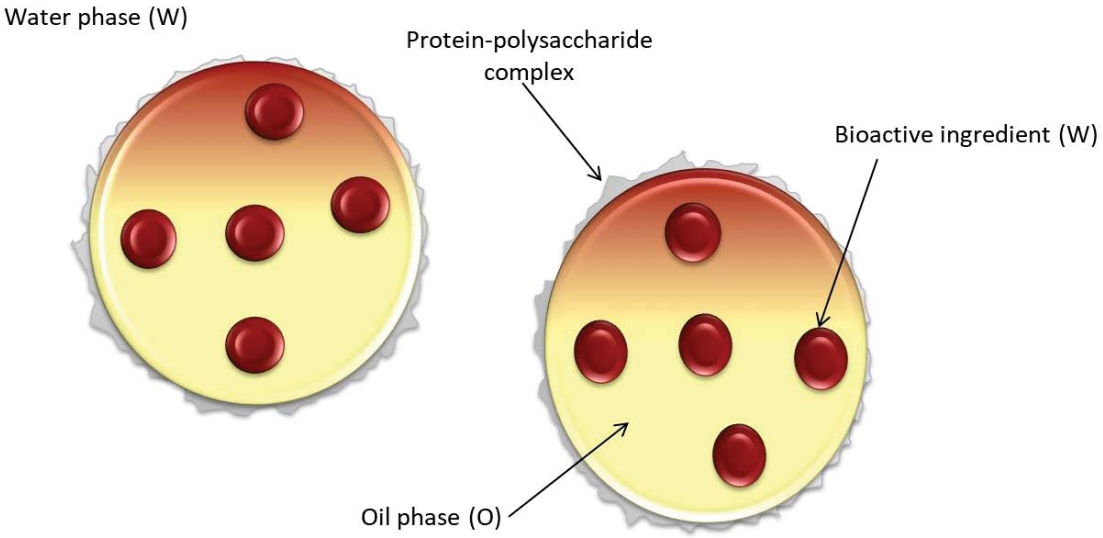
Chapter 6

Stabilization of W/O/W multiple emulsions loaded with *Hibiscus sabdariffa* extract through protein-polysaccharide complexes



LWT- Food Science and Technology

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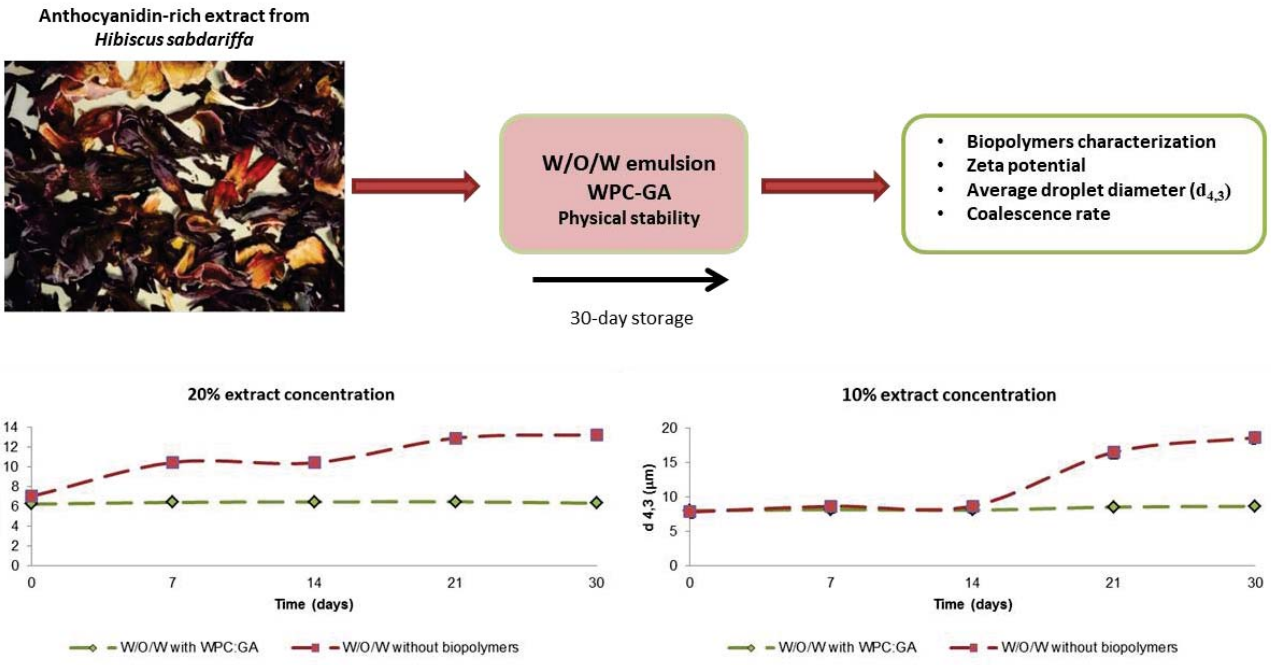
Stabilization of W/O/W multiple emulsion loaded with *Hibiscus sabdariffa* extract through protein-polysaccharide complexes

Abstract



In this work, a hydrophilic anthocyanidin-rich extract from *Hibiscus Sabdariffa (H. sabdariffa)* was vehiculized in the inner phase of a water/oil/water emulsion and its physical stability was improved by means of the reinforcement of both interfaces, the oil-water and the water-oil one, with whey protein concentrate (WPC)-arabic gum (AG) complexes. To this end, the main critical parameters involved on destabilization mechanisms for emulsions have been considered, e.g. the active ingredient concentration, the emulsifier amount and ratio, the biopolymers concentration and ratio (WPC:AG), and the pH value during emulsification. Stable and constant values of average droplet volume diameter ($d_{4,3}$) around 6.47-8.54 μm after 30 d of storage at room temperature were obtained for multiple emulsions containing 20 and 10 g/100g

of extract concentration, respectively. The pH was found to play an important role in the long-term stability. Indeed, the higher long-term stability parameters were achieved at pH of 4.5, value in which the WPC and GA result in maximum complex formation. Thus, the stabilization of water/oil/water multiple emulsions containing *H. sabdariffa* extract through WPC and AG complexes was confirmed to be a potential strategy to improve physical stability over time.

Keywords: *Hibiscus sabdariffa*; emulsion; stability; protein-polysaccharide complexes; antioxidant.

Introduction

Hibiscus sabdariffa (Malvaceae family) is a tropical plant commonly used in the preparation of herbal drinks, hot and cold beverages, because of its antioxidant function in humans. Besides this, recent studies have shown that these extracts can help reduce some diseases as diabetes mellitus, cancer, dyslipidemia and hypertension (Fernandez-arroyo, Camps, Menendez, & Joven, 2015; Gurrola-Diaz et al., 2010; Mohamed, Shing, Idris, Budin, & Zainalabidin, 2013). These functional characteristics described for *H. sabddariffa* result from its composition since this plant contains different antioxidant compounds, mainly flavonoids and anthocyanins (Cid-Ortega & Guerrero-Beltran, 2015). The antioxidant activity of this extract has been investigated in several studies. In this sense, Frank et al. (2012) carried out a randomized study to evaluate the impact of *H. sabdariffa* extract on the systemic antioxidant status. These researchers measured the antioxidant activity through the ferric reducing ability of plasma (FRAP), and showed that this extract can help to improve the systemic antioxidant potential and decrease the oxidative stress in humans. In addition, after its

consumption, urinary hippuric acid excretion was reported to increase due to a high microbial biotransformation of the ingested extract.

However, the proven bioactivity for *H. sabdariffa* extracts is very short-term due to rapid oxidation or insufficient gastric residence time, low permeability and/or solubility within the gut. Indeed, polyphenols are very unstable under typical food processing and storage conditions (e.g., temperature, oxygen, light exposure, etc), or through the gastrointestinal tract (e.g., pH, enzymes, interaction with other nutrients, etc) leading to partial or even total loss that restricts their application in products for human consumption (Munin & Edwards-Lévy, 2011). In this regard, Aurelio et al. [(2008)] showed that the bioactive components found in *H. sabdariffa* may be degraded during cooking process, losing many of their health properties.

Therefore, in order to establish a vehicle to incorporate antioxidants from *H. sabdariffa* extracts in food matrices it is necessary to reduce or prevent the degradation of flavonoids and to develop products capable to protect and deliver high enough concentration of these compounds, improving their bioactivity in parallel.

Within this context, multiple emulsions are a promising technology to accomplish this challenge and entrap/protect the bioactive ingredients of this extract. Water-in-oil-in water ($W_1/O/W_2$) emulsions consist of small water droplets contained within larger oil droplets that are themselves dispersed within a water continuous phase. Consequently, there are two different interfaces (W_1-O and O-W_2) that need to be stabilized by an oil-soluble emulsifier and a water-soluble emulsifier, respectively. Thereby, this structured delivery system can be used for protection, encapsulation and release of hydrophilic and hydrophobic active compounds (McClements, 2015;

Pimentel-Moral, Verardo, Robert, & Segura- Carretero, Martínez-Férez, 2016). Multiple emulsions have already demonstrated to be able to protect and gradually release bioactive compounds. For example, Jiménez-Alvarado et al., (2009) studied the encapsulation of ferrous bisglycinate in the inner aqueous phase of a $W_1/O/W_2$ emulsion stabilized by protein-polysaccharide complexes with good stability, encapsulation efficiencies, protection against oxidation, and slow release rates. More recently, Akhtar, Murray, Afeisume, & Khew, (2014) encapsulated successfully rutin and anthocyanins within the internal aqueous phase of W/O/W multiple emulsions using spinning disc reactor technology, giving an encapsulation efficiency of > 80 %.

Nevertheless, despite their potential, the application of multiple emulsions has been limited by their inherent thermodynamic instability. This natural instability needs to be overcome in order to definitely achieve potential application in the food or pharmaceuticals areas. To this end, multiple emulsions are often stabilized using a combination of hydrophilic and hydrophobic surfactants. In addition to this, a potential strategy is the reinforcement of the oil-water and water-oil interfaces. Currently, mixtures of biopolymers, especially proteins and polysaccharides, are drawing more attention in emulsion stabilization due to the fact that these complexes may improve the resistance of emulsions to environmental stresses such as pH, ionic strength, and temperature. In addition to this, proteins and polysaccharides form, under specific situations, complexes that improve their functional characteristics in comparison with proteins and polysaccharides alone (Dickinson, 2011). Experimental studies indicate that electrostatic interaction could lead to the formation of molecular complexes when proteins and polysaccharides are oppositely charged and contribute in the stabilization

of interfaces and emulsions (Dickinson, 2008). These authors designed food grade multiple emulsions containing caffeine using whey protein:carboxymethylcellulose and whey peptides:carboxymethylcellulose binary soluble complexes, and a whey protein:carboxymethylcellulose:whey peptides ternary soluble complex exhibiting long term stability and different rheological properties. Murillo-Martínez, Pedroza-Islas, Lobato-Calleros, Martínez-Ferez, & Vernon-Carter, (2011) studied the electrostatic charges between low-methoxyl pectin (LMP) - Whey protein isolate (WPI) and carboxymethylcellulose (CMC) –WPI to stabilize water-in-oil-in-water double emulsions.

The current study addresses the development and improvement of the physical stability of a multiple W/O/W emulsion loaded with a hydrophilic anthocyanidin-rich extract from *H. sabdariffa* in the inner aqueous phase. To this end, the emulsifiers amount and ratio, the extract concentration and the influence of protein-polysaccharide complexes (whey protein concentrate-arabic gum) in the stabilization of both interfaces were studied. To the author's knowledge, there are no previous studies available in the scientific literature on the incorporation of all the bioactive compounds from *H. sabdariffa* extracts into the inner phase of a W/O/W emulsion, which entails a greater complexity in the formulation, via the two-stage emulsification process.

Material and methods

Materials

First, water soluble *H. sabdariffa* extract containing a concentration of anthocyanidins equal to 10 g/100g was incorporated into the inner phase of developed

double emulsions. The oil phase of the emulsions (O) was composed by medium chain triglycerides as caprylic/capric triglyceride (MCT), purchased from Fagron Iberica, S.A.U. (Barcelona, Spain). The hydrophobic emulsifier was Sorbitane monooleate (Span 80), whereas Polyethylene glycol sorbitan monolaurate (Tween 20) was chosen as the hydrophilic one (both from Merck, Hohenbrunn, Germany).

The protein-polysaccharide complexes used were whey protein concentrate (WPC) with a reported protein content of 60 g/100g on dry basis, and arabic gum (AG). Both were obtained from Helm Iberica S.A. (Alcobendas, Madrid). To prevent the microbiological contamination of these biopolymers, 0.01 g/100g sodium azide was used. All water used in the experiments was distilled and deionized.

Methods

Development of primary W_1/O emulsion

Firstly, a W_1/O emulsion was formulated with a dispersed phase mass fraction ($\varphi_{W1/O}$) of 0.3. The total surfactants concentration assayed were 8, 10 and 12 g/100g, whereas the OS:WS (oil-soluble surfactant:water-soluble surfactant) mass ratio studied were 7:3, 6:4, 5:5 and 4:6. The dispersed phase in the inner phase consisted of an aqueous solution containing 10 or 20 g/100g *H. sabdariffa* extract. All the primary emulsions were carried out using a rotor stator system (Ultra-Turrax IKA T25) at 12000 rpm for 5 minutes (20 ºC).

Particle size measurement and coalescence rate of the primary W_1/O emulsions

Emulsion stability was determined by measuring the mean droplet size after its preparation and during storage at 25 ºC for 30 days. The droplet size distribution was

measured by laser diffraction using a Malvern Particle Size Analyser (Mastersizer 2000S, Malvern Instruments Ltd, Malvern, UK). The optical parameters selected were a dispersed-phase refractive index of 1.333 (water refractive index) and a continuous-phase refractive index of 1.440 (MCT refractive index). Droplet size measurements were carried out in triplicate on each emulsion and the results were reported as average droplet volume diameter ($d_{4,3}$). This can be represented as indicated in eq. 1

$$d_{4,3} = \sum n_i d_i^4 \Big/ \sum n_i d_i^3 \qquad \text{Eq. (1)}$$

Regarding the coalescence rate (*Kc*), this parameter largely follows a first-order kinetics and can be represented as shows in eq. 2 (Bosquez-Molina, Guerrero-Legarreta, & Vernon-Carter, 2003; Sherman 1969)

$$\frac{Nt}{N_0} = e^{-Kc} \cdot t \qquad \text{Eq. (2)}$$

where N_t is the number of droplets at time t (d) and N_0 is the corresponding ones at initial time. Therefore, Kc can be calculated from the slope of $ln(N_t/N_0)$ versus t plot.

The relationship between the emulsion droplet number, N_t, and the average droplet volume diameter, $d_{4,3}$, fits the following equation (3):

$$Nt = \frac{6\phi 10^{12}}{\pi \left(d_{4,3}\right)} \qquad \text{Eq. (3)}$$

where φ is the dispersed phase mass fraction.

Development of multiple $W_1/O/W_2$ emulsions

The primary emulsions (W_1/O) with the lowest coalescence rate for both *H. sabdariffa* aqueous solution extract concentrations assayed (10 and 20 g/100g) were further dispersed in aqueous binary biopolymers mixtures (WPC:AG), leading to the development of $(W_1/O/W_2)_{a,b,c}$ multiple emulsions. The subscript *a* denotes the extract concentration, the subscript *b* indicates the total biopolymers concentration and the subscript *c* refers to the protein-polysaccharide ratio. The dispersed phase mass fraction (W/O) in the multiple emulsions ($\varphi_{W1/O/W2}$) was 0.2. The total biopolymers concentrations assayed were 3, 5 and 7 g/100g whereas the WPC:AG mass ratio was 3:1, 2:1 and 1:1. Moreover, 0.01 g/100g sodium azide was always added to prevent bacterial contamination. The $W_1/O/W_2$ multiple emulsion was carried out using a rotor stator (Ultra-Turrax IKA T25) at 4500 rpm for 5 minutes (20 ºC).

Particle size measurement, zeta potential and coalescence rate of the $W_1/O/W_2$ emulsions

The optical parameters to measure the particle size and the coalescence rate of the double emulsion were set as follows: dispersed phase refractive index 1.440 (W_1/O) and continuous phase refractive index 1.333. The zeta potential of the double emulsions was determined using a Malvern Zetasizer Nano ZS (Malvern Instruments Ltd, UK).

Biopolymers characterization

Whey protein concentrate (WPC) and arabic gum (AG) were characterised by measuring the zeta potential of each biopolymer separately in a range of pH (2-12) using a Nano ZS Malvern Zetasizer. To this end, 5% aqueous solutions (w/w) of WPC

and AG were prepared and stored at 4 ºC for 24 h to enhance complete hydration. Adjustment of the solutions pH was carried out with 0.1mol/L HCl or 0.1mol/L NaOH. The pH at which there is a maximum difference of the electrostatic charges between WPC and AG was determined.

Results and discussion

Biopolymers characterization

The zeta potential measured for each biopolymer is shown in **Figure 1**. As it can be seen, the isoelectric point (pI) for WPC was 4.5, while the pH value at which the arabic gum lost the capacity to ionize its carboxyl groups was approximately 2.2. Moreover, the highest strength of the electrostatic interaction (SEI) values between oppositely charged polyelectrolytes indicate the range of pH where the attractions of these biopolymers could be strongest (Weinbreck et al., 2004). In this regard, the pH value upon which the WPC and AG showed more opposite charge and maximum complex formation was found to be equal to 3.5.

