Encapsulation of Antioxidants from *TheoBroma Cacao L.* for food applications: *in vitro* Bioaccessibility and Kinetic release profile

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Dedicated to my family, and friends for always supporting, helping, and loving me.

When I realized that my fears
threatened to take a toll on my work,
I decided I had to deal with them
Relax don’t be so hard with you
I actually disagree
You have to practice, become better
and get rid of the box

Christoph Niemann

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TABLE OF CONTENT

LIST OF TABLES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

LIST OF FIGURES

CHAPTER 1

CHAPTER 2

CHAPTER 3

CHAPTER 4

CHAPTER 5

CHAPTER 6

CHAPTER 7

RESUMEN

TÍTULO: ENCAPSULATION OF ANTIOXIDANTS FROM THEOBROMA CACAO L. FOR FOOD APPLICATIONS: IN VITRO BIOACCESSIBILITY AND KINETIC RELEASE PROFILE*

AUTOR: SAID TORO URIBE**

PALABRAS CLAVES: PROCIANIDINAS DE CACAO, PURIFICACIÓN, CARACTERIZACIÓN, ENCAPSULACIÓN, DIGESTIÓN *IN VITRO.*

DESCRIPCIÓN:

El objetivo de este estudio es la encapsulación en liposomas de los metabolitos secundarios del cacao para mejorar su bioaccesibilidad *in-vitro*. Así, las condiciones más favorables para inhibir la acción de las enzimas fueron 70 mM de solución inhibitoria a 96 °C durante 6.4min, alcanzando 93.3% de reducción. Las condiciones optimizadas de extracción permitieron una alta recuperación de flavonoides totales (88.9 ± 0.8 mg $ECEg^{-1}$), así como establecer una nueva estrategia para evitar el desengrasado (tamaño de partícula < 0.2 mm y ultrasonicación por 20 min). La caracterización del extracto de cacao por medio de las fases HILIC, RP-LC, así como total online-modulación HILIC×RP acoplado a masas confirmó que el extracto es conformado hasta tetradecameros, siendo teobromina, (-)-epicatequina, y trimeros los más abundantes. Adicionalmente, purificación del extracto de cacao se logró usando una columna Diol. Formulación efectiva de nano-liposomas fue obtenida por sonicación a 75% amplitud por 7 min. Los resultados muestran que la actividad antioxidante en los liposomas es dependiente de la concentración y del pH, y la habilidad de inhibir la oxidación lipídica incrementa con el grado de polimerización. La estabilidad bajo la digestión *in-vitro* de los compuestos del cacao demostró una liberación controlada, dependencia de la estructura del polifenol, alta bioaccesibilidad y actividad antioxidante para los polifenoles encapsulados que no encapsulados. Análisis por UHPLC-DAD-QTOF-MS confirmó que el mecanismo de digestión de las procianidinas consistió principalmente de depolimerización y transformación. Imágenes por TEM muestra cambios significativos en la bicapa lipídica durante la fase gástrica y duodenal, efectos como hinchamiento, reducción de lamelaridad, complejos con proteínas, cambios en la permeabilidad, tamaño de partícula, y ruptura de la bicapa lipídica.

Está tesis doctoral contribuye a un mejor conocimiento del potencial uso de procianidinas como aditivo alimentario, así como las aplicaciones de implementar liposomas como transportador de los polifenoles del cacao.

*Tesis Doctoral

** Facultad de Ingeniería Físico-Química, Escuela de Ingeniería Química, Director: Luis Javier López Giraldo, Bioquímica, Química en Tecnología de Alimentos.

ABSTRACT

TITLE: ENCAPSULATION OF ANTIOXIDANTS FROM THEOBROMA CACAO L. FOR FOOD APPLICATIONS: *IN VITRO* BIOACCESSIBILITY AND KINETIC RELEASE PROFILE*

AUTHOR: SAID TORO URIBE**

KEYWORDS: COCOA PROCYANIDINS, ISOLATION, CHARACTERIZATION, ENCAPSULATION, IN VITRO DIGESTION.

DESCRIPTION:

The purpose of this study was to encapsulate cocoa secondary metabolites into liposomes to enhance *in-vitro* bioaccessibility. Therefore, the most favorable conditions to inhibit the action of oxidase enzyme on total polyphenols were 70 mM inhibitory solution at 96 ºC for 6.4 min, which allowed to reduce by 93.3% PPO activity. The optimized extraction conditions allowed not only to recovery high content of flavonoids (88.9 ± 0.8 mg $ECEg^{-1}$), but also to establish a new strategy to avoid the degreasing process (cocoa particle size < 0.2 mm, ultrasonicated for 30 min). Characterization of cocoa extract was performed by using HILIC- and RP-LC-phases coupled to tandem mass spectrometry detectors. On-line-modulated comprehensive HILIC×RP was also developed and confirmed that cocoa extract consisted up to tetradecamers, being theobromine, (-)-epicatechin, and trimers the most abundant. Moreover, effective fractionation of cocoa-procyanidins by increasing degree of polymerization were achieved using a diol-column.

An effective nano-liposome formulation for cocoa-procyanidins was obtained by sonication at 75% amplitude for 7 min. Results showed that the antioxidant activity of liposomes is dose- and pH-dependent, and the ability to inhibit the lipid oxidation increased with increasing molecular size. The stability under *in-vitro* digestion of cocoa-components showed *in-vitro* sustained release profile, burst effect, polyphenol structure-dependent, higher bioaccessibility and greater antioxidant activity for those polyphenols loaded-liposomes than those in non-encapsulated form. UHPLC-DAD-QTOF-MS analysis confirmed that the digestion mechanism of procyanidins consisted mainly on depolymerization process and transformation during digestion. TEM images demonstrated significant changes of lipid bilayer mainly during gastric and duodenal stages, effects such as swelling, lamellarity reduction, protein-complex interaction, permeability, particle size, and rupture of lipid bilayer were observed.

This Ph.D. dissertation contribute to increasing the knowledge on the potential use of procyanidins as a food additive as well as the promising applications of liposomes as carrier system for cocoa polyphenols.

*Doctoral Dissertation Thesis

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1. INTRODUCTION

1.1. Food and health challenges

Some of the most important challenges in the world are related to climate changes, growing world population, economic growth inclusive, food and water access, energy solutions, health, nutrition crisis, environmental pollutants, growing disease pandemics, among other (Khoo & Knorr, 2014). In particular for health and nutrition scenario, World Health Organization (WHO, 2018) and Food and Administration Organization (P. Liu, 2017) attempt to a) improve the access of clean water and food, b) increase the knowledge of healthy behaviors, thus improving the longevity and quality of life, c) ensure food hygiene, d) development of new vaccine to protect people from several viruses and infectious diseases (e.g., HIV/AIDS, malaria, tuberculosis), e) eradicate extreme poverty and hunger, thus reducing the malnutrition, f) reduce of risk of death, in particular for children, and so on. All these scenarios impact the agriculture and food chain directly or indirectly.