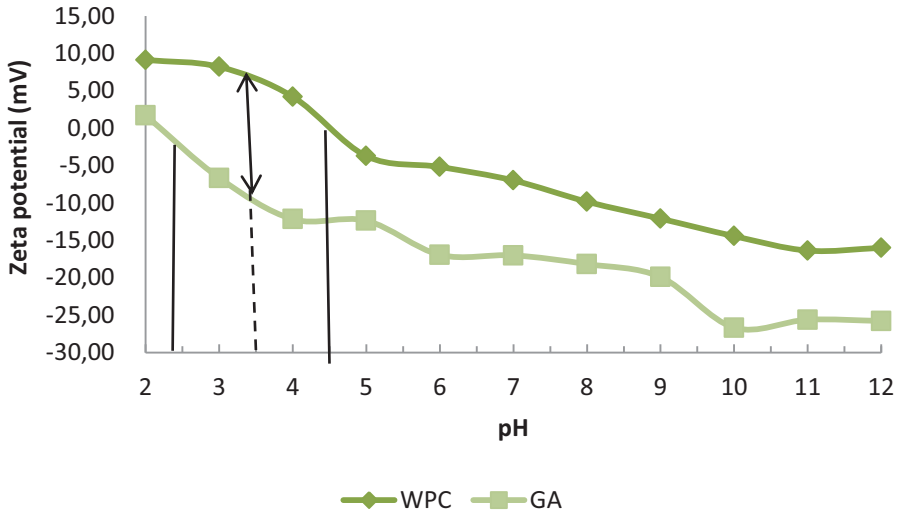


Figure 1. Zeta potential as a function of pH for WPC and GA solutions.

These results are similar to those reported by other researchers (Jiménez-Alvarado et al., 2009). Besides, other research works relied on the zeta potential to enhance the stability of emulsions: e.g. Hernández-Marín et al. (2013) studied the interaction pH values to generate whey protein concentrate-carboxymethylcellulose soluble complexes, whereas Ruiz-Ramos et al. (2006) stabilized white mineral oil-in-water emulsions with different ratios of the oppositely charged mesquite gum and chitosan polyelectrolytes. A higher proportion of chitosan in the biopolymer blend was found to increase the pH and mean volume average droplet diameter, as there were more oppositely charged molecules available for electrostatic interactions, leading to the formation of an apparently higher molecular weight complex.

Stability of the (W_1/O) primary emulsion

The effect of the emulsifiers concentration and OS:WS mass ratio on the $d_{4,3}$ of the primary emulsions developed is shown in **Figure 2.** After one month storage at room temperature, the $d_{4,3}$ was found to decrease when the total emulsifiers concentration was incremented from 8 to 10 or 12 g/100g and the OS concentration was reduced (from 7:3 to 4:6). At surfactants concentration of 8 g/100g the difference between 20 (panel A) and 10 g/100g *H. sabdariffa* extract concentration (panel B) was not significant. However, the increase of surfactant concentration (10 and 12 g/100g) showed different behaviour depending on the extract concentration, being less pronounced the reduction of $d_{4,3}$ in the case of 10 g/100g. Surfactants usually act to reduce the interfacial free energy. Therefore, at a higher concentration of surfactants the droplets size during the emulsification would be lower and there would be a minor increase in the droplets size during storage. However, at low emulsifier concentration,

most of the individual droplets formed during the emulsification are not retained in the final emulsion, resulting in a larger particle size and lower stability during storage (Dickinson, 2009).

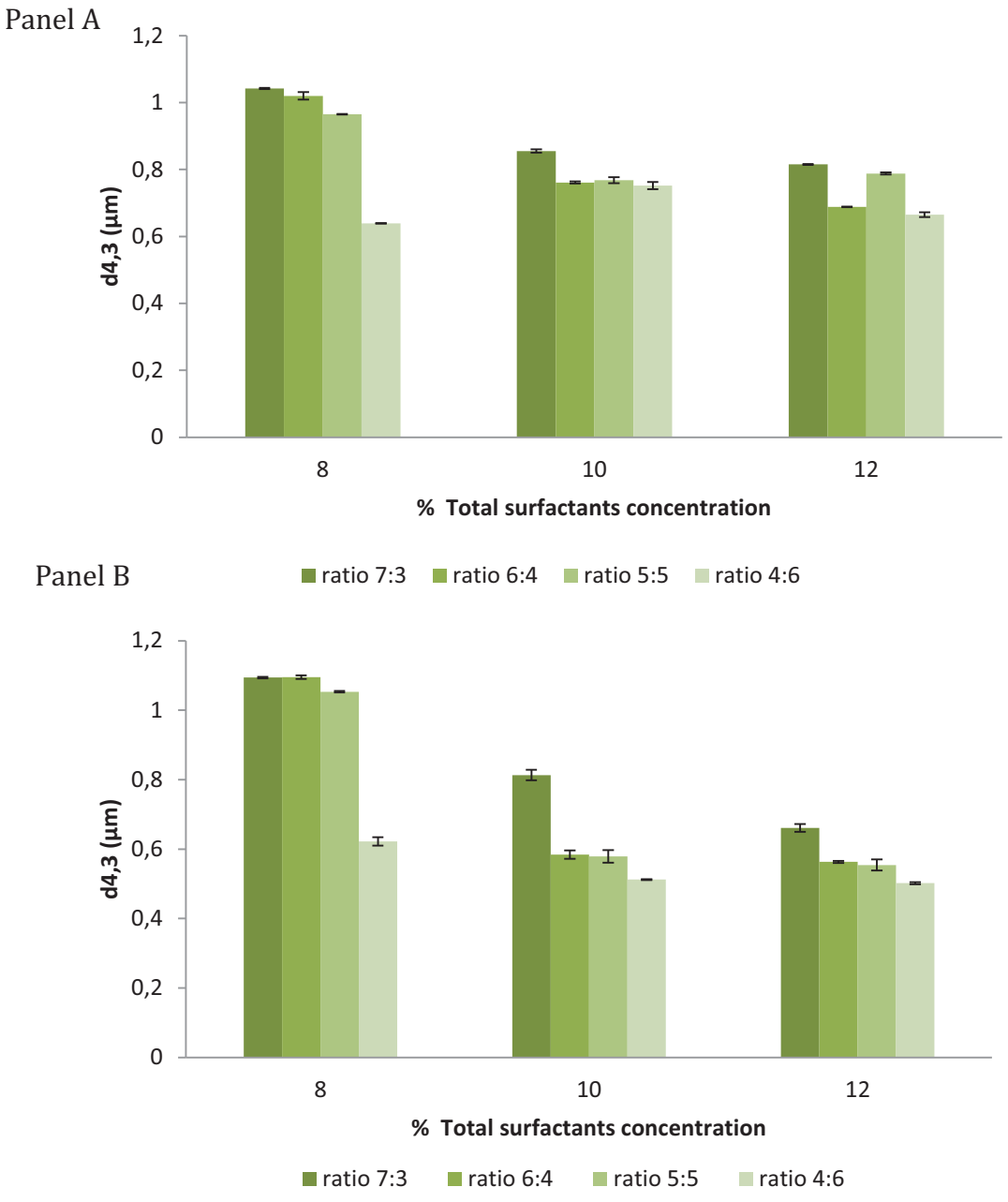


Figure 2. Volume mean diameter ($d_{4,3}$) after 30 days at room temperature as a function of the mass ratio and surfactants concentration. A) 20% *H.sabdariffa* extract, B) 10% *H.sabdariffa* extract.

These results agree with the results reported by Sadeghi et al., (2013) who observed that the droplets size decreased by increasing the surfactants concentration in water in *Soroush* crude oil emulsions, upon keeping the same stirring speed. In another research work, several types of sunflower lecithins were examined on the stability of W/O emulsions. They showed that the emulsion droplet size was dependent on the surfactant concentration: at 0.1 w t% lecithin, the droplet size was similar to the size of an emulsion with no emulsifier, while at 1.0 wt % the droplets significantly decreased in size (Pan, Tomás, & Añón, 2002). Also, Jiménez-Alvarado et al., (2009) reported a smaller mean volume diameter when the OS concentration decreased in the formation of W/O emulsions to entrap ferrous bisglycinate.

On the other hand, the coalescence rate confirmed these results. The $Kc_{(W1/O)}$ values of the primary emulsions are reported in **Table 1**. The lowest $Kc_{(W1/O)}$ for 20% and 10 g/100g extract concentration emulsions was achieved with a 4:6 OS:WS mass ratio and 12 g/100g total emulsifiers concentration.

Surfactant concentration	OS:WS ratio	Kc $_{(W1/O)}$ (1 / s) (20% extract concentration)	Kc $_{(W1/O)}$ (1 / s) (10% extract concentration)
8%	7:3	$3.48 \cdot 10^{-11}$	$4.66 \cdot 10^{-11}$
	6:4	$2.22 \cdot 10^{-11}$	$3{,}41 \cdot 10^{-11}$
	5:5	$1.64 \cdot 10^{-11}$	$3.63 \cdot 10^{-11}$
	4:6	$1.93 \cdot 10^{-11}$	$1.84 \cdot 10^{-11}$
10%	7:3	$2.73 \cdot 10^{-11}$	$4.19 \cdot 10^{-11}$
	6:4	$1.52 \cdot 10^{-11}$	$2.33 \cdot 10^{-11}$
	5:5	$9.28 \cdot 10^{-12}$	$1.86 \cdot 10^{-11}$
	4:6	$7.10 \cdot 10^{-12}$	$2.12 \cdot 10^{-11}$
12%	7:3	$1.91 \cdot 10^{-11}$	$2.44 \cdot 10^{-11}$
	6:4	$7.74 \cdot 10^{-12}$	$1.70 \cdot 10^{-11}$
	5:5	$1.01 \cdot 10^{-11}$	$2.18 \cdot 10^{-11}$
	4:6	$4.76 \cdot 10^{-12}$	$1.51 \cdot 10^{-11}$

Table1. Coalescence rates of the primary emulsions (W_1/O)

Stability of the $W_1/O/W_2$ emulsions

The WPC:AG mixture affected significantly the $d_{4,3}$ of the $(W/O/W)_{a,b,c}$ multiple emulsions. As it can be seen in **Figure 3**, the values of $d_{4,3}$ were always lower than the multiple emulsions without WPC:AG for both extracts concentration. Moreover, they were maintained constant in time for every biopolymer concentration in the case of 10 g/100g extract concentration (panel B), whereas in the case of 20 g/100g extract concentration only the set of 3 g/100g biopolymer concentration was noted to maintain constant values (panel A).

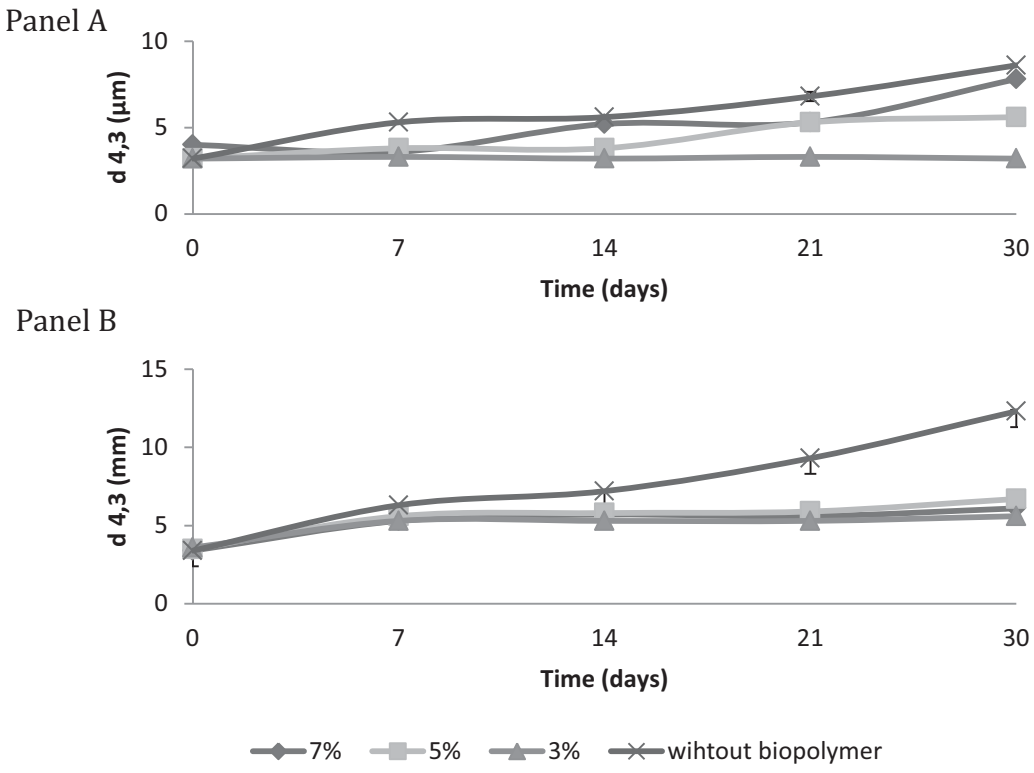


Figure 3. Evolution of $d_{4,3}$ for (W/O/W)a,b,c multiple emulsions in time as a function of total biopolymers concentration and 2:1 WPC:AG mass ratio. A) 20% *H.sabdariffa* extract concentration, B) 10% *H. sabdariffa* extract concentration.

As shown in **Figure 3**, the different biopolymers concentration was not significant in the case of $(W/O/W)_{10,x,x}$ emulsions. On the other hand, $(W/O/W)_{20,3,x}$ emulsions showed better results on $d_{4,3}$ than $(W/O/W)_{20,5,x}$ and $(W/O/W)_{20,7,x}$. In parallel, the influence of the WPC:AG mass ratio on the $d_{4,3}$ was also studied (**Figure 4**). It can be observed that for $(W/O/W)_{20,x,x}$ emulsions (Panel A), the lower size values were found for 3 g/100g biopolymer concentration, without significant influence of the WPC:AG ratio. On the contrary, in the case of $(W/O/W)_{10,x,x}$ emulsions (Panel B), the biopolymer concentration did not lead to any substantial improvement in any WPC:AG mass ratio.

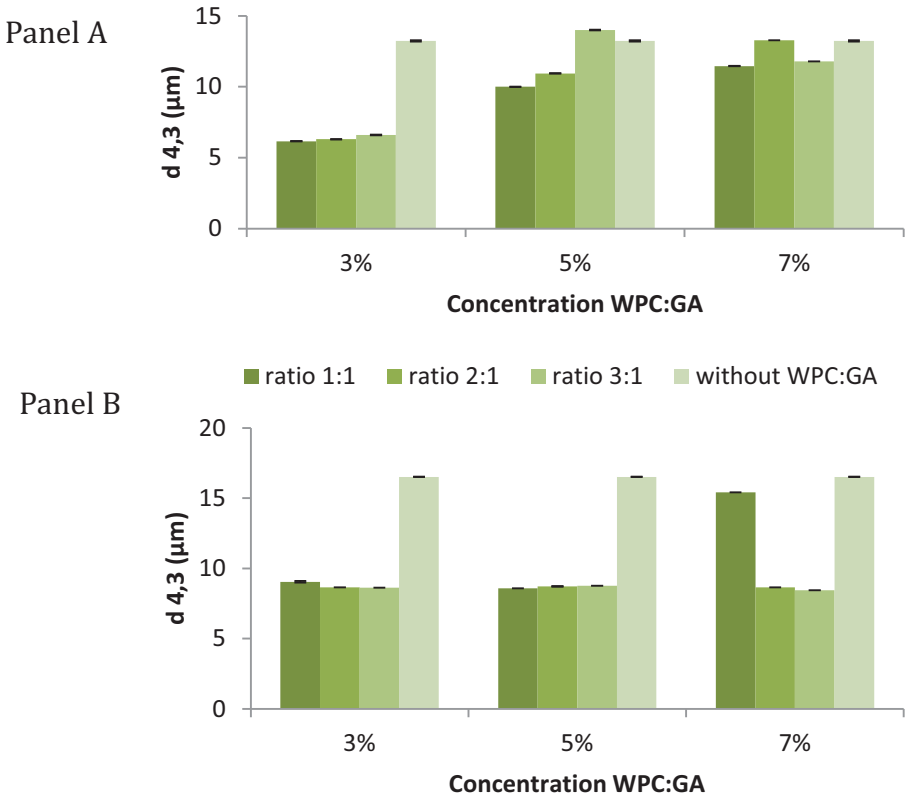


Figure 4. Volume mean diameter ($d_{4,3}$) as a function of the mass ratio; respectively, and concentration of WPC:AG mixture after 30-day storage at room temperature. A) $(W_1/O/W_2)_{20\%}$ emulsions B) $(W_1/O/W_2)_{10\%}$ emulsions.

This behaviour can be explained by the natural acid pH of *H. sabdariffa* extract, which helps decrease the pH and enhances the formation of WPC-GA complexes. In order to clarify the influence of the pH on the stability, the pH of all $(W/O/W)_{a,b,c}$ multiple emulsions were measured. $(W/O/W)_{10,x,x}$ emulsions exhibited similar pH values around 5.6 ± 0.3 whereas the pH of the $(W/O/W)_{20,x,x}$ emulsions was lower: 5 ± 0.09 for $(W/O/W)_{20,7,x}$ and $(W/O/W)_{20,5,x}$ multiple emulsions and values of 4.7 ± 0.17 in the case of $(W/O/W)_{20,3,x}$ emulsions. Thus, it is noticeable the influence of the extract concentration on the final pH. The latter pH value was the closest to 3.5, previously reported as the pH value upon which the WPC and AG showed opposite charge and maximum complex formation, providing as a consequence greater biopolymers interaction and better emulsion stabilization. Moreover, it is very important to notice that the isoelectric point of WPC is 4.5, and that pH below this value could cause its precipitation and therefore the destruction of the formed complexes.

In order to check this lack of stability at values below 4.5, multiple emulsions at pH 3.5 were developed and the mean volume diameter and zeta potential were measured. As it can be seen in **Table 2**, emulsions at pH of 3.5 result in higher values of $d_{4,3}$ and Kc and lower signal of the Z potential. It is worth reminding that emulsions with zeta potential greater than an absolute value of 25 mV are usually considered stable (Nanocomposix, 2012).

W/O/W emulsion at pH > 4.5				W/O/W emulsion at pH= 3.5			
$(W_1/O/W_2)_{a,b,c}$	$d_{4,3}$(m)	Kc (1/s)	Z potential abs value (mV)	$(W_1/O/W_2)_{a,b,c}$	$d_{4,3}$(m)	Kc (1/s)	Z potential abs value (mV)
$(W_1/O/W_2)_{20\%,7\%,1:1}$	1.15	$3.71\cdot10^{-10}$	24.8	$(W_1/O/W_2)_{20\%,7\%,1:1}$	1.35	$3.36\cdot10^{-10}$	19.6
$(W_1/O/W_2)_{20\%,7\%,2:1}$	1.33	$2.30\cdot10^{-10}$	32.7	$(W_1/O/W_2)_{20\%,7\%,2:1}$	2.07	$1.57\cdot10^{-9}$	14.0
$(W_1/O/W_2)_{20\%,7\%,3:1}$	1.18	$2.10\cdot10^{-10}$	30.8	$(W_1/O/W_2)_{20\%,7\%,3:1}$	3.35	$4.99\cdot10^{-9}$	8.93
$(W_1/O/W_2)_{20\%,5\%,1:1}$	1.00	$1.68\cdot10^{-10}$	29.8	$(W_1/O/W_2)_{20\%,5\%,1:1}$	1.14	$3.62\cdot10^{-10}$	15.3
$(W_1/O/W_2)_{20\%,5\%,2:1}$	1.09	$1.45\cdot10^{-10}$	29.6	$(W_1/O/W_2)_{20\%,5\%,2:1}$	2.45	$5.93\cdot10^{-10}$	13.2
$(W_1/O/W_2)_{20\%,5\%,3:1}$	1.40	$2.58\cdot10^{-10}$	29.8	$(W_1/O/W_2)_{20\%,5\%,3:1}$	1.91	$1.54\cdot10^{-10}$	9.3
$(W_1/O/W_2)_{20\%,3\%,1:1}$	0.61	$1.25\cdot10^{-10}$	27.8	$(W_1/O/W_2)_{20\%,3\%,1:1}$	0.99	$1.40\cdot10^{-10}$	16.3
$(W_1/O/W_2)_{20\%,3\%,2:1}$	0.63	$1.20\cdot10^{-10}$	25.7	$(W_1/O/W_2)_{20\%,3\%,2:1}$	1.95	$7.15\cdot10^{-10}$	14.5
$(W_1/O/W_2)_{20\%,3\%,3:1}$	0.66	$1.24\cdot10^{-10}$	27.8	$(W_1/O/W_2)_{20\%,3\%,3:1}$	2.01	$1.13\cdot10^{-9}$	10.8
$(W_1/O/W_2)_{10\%,7\%,1:1}$	1.54	$4.50\cdot10^{-10}$	37.0	$(W_1/O/W_2)_{10\%,7\%,1:1}$	1.61	$3.63\cdot10^{-10}$	14.1
$(W_1/O/W_2)_{10\%,7\%,2:1}$	0.84	$4.76\cdot10^{-10}$	31.2	$(W_1/O/W_2)_{10\%,7\%,2:1}$	2.84	$2.85\cdot10^{-10}$	5.63
$(W_1/O/W_2)_{10\%,7\%,3:1}$	0.85	$5.70\cdot10^{-10}$	30.1	$(W_1/O/W_2)_{10\%,7\%,3:1}$	1.83	$1.70\cdot10^{-11}$	10.8
$(W_1/O/W_2)_{10\%,5\%,1:1}$	0.86	$3.99\cdot10^{-10}$	29.2	$(W_1/O/W_2)_{10\%,5\%,1:1}$	1.56	$3.13\cdot10^{-10}$	14.0
$(W_1/O/W_2)_{10\%,5\%,2:1}$	0.89	$2.40\cdot10^{-10}$	24.6	$(W_1/O/W_2)_{10\%,5\%,2:1}$	2.49	$2.02\cdot10^{-9}$	9.85
$(W_1/O/W_2)_{10\%,5\%,3:1}$	0.88	$2.64\cdot10^{-10}$	30.4	$(W_1/O/W_2)_{10\%,5\%,3:1}$	1.77	$3.24\cdot10^{-10}$	13.9
$(W_1/O/W_2)_{10\%,3\%,1:1}$	0.91	$3.33\cdot10^{-10}$	30.8	$(W_1/O/W_2)_{10\%,3\%,1:1}$	0.97	$3.40\cdot10^{-10}$	17.0
$(W_1/O/W_2)_{10\%,3\%,2:1}$	0.87	$3.28\cdot10^{-10}$	21.9	$(W_1/O/W_2)_{10\%,3\%,2:1}$	1.98	$3.47\cdot10^{-10}$	14.2
$(W_1/O/W_2)_{10\%,3\%,3:1}$	0.86	$3.12\cdot10^{-10}$	32.0	$(W_1/O/W_2)_{10\%,3\%,3:1}$	2.66	$3.17\cdot10^{-10}$	9.05

Table2. Stability values of W/O/W emulsions at pH 3.5 in comparison with emulsions at pH above than 4.5. The subscript *a* denotes the extract concentration, the subscript *b* indicates the total biopolymers concentration and the subscript *c* refers to the protein-polysaccharide ratio.

When the pH is close to the isoelectric point of the protein (but not less), the emulsion system with protein-polysaccharide complex always exhibited a more pronounced electrostatic repulsion and prevented aggregation and destabilization. Others authors have observed analogous results. For instance, Park et al. (2015) studied the effect of chondroitin sulfate on emulsion stability with bovine serum albumin at chondroitin sulfate concentrations ranging from 0 to 0.1% (w/w) and different pH values. The complex was formed and prevented phase-separation when the pH was near the protein isoelectric point. Chuah et al., (2014) observed the effect of pH on $d_{3,2}$ of beta-lactoglobulin-carboxymethylcellulose emulsions. They observed that nearby the isoelectric point of beta-lactoglobulin emulsion samples no flocculation was observed to occur even after one week of storage.

Summing up, upon the adequate conditions the stabilization of W/O/W multiple emulsions containing *H. sabdariffa* extract through whey protein concentrate (WPC) and arabic gum (AG) complexes could be a potential and feasible strategy to improve the physical stability in time.

Conclusions

In this research work a method to entrap an aqueous *Hibiscus sabdariffa* extract into the inner phase of a double emulsion was developed. To reinforce the stability of this thermodynamically unstable system, a strategy based on the reinforcement of the oil-water and water-oil interfaces by means of whey protein concentrate - arabic gum (WPC-AG) complexes has been used.

The pH value upon which the WPC and AG showed opposite charge and maximum complex formation was found to be equal to 3.5. However, this value is limited by the

isoelectric point of WPC (4.5), since lower values could cause its precipitation and therefore the destruction of the formed complexes. The lowest coalescence rate for the primary W_1/O emulsion was achieved with a 4:6 OS:WS mass ratio and 12 g/100g total emulsifiers concentration. In the case of $(W/O/W)_{20,x,x}$, lower size values were found for 3 g/100g of biopolymer concentration, with no significant influence of the WPC:AG mass ratio. On the contrary, in the case of $(W/O/W)_{10,x,x}$ emulsions, the different biopolymer concentration examined did not result in any substantial improvement for any WPC:AG ratio.

For all the multiple emulsions formulated and stabilized by WPC:AG, the $d_{4,3}$ was always lower than multiple emulsions without WPC:AG for both extract concentration and it was observed to be maintained constant over a period of 30 days at room temperature.

To sum up, the stabilization of $W_1/O/W_2$ multiple emulsions containing *Hibiscus sabdariffa* extract through whey protein concentrate (WPC) and arabic gum (AG) complexes could be a potential and feasible strategy to improve the physical stability in time.

Acknowledgments

This work was supported by the Research Group TEP025 “Technologies for Chemical and Biochemical Processes” from the Chemical Engineering Department of the University of Granada. We would like to thank the Applied Physics Department (specially Prof. Angel Delgado) of the University of Granada for making possible to use

the Malvern Mastersizer. The Ministry of Education, Culture and Sport (MECD) is deeply acknowledged for supporting the grant FPU15/01125 of Ms. Pimentel-Moral.

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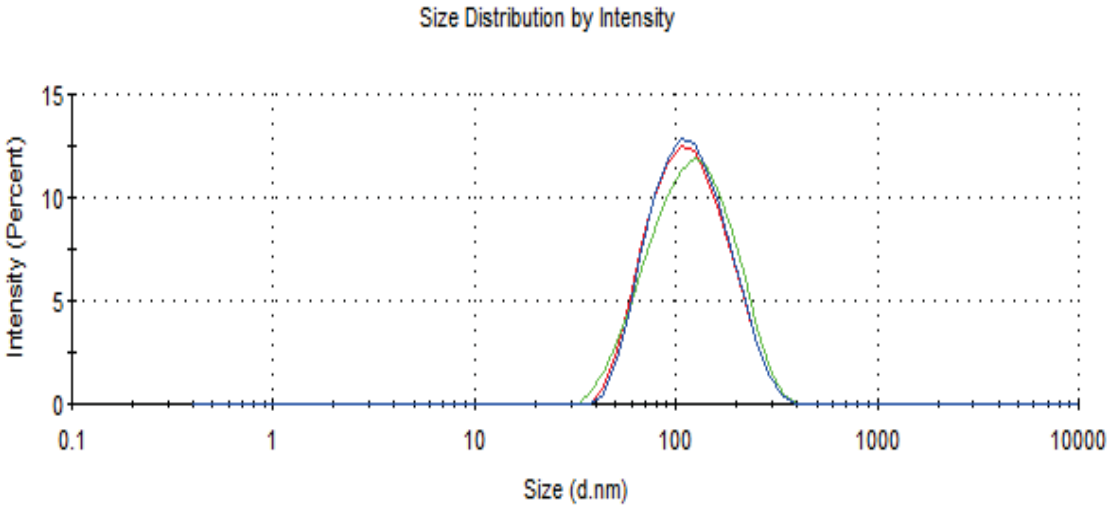
Chapter 7

Polyphenols-enriched *Hibiscus sabdariffa* extract-loaded Nanostructured lipid carriers (NLC): Optimization by multi-response surface methodology



Journal of drug delivery and science technology

Pimentel-Moral, Sandra, Teixeira, Maria du Ceu, Fernandes, Ana Rita, Borrás-Linares Isabel, Arráez-Román, David, Martínez-Férez, Antonio, Segura-Carretero, Antonio, Souto, Eliana.

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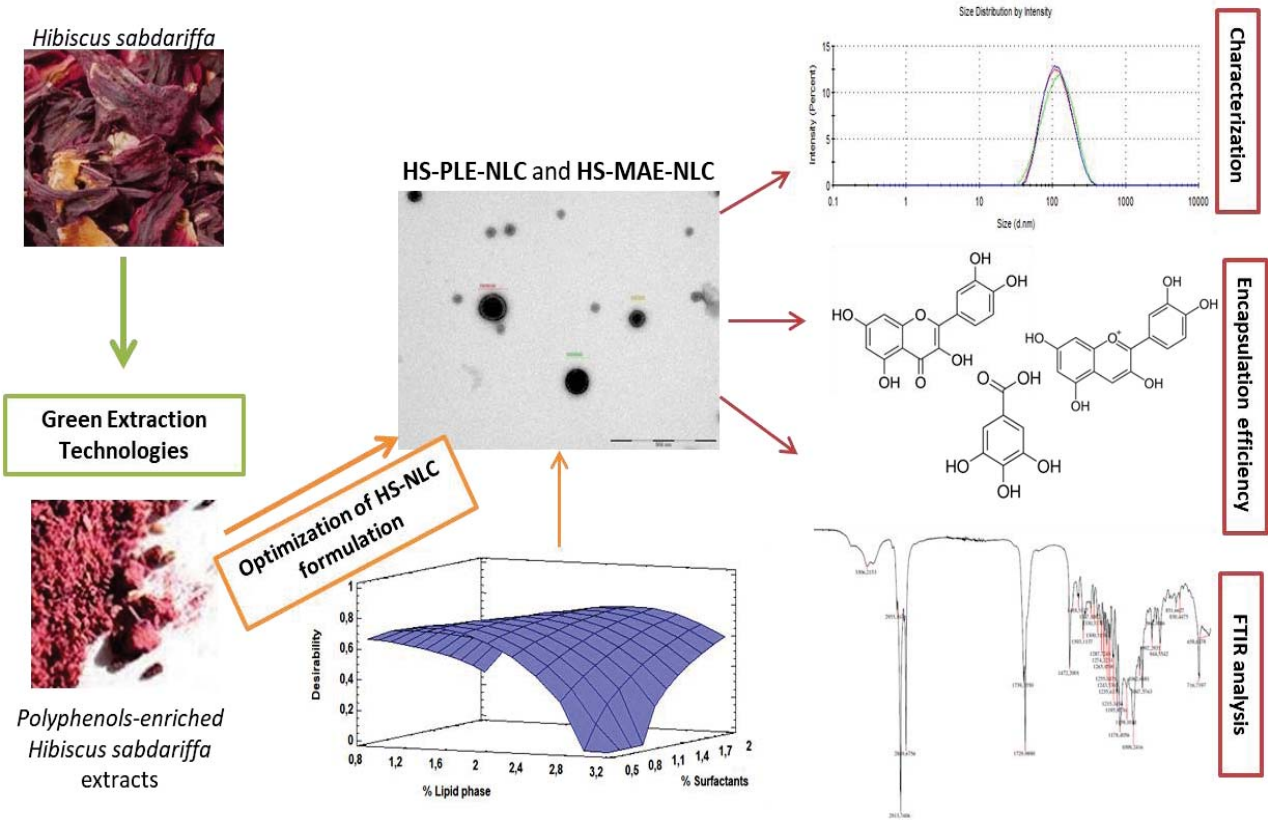
UNIVERSIDADE DE COIMBRA

Polyphenols-enriched *Hibiscus sabdariffa* extract-loaded Nanostructured lipid carriers (NLC): Optimization by multi-response surface methodology

Abstract



Nanoparticles are commonly used to improve the bioavailability of several bioactive compounds. In the present work, nanostructured lipid carriers (NLC) have been optimized by response-surface methodology to protect phenolic compounds from *Hibiscus sabdariffa*. The influence of lipid and surfactant concentration on the mean particle size, polydispersity index and zeta potential of NLC was analyzed. The results showed that the effect of both independent variables was statistically significantly on the selected responses. The optimized formulation obtained from the predicted model was composed of 2.21 % (w/w) of lipid phase and 1.93 % (w/w) of

surfactant, with a desirability function of 0.77. The loading of polyphenol-enriched *Hibiscus sabdariffa* extract - obtained by microwave assisted extraction or by pressurized liquid extraction -within the NLC resulted in particles with a mean size of 470 ± 8 nm (encapsulation efficiency of 52.9 ± 0.9 % for quercetin and 60 ± 2 % for anthocyanins) and 344 ± 12 nm (encapsulationefficiency of 93 ± 3 % for quercetin and 84 ± 4 % for anthocyanins), respectively. Transmission electron microscopy images and Fourier transformed infra-red analysis of extracts-loaded NLC confirmed that interaction between lipids and polyphenols improved the physicochemical stability of the particles. Our findings highlight the added value of NLC as a delivery system for phenolic compounds extracted from *Hibiscus sabdariffa.*

Keywords: nanostructured lipid carrier; *Hibiscus sabdariffa*; response surface methodology; encapsulation; polyphenols; nanoparticles

Introduction

In the last years, lipid nanoparticles have been used in food industry with the purpose of improving the bioavailability of bioactive compounds. Nanoemulsions, liposomes, solid lipid nanoparticles (SLN) and nanostructured lipid carriers (NLC) [1], are described to modify the release profile of loaded pharmaceutics and nutraceutics, and exhibit targeted delivery properties [2-6]. Among different lipid nanoparticles, NLC offer additional advantages e.g. higher loading capacity and longer physicochemical stability attributed to the mixture of solid and liquid lipids composing their lipid matrix [6, 7]. NLC are characterized by a partially crystallized lipid matrix with a low-ordered structure that limits the risk of drug expulsion during storage time [8,9]. Among the

different methods described for the production of lipid nanoparticles [10], double W/O/W emulsion has been recommended for the loading of hydrophilic compounds. Double emulsion method is based on the solubilization of the drug in the internal phase of a W/O emulsion in which the organic phase is composed of melted bend of lipids, followed by the dispersion of the hot W/O emulsion in an aqueous surfactant solution. The cooling of the dispersion allows the lipids to recrystallize to obtain NLC. Ultrasonication may also be applied to reduce the polydispersity of the final lipid nanoparticle formulation [11, 12].

To obtain a stable NLC dispersion, the influence of the lipid and surfactant concentrations (independent variables) on the mean particle size (Z-Ave), polydispersity index (PDI) and zeta potential (ZP) (dependent variables) will have to be evaluated. Response surface methodology (RSM) is a multivariate statistical technique that allows the study of several factors in order to produce the best possible response [13]. To apply RSM, an experimental design defining a specific set of combinations for the levels of variables that must be applied experimentally to obtain the responses, is required. Moreover, to approximate a response function to experimental data, experimental designs for quadratic response surfaces should be used, such as three-level factorial, Box-Behnken, or central composite design. Such approaches allow the description of the behavior of the data in order to make statistical predictions.