Indeed, the world´s population is expected to grow to almost 10 billion by 2050, boosting agricultural demand (likely 50 percent compared to 2013) (UN FAO, 2017). Income growth will drive to structural change in the economy. Therefore, a significant increase in the consumption of meat, fruits, and vegetables (Rosegrant & Cline, 2003) is expected. These phenomena will be responsible for important challenges across regions, in particular for the major agroindustrial countries. To satisfy the increasing demand, new strategies have to be considered since more intense agriculture practices will raise greenhouse gas emissions, deforestation, overused land, which will contribute to global warming and land degradation (Oertel, Matschullat, Zurba, Zimmermann, & Erasmi, 2016). To ensure food availability for everyone, sustainable agricultural development, and collective responsibility to reduce the food- and agroindustrial waste and

overconsumption self-consciousness needs to be addressed (Lim, 2017). Sustainable development, not only in environmental aspects but also in social and economic point of view are in line with the recent Colombian National Plan of Science, Technology, and Innovation (Libro Verde 2030) (COLCIENCIAS, 2018).

To address the emerging health issues, countries have delivered national food, nutrition, and health guidelines and regulations (Khoo & Knorr, 2014), such as to reduce the consumption of sodium, cholesterol, saturated fats, sugar, and increase the intake of vegetables, fruits, and whole grains as well as maintaining healthy lifestyle habits (German & Dillard, 2004; Havas, Dickinson, & Wilson, 2007; Pronk et al., 2004). In fact, integrated role between food and nutrition has been observed, for instance, inadequate or excessive consumption of dietary ingredients leading to the development of many diseases and their associated risk factors, such as obesity and low immune system (Kau, Ahern, Griffin, Goodman, & Gordon, 2011; Ohlhorst et al., 2013). In this perspective, evidence of the nutritional value of foods and its interaction with gut microbiota, gut microbiome, and relationship with the host status and immune system, together with the transmission across generations, is currently a hot topic (Flint, Scott, Louis, & Duncan, 2012; Kau et al., 2011; Laparra & Sanz, 2010). A clear example of this latter point is the presence of microbial β-porphyranase (a microorganism that can process porphyran derived from marine red algae) in Japanese population instead of U.S. population (Kau et al., 2011).

Plant and animal foods are the primary vehicles providing both macronutrients (proteins, fat, and carbohydrates) and micronutrients to human beings. Macronutrients are used for the body as the primary source of energy; nevertheless, large fat and carbohydrate (dietary fibers, sugar, and starches) intake have an adverse effect on our health in terms of increasing lipogenesis, satiety response, obesity-related conditions, and cardiovascular issues (Bellissimo & Akhavan, 2015;

Hirsch, 1995). On the other hand, micronutrients (e.g., Mg, Cu, Fe, Zn, vitamins, and other derived antioxidants) also play an essential role in the prevention of human diseases (Evans & Halliwell, 2001); in this sense, the positive impact of micronutrients in human health is well known, for example: (a) vitamin E to protect against lipid peroxidation (Huang et al., 2002), (b) vitamin C to scavenge radicals species (Frei, 1994) and for prevention of common cold (Hemilä & Chalker, 2013), (c) Cu for regulating mitochondrial functions and Zn for protein synthesis, energy production and maintenance of structural integrity of biomembranes (Hänsch & Mendel, 2009), and (d) Fe in the hemoglobin synthesis, oxygen transport and DNA synthesis (Abbaspour, Hurrell, & Kelishadi, 2014). In addition, secondary metabolites from plants such as phenolic compounds (e.g., catechins and carotenoids) are widely consumed on our diet, might greatly contribute to the antioxidant activity by reducing peroxyl (ROO•), hydroxyl (•OH), and superoxide (O_2^-) radicals, thus exerting a positive action on health status to prevent cellular oxidative stress, cellular apoptosis and proliferation, enzyme activities regulation, and so on (Birben, Sahiner, Sackesen, Erzurum, & Kalayci, 2012; Martín et al., 2010; Watson, Preedy, & Zibadi, 2012).

In general, insufficient intake of both macronutrients and/or micronutrients gives rise to altered metabolism and increased oxidative stress which is implicated in the pathogenesis of human infections such as VIH, hepatitis, influenza, among others (Semba & Tang, 1999). The complexity of these issues will require both public and private efforts and multidisciplinary experts across all sectors and areas involved (e.g., nutrition, medicine, lifestyle behavior, and food science and technology) to come up with scientific solutions and industrial implementations.

1.2. Cocoa as a functional food

Today foods are not only intended to satisfy hunger and provide humans with necessary nutrients but also to prevent nutrition-related diseases and increase the physical and mental well-being of consumers .(Menrad, 2003; Siró, Kápolna, Kápolna, & Lugasi, 2008; Vergari, Tibuzzi, & Basile, 2010). Thereby, foods and vegetables (e.g., whole, fortified, or enriched foods) have now assumed the role of "functional foods"(Gul, Singh, & Jabeen, 2016), which are defined as food that provides a health benefit beyond that of the traditional nutrient it contains (Frewer, Scholderer, & Lambert, 2003). This new trend represented values at US$ of 168 billion in a global market (R. S. Khan, Grigor, Winger, & Win, 2013).

Over the last two decades, food and pharmaceutical industries have been developed and launched new food products that offer bioactive compounds with potential benefits for the prevention of certain diseases (Kris-Etherton et al., 2002; Siró et al., 2008). Indeed, phenolic compounds are naturally occurring compounds found largely in the plant kingdom (El Gharras, 2009). These compounds are secondary metabolites that play an essential role on plants and fruits, e.g., defense against ultraviolet radiation or aggression by pathogens and contribute to the bitterness, astringency, color, flavor, odor and oxidative stability of foods (Beckman, 2000; Pandey & Rizvi, 2009). Among polyphenol-rich food, apples, garlic, onions, tomatoes, carrots, grape seed, cocoa, tea and their derivate products are included (Hounsome, Hounsome, Tomos, & Edwards-Jones, 2008; J. Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010; Won Leen, Kim, Jun, Jun Lee, & Yong Lee, 2003). It has been described that cocoa contained much higher levels of total phenolics and total flavonoids than black and green tea, and red wine (Lee, Kim, Lee, & Lee, 2003). These findings are in line with Phenol-Explorer database, where cocoa is the fourth most abundant dietary source of polyphenols with only cloves (1^{st}), peppermint (2^{nd}) and star anise (3^{rd}) having higher

polyphenol contents (J. Pérez-Jiménez et al., 2010). Thereby, the scope of this work will be related to cocoa polyphenols and its future applications.

Cocoa is a larger crop cultivated worldwide which constitute a major global commodity, in particular for the countries that lie on the earth's equator. In 2016, world production of cocoa beans was 4.46 million and the major producing countries were Ivory Coast (32.9%), Ghana (19.2%), Indonesia (14.7%), Cameroon (6.5%), Nigeria (5.3%), Brazil (4.8%), Ecuador (3.9%), Peru (2.4%), Dominic Republic (1.8), and Colombia (1.3%) (FAOSTAT, 2018). Interestingly, Colombia has increased the cocoa bean production substantially in the last five years; for instance, 49420 to 60535 tons from 2012 to 2017 (FEDECACAO, 2018). There is also a governmental initiative (Law 896-2017) to switch from cocaine to cocoa and to join forces with the private sector to enhance Colombia's competitiveness at international level (Decreto/Ley 896, 2017; Presidencia de la República de Colombia, 2017).