Hibiscus sabdariffa (H. sabdariffa) is a tropical plant belonging to *Malvaceae* family, which use in pharmaceutical and food industries has been continuously increasing attributed to its beneficial effects in human health. Its composition in organic acids, anthocyanins, phenolic acids, polysaccharides and flavonoids [14], reasons its use in the management of chronic diseases, such as hypertension, diabetes

or even obesity [15-17]. As it can be used "beyond the diet, before drugs" [18], extracts of *H. sabdariffa* may be considered in formulation of oral nutraceuticals.

It is possible to increase the content of these bioactive compounds in extracts of *H. sabdariffa* when obtained by microwave assisted extraction (MAE) or pressurized liquid extraction (PLE) techniques. These environmental-friendly extraction methods allow to obtain extracts with higher bioactivity [19]. However, the bioactive compounds from *H. sabdariffa* are sensitive to light, oxygen, and temperature, which may compromise their bioavailability and limit their therapeutic efficacy [20]. NLC have been proposed to load polyphenol-enriched extracts from *H. sabdariffa* to improve the bioavailability of these bioactive compounds.

The aim of this work was the design and optimization of a novel NLC formulation produced by combining of double emulsion and ultrasonication techniques for the loading of two types of polyphenol-enriched extracts of *H. sabdariffa* (extracted by MAE or by PLE). For that purpose, an experimental design was performed by RSM to evaluate the influence of the formulation parameters (i.e. percentage of lipid phase and surfactant) on the quality parameters of the NLC dispersion (i.e. mean particle size, PDI and ZP). Furthermore, morphological examination by TEM and FTIR assays were also carried out for the *H. sabdariffa* loaded-NLC (HS-NLC).

Material and methods

Samples and reagents

The calyces of *H. sabdariffa* were collected from Monteloeder (Elche, Spain). These calyces were ground using an ultra-centrifugal mill ZM200 (Retsch GmbH, Haan,

Germany) getting a powder < 2 mm which was stored in darkness in a dry place until treatment.

For the extractions, ethanol ≥ 99.8% was purchased from VWR International S.A.S. (Fontenay-sous-Bois, France). Sea sand was obtained from Panreac (Barcelona, Spain). Ultrapure water was obtained from a Milli-Q system (Millipore, Bedford, MA, USA). For the development of HS-NLCs, the lipid phase was composed of a mixture of solid lipid (Biograpress^{TM} Vegetal BM 297 ATO) kindly provided by Gatefossé (France) and lipid liquid (Soybean oil) purchased from Sigma (Darmstadt, Germany). The surfactants used were Tween 80 and Span 80, both from Merck (Hohenbrunn, Germany).

Extraction of phenolic compounds from *H. sabdariffa* by Microwave-Assisted Extraction (MAE) and Pressurized liquid extraction (PLE)

Two different extracts were obtained by two different green technologies, applying distinct extraction conditions, i.e. for MAE (percentage of ethanol, time and temperature) [21] and for PLE (percentage of ethanol and temperature). Briefly, MAE was carried out in a microwave extraction reactor (Anton Paar GmbH, Graz, Austria) at 164ºC, 60% of ethanol-water (v/v) and a total extraction time of 22 min. PLE was developed using a Dionex ASE 350 extractor (Dionex Corp., Sunnyvale, CA, USA) at 200ºC and 100 % ethanol (v/v). After cooling, samples were centrifuged in a centrifuge (Sorvall ST 16 R, Thermo Scientific, Leicestershire, UK) and the supernatant was collected and evaporated at 35°C to dryness in a Savan SC250EXP Speed-Vac (Thermo Scientific, Leicestershire, UK).

Production of *H. sabdariffa* lipid nanoparticles (HS-NLC)

The production of *H. sabdariffa* extracts-loaded NLC (HS-NLC) followed the method described by Fangueiro et al. [23] with some modifications. The inner aqueous phase was prepared dissolving 10 mg of *H. sabdariffa* extract from MAE or PLE in 1.25 ml of ultra-purified water. For blank and for RSM study only the ultra-purified water was used. The lipid phase was composed of a mixture of solid lipid and liquid lipid in the ratio 70:30 and melted at 60 ºC. The inner aqueous phase was added to the lipid phase to produce the primary emulsion homogenized using a rotor stator system (Ultra-Turrax IKA T18) at 11000 rpm, 90s. Then, this pre-emulsion and a water-surfactants mixture (ratio Tween 80:Span80, 90:10) was homogenized at 11000 rpm, 3 min by Ultra-Turrax and then sonicated for 3 min with a sonication probe (6 mm diameter) by means of an Ultrasonic processor VCX500 (Sonics, Switzerland). A power output with amplitude of 90% was applied. The volume of the final formulation was 25 mL. HS-NLC from MAE (HS-MAE-NLC) and HS-NLC from PLE (HS-PLE-NLC) were then obtained.

Optimization of NLC formulation by response surface methodology (RSM)

RSM is a compilation of mathematical and statistical techniques based on the fit of a polynomial equation to the experimental data, in order to describe the behavior of a data set with the aim to make statistical predictions [24]. In this study, the independent variables considered to optimize the NLC formulation were the percentage of lipid phase (0.8-3% w/w) and percentage of surfactants (0.5-2% w/w) and the factor responses were the mean particle size (measured in nm), polydispersity index (PDI) and zeta potential (measured in mV).

To study the effect of percentage of lipid phase and surfactants on the abovementioned responses, a full factorial experimental design at three levels (3^2)

with 3 central points which included 12 experimental points was employed. The parameters of the model (analysis of variance, ANOVA), optimal formulation condition and plot of response surface were estimated by using Statgraphics Centurion software XVI provided by Statpoint Technologies (Warrenton, VA, USA). To verify the suitability of the quadratic equation for predicting the optimum formulation, the verification experiment was carried out under optimum conditions and these conditions were applied for the loading of MAE and PLE *H. sabdariffa* extract.

NLC characterization

The mean particle size and PDI were determined by dynamic light scattering (DLS) using a particle size analyzer (DLS, Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). For each measurement, the NLC dispersions were diluted in Milli Q water (1:10) to an appropriate concentration to avoid multiple scattering. Mean diameter and PDI of NLC dispersions were determined in triplicate. ZP measurements were runs by electrophoretic light scattering (ELS) using a Nano Zeta Potential Analyzer (DLS, Zetasizer Nano ZS, Malvern Instruments, Malvern, UK Measurements) in triplicate at 25 ºC. The dilution used was (1:10).

Quercetin, anthocyanins and total phenolic compounds entrapped

The encapsulation efficiency (EE) of quercetin, anthocyanins and total phenolic compounds (TPC) from *H. sabdariffa* extract in optimal NLC formulation was determined by indirect procedure. Dispersions were firstly centrifuged at 15000 rpm for 30 min, at 25°C, clearly separating the supernatant from the lipid. The non-encapsulated quercetin and anthocyanins in the supernatant obtained after centrifugation were measured by UV spectroscopy at 300 and 520 nm, respectively. To determine TPC, Folin-Ciocalteu assay was employed [22]. Briefly, 10 µL of the

supernatant from HS-MAE-NLC and HS-PLE-NLC were mixed in 50 µL of Folin-Ciocalteu reagent. After 10 min, 150 µL of 2% (w/v) Na_2CO_3 were added and the volume was made up to 1 mL with water. The mixture was incubated for 2 h at room temperature in darkness and, then, 200 µL of the mixture was transferred into a well of a microplate. The absorbance was measured at 760 nm by a Synergy Mx Monochromator-Based Multi-Mode Micro plate reader, by Bio-Tek Instruments Inc. (Winooski, VT, USA). The results were compared to a gallic acid calibration curve (25-500 µg/mL) built in the same manner. The total phenolic content was measured in triplicate and calculated as mean ± SD (n = 3) and expressed as µg of gallic acid per g of sample.

The EE of these compounds was calculated applying the following equation:

$$\% \text{ EE} = \frac{\text{Total amount of bioactive compound (g)} - \text{Amount of bioactive compound in supernatant (g)}}{\text{Total amount of biactive compound (g)}} \times 100$$

Morphological characterization

Transmission electron microscopy (TEM) analysis was performed for the morphological observation of the obtained optimized NLC formulations (HS-MAE-NLC and HS-PLE-NLC) using a JEOL-JEM1010 instrument (JEOL Ltd., Tokyo, Japan). The dispersions were diluted with distilled water, dropped into copper grids and dried. Then, the particles' surface was stained with 0.2% (w/v %) phosphotungstic acid and dried. The analysis was performed at an accelerating voltage of 80 kV.

Fourier Transform Infrared Spectroscopy

The infrared spectra of lyophilized samples of HS-NLC formulations were scanned on a Fourier transform infrared (FTIR) spectrophotometer (Spectrum 400;

Perkin Elmer, USA), by using circular KBr cell window, 0,05 mm round Teflon spacers, Spectrum 10 software (Perkin Elmer, USA), at frequency range between 4000 and 600 cm^{-1}.

Results and discussion

NLC characterization and optimization

NLC formulations composed of a blend of Biograpress™ Vegetal BM 297 ATO and soybean oil, and stabilized by a surfactant combination of Tween 80 and Span 80, have been produced. Twelve experimental runs were carried out using central composite design **(Table 1)**. The concentration of lipids and surfactants was set up as independent variable since it is the most relevant parameter influencing the immediate and long-term stability of NLC [25]. Mean particle size, PDI and ZP were set as the dependent variables followed by the analysis of variance (ANOVA) of the experimental data with 95% confident level **(Table 2)**.

Code	% Lipid phase	% Surfactants	Size	PDI	Z -Potential
NLC-1	0.8	0.5	87 ± 2	0.24 ± 0.02	-25 ± 1
NLC-2	0.8	1.25	66.5 ± 0.3	0.19 ± 0.02	-23 ± 2
NLC-3	0.8	2	120 ± 2	0.29 ± 0.01	-21 ± 1
NLC-4	1.9	0.5	149.0 ± 0.5	0.211 ± 0.000	-26.5 ± 0.8
NLC-5	1.9	1.25	120.4 ± 0.9	0.18 ± 0.02	-26 ± 2
NLC-6	1.9	2	102.6 ± 0.4	0.14 ± 0.02	-26 ± 2
NLC-7	3	0.5	294 ± 2	0.39 ± 0.01	-29.1 ± 0.6
NLC-8	3	1.25	205.7 ± 0.7	0.35 ± 0.01	-26.9 ± 0.5
NLC-9	3	2	134 ± 1	0.17 ± 0.02	-24.7 ± 0.9
NLC-10	1.9	1.25	131 ± 0.5	0.193 ± 0.01	-27.5 ± 1
NLC-11	1.9	1.25	120 ± 1	0.156 ± 0.000	-26± 1
NLC-12	1.9	1.25	125.6 ± 2	0.187 ± 0.000	-27 ± 0.3

Table 1. Full factorial experimental design at three levels (3^2) with 3 central points and observed responses under different experimental conditions.

Source	Particle Size (nm)					PDI					Zeta Potential (mV)				
	SS	DF	MS	F-value	p	SS	DF	MS	F-value	p	SS	DF	MS	F-value	p
A	21624.0	1	21624.0	808.17	**0.0001**[a]	0.00602	1	0.00602	22.85	**0.0174**[a]	22.815	1	22.815	40.56	**0.0078**[a]
B	5011.26	1	5011.26	187.29	**0.0008**[a]	0.00968	1	0.00968	36.76	**0.0090**[a]	13.2017	1	13.2017	23.47	**0.0168**[a]
A:A	1338.03	1	1338.03	50.01	**0.0058**[a]	0.02362	1	0.02362	89.48	**0.0025**[a]	5.9042	1	5.9042	10.49	**0.0479**[a]
A:B	9312.25	1	9312.25	348.03	**0.0003**[a]	0.01822	1	0.01822	69.21	**0.0036**[a]	0.04	1	0.04	0.07	0.8070
B:B	390.427	1	390.427	14.59	**0.0316**[a]	6.6 E-7	1	6.6 E-7	0.00	0.9630	0.09375	1	0.09375	0.17	0.7105
Lack of fit	668.84	3	222.947	8.33	0.0576	0.00709	3	0.00236	8.98	0.0522	4.65708	3	1.55236	2.76	0.2133
Pure error	80.27	3	26.7567			0.00079	3	0.00026			1.6875	3	0.5625		
Total	39183.2	11				0.06822	11				49.7025	11			
R^2	0.98088					0.88436					0.872349				
Adj. R^2	0.96495					0.78041					0.765973				

Table 2 Analysis of variance (ANOVA) of the regression model. A = % Lipid phase; B = % Surfactants; SS = sum of squares; DF= degrees of freedom; MS = mean square; R^2 = Quadratic correlation coefficient; [a] Significant ($p < 0.050$)

Obtained NLC exhibited a particle size ranging from 66.5 ± 0.3 nm to 294 ± 2 nm. In order to achieve a small particle size in the freshly prepared NLC dispersion (thereby limiting the risk of destabilization by creaming or sedimentation due to Brownian motion [26]), during the production the hot w/o/w emulsion should also exhibit a small droplet size. The particle size was significantly influenced by the percentage of lipid phase (A) and percentage of surfactants (B) (p-value < 0.5) **(Figure 1 (a) and Table 2).** According to the suggested model, the optimum theoretical values to minimize particle size obtained by the quadratic polynomial equation were 0.8 % (w/w) lipid phase and 0.65 % (w/w) surfactant.

$$\text{Particle Size} = 26.7454 + 57.3347*A + 18.8101*B + 18.5124*A^2 - 58.4848*A*B + 21.5111*B^2$$

The effect of lipid phase on the droplet size of o/w emulsions stabilized with whey protein isolate (WPI) and xanthan gum was studied by Sun & Gunasekaran, [27], who reported that droplet size decreased with increasing the WPI concentration or decreasing the oil phase volume fraction. The formulation composed of 0.2% (w/w) WPI and 40% (v/v) oil exhibited the highest mean droplet size (1.65 µm), whereas that composed of 2% (w/w) WPI and 5% (v/v) oil the smallest (0.60 µm).

With respect to the particle size distribution, the smaller the PDI, the higher the long-term stability. PDI is 0 for ideally monodisperse particles, a PDI of 0.100 still indicates a relatively narrow distribution, values of up to 0.250 are reported for parenteral fat emulsions and a PDI > 0.500 indicates a very broad distribution. In our work, the smallest PDI obtained in the experimental design was 0.140 ± 0.02 and the

highest value was 0.900 ± 0.01. In addition, the effect of both independent variables was significant (p-value < 0.05). However, unlike in the case of particle size, surfactant concentration was the most influential factor **(Figure 1 (b))**. In this case, a higher percentage of surfactant contributed for a lower PDI value, being 2% (w/w) surfactant and 2.1% (w/w) lipid phase the optimum predicted values to minimize PDI according to the following quadratic equation:

$$PDI = 0.274987 - 0.164146*A + 0.104121*B + 0.077686*\%\ A^2 - 0.0818182*A*B$$

The model equation provides only the significant parameters to reduce the correlation deviation. Thus, as shown in **Table 2**, the quadratic effect for percentage of surfactants was not significant (p-value > 0.05).

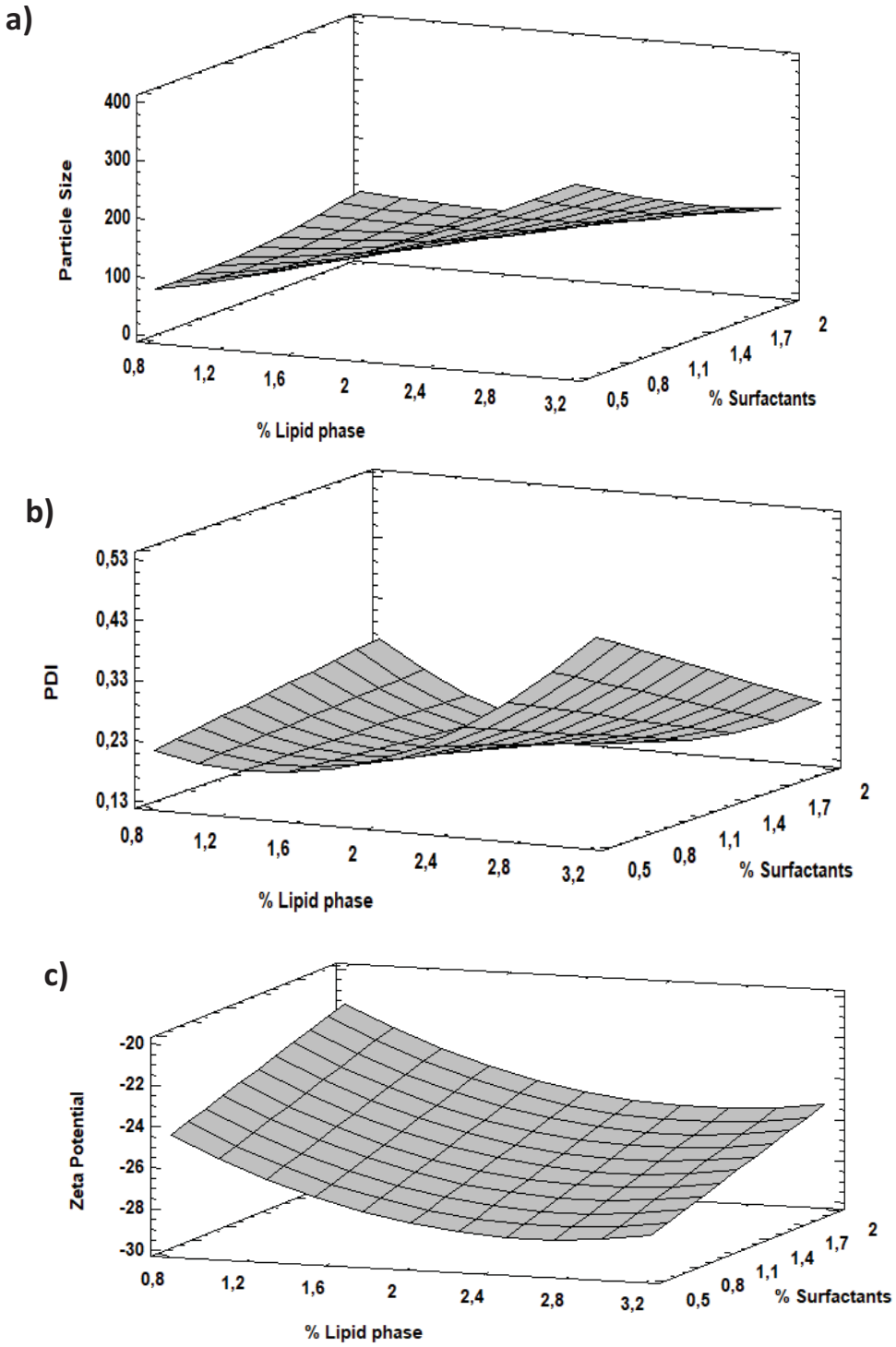


Figure 1. 3D plots of response surface for the effect of % lipid phase and % surfactant on the response selected: a) particle size (nm), b) PDI, c) zeta potential (mV).

With respect to the ZP, this parameter is an indirect measure of the surface electrical charge, which translates the particle stability in dispersion. Values lower than -25 mV and higher than 25 mV indicate physical stability of the dispersions over time [28, 29]. Except for NLC-2 and NLC-3, our experimental runs exhibited a potential zeta higher than |25 mV|, for which both independent variables had a significant effect. The decrease of the percentage of surfactant and increase of the percentage of lipid phase contributed to the increase of the zeta potential values **(Figure 1 (c))**. The optimum theoretical values were 0.5 % (w/w) surfactant and 2.7 % (w/w) lipid phase calculated keeping only the significant parameters in the following quadratic equation:

$$\text{Zeta Potential} = -20.9797 - 6.6405*A + 1.7778*B + 1.28099*A^2$$

The obtained parameters of ANOVA enabled to confirm that this model provided a good approximation to the experimental conditions. The polynomial model equation for particle size, PDI and ZP were expressed by the regression coefficient (R^2 = 0.98404). (R^2 = 0.894771) and (R^2 = 0.872349) respectively, demonstrating a good correlation between the experimental and theoretical data. The lack of fit was shown to be not significant (p-value > 0.05) for all responses, which indicates that this model is suitable for the data observed at the 95% confidence level. **Table 3** summarizes the coefficient of variation (CV) between experimental and theoretical results resulting in low CV values for all studied responses.

Run	Factor A	Factor B	Particle size (nm)			PDI			Zeta Potential (mV)		
	% Lipid phase	% Surfactants	Experimental	Predicted	CV %	Experimental	Predicted	CV %	Experimental	Predicted	CV %
1	1.9	1.25	131.0	120.7	5.787	0.193	0.178	5.730	27.5	26.5	2.619
2	1.9	1.25	120.0	120.7	0.411	0.156	0.178	9.311	26.0	26.5	1.347
3	1.9	1.25	125.6	120.7	3.464	0.187	0.178	3.484	27.0	26.5	1.322
4	1.9	2.00	102.6	103.9	0.919	0.14	0.137	1.274	26.0	25.0	2.726
5	0.8	1.25	66.5	83.1	11.738	0.19	0.240	16.444	23.0	23.0	0.000
6	3.0	2.00	134.0	138.1	2.899	0.17	0.196	9.927	24.7	25.4	2.022
7	3.0	1.25	205.7	203.2	1.768	0.35	0.303	10.101	26.9	26.9	0.000
8	1.9	0.5	149.0	161.7	8.980	0.211	0.218	2.253	26.5	28.0	3.850
9	1.9	1.25	120.4	120.7	0.212	0.18	0.178	0.923	26.0	26.5	1.347
10	3.0	0.50	294.0	292.4	1.131	0.39	0.411	3.708	29.1	28.4	1.763
11	0.8	0.50	87.0	78.85	5.763	0.24	0.213	8.540	25.0	24.5	1.477
12	0.8	2.00	120.0	114.55	3.853	0.29	0.267	5.752	21.0	21.5	1.719

Table 3. Predicted and observed values of each individual response according to the polynomial model equations

A multi-response surface methodology was applied to achieve the optimum NLC formulation conditions taking into account the particle size, PDI and ZP altogether. The optimum NLC formulation condition given by the model was 2.21 % (w/w) lipid phase and 1.93 % (w/w) surfactantS. These optimal conditions and predicted values were determined on the basis of the desirability function, which was 0.77 **(Figure 2)**.

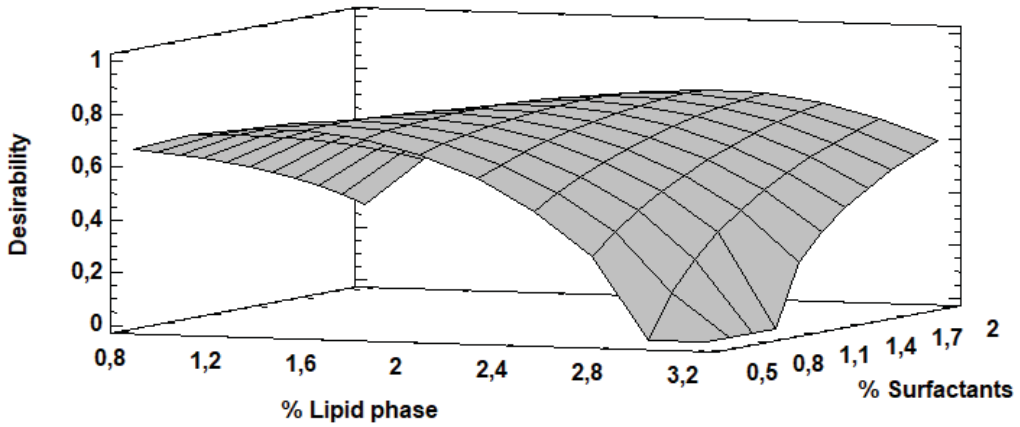


Figure 2. 3D plots of multi-response surface for the effect of % lipid phase and % surfactant on the overall desirability

To verify the suitability of the model, this formulation was produced. **Table 4** summarizes the optimal formulation responses obtained experimentally and compared to predicted values, while **Figure 3** shows the optimum size values measured by dynamic light scattering (DLS).

Optimized desirability = 0.77			
Response	**Predicted**	**Experimental**	**CV %**
Size (nm)	109.6	107 ± 0.4	1.70
PDI	0.174	0.163 ± 0.010	4.62
Zeta Potential (mV)	-25.6	-25.8 ± 0.9	0.64
Optimum formulation condition: 2.21 % Lipid phase and 1.93 % surfactants			

Table 4. Optimum formulation condition by multi-response surface methodology

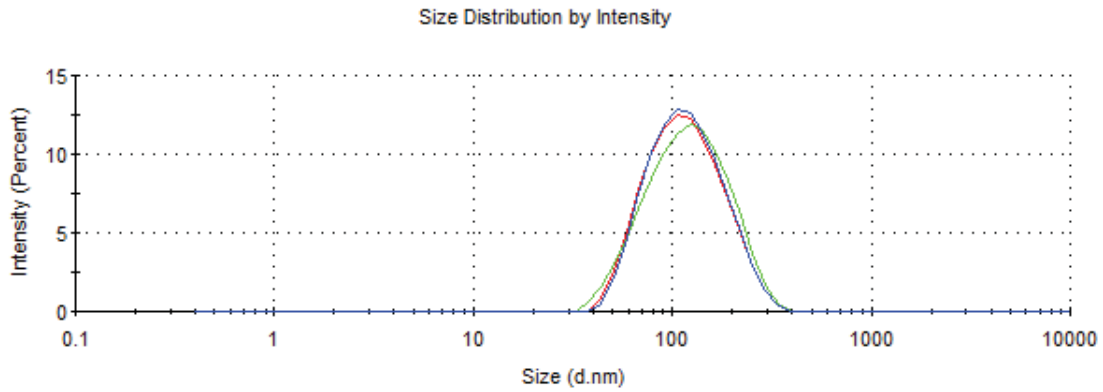


Figure 3. Particle size in empty optimum NLC measured by dynamic light scattering (DLS) to verify the predicted model

Encapsulation of *Hibiscus sabdariffa* extract into optimum NLC formulation

For the loading of *H. sabdariffa* extracts obtained by MAE or by PLE, 10 mg of each extract was diluted in the inner aqueous phase to produce HS-MAE-NLC and HS-PLE-NLC. The NLC were then characterized for their mean particle size, ZP and PDI. The loading of *H. sabdariffa* extracts in NLC strongly influenced the mean particle size. A higher particle size for HS-MAE-NLC (470 ± 8 nm) compared to HS-PLE-NLC (344 nm ± 12) **(Table 5)**. A similar behavior was observed for PDI (0.47 ± 0.01 for HS-MAE-NLC and 0.34 ± 0.06 for HS-PLE-NLC). For the ZP values, results were very similar for both HS-NLC and NLC without extract, and differences recorded for HS-MAE-NLC and HS-PLE-NLC were not statistically significant. Our results confirm that the developed HS-NLC have no tendency to aggregate attributed to the repulsion forces promoted by the high surface electrical charge. It has been reported that size and surface charge of nanoparticles play a key role in the intestinal permeability, thereby influencing the oral bioavailability of bioactive compounds [30]. HS-MAE-NLC and HS-PLE-NLC could be suitable for oral administration as their mean particle size is lower than 500 nm, which

promotes their absorption by intestinal enterocytes by endocytosis [31, 32]. Literature revises several examples of the successful loading of polyphenolos in lipid nanoparticles, with a particulas emphasis for oral administration [33-35]. Tea polyphenols (TPP) have been successfully loaded in solid lipid nanoparticles (SLN) of mean size of about 500 nm (ZP of -15 mV), which were then administered to Swiss Albino mice [36]. The oral administration of TPP-SLN in Ehrlich ascetic carcinoma bearing mice resulted in a significant increase of plasma hemoglobin, glucose, superoxide dismutase and catalase, when compared to control mice. This study highlights the added value of lipid nanoparticles in improving the oral bioavailability of polyphenols, which has been well documented in the literature [34]. Lipid nanoparticles with a mean size between 100 and 250 nm have been loaded with curcuminoids (the active substances in turmeric) exhibiting modified release profile and increased the absorption of the drugs by the gastrointestinal tract [37]. More recently, essential oil containing the monoterpene citral has been loaded in NLC and confirmed for the first time to exhibit anti-inflammatory activity in a RAW 264.7 cell line and strong cytotoxic effect in two cell models (non-tumoral HaCaT and tumoral A431), highlighting the synergistic effect of NLC in vitro [5]. Other Mediterranean essential oils (e.g. Rosmarinus officinalis L., Thymus capitatus, Lavandula and Origanum vulgare), were also loaded to produce NLC with enhanced antioxidant and anti-inflammatory activities in RAW 264.7 cell line [7]. The poorly water-solubility profile of linalool was also reported to be improved by the loading in NLC [3, 38].

	Size (nm)	PDI	ZP (mV)	% EE (Quercitin)	%EE (Anthocyanins)	% TPC retained
HS-MAE-NLC	470 ± 8[a]	0.47 ± 0.01[a]	- 26.3 ± 0.9[a]	52.9 % ± 0.9[a]	60 % ± 2[a]	55 % ± 4[a]
HS-PLE-NLC	344 ± 12[b]	0.34 ± 0.06[b]	- 25.7 ± 0.5[a]	93 % ± 3[b]	84 % ± 4[b]	73 % ± 6[b]

Table 5. HS-NLC characterization. Mean values superscripted by different letters indicate significantly different values ($p < 0.05$).

With respect to the encapsulation efficiency (% EE), 52.9 ± 0.9% and 60 ± 2% quercetin and anthocyanins, respectively, in HS-MAE-NLC. In HS-PLE-NLC, the % EE was higher i.e. 93 ± 3% for quercetin and 84 ± 4% for anthocyanins. These results are in agreement with the data obtained for TPC measured by Folin-Ciocalteu. For HS-MAE-NLC, the TPC retained was 55 ± 4% while for HS-PLE-NLC the percentage of loaded TPC was 73 ± 6%.

The formulation of *H. sabdariffa* extract obtained by PLE in NLC showed better results in terms of particle size, PDI and % EE than NLC loaded with *H. sabdariffa* extract by MAE ($p < 0.05$). The better results reported for the NLC loading *H. sabdariffa* extract obtained by PLE were attributed to the higher ethanol content used for the production of the extract (100% ethanol). This may offer higher affinity to the lipid matrix thereby contributing to the higher % EE, as well as to a better emulsifying process resulting in a lower particle size and PDI.

TEM analysis

The shape and size of NLC were analysed with the help of TEM (Fig. 4). Results reveal that HS-MAE-NLC and HS-PLE-NLC have uniform spherical shape with narrow particle size distribution.

a) HS-MAE-NLC b) HS-PLE-NLC

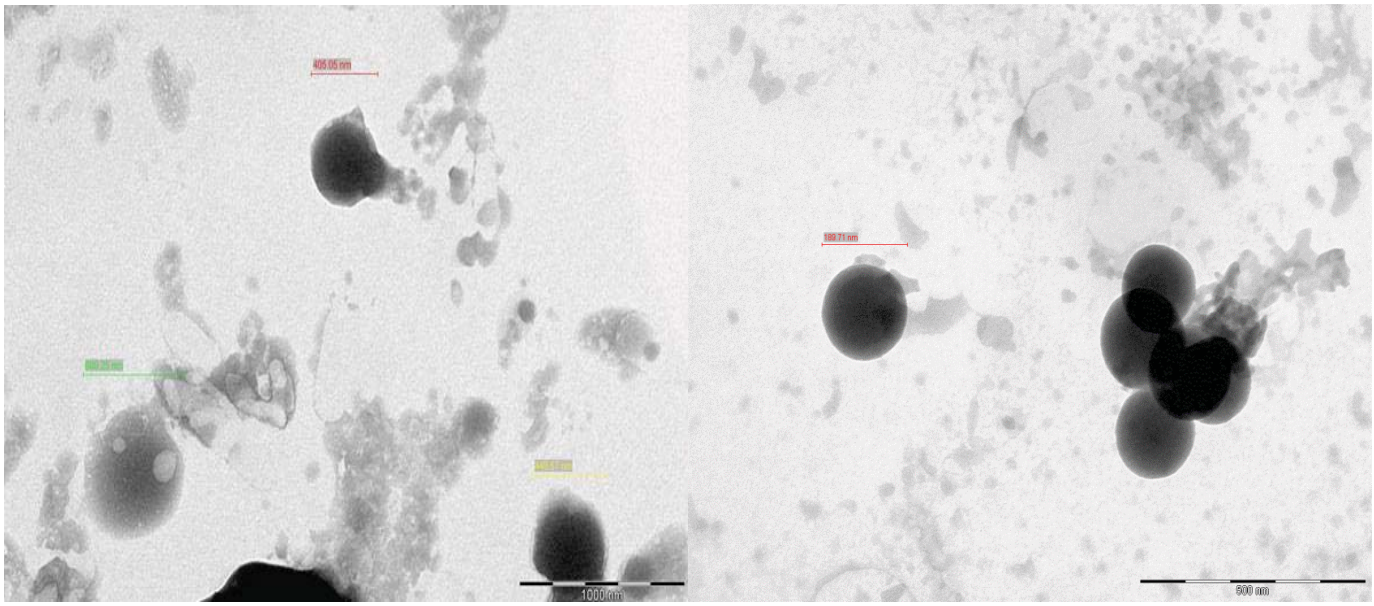


Figure 4. TEM images of HS-MAE-NLC (a) and HS-PLE-NLC (b)

FTIR Analysis

FTIR studies were conducted as a supplementary technique to confirm the presence of polyphenols from *H. sabdariffa* extracts within the lipid matrix. FTIR spectra of both extracts showed a characteristic peak in the region 3300 cm^{-1} and 2900 cm^{-1} which represents O-H and C-H stretching respectively of aromatic compounds. Both extracts showed bands around 1700 cm^{-1} corresponding to C=O stretching vibration and OH-H bending vibration (859 cm^{-1}) of the hydrogen bond which are related to carboxylic acid group. The region 1600 cm^{-1} represents C=C stretching from aromatic compounds. The absorption band at 1398 cm^{-1} was assigned to methylene group (CH_3) and the region 1180 cm^{-1} and 950 cm^{-1} was assigned to C-O vibrations from phenolic alcohols. Finally, the absorption band 709 cm^{-1} represents C-H stretching form aromatic compounds.

Significant changes were observed in the spectra of loaded NLC **(Figure 5)**. The most significant changes were identified in the finger print region, which revealed the presence of *H. sabdariffa* extract within the lipid matrix. The absorption band at 2900 cm^{-1} and 1700 cm^{-1} showed a stronger signal due to the C-H and C=O vibrations from the lipids used in the formulation [39]. These results corroborate the physicochemical stability reported for our NLC.