Cocoa beans belong to the genus *Theobroma cacao*, where four different varieties are known as Criollo, Forastero, Trinitary (a hybrid of Criollo and Forastero), and Nacional (Afoakwa, Paterson, Fowler, & Ryan, 2008). Criollo beans are characterized by their fine flavor and aroma and are typically grown in South America and Central America. Nacional is known as fine flavor cocoa and is produced mainly in the West Indies and Ecuador's regions (Afoakwa, 2012; Aprotosoaie, Luca, & Miron, 2016). Forastero beans had a stronger flavor than Criollo beans and are grown primarily in Africa and Brazil (Adomako, 1983). Trinitarian (hybrid of Criollo and Forastero) has the advantages of Forastero in terms of lower vulnerability to diseases and higher yields as well as the advantages of Criollo variety with better organoleptic acceptance (Adomako, 1983).

It is well known that cocoa-derived products are extensively used in food and pharmaceutical industries. The main uses of cocoa is the production of chocolate, confectionery products, flavored drinks and desserts, cocoa butter, among others (Wollgast & Anklam, 2000). In fact, the chocolate industry is estimated at nearly US$22.4 billion only in the US, where the leading companies are Hershey and Mars constituting 44 and 29%, respectively (The statista-portal, 2017). Nevertheless, during the chocolate process the beans are subjected to several steps (e.g., fermentation, drying, alkalization and roasting), which negatively impact the concentration of cocoa polyphenols; for instance, more than 85 % of initial monomers are reduced during the fermentation, drying and alkalization, therefore, their antiradical activity is also reduced by 57 % (Di Mattia et al., 2013; Miller et al., 2008).

It has been published that cocoa is composed mainly by flavanol monomers, procyanidins oligomers (B-type, -(epi)catechin as base unit- and to less extent A-type) from dimers to tridecamers, which constitute about the 60% of total polyphenol content (Jalil & Ismail, 2008; Pedan, Weber, et al., 2017; Toro-Uribe, Montero, López-Giraldo, Ibáñez, & Herrero, 2018). Other minor polyphenol constituents such as quercetin, naringenin, apigenin, luteolin, kaempferol, clovamide, and some anthocyanins have also been reported in the literature (Rodríguez-Carrasco, Gaspari, Graziani, Sandini, & Ritieni, 2018; Sánchez-Rabaneda et al., 2003), which give the raw cocoa bean its purple color (Dreosti, 2000). Furthermore, cocoa beans contain a high amount of alkaloids, mainly theobromine and caffeine with concentrations ranging from 7.1-9.7 mg/g and 0.7-1.8, mg/g, respectively (Carrillo, Londoño-Londoño, & Gil, 2014; Caudle & Bell, 2000; Senanayake & Wijesekera, 1971).

1.3. Flavonoids

Phenolic compounds constitute the most numerous secondary metabolites occurring on plant kingdom (Rasouli, Farzaei, & Khodarahmi, 2017). It is estimated that 100,000 to 200,000

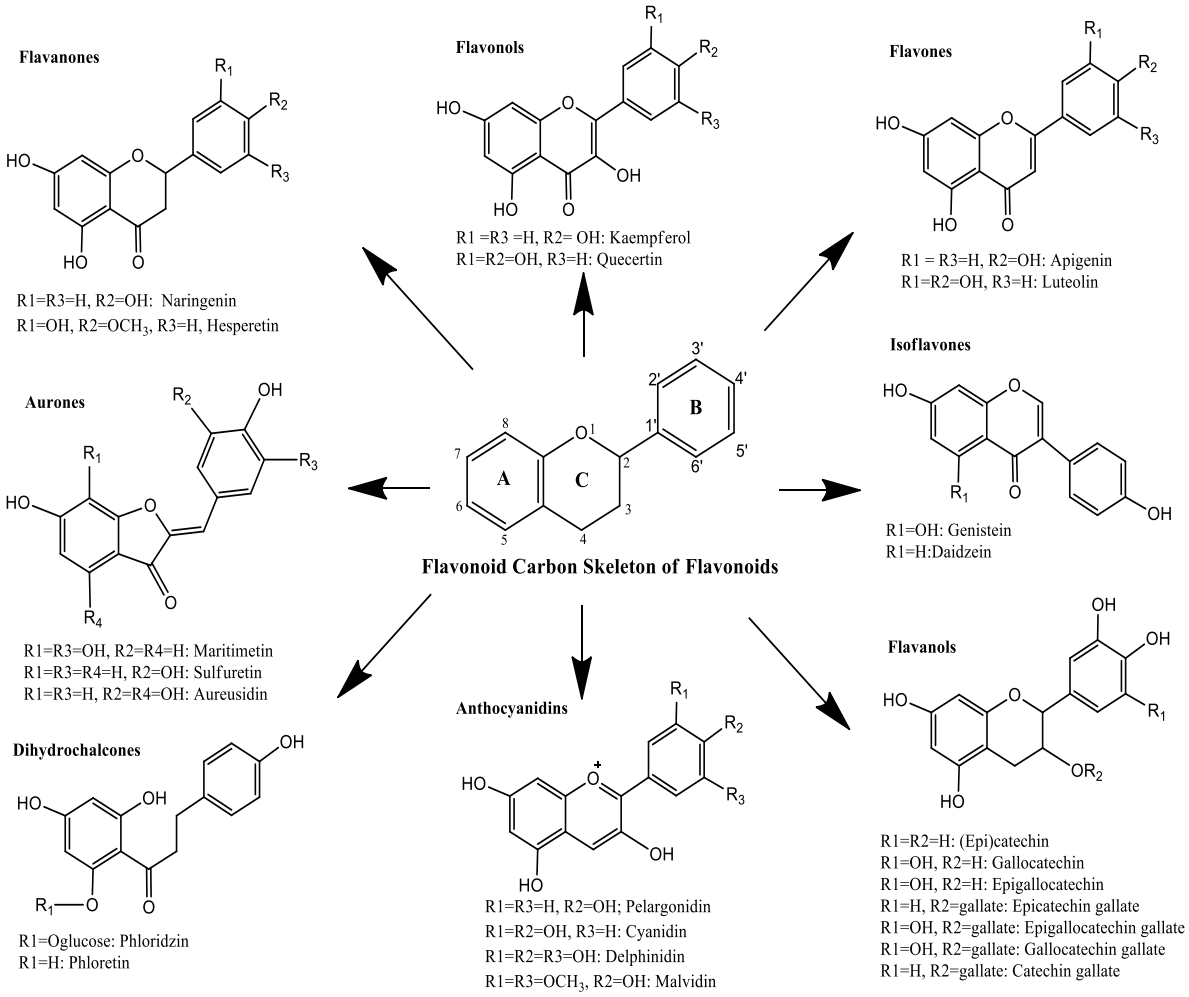


Figure 6. Chemical structure of main sub-groups of flavonoids.