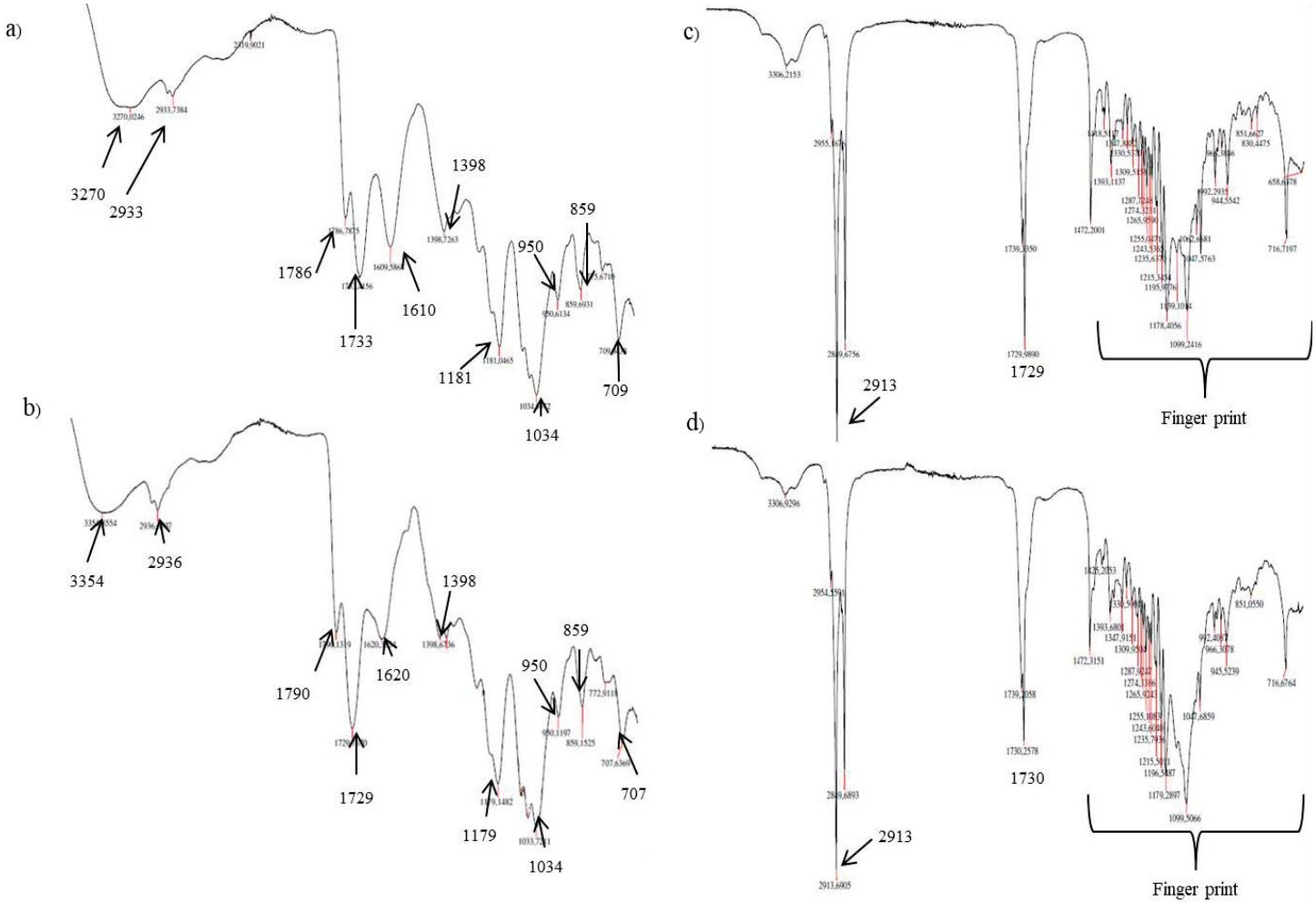


Figure 5. FTIR spectrum: a) MAE extract, b) PLE extract c) HS-MAE-NLC, d) HS-PLE-NLC

Conclusion

This study reports an approach to use a 3-level 2-factor factorial design in the optimization of NLC formulations produced with different percentages of lipid phase and surfactants to incorporate phenolic compounds from *H. sabdariffa*. The predicted model obtained by RSM demonstrated to be suitable to evaluate the influence of independent variables on the response variables (particle size, PDI and ZP). Moreover, it was demonstrated that polyphenol-enriched *H. sabdariffa* extracts obtained by two different green extraction technologies can be successfully loaded in optimized NLC. NLC loading PLE extract showed lower particle size and higher encapsulation efficiency for quercetin, anthocyanins and phenolic compounds. The loading of phenolic compounds from H. sabdariffa within the developed NLC was also confirmed by FTIR.

Acknowledgments

This work was supported by the Research group AGR274 “Bioactive ingredients” from the Analytical Chemistry Department of the University of Granada, “REQUIMTE/LAQV” group of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra. Also, the authors would like to thank the financial support received from the Spanish Ministry of Economy and Competitiveness (MINECO) (project AGL2015-67995-C3-2), from Portuguese Science and Technology Foundation (FCT/MCT) and from European Funds (PRODER/COMPETE) under the project M-ERA-NET/0004/2015-PAIRED, co-financed by FEDER, under the Partnership Agreement PT2020. We would like to thank the Ministry of Education, Culture and Sport (MECD) for supporting the grant FPU15/01125 of SPM and the stay in the University of

Coimbra supported by FPU short – term stay. MCT wishes to acknowledge FCT and Dendropharma - Investigação E Serviços De Intervenção Farmacêutica, Sociedade Unipessoal Lda. for the individual fellowship (PD/BDE/135086/2017).

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Chapter 8

Hibiscus sabdariffa – loaded nanostructured lipid carriers for development of diary beverage

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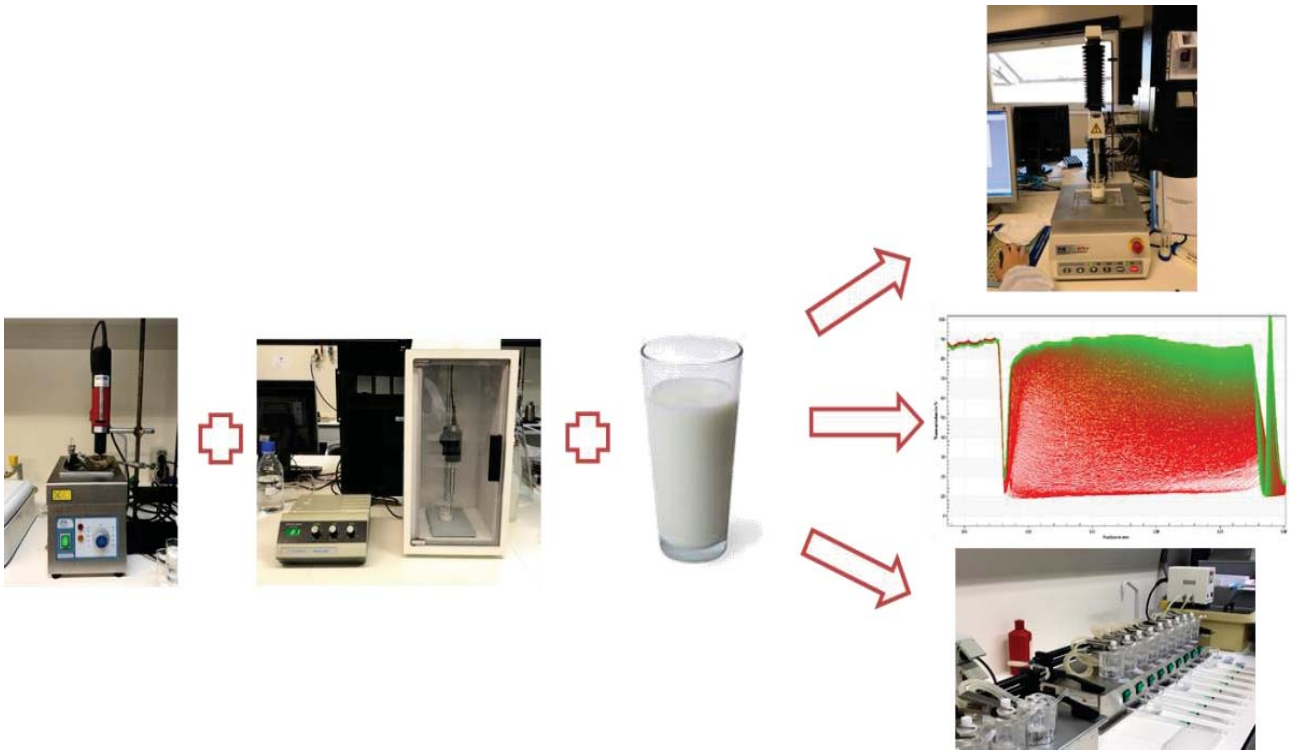
UNIVERSIDADE DE COIMBRA

Hibiscus sabdariffa – loaded nanostructured lipid carriers for development of diary beverage

Abstract



The growing interest in herbal based dairy foods is due to plant extracts are a source of potential antioxidant agents which have positive effect on a wide of variety of diseases. Within this field, *Hibiscus sabdariffa (H. sabdariffa)* is a tropical plant with high content of phenolic compounds such as quercetin and anthocyanins and with demonstrated bioactivity that makes that this plant is suitable for incorporation in functional dairy beverages. Nevertheless, many of these phenolic compounds are not stable under environmental conditions. Thus, in this work, two polyphenol – enriched extracts from *H. sabdariffa* obtained by microwave – assisted extraction (MAE) and pressurized liquid extraction (PLE) were entrapped by nanostructured lipid carriers

(NLC) and incorporated into dairy beverage for the evaluation of the long-term stability, texture properties and *in vitro* release study. The results showed that these enriched milks were a stable suspension due to protein matrix provides a stabilization of lipid nanoparticles. In addition, the *in vitro* release study of quercetin and anthocyanins from dairy beverage with these *H. sabdariffa* nanoparticles was slower than milk with free extract. Therefore, NLC has demonstrated to be a potential technology that improves the bioaccessibility and bioavailability of bioactive ingredients from *H. sabdariffa.*

Keywords: *Hibiscus sabdariffa*; encapsulation; dairy beverage; quercetin; anthocyanins; release

Introduction

The food industry is one of the most important areas of the economy in the European Union, playing a main role for the processing of agricultural raw materials. In addition, during the last decade, the requirements of consumers in healthy food have increased considerably due to the knowledge about nutrients and their effect to prevent diseases and to improve physical and mental well-being. In this regard, the introduction of functional foods in the market has been highly satisfactory in terms of acceptability. Therefore, functional foods are an interesting sector of research and innovation in the food industry (Bigliardi & Galati, 2013).

Among functional foods, dairy-based functional foods account for nearly 43% of the market because of the ease of this food matrix to incorporate probiotics or other bioactive compounds, demonstrating health benefits beyond their basic nutritional value (Marsh, Hill, Ross, & Cotter, 2014). Hence, these products can be enriched with bioactive ingredients belonging from dairy and nondairy sources, being the probiotics

the main bioactive components of fermented functional dairy foods. Besides that, dairy beverages have been enriched with other compounds with healthy benefits such as omega-3 fatty acids, phytosterols, vitamins, minerals, fiber and phytochemicals including phenolic compounds (Benito et al., 2006; Boroski et al., 2012; Casas-Agustench et al., 2012; Castro, Monteiro, Barroso, & Bertolami, 2007). Indeed, several researches have incorporated plant extracts such as *Panax quinquefolius L*. (American ginseng), green tea, black tea or soybean into milk beverage (Tárrega et al., 2012; Zhao & Shah, 2015). The growing interest in herbal based dairy foods is due to plant extracts are a source of potential antioxidant agents which have positive effect on a wide of variety of diseases i.e. diabetes, obesity, cardiovascular diseases, inflammatory diseases, among others (Petti & Scully, 2009). Within this field, *Hibiscus sabdariffa (H. sabdariffa)* is a tropical plant with high content of phenolic compounds (phenolic acids and flavonoids such as quercetin and anthocyanins) and demonstrated bioactivity (Patel, 2014). These characteristics makes that this plant is suitable for incorporation in functional dairy beverages.

Nevertheless, many of these phenolic compounds are not stable under environmental conditions (oxygen, light, temperature), limiting their application and incorporation in beverages. Moreover, these components are modified or degraded in the gastrointestinal tract when these are consumed. Hence, to ensure the stability of these functional ingredients is necessary. To this end, nanoencapsulation involves the incorporation of food ingredients in small capsules, protecting them from environmental and gastrointestinal condition and allowing their delivery in the specific place that they carry out their function (Gibbs, Kermasha, Alli, & Mulligan, 1999). In

this way, nano – lipid carriers (NLC) is a lipid based encapsulation technique that consists of lipid droplets crystalized which contains the bioactive compounds. Therefore, as well as all lipid encapsulation system, NLC are to be able to incorporate hydrophobic and hydrophilic compounds due to their chemical structure. In addition, NLC is a novel system that solves the limitations for oral administration of other lipid encapsulation technologies like liposomes or solid-lipid nanoparticles (SLN) (Müller, Radtke, & Wissing, 2002). Thus, phenolic compounds from *H. sabdariffa* could be vehiculized by NLC, allowing a good stability for incorporation in a dairy beverage.

On the other hand, for a successful incorporation of bioactive ingredients encapsulated into food matrices, the release rate of these compounds is an important factor that should be borne in mind to ensure the bioavailability and beneficial effects. In particular, the design of colloidal nanosystem provides chemical structure modifications that improve the bioaccessibility and bioavailability of bioactive ingredients (Rein et al., 2013). Thus, the aims of this study were 1) to encapsulate two polyphenol – enriched extracts from *H. sabdariffa* obtained by microwave – assisted extraction (MAE) and pressurized liquid extraction (PLE) by NLC and incorporate into a dairy beverage; 2) to evaluate the long-term stability and texture properties of these enriched milks and 4) to monitor the *in vitro* absorption of quercetin and anthocyanins from milk that contains *H.sabdariffa* – loaded nanoparticles.

Materials and methods

Samples and reagents

The calyces of *H. sabdariffa* were collected from Monteloeder (Elche, Spain) and homogenized by an ultra-centrifugal mill ZM200 (Retsch GmbH, Haan, Germany).

For extractions, absolute ethanol was purchased from VWR international S.A.S. (Fontenay-sous-Bois, France). Sea sand was from Panreac (Barcelona, Spain). Ultrapure water was from a Milli-Q system (Millipore, Bedford, MA, USA).

For development of lipid nanoparticles, the lipid phase was composed of a mixture of solid lipid (BiograpressTM Vegetal BM 297 ATO) kindly provided by Gatefossé (France) and lipid liquid (Soybean oil) purchased from Sigma (Darmstadt, Germany). The surfactants used were Tween 80 and Span 80, both from Merck (Hohenbrunn, Germany). These nanoparticles were incorporate into skimmed milk from Mimosa (Porto, Portugal).

For in vitro release study, phosphate buffered saline (PBS) was composed of disodium hydrogen phosphate and sodium chloride, both purchased from Sigma (Darmstadt, Germany). This solution was adjusted to pH 7.4.

Production of polyphenol – enriched extracts from *H. sabdariffa*

Microwave – assisted extraction (MAE) and pressurized liquid extraction (PLE) were performed to obtain two polyphenol – enriched extracts. Extract conditions used for MAE and PLE had been described previously (Pimentel-moral, Borrás-linares, & Lozano-sánchez, 2018). Hence, PLE conditions were 200 ºC and 100 % (v/v) ethanol using a Dionex ASE 350 extractor (Dionex Corp., Sunnyvale, CA, USA) and MAE conditions were 164 ºC, 60% ethanol (v/v) and a total extraction time of 22 min using

a microwave extraction reactor (Anton Paar GmbH, Graz, Austria). Both extracts were dried later.

Preparation of *H. sabdariffa* – loaded nanostructured lipid carriers (HS-NLC)

This formulation was developed based on a previous study (Pimentel-Moral et al., 2019). The optimum percentage of lipid phase (2.21 % (w/w)) and surfactant amount (1.93 % (w/w)) were applied to obtain HS-NLC from microwave - assisted *H. sabdariffa* extract (HS-MAE-NLC) and from pressurized – liquid *H. sabdariffa* extract (HS-PLE-NLC). Thus, 10 mg of *H. sabdariffa* extract from MAE or PLE were dissolved in 1.25 mL of ultra-purified water and incorporated into the inner aqueous phase by W/O/W double emulsion. This inner phase was added to the lipid phase composed of a liquid lipid (30% (v/v)) and a solid lipid (70% (w/w)) which was melted at 60 ºC. This mixture was homogenized at 11000 rpm, 90 s by Ultra – Turrax and added to the external aqueous phase composed of water-surfactants mixture (ratio Tween 80:Span80, 90:10). Finally, the double emulsion was performed at 11000 rpm for 3 min and sonicated at 90 % amplitude with a sonication probe using an Ultrasonic processor VCX500 (Sonics, Switzerland). The obtained warm nanoemulsions were quickly solidified in ice bath.

Beverages formulation

The dairy beverages were formulated based on (Badejo, Damilare, & Ojuade, 2014) combining the developed HS-NLC and the skimmed milk in a 70:20:10 ratio. For example, HS-MAE-NLC beverage was prepared by combining 10 mL of HS-MAE-NLC, 20 mL of water, and 70 mL of skimmed milk. These formulations were stirred for 10 min.

On the other hand, to compare the *in vitro* delivery of *H. sabdariffa* encapsulated and without be encapsulated, a beverage with *H. sabdariffa* extract was incorporated in milk in a free-form. To that end, 10 mg of powdered extract was diluted in 25 mL of water. Then, 70:20:10 ratio was applied.

Long-term stability of HS-NLC and HS-NLC dairy beverages

A simulated long-term stability of empty NLC, HS-MAE-NLC and HS-PLE-NLC developed and their incorporation in milk was carried out by LUMiSizer (LUM GmbH, Dias de Sousa, Portugal). This analytic centrifuge accelerates the destabilization by sedimentation or creaming phenomena employing the STEP^{TM} – Technology (Space – and Time – resolved Extinction Profiles). Firstly, the samples were stored at 4 ºC, 22 ºC and 40 ºC for 48 h. After this time, the samples were placed in rectangular test-tubes (optical path of 2 mm) and exposed to centrifugal force under 4000 rpm, 25 ºC for 2:46 h. Each 10 s, the samples were analyzed resulting in 1000 different profiles. Therefore, this mechanism allow evaluate the effect of temperature on formulation stability.

Textural properties of new functional beverages

The texture of dairy beverages with HS-NLC and the skimmed milk without these nanoparticles were measured using a texture analyzer (TA, XTplus; Stable Micro System, UK) with a 7 mm blade. The parameters of the texture analyzer were as follows: pretest and post-test speed, 2 mm/s; blade movement speed, 0.2 mm/s; and mov-ing distance, 5 mm. All measurements were carried out at 10 ºC in triplicate.

In vitro release study

For absorption study, static Franz glass cells were used. This method consists of donor and receptor chambers between which a membrane is positioned (Franz, 1975). The receptor chamber was composed with 5 mL of phosphate-buffered saline (PBS) commonly used in biological researches to describe the release behavior of oral formulations at pH 7.4. Cellulose membranes with an average pore size 0.6 µm were used. The receptor chamber was maintained at 37 ºC in order to simulate the surface intestinal temperature. Each cell contained a magnetic bar and was stirred during the experiment. 1mL of each sample (HS-MAE-NLC in milk, HS-PLE-NLC in milk, and free *H. sabdariffa* extract in milk) were introduced into donor chamber and after 15, 30 min, 1, 2, 4, 6, 8 and 24 h, 200 µL were taken. After each sample selection, the Franz cells were filled up with receptor medium.

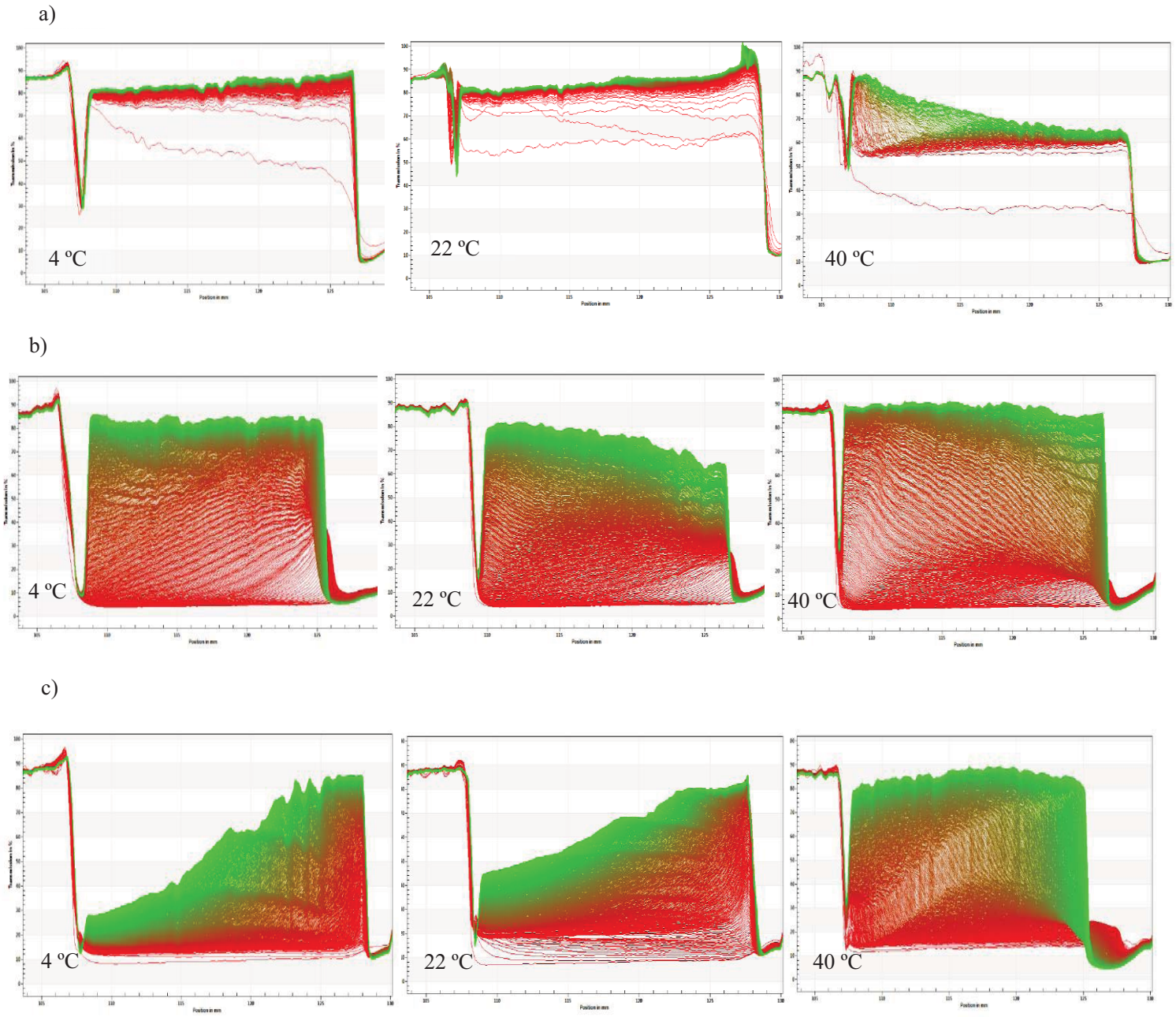
The absorbance of samples collected was measured at 300 nm for determination of quercetin and at 520 nm for determination of anthocyanins. Thus, a release study of these phenolic compounds from *H. sabdariffa* beverage formulation could be studied over time.

Results and discussion

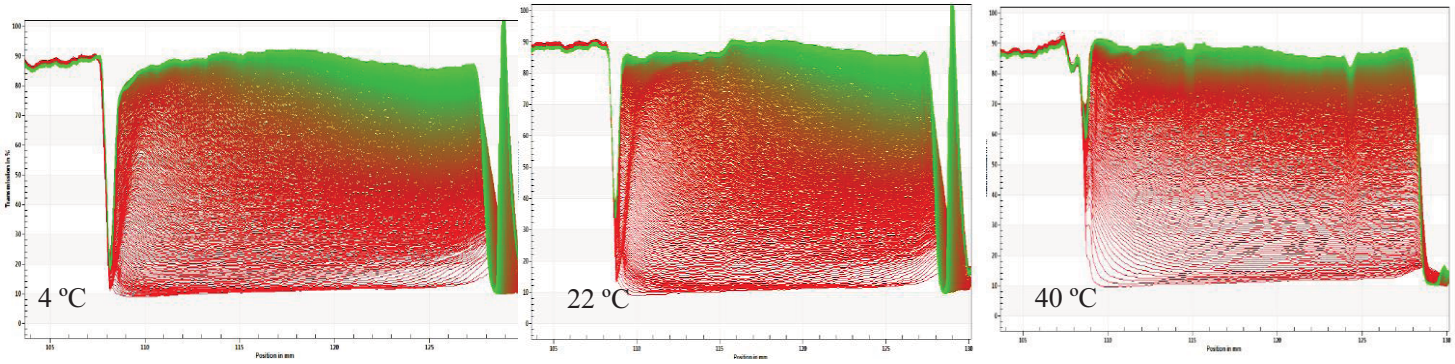
Long-term accelerated formulation stability

LUMiSizer has become an instrument of choice for the analysis of long-term stability of nanosuspensions due to allows to measure the intensity of the transmitted light during centrifugation, as a function of time and position, over the entire sample length (Fernandes et al., 2017). The progression of the transmission profiles provides

information about the kinetics nanoparticles behavior and shelf-life estimation. Thus, the instability phenomena are related to a migration of particles (sedimentation or creaming) when centrifugal acceleration is applied. In the present study, the transmission profiles of empty NLC, HS-MAE-NLC and HS-ASE-NLC nanoparticles and the dairy beverages with these nanoparticles incorporated and stored for 48 h at different temperatures are shown in **Figure 1**.



d)



e)

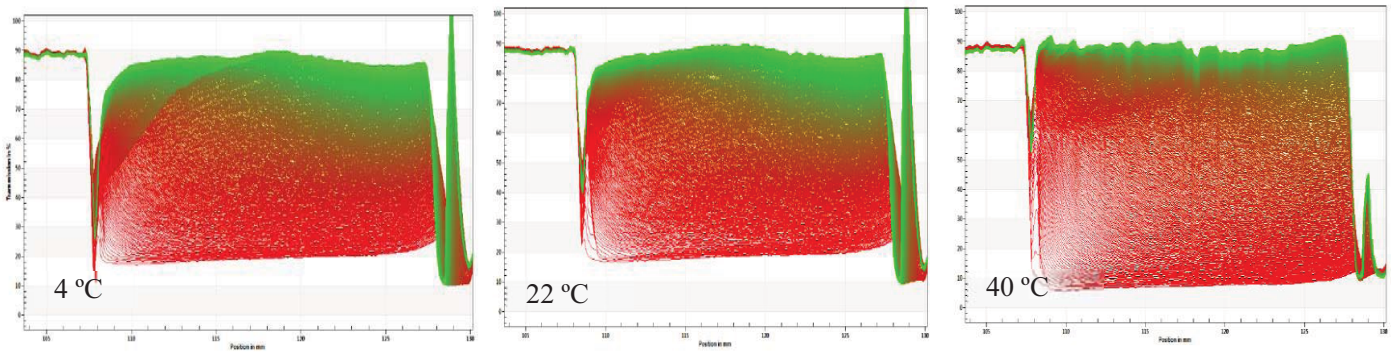


Figure 1. Long – term stability of empty NLC (a), HS-MAE-NLC (b), HS-PLE-NLC (c), HS-MAE-NLC milk (d) and HS-PLE-NLC milk (e)

In the case of NLC formulation without extract, particles with different sizes are observed. In comparison to HS-MAE-NLC and HS-PLE-NLC profiles, NLC without extract showed a multimodal particle size distribution, being the most unstable formulation. Therefore, *H. sabdariffa* extract provides a better long – term stability. It could be due to the lipid – polyphenol interaction because polyphenols are able to be distributed at the oil / tensioactive interface providing a protective antioxidant effect in aqueous media and on lipid phase (Mignet, Seguin, & Chabot, 2013). This was proved in a

previous study, in which epigallocatechin gallate (EGCG) has been shown to interact with the phosphate of dimyristoyl-phosphatidylcholine (DMPC) lipid models using 31P NMR (Kumazawa et al., 2004). In addition, the spacing between the profiles in NLC (**Figure 1 (a)**) decreased over time possibly and the particles tend to migrate more slowly. It could be caused by particle aggregation. At 4 ºC, creaming was the mechanism of destabilization more prominent, although at higher temperatures, the sedimentation showed a strong tendency.

For HS-MAE-NLC and HS-PLE-NLC a homogeneous particle size distribution was observed suggested by the symmetrical spacing in the profiles. Therefore, higher stability was obtained. In addition, for HS-MAE-NLC similar profiles were showed at different temperatures although there was a slight higher tendency toward the creaming mechanism at 4 ºC that went gradually down and increased the sedimentation process at higher temperatures. In contrast, as is shown in **Figure 1 (c)**, this phenomenon was more pronounced in HS-PLE-NLC.

Respect to formulated milk with HS-MAE-NLC and HS-PLE-NLC showed similar behavior. At 4 ºC and 22 ºC a creaming process was found in both beverages and at 40 ºC sedimentation mechanism increased. However, these instability mechanisms were less pronounced when these nanoparticles were incorporate into a dairy beverage providing higher stability. This could be because protein from milk could stabilize the final lipid nanoparticles formulation. Previously, has been demonstrated that many proteins are surface-active molecules that can be used as emulsifiers and improve the stability of emulsion formulation (McClements, 2004). Thus, milk-proteins can produce

adsorbed layer on the oil droplets, which create a physical barrier to coalescence (Singh, 2011; Wilde, Mackie, Husband, Gunning, & Morris, 2004).

On the other hand, instability index was measured to compare these functional beverages. The results obtained showed that higher temperature influenced slightly on the stability of HS-MAE-NLC milk and HS-PLE-NLC milk. Besides that, HS-PLE-NLC beverage showed to be more stable than HS-MAE-NLC beverage **(Figure 2)**.

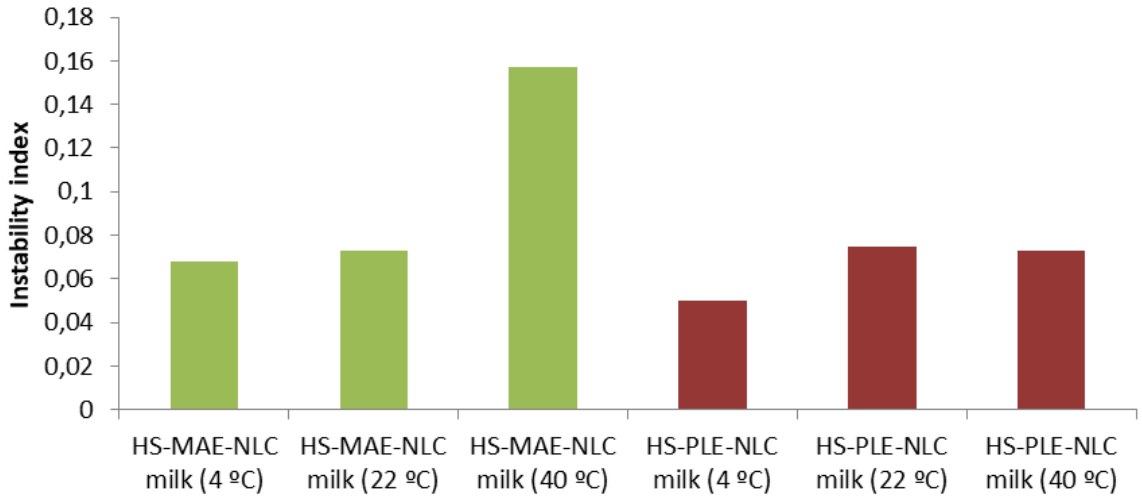


Figure 2. Instability index of HS-NLC beverages

Textural properties of new functional dairy beverages

Texture is one of the key quality attributes used in the food industry to assess product quality and acceptability. For processed foods, texture properties is important for the control of processing operations such as heating, frying and drying to attain desired quality attributes of the end product. Therefore, food formulation could be related to desirable or undesirable changes in texture. To measure the texture there are sensory and instrumental assays. However, sensory methods are subjective

measured which disadvantages such as level of training received by the participants, time consumed or the high cost, have limited their use against instrumental approaches (Chen & Opara, 2013; Nishinari & Fang, 2018).

Thus, in the current work, an instrumental texture analyzer was used to measure the firmness, the consistency, the cohesiveness and the index of viscosity of skimmed milk and skimmed milk with HS-MAE-NLC and HS-PLE-NLC. As is showed in **Table 1**, when nanoparticles were incorporate into dairy beverage, firmness, consistency, cohesiveness and index of viscosity obtained higher values. Indeed, significant differences ($p < 0.05$) were observed between skimmed milk without nanoparticles and with them.

	Firmness (g)	Consistency (g/sec)	Cohesiveness (g)	Index of Viscosity (g/ sec)
Skimmed milk (Control)	10.8 ± 0.4^{a}	32.9 ± 0.6^{a}	-8.7 ± 0.7^{a}	25.8 ± 0.4^{a}
HS-PLE-NLC milk	18.9 ± 0.9^{b}	56 ± 2^{b}	-13.2 ± 0.8^{b}	40 ± 1^{b}
HS-MAE-NLC milk	25 ± 1^{c}	89 ± 2^{c}	-15.9 ± 0.8^{c}	41 ± 1^{b}

Table 1 Textural properties of fortified milk. Values with different letters are significantly different ($p < 0.05$).

Besides that, higher values were found for HS-MAE-NLC beverage compared to HS-PLE-NLC drink. Between both, significant differences were appreciated in firmness, consistency and cohesiveness. However, the index of viscosity did not show significant differences. This increase of these parameters could be attributed to the supplementation and interaction of these nanoparticles with the protein matrix of the milk (Damin, Alcântara, Nunes, & Oliveira, 2009). These results are according to previous researches. In a previous work, a texture analysis of fortified yogurt with nano and micro sized calcium, iron and zinc was measured and the results showed that the

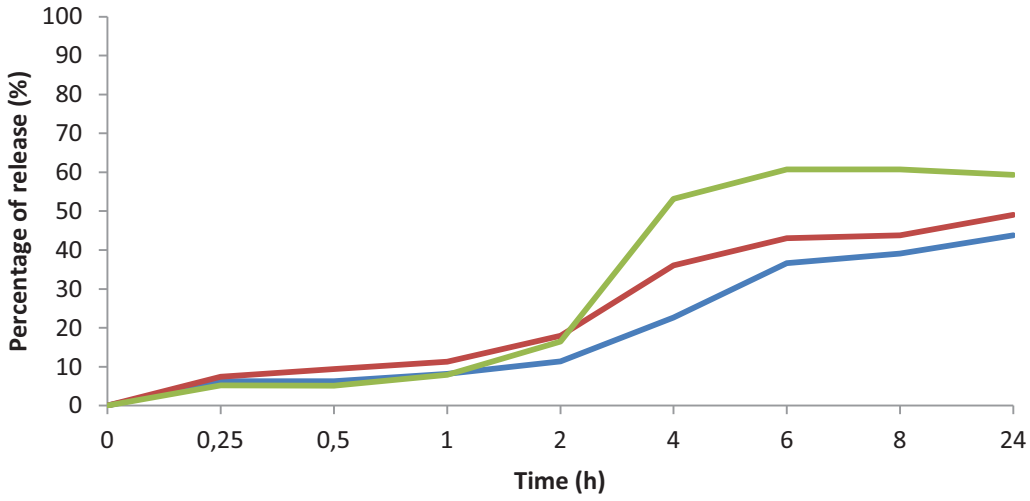
yogurts fortified with calcium and zinc nanoparticles increased their consistency and firmness (Santillán-Urquiza, Méndez-Rojas, & Vélez-Ruiz, 2017).