secondary metabolites exist (Belščak-Cvitanović, Durgo, Huđek, Bačun-Družina, & Komes, 2018; Pereira, Valentão, Pereira, & Andrade, 2009), which more than 8,000 are identified as flavonoids (Duthie, Gardner, & Kyle, 2003; Harborne & Williams, 2000). Although phenolic compounds are chemically characterized by containing phenolic structural features, they are

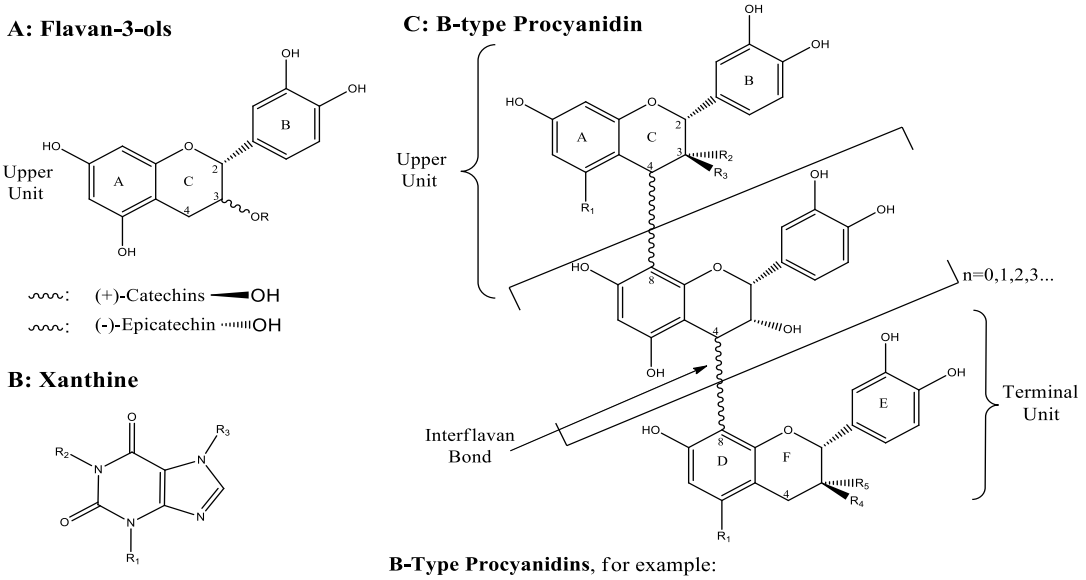
classified in sub-groups as a function of sources of origin, natural distribution, biological function, and chemical structure as follows: phenolic acids, flavonoids, stilbenes, lignans, and others (Belščak-Cvitanović et al., 2018; Bravo, 2009). Flavonoids consist of two aromatic rings (A and B) connected by an oxygen-containing pyrene ring (C) (**Figure 6**) and constitute the most plentiful group of polyphenols (Wollgast & Anklam, 2000). The position of the chroman-aromatic linkage determines benzopyran class, for it, these compounds are divided into several subgroup classes such as dihydrochalcones, aurones, flavones, flavonols, flavanones, flavanols, anthocyanidins, isoflavonoids, and proanthocyanidins (Harborne, 1991; Wollgast & Anklam, 2000) (**Figure 6**). Although these compounds belong to the same family, their biological and chemical properties are quite different (Erlund, 2004). In the present work, only proanthocyanidins sub-group has been studied.

1.3.1 Proanthocyanidins. The flavonoid skeletal of proanthocyanidins (PAs) is subdivided into individual families depending upon the degree of hydroxylation, for example, proguibourtinidin, promelacacidin, prorobinetinidin (resorcinol family); propelargonidins, procyanidins, and prodelphinidins (phloroglucinol family) (Harborne, 1991; Wollgast & Anklam, 2000). The latter two are the most commonly found in nature (Monagas, Quintanilla-López, Gómez-Cordovés, Bartolomé, & Lebrón-Aguilar, 2010).

Procyanidins (PCs) consist of flavan-3-ols units such as (+)-catechin and/or (–)-epicatechin linked through $C_4 \rightarrow C_8$ interflavanoid linkage but also extent through a $C_4 \rightarrow C_6$ bonds, both are referred to B–type PCs (Domínguez-Rodríguez, Marina, & Plaza, 2017; F. He, Pan, Shi, & Duan, 2008; Kimura, Ogawa, Akihiro, & Yokota, 2011; H. J. Li & Deinzer, 2008). Additionally, proanthocyanidins have two linkages $C_4 \rightarrow C_8$ bond plus a $C_2 \rightarrow O \rightarrow C_7$ or $C_2 \rightarrow O \rightarrow C_5$ ether bond, which is denoted as A-type (Neilson & Ferruzzi, 2011). These flavan-3-ols compounds can also

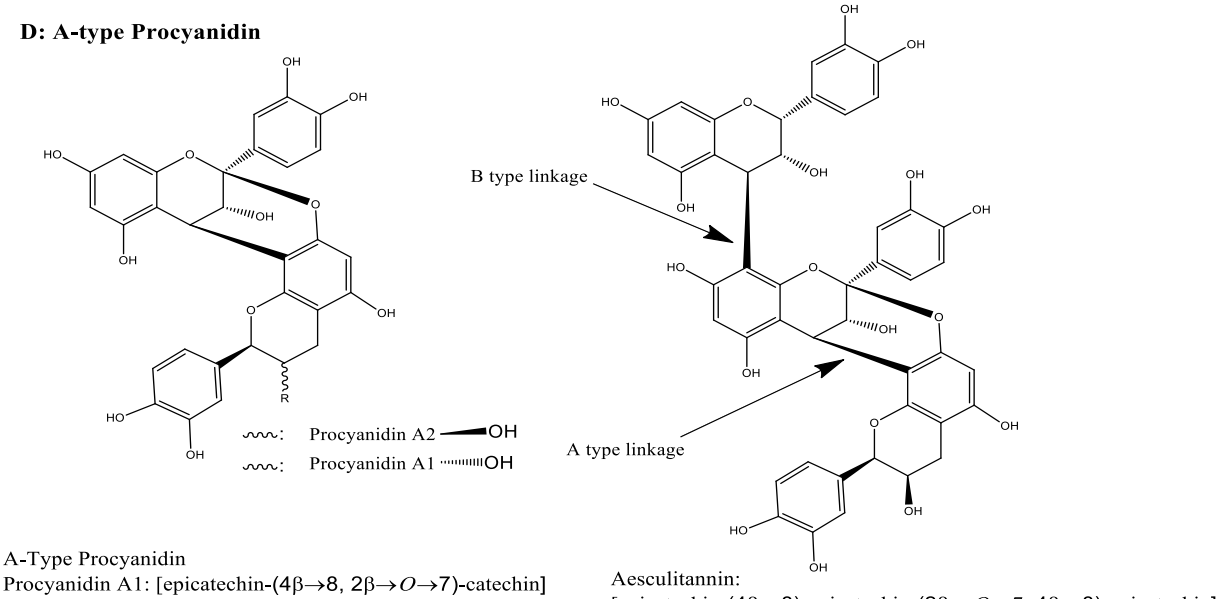
be esterified with gallic acid and glucose moieties (Glavnik, Vovk, & Albreht, 2017), therefore increasing the structural diversity of PCs. Examples of B-type include procyanidin linked by two $C_4 \rightarrow C_8$ such as B1 to B4, and dimers B5 to B8 with $C_4 \rightarrow C_6$, trimer C1 (bound by two $C_4 \rightarrow C_8$), and tetramer Cinnamtannin A2. A-type contains, e.g., procyanidin A1, A2, Cinnamtannin B1, and Aescultannin C (**Figure 2**). The nature of proanthocyanidins is more complicated than we thought; indeed, the condensation of catechin and epicatechins into PAs can form stereoisomers because of different patterns can link the C_6 and C_8 positions (e.g., C_6-C_6, C_8-C_8, and C_6-C_8 together with R and S linkages). Thus the polymers have a complex structure (F. He et al., 2008). Considering only molecules with catechin and epicatechin, the number of asymmetric carbon atoms, and the possible forms of rotation around the interflavan bonds (C_4–C_8 or C_4–C_6), theoretically the potential number of stereoisomers and rotational isomers is $2^{(5*DP-3)}$(S. Zhang et al., 2017). For example, 128, 4096 and 131072 combinations for dimers, trimers and tetramers could be found, respectively.

1.3.2. Cocoa polyphenols: a brief overview. Cocoa has been identified as a polyphenol-rich food since the 1960s (Robinson, Ranalli, & Phillips, 1961). Several authors have characterized the main polyphenols contained in cocoa and cocoa-based products, together with quality and chemical differences between cocoa varieties. For example, Carrillo et al., (2014) and Perea (2013) studied the concentration of alkaloids, monomers ((+)-catechin, (-)-epicatechin), as well as the primary macronutrients from more than 18 clones cultivated in Colombia. These studies confirmed that cocoa-genotypic type influences the chemical composition (e.g., concentration of catechins, procyanidins, and alkaloids). Theobromine constitutes the principal alkaloid in cocoa sample; however, there is not a universal ratio between theobromine/caffeine because of genetic variety-dependence. Thereby, the relationship between these compounds is tentatively classified in 15-10, 10-5, and 2-1 for Forastero, Trinitary, and Criollo, respectively (Hasing, 2004). Furthermore, the content of epicatechin from unfermented cacao is about 20 times higher than catechin. Therefore, catechin and epicatechin content range from 2.8-9.4% and 9.1-21.8 % in total polyphenol, respectively (Loureiro et al., 2017).



Caffeine: R1=CH3; R2= CH3; R3= CH3
Theobromine: R1= CH3; R2= H; R3=CH3
Theophyllie: R1= CH3; R2= CH3; R3=H
Paraxanthine: R1=H; R2=CH3; R3=CH3

B-Type Procyanidins, for example:
B1: n=0 ; R1=OH; R2=OH; R3= H; R4=OH; R5=H, [epicatechin-(4β→8)-catechin]
B2: n=0; R1=OH; R2=OH; R3=H; R4=H; R5=OH, [epicatechin-(4β→8)-epicatechin]
C1: n=1; R1= OH; R2= OH; R3=H; R4= H; R5=OH, [epicatechin-(4β→8)-epicatechin-(4β→8)-epicatechin]



A-Type Procyanidin
Procyanidin A1: [epicatechin-(4β→8, 2β→*O*→7)-catechin]
Procyanidin A2: [epicatechin-(4β→8, 2β→*O*→7)-epicatechin]

Aesculitannin:
[epicatechin-(4β→8)-epicatechin-(2β→*O*→7, 4β→8)-epicatechin]

Figure 7. Structure of (A) catechins, (B) alkaloids, (C), B-type procyanidins, and (D) A-type procyanidins from cocoa beans.

Owing the number of scientific works and reviews found in the literature for cocoa polyphenols, the most remarkable works regarding flavonoid composition and methods of analysis (e.g., HPLC,

MS, NMR), together with the extraction method for cocoa-based products are summarized in **Table 7** and **Table 2.** In general, quantification of total polyphenols, total flavonoids, and total flavan-3-ols is commonly based on spectrophotometric assays such as Folin-Ciocalteu method (Singleton, Orthofer, & Lamuela-Raventós, 1998a), flavonoid-aluminum chloride reaction (Zhishen, Mengcheng, & Jianming, 1999), and vanillin assay (Sun, Ricardo-da-Silva, & Spranger, 1998), respectively. Quantification of both degree of polymerization and individual compounds is carried out using HPLC by either normal phase (NP) (Gu, House, Wu, Ou, & Prior, 2006; Miller et al., 2006) and reversed phase (RP) (Tomas-Barberán et al., 2007; Toro-Uribe, Montero, et al., 2018) coupled, in most cases, to fluorescence detector (230/321 nm, excitation/emission) (Robbins et al., 2009) and UV detection at 280 nm (Adamson et al., 1999; Wollgast & Anklam, 2000). Recently, new strategies have been employed to enhance further characterization of main isomers on cocoa samples. To do so, comprehensive LC (LC×LC) have been previously assayed by Kalili & De Villiers (2013a,b) and Toro-Uribe et al., (2018) who reported simultaneous separation of catechins by increasing the degree of polymerization, and good resolution of isomeric compounds. Further information of this method can be found in **Chapter 3**; moreover, further insight on LC×LC separation mechanisms can be found in François, Sandra, & Sandra (2009), Dugo et al., (2008) and Jandera (2012).

LC analysis using non-polar C_{18}-bonded silica enables the separation of alkaloids, catechins and few oligomers with high resolution and sensitivity, where the retention of non-polar compounds is stronger than that of polar compounds (Kostiainen & Kauppila, 2009). However, there are some limitations, for instance, reverse-phase methodologies are quite ineffective in separating higher oligomers (> trimer) and their main isomers, with these compounds eluting as a large unresolved hump (Adamson et al., 1999).

Table 7. Overview of phenolic acids, alkaloids, and flavonoids from cocoa beans.