In vitro release test

Is known that nanocarriers can enhance drug penetration and permeation (Porter, Pouton, Cuine, & Charman, 2008). An important step for product development is the *in vitro* release testing, which is an analytical tool to obtain information on drug release mechanism and kinetics and allows the establishment of an *in vivo / in vitro* correlation.

In the present study, quercetin and anthocyanins delivery from HS-MAE-NLC and HS-PLE-NLC dairy beverages were assayed. Thus, for quercetin, the *in vitro* release was slower in HS-NLC milks compared to when *H. sabdariffa* extract was incorporate into milk in the free form. Under intestinal condition (pH 7.4), quercetin release from free *H. sabdariffa* extract drink was greatly increased from 5.2 % to 53.1 % in 4 h as is showed in **Figure 3**. Nevertheless, quercetin release from HS-PLE-NLC and HS-MAE-NLC milks was slightly increased from 6.3 % and 5.9 % to 36.0 % and 22.7 %, respectively. Therefore, the slowest release of quercetin was found for HS-MAE-NLC dairy beverage. In the same way, similar results were obtained for anthocyanins release. For this group of compounds, the delivery was lower when *H. sabdariffa* extract was encapsulated. Indeed, a release of 100% was found at 4 h while for HS-PLE-NLC and HS-MAE-NLC drinks, 92.5 % and 31.1 % was delivered at this time, respectively. Therefore, a lower release of anthocyanins in HS-MAE-NLC enriched milk was found.

A)



B)

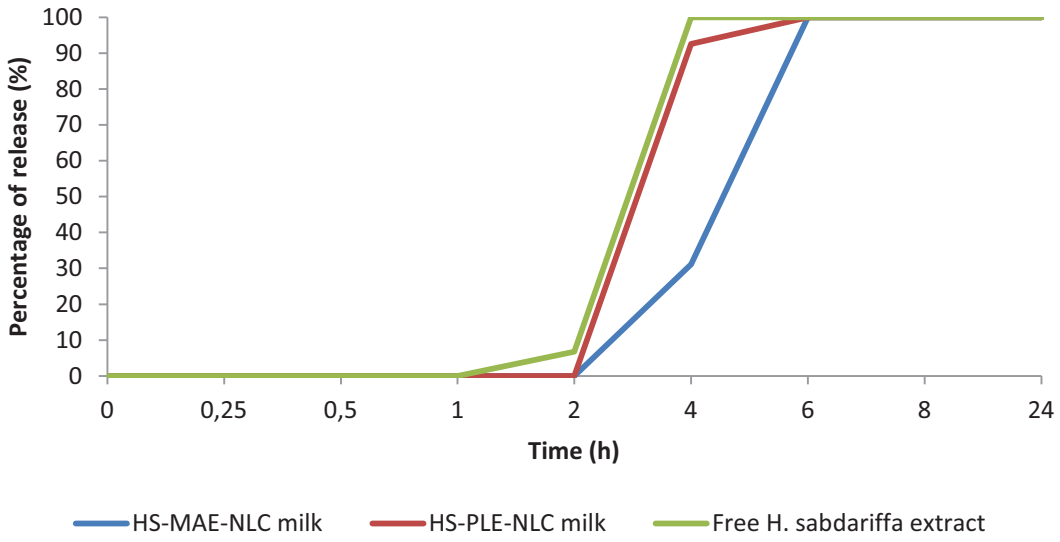


Figure 3. In vitro release of quercetin (A) and anthocyanins (B) from fortified milks with HS-MAE-NLC and HS-PLE-NLC

In a previous research, quercetin was loaded in cationic nanostructured lipid carrier (QR-CNLC) and the *in vitro* release assay was carried out. The delivery of quercetin was much faster than QR-CNLC and only 50 % of quercetin was released from QR-CNLC after 24 h (Liu et al., 2014). Similar results were found in the current

work, where only 43 % and 49 % of quercetin was released from HS-MAE-NLC and HS-PLE-NLC dairy beverages after 24 h.

On the other hand, quercetin has been already loaded by SLN (QT-SLN) to enhance gastrointestinal absorption (Li et al., 2009). However, the *in vitro* release studies showed that 50 % of quercetin from QT-SLN was released within 6 h showing a faster delivery than the present work. This could be because NLC are the second generation SLN which provides better loading capacity for drug accommodation than SLN (Pimentel-Moral et al., 2018). Thus, NLC unlike SLN, contain lipid droplets that are partially crystallized and have a less-ordered crystalline structure or an amorphous solid structure preventing the risk of expulsion of drug entrapped (Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2013).

In other work, quercetin was entrapped in poly-$_{D,L}$-lactide (PLA) nanoparticles (Kumari, Yadav, Pakade, Singh, & Yadav, 2010). However, the release profile, showed a fast burst release of quercetin (40-45%) within 0-0.5 h although the complete release was set at 72 h.

Respect to anthocyanins, the release behavior of these bioactive compounds is dependent on pH and temperature, due to them are easily degradable at high pH and temperature (Loypimai, Moongngarm, & Chottanom, 2016). Thus, at 37 ºC and pH 7.4 (intestinal conditions), anthocyanins are quickly released showing a complete delivery within 4 h for free *H. sabdariffa* extract beverage. Nevertheless, for HS-MAE-NLC and HS-PLE-NLC dairy drink, a complete release was found within 6 h, achieving a slower release profile. Indeed, in a previous work, anthocyanins were entrapped in SLN and the release and short-term stability was measured (Ravanfar, Tamaddon, Niakousari, &

Moein, 2016). In this study was demonstrated that the percent of release of anthocyanins from anthocyanins SLN was higher at pH = 7.4 than at pH = 3. In addition, the short-term stability was investigated in PBS (pH= 7.4) at 25, 40 and 50 ºC showing that the most appropriate condition for anthocyanin storage is at lower temperatures (T < 25 ºC). However, anthocyanin SLN increased the apparent short-term accelerated stability of anthocyanins against relatively high pH and temperature.

Therefore, NLC has demonstrated to be a potential technology for entrapment of flavonoids such as quercetin and anthocyanins exhibiting a controlled delivery. The release profiles revealed that polyphenol-enriched *H. sabdariffa* extract can become entrapped in NLC and successful incorporate in dairy products.

Conclusions

In present study, a novel enriched diary beverage with *H. sabdariffa* – loaded nanostructured lipid carriers provided a promise physicochemical property for oral delivery. This delivery system demonstrated to be a stable suspension into beverage due to protein matrix from milk, which provides a stabilization of lipid nanoparticles. In addition, the *in vitro* release study of quercetin and anthocyanins from enriched milks with these *H. sabdariffa* nanoparticles was slower than enriched milk with free extract. Therefore, there is a clear need for further research in this area to have a better understanding of the development of functional dairy beverages.

Acknowledgments

This work was supported by the Research group AGR274 “Bioactive ingredients” from the Analytical Chemistry Department of the University of Granada

and “REQUIMTE/LAQV” group of Pharmaceutical Technology, Faculty of Pharmacy, University of Coimbra. Also, the authors would like to thank the financial support received from the Spanish Ministry of Economy and Competitiveness (MINECO) (project AGL2015-67995-C3-2), from Portuguese Science and Technology Foundation (FCT/MCT) and from European Funds (PRODER/COMPETE) under the project references M-ERA-NET/0004/2015-PAIRED and UID/QUI/50006/2013, co-financed by FEDER, under the Partnership Agreement PT2020. We would like to thank the Ministry of Education, Culture and Sport (MECD) for supporting the grant FPU15/01125 of SPM and the stay in the University of Coimbra supported by FPU short – term stay. MCT wishes to acknowledge FCT and Dendropharma - Investigação E Serviços De Intervenção Farmacêutica, Sociedade Unipessoal Lda. for the individual fellowship (PD/BDE/135086/2017).

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Conclusions/ Conclusiones

❖ CONCLUSIONS

1. A response-surface methodology was applied to evaluate the impact of temperature (50–150 °C), composition of extraction solvent (15–75% EtOH) and extraction time (5–20 min) on the extraction yield and individual compounds concentrations from *Hibiscus sabdariffa* under microwave assisted extraction. In addition, the individual compounds concentrations were determined by HPLC-ESI-TOF-MS. A maximum of 30 and a minimum of 24 compounds were identified and quantified in the studied *H. sabdariffa* MAE extracts. The optimal extraction conditions which allowed the highest extraction yield were 164 °C, 60% ethanol and 22 min. Nevertheless, at higher temperatures (above 100 °C) some compounds with more thermo-labile nature like glycoside flavonoids were degraded.
2. Pressurized Liquid Extraction (PLE) was employed to recovery phenolic compounds from *Hibiscus sabdariffa.* To this end, response-surface methodology was employed to evaluate the significance of temperature and solvent composition on the PLE efficiency. In addition, qualitative and quantitative composition was determined by HPLC-ESI-QTOF-MS. The optimum condition was obtained at 200 ºC and 100 % of ethanol. Thus, higher temperature obtained higher phytochemicals content, although lower cyanidin-3-sambubioside content was obtained at higher temperature.

3. A response-surface methodology was designed to found the optimal SFE extraction conditions for the recovery of phenolic compounds from *Hibiscus sabdariffa*. The extraction parameters such as temperature, pressure of CO_2 and percentage of co-solvent were evaluated on the chemical composition and individual compound concentration was determined by HPLC-ESI-TOF-MS. Thus, for phenolic compounds recovery the optimum condition was obtained at 64 °C, 391 bar and 16.7 % of ethanol, whereas for organic acids was at 47 °C, 15.9 % of co-solvent and 81 bar.
4. Recent advances of lipid nanocarriers commonly used for the encapsulation of polyphenols have been described highlighting their added value to increase bioavailability and bioactivity of this group of compounds. Thus, these nanolipid-based systems were classified in nanoemulsions, liposomes and advanced lipid nanosystems such as solid lipid particles or nanostructed lipid carriers. In addition, lipid nanocarriers are also a potential technique for polyphenol encapsulation in order to improve their bioavailability and interactions with physiological targets.
5. New functional oils (extra virgin olive oil, EVOO and sunflower oil, SO) containing antioxidants from *Hibiscus sabdariffa* extract were developed by W/O emulsion and their physical and chemical stability were measured over time. EVOO emulsions were chemically more stable over time than SO emulsions in terms of total phenolic content (TPC), antioxidant activity and chemical composition measured by HPLC-ESI.TOF-MS.

6. *Hibiscus sabdariffa* extract was vehiculized in the inner phase of a W/O/W emulsion and its physical stability was improved with whey protein concentrate (WPC)-arabic gum (AG) complexes. Stable and constant values of average droplet volume diameter ($D_{4,3}$) around 6.47-8.54 µm after 30 d of storage at room temperature. In addition, pH played an important role in the long-term stability achieving the highest stability at pH 4.5, value in which the WPC and GA result in maximum complex formation.
7. Nanostructured lipid carriers (NLC) were optimized by response-surface methodology to protect phenolic compounds from *Hibiscus sabdariffa*. The optimized formulation obtained from the predicted model was composed of 2.21 % (w/w) of lipid phase and 1.93 % (w/w) of surfactants, with a desirability function of 0.77. The loading of polyphenol-enriched *Hibiscus sabdariffa* extract - obtained by microwave assisted extraction (MAE) or by pressurized liquid extraction (PLE) -within the NLC resulted in particles with a mean size of 470 ± 8 nm (encapsulation efficiency of 52.9 ± 0.9 % for quercetin and 60 ± 2 % for anthocyanins) and 344 ± 12 nm (encapsulationefficiency of 93 ± 3 % for quercetin and 84 ± 4 % for anthocyanins), respectively.
8. Two polyphenol – enriched extracts from *H. sabdariffa* obtained by microwave – assisted extraction (MAE) and pressurized liquid extraction (PLE) were entrapped by nanostructured lipid carriers (NLC) and incorporated into dairy beverage for the evaluation of the long-term stability, texture properties and in vitro release study. The results showed that these enriched milks were a stable suspension due to protein matrix

provides a stabilization of lipid nanoparticles. In addition, the in vitro release study of quercetin and anthocyanins from dairy beverage with these *H. sabdariffa* nanoparticles was slower than milk with free extract.

❖ CONCLUSIONES

1. Se empleó la metodología de superficie-respuesta para evaluar el impacto de la temperatura (50-150 °C), la composición del disolvente de extracción (15 – 75 % EtOH), y el tiempo de extracción (5 – 20 min) sobre el rendimiento y la concentración de cada compuesto procedente de *Hibiscus sabdariffa* extraídos con microondas. Además, la concentración individual de cada compuesto fue determinada por HPLC-ESI-TOF-MS. Un máximo de 30 y un mínimo de 24 compuestos fueron identificados y cuantificados en los extractos. La condición de extracción óptima que mostró mayor rendimiento fue 164 °C, 60 % etanol y 22 minutos. Sin embargo, a temperaturas mayores de 100 °C, algunos compuestos más termolábiles, como los flavonoides glicósidos se degradaron.
2. Extracción por fluidos presurizados (PLE) se utilizó para obtener los compuestos fenólicos procedentes de *Hibiscus sabdariffa*. Para este fin, se utilizó la metodología de superficie-respuesta para evaluar el impacto de la temperatura y la composición del disolvente de extracción en la eficiencia del PLE. Además, la composición de estos extractos fue determinada por HPLC-ESI-QTOF-MS. La condición óptima de extracción fue a 200 °C y 100 % de etanol. Así a mayor temperatura se obtuvo mayor contenido de compuestos fenólicos, aunque para el compuesto cyanidin-3-sambubioside se obtuvo menos cantidad a altas temperaturas.
3. Una metodología de superficie-respuesta fue diseñada para encontrar la condición óptima de extracción por SFE para la obtención de compuestos fenólicos procedentes de *Hibiscus sabdariffa*. Los parámetros de extracción evaluados fueron la temperatura, la presión de CO_2 y el porcentaje de co-

solvente. La composición química y la concentración individual de cada compuesto fue determinada por HPLC-ESI-TOF-MS. Así, para la obtención de compuestos fenólicos la condición de extracción óptima fue a 64 °C, 391 bar y 16.7 % de co-solvente, mientras que para la obtención de ácidos orgánicos, la mejor extracción tuvo lugar a las condiciones de 47 °C, 15.9 % de co-solvente y 81 bar.

4. Se han descrito los recientes avances sobre nanocarriers lipídicos que son usados para la encapsulación de polifenoles, aumentando su biodisponibilidad y bioactividad. Así, estos sistemas lipídicos se clasificaron en emulsiones, liposomas y nanosistemas lipídicos avanzados tales como las partículas sólidas lipídicas o nanocarriers lipídicos. Además, estos sistemas demostraron ser una técnica potencial para la encapsulación de compuestos fenólicos y mejorar su biodisponibilidad ejerciendo su función fisiológica.
5. Se formularon nuevos aceites funcionales que contenían aceite de oliva extra virgen (EVOO) o aceite de girasol (SO) y antioxidantes procedentes de *Hibiscus sabdariffa*. Su desarrollo fue llevado a cabo mediante emulsiones W/O y su estabilidad física y química fue medida en el tiempo. Emulsiones EVOO mostraron ser más estables químicamente en el tiempo que las emulsiones SO en términos de contenido total de compuestos fenólicos (TPC), actividad antioxidante y composición química medida mediante HPLC-ESI-TOF-MS.
6. Un extracto de *Hibiscus sabdariffa* fue vehiculizado en la fase acuosa interna de una emulsión W/O/W y su estabilidad física se mejoró cuando complejos de concentrado de proteína (WPC)-goma arábiga (AG) fueron añadidos. El diámetro de partícula en volumen ($D_{4,3}$) se mantuvo constante después de 30

días (6.47-8.54 µm). Además, se demostró que el pH juega un papel importante en la estabilidad de las emulsiones, consiguiendo la mejor estabilidad a un pH de 4.5, valor en el que WPC y GA forma un mayor complejo.

7. Se optimizó la formulación de nanocarriers lipídicos (NLC) mediante la metodología de superficie-respuesta, para proteger los compuestos fenólicos procedentes de *Hibiscus sabdariffa*. La formulación óptima predicha por el modelo estaba compuesta por 2.21 % (w/w) de fase lipídica y 1.93 % (w/w) de emulgentes con una función de deseabilidad de 0.77. Los compuestos fenólicos procedentes de extractos de *Hibiscus sabdariffa* obtenidos mediante MAE y PLE e incorporados a NLC, resultaron en partículas con tamaños de 470 ± 8 nm (eficiencia de encapsulación de 52.9 ± 0.9 % para quercetina and 60 ± 2 % para antocianinas) y 344 ± 12 nm (eficiencia de encapsulación de 93 ± 3 % para quercetina and 84 ± 4 % para antocianinas) respectivamente.
8. Dos extractos de *Hibiscus sabdariffa* obtenidos mediante MAE y PLE y encapsulados mediante NLC fueron incorporados en una bebida láctea para la evaluación de su estabilidad física, su textura y la liberación *in vitro*. Los resultados mostraron que estas leches enriquecidas fueron suspensiones estables debido a las proteínas procedentes de la matriz. Además la liberación *in vitro* de quercitina y antiocianinas de la bebida láctea con nanopartículas de *H. sabdariffa* fue menor que la bebida que contenía el extracto sin encapsular.