Phenolic Acids
Gallic acid
Chlorogenic acid
Ferulic acid
Caffeic acid
Coumaric acid
Protocatechuic acid
Alkaloids
Caffeine
Theobromine
Flavanols
(+)-Catechin
(-)-Catechin
(-)-Epicatechin
(+)-Epigallocatechin
(+)-Gallocatechin
Flavonols
Quercetin
Quercetin-3-*O*-rutinoside (rutin)
3´-Methylquercetin
Quercetin 3-*O*-galactoside (hyperoside)
Quercetin-3-*O*-rhamnoside (quercitrin)
Quercetin-3-*O*-*β*-D-glucopuranoside
Quercetin-3-*O*-*β*-D-arabinopyranoside
Quercetin-3-α-L-arabinoside
Quercetin-3-*O*-glucoside (isoquercitrin)
Quercetin-3-*O*-arabinoside
Kaempherol
Kaempherol 3-*O*-Rutinoside
Kaempherol 7- *O*-Neohesperoside
Kaempherol 3-*O*-Glucoside
Isorhamnetin

Procyanidins
Procyanidin B1
Procyanidin B2
Procyanidin B3
Procyanidin B4
Procyanidin B5
Procyanidin C1
Procyanidin D
Anthocyanins
Cyanidin-3-α-L-arabinoside
Cyanidin-3-β-D-galactoside
Cyanidin-3- β -D-arabinoside
Flavanones
Naringenin-7-rhamnosidoglucoside
Naringenin
Naringenin 7-*O*-Glucoside
Naringenin-7-*O*-neohesperidoside (naringin)
Kaempferol-7-*O*-neohesperidoside
Flavonoids
Apigenin
Luteolin
Luteolin 7-O-Glucoside
Apigenin 8-C-Glucoside (vitexin)
Amentoflavone
Apigenin 6-C-Glucoside (isovitexin)
Apigenin 7-O-Glucoside
Luteolin 6-C-Glucoside (isoorientin)
Apigenin 7-rutinoside (isorhoifolin)
Luteolin 8-C-Glucoside (Orientin)
Biapeginin

On the other hand, normal-phase methodologies allow the separation of procyanidin according to their degree of polymerization but require the use of toxic solvents such as dichloromethane-methanol-formic acid-water mixtures (Rigaud, Escribano-Bailon, Prieur, Souquet, & Cheynier,

1993) and hexane-acetone mixtures (Yanagida et al., 2000). HILIC stationary phase has proven to be a useful alternative in the analysis of procyanidins; in fact, the compounds (higher affinity for polar than non-polar) are eluted in order of increasing polarity with water-organic solvent mixtures as eluent and a gradient with decreasing organic solvent content (Kostiainen & Kauppila, 2009). Thereby, high sensitivity and resolution up to dodecamers employing acetonitrile-acetic acid and methanol-water-acetic acid mixtures through HILIC column have been reported (Robbins et al., 2009).

Acidification of mobile phase also play an essential role on the retention, peak shape, resolution and separation of procyanidins depending on the occurrence of protonation or dissociation (Wollgast & Anklam, 2000). Therefore, formic acid, trifluoroacetic acid, acetic acid, and ammonium acetate and formate are the most used (Cuyckens & Claeys, 2004). Overall, a lower concentration of formic acid (≤ 0.1 %) seems to be preferable because is not a strong ion-pair agent and it does not suppress MS ionization (Annesley, 2003; Kostiainen & Kauppila, 2009).

As it can be seen in **Table 2**, mass spectrometry is highly used because of its high sensitivity, possibilities of coupling with liquid chromatography and the availability of powerful tandem mass spectrometric techniques (Cuyckens & Claeys, 2004). Electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI), coupled to LC have been previously tested (Rauha, Vuorela, & Kostiainen, 2001). These can be carried out in positive and negative ion mode, where the last one provides the highest sensitivity (Fabre, Rustan, De Hoffmann, & Quetin-Leclercq, 2001). Other works also employed matrix-assisted-laser-desorption/ionization (MALDI) and time-of-flight (TOF) for proanthocyanidins analysis (Guaratini et al., 2014; Perret, Pezet, & Tabacchi, 2003). Additionally, direct coupling of an NMR instrument to an LC-UV-MS is used in less extent and offers a unique and very powerful tool (but also very expensive), for the

identification of complex unknown polyphenolic compounds of plant origin (Cuyckens & Claeys, 2004; Hansen et al., 1999).

Table 2 shows several differences among total polyphenol content, concentration of procyanidins, lipid fat removal process, methodologies and solvents for polyphenols' recovery from cocoa beans and related products. Overall, chemical profile from cocoa is vulnerable to several conditions, for instance, plant variety and genotype, geographical and climate growing conditions (N. Khan et al., 2014). Cocoa process for chocolate manufacture also impacts total concentration of flavonoids, thus its antioxidant activity (Di Mattia et al., 2013; Miller et al., 2008). Moreover, sample preparation (e.g., classical isolation and solid-phase extraction), extraction procedure (e.g., solid-liquid extraction, sonication, Soxhlet, high-pressure fluids, and type and concentration of solvent), and analytical techniques (e.g., spectrophotometry, liquid chromatography, nuclear magnetic resonance, and mass spectrometry) influence the magnitude of the analysis. Further information related to characterization and quantification of main polyphenols from cocoa and cocoa-based products as well as antioxidant activities in different model systems are described in **Chapters 1 - 7.**

Table 8. Overview of methods of analysis, identification, and quantification of procyanidins from cocoa beans and related compounds.

Sample	Lipid Removal Solvent	Method/Solvent for polyphenols' recovery	Total Polyphenols[a] (mgGAE/g)	Antioxidant Activity[b] (µmolTrolox/g)	Analysis method and Detection	Procyanidins[c] (mg/g)	DP[d]	Ref.
Cocoa beans	-	S-L extraction 50% Ethanol	-	1201.5[b]	NP-HPLC-ESI-MS FL, 230/321nm	50.1	14	(Toro-Uribe, López-Giraldo, & Decker, 2018)
Cocoa beans	-	S-L extraction 50% Ethanol	-	-	Comprehensive LC×LC HILIC-RP-LC-ESI-MS	-	8	(Toro-Uribe, Montero, et al., 2018)
Chocolate (several genotypes)	Hexane	S-L extraction 80% Methanol	-	-	RP-UHPLC-ESI-Q-Orbitrap-MS	0.8 – 1.9		(Rodríguez-Carrasco et al., 2018)
Cocoa beans	Petroleum ether	-	7.8 - 21.3	0.2 – 0.9[e]	RP-HPLC UV, 280nm			(Quiroz-Reyes & Fogliano, 2018)
Cocoa Liquor (different percent)	Petroleum ether	S-L extraction 70/29.5/0.5 v/v/v AWA	-	537.0 – 961.1[b]	UHPL-ESI-Q-TOF-MS UV, 280 nm	8.4 – 13.6	-	(Żyżelewicz et al., 2018)
Cocoa pod husk	-	Supercritical Fluid CO_2 + ethanol	12.9	0.2[b]	-	-	-	(Valadez-Carm

								ona et al., 2018)
Cocoa beans and chocolate	Hexane	S-L extraction 50 % Acetone			NP-HPLC UV, 275nm	5.7 – 30.0	13	(Pedan, Fischer, Bernath, Hühn, & Rohn, 2017)
Cocoa bean (several genotypes)	Hexane	S-L extraction 50% Acetone	50.6 – 94.3	-	RP-HPLC-ESI-MS	-	6	(Pedan, Weber, et al., 2017)
Cocoa bean Chocolate	Hexane	S-L extraction 70/29.5/0.5 v/v/v AWA	6.3-65.2	0.2-2.3[g] 1.3 – 11.4[g]	-	-	-	(Batista, de Andrade, Ramos, Dias, & Schwan, 2016)
Cocoa beans (fermented)	Hexane	S-L extraction Methanol, Ethanol, Acetone, 1-Propanol, 2.Propanol	88 - 116	-	RP-HPLC-ESI-QqQ-MS	-	4	(Pedan, Fischer, & Rohn, 2016)
Cocoa beans	Hexane	S-L extraction 70 % Acetone Preparative isolation[f]	-	-	NP-HPLC RP-HPLC-ESI-MS UV, 280 nm NMR	-	9	(Esatbeyoglu, Wray, & Winterhalter, 2015)

Cocoa beans (roasted)	Hexane	Sonication bath 70/29.5/0.5 v/v/v AWA	-	-	NP-HPLC-ESI-MS UV, 280 nm	12.3	10	(Ioannone, F. Mattia, C. D. di Gregorio, M. de Sergi, M. Serafini, M. Sacchetti, 2015)
Chocolate	Hexane	Sonication bath 70/29.5/0.5 v/v/v AWA	2.0 – 36.9	68.5 – 790.5[b]	RP-HPLC UV, 278 nm	0.5 – 7.4[h]	-	(Todorovic et al., 2015)

Sample	Lipid Removal Solvent	Method/Solvent for polyphenols' recovery	Total Polyphenols[a] (mgGAE/g)	Antioxidant Activity[b] (µmolTrolox/g)	Analysis method and Detection	Procyanidins[c] (mg/g)	DP[d]	Ref.
Cocoa beans (several genotypes)	Hexane	Sonication bath 60% Propanol	45.3 – 70.1	387.3 – 639.5[b]	RP-HPLC UV, 280 nm			(Carrillo et al., 2014)
Cocoa beans (several genotypes)	-	S-L extraction 80% Ethanol	49.6 – 74.5	1314.4 – 1587.5[b]	-	-	-	(Perea, 2013)
Cocoa beans	Hexane	Pressurized Liquid Ext. Solid-phase separation 50 % Acetone	-	-	RP-HPLC-ESI-QqQ-MS UV 280 nm	1.7 – 7.8	3	(Kothe, Zimmermann, & Galensa, 2013)
Cocoa bean shell	Petroleum ether Hexane	Microwave extraction 80% acetone 80% ethanol 80% methanol Water	17.2-41.8	-	-	-	-	(Nsor-Atindana, Zhong, Mothibe, Bangoura, & Lagnika, 2012)
Cocoa liquor, Cocoa powder Chocolate	Hexane	Sonication bath 70% Methanol	5.0 – 34.0	5.2 – 11.8[i] 3.8 – 20.5[j] 2.3 – 29.2[k]	RP-HPLC UV, 280 nm	0.2 -4.8[h]	-	(Belščak, Komes, Horžić, Ganić, & Karlović, 2009)
Cocoa powder and chocolate	Hexane	Sonication Solid-phase extraction 70/29.5/0.5 v/v/v AWA	-	-	NP-HPLC FL, 230/321 nm	1.55 – 43.7	10	(Robbins et al., 2009)
Cocoa bean	Hexane	S-L extraction 70% acetone			Offline LCxLC HILIC-RP-LC-ESI-MS		12	(Kalili & de Villiers, 2009)

Cocoa beans	-	Soxhlet extraction 70% Acetone	93.2 – 139.3	-	NP-HPLC-API-MS UV 280 nm	20.0 – 128.9		(Cienfuegos-Jovellanos et al., 2009)
Cocoa beans	Hexane	Supercritical Fluid CO_2 and ethanol	-	-	NP-HPLC-UV	-	10	(Sarmento et al., 2008)
Cocoa beans (several genotypes)	Petroleum ether	Sonication bath 70% Acetone	6.4 – 8.4	-	RP-HPLC-ESI-MS UV 280 nm	23.0 - 119.8		(Tomas-Barberán et al., 2007)
Cocoa powder and chocolate (several genotypes)	-	-	3.3 – 60.2	57.5 – 875.0[b]	NP-HPLC-MS FL, 280/310 nm	0.4 – 23.7	-	(Miller et al., 2006)
Cocoa bean	Hexane	Sonication 70% Acetone	-	-	NP-HPLC-ESI-MS FL, 276/316 nm	-	14	(Kelm, et al., 006)
Cocoa powder and chocolate	Hexane	Sonication 70/29.5/0.5 v/v/v AWA	-	80.0 – 826.0[b]	NP-HPLC-ESI-MS FL, 276/316 nm	3.1 – 48.7	6	(Gu et al., 2006)
Chocolate and Cocoa	Hexane	S-L extraction Sephadex	-	67.4 – 520.1[b]	NP-HPLC FL, 276/316	0.7-19.4[L]	10	(Adamson et al., 1999)

1.3.3. Polyphenol oxidase in cocoa and its effect on polyphenols content. Prior to the extraction, separation, and characterization, it is essential to evaluate the enzymatic action on total polyphenol content. Indeed, the role of enzymatic and non-enzymatic reactions in the physicochemical changes (e.g., color, texture, flavor, and taste) from cocoa beans during the cocoa processing is widely known (Camu et al., 2008; López-Nicolás & García-Carmona, 2009). Polyphenol oxidase (PPO), invertase, and protease are the most important enzymes (Macedo, Rocha, Ribeiro, Soares, & Bispo, 2016), while the most common type of non-enzymatic browning is the Maillard reaction (Sacchetti et al., 2016). In particular, PPO is a copper-associated enzyme with two binding sites for phenolic substrates (Steffens, Harel, & Hunt, 1994), that accelerates oxidation and degradation of polyphenols and their derivates (F. Li, Chen, Zhang, & Fu, 2017). Overall, PPO catalyzes the hydroxylation of *o*-monophenols forming *o*-diphenols (monophenolase activity), and then the *o*-dihydroxyphenols to *o*-quinones (diphenolase activity) (De la Rosa, Alvarez-Parrilla, & González-Aguilar, 2009; Q. He, Luo, & Chen, 2008) (**Figure 8**). Maximum PPO activity is well-described in the literature, with optimum pH and temperature are between 6.5 – 7.5 and 25 – 35 ºC, respectively (Ayaz, Demir, Torun, Kolcuoglu, & Colak, 2008; Cheema & Sommerhalter, 2015; de Oliveira Carvalho & Orlanda, 2017).

The action of PPO is activated when plant tissues undergo physical damage such as cutting or blending (C. Fang, 2007), which reduces the shelf life of many processed foods, but is desired in tea, and cocoa process (e.g., to reduce the bitterness and astringency) (Misnawi, Selamat, Bakar, & Saari, 2002). Substrates for this reaction include simple phenols, such as catechol, caffeic acid, chlorogenic acid, gallic acid, and catechins (J. Zhang, Wang, & Mi, 2011). Moreover, quinones formed during PPO-oxidation reactions may undergo redox recycling, which generates free radicals, and can damage DNA, proteins, amino acids or lipids (Yoruk & Marshall, 2003).

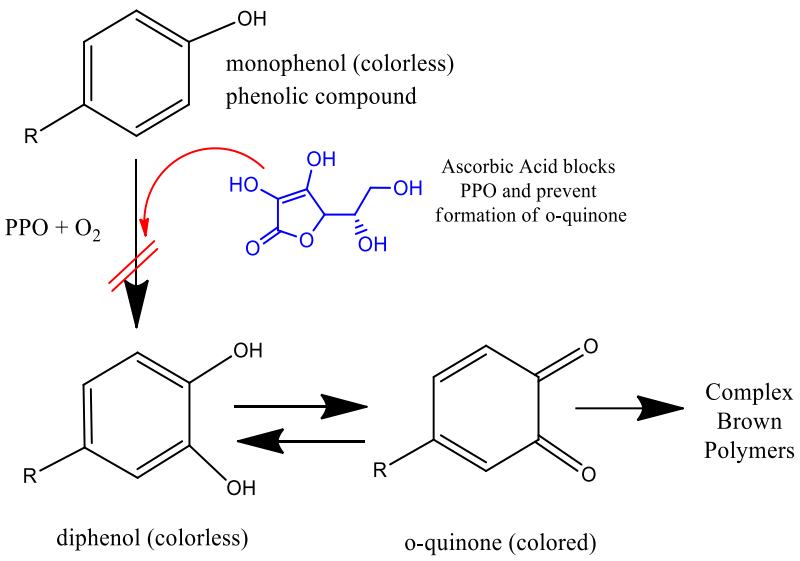


Figure 8. Enzymatic oxidation of phenols compounds catalyzed by polyphenol oxidase enzyme.

It is widely reported in the literature that epicatechin, catechin and their polymeric structures are also substrates of PPO (Ho, 1999; Kiewning, Wollseifen, & Schmitz-Eiberger, 2013; López-Serrano & Ros Barceló, 2002; Wuyts, De Waele, & Swennen, 2006). Main selected works concerning enzymatic inhibition are summarized in **Table 9**. Conventional techniques consisted of heat treatment (also known as blanching process). These methods are straightforward and cheap; they consist on dipping the sample into a solution containing the inhibitor solution at high temperature (70 – 125 ºC) (Bradbury et al., 2011; Pons-Andreu et al., 2008; El-Shimi, 1993; López-Malo, Palou, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1998; Schweiggert, Schieber, & Carle, 2005) for several minutes (0 – 180 min) (Bradbury et al., 2011; Pons-Andreu et al., 2008; Chutintrasri & Noomhorm, 2006; El-Shimi, 1993; López-Malo et al., 1998). Thermal inactivation profiles of PPO follow first-order reaction kinetics (Chutintrasri & Noomhorm, 2006). Subsequently, the enzyme activity is measured by either melanosis index scale or preferentially by total specific content (Pons-Andreu et al., 2008; Yuan, Lv, Tang, Zhang, & Sun, 2016). In addition,

several chemical inhibitors of browning are carboxylic acids such as oxalic and oxalacetic acids, ascorbic acid derivatives or thiol-containing compounds such as cysteine, glutathione, and N-acetylcysteine. However, many of these reagents have been restricted (e.g., sulfites) because of their adverse effect on human health (Ali, El-Gizawy, El-Bassiouny, & Saleh, 2015). In general, at higher inhibitor concentration, lower polyphenol oxidase, and phenolase activity (El-Shimi, 1993). Up to date, ascorbic acid (food additive E-300) and L-cysteine (food additive E-920) are approved in foods by commission regulation EU No. 1129/2011. On the other hand, emerging technologies include high hydrostatic pressure treatment (López-Malo et al., 1998; Rastogi, Eshtiaghi, & Knorr, 2009) and ultraviolet light (UV) radiation (Lante, Tinello, & Nicoletto, 2016), which are gaining interest as non-thermal technologies. Indeed, several studies have confirmed its effectiveness as PPO inhibitors, but they are beyond the scope of this work. Overall, conventional and non-thermal techniques have some drawbacks, for example, UV-C radiation (100 -280 nm) application is limited because of possible adverse effects in food including alteration of sensory quality attributes such as colour (Lante et al., 2016); and the inhibitory effect of chemicals (e.g., ascorbic acid) is temporary; however, combination of ascorbic acid with other reagents (e.g., citric acid, L-cysteine) has shown synergic and prolonged inhibitory effect (Dudley & Hotchkiss, 1989; Siddiq & Dolan, 2017; Yoruk & Marshall, 2003).

As state in **Table 9**, higher denaturation of enzymes (≥70 %) is achieved at high temperatures. However, little is known about the heat effect on the stability of polyphenols. In this context, optimal conditions to enhance enzyme inhibition together with a correlation between the total polyphenol content during PPO denaturation have not been studied yet and has been one of the goals of the present work (**Chapter 1)**.

Table 9. Summary of main studies focusing on enzyme inhibition from several plant sources.

Food matrix	Inactivation Method / Conditions	Enzyme Assays	Inhibitor	Enzyme Inactivation	Ref.
Apples Pears	UV-radiation treatment 0 - 60 min 25 ºC	Polyphenoloxidase	UV-A light (390nm)	Color measurement PPO ≤ 98.2 %	(Lante et al., 2016)
Strawberry purées	Dipping in inhibitor solution Time and temperature not available	Polyphenoloxidase	0.08 % L-cysteine, 0.3 % L-cysteine-HCl 2.5 % Citric acid, 0.1% Papain, 0.1 % Pepsin 0.1 % Bromelain	PPO ≥ 99 % ≥ 95 % ≥ 70 % Ineffective ≤ 20 % Ineffective	(Holzwarth, Wittig, Carle, & Kammerer, 2013)
Cocoa beans	Thermal Treatment 20 -45 min 70 -125 ºC	Polyphenoloxidase	Water	PPO 70 - 95 %	(US Patent 8048469B2, Nov 1, 2011, n.d.)
Cocoa beans	Thermal Treatment 3 - 15 min 85 -100 ºC	Melanosis scale	Water	Color index scale: No browning to slight browning	(US Patent 0193629 A1, Aug 14, 2008, n.d.)
Pineapple puree	Thermal Treatment 5 -30 min 40 -90 ºC	Polyphenoloxidase	Water	PPO ≥ 98 %	(Chutintrasri & Noomhorm, 2006)
Paprika Chili Powder	Thermal Treatment 1 -10 min 80 – 100 ºC	Peroxidase Protein determination Polyphenoloxidase Lipoxygenase	Water	POD ≤ 98.2 %	(Schweiggert et al., 2005)
Red grapes	High hydrostatic pressure treatment 10 – 600 MPa	Polyphenoloxidase Peroxidase	Water	POD 55.8 % PPO 41.9 %	(Rastogi, Eshtiaghi, & Knorr, 1999)

	0 – 60 ºC				
Litchi fruit	Dipping in inhibitor solution Time and temperature not available	Polyphenoloxidase	10 - 100 $mmolL^{-1}$ Glutathione+ Citric acid	PPO 80-85 %	(Jiang, Fu, Zauberman, & Fuchs, 1999)
Avocado puree	High hydrostatic pressure treatment 0 – 30 min 5 – 25 ºC 345 – 689 MPa	Polyphenoloxidase	Water	PPO ≤ 45 %	(López-Malo et al., 1998)
Apple slices	0 – 3 h pH 1 – 7 30 - 80 ºC	Polyphenoloxidase	0.5 -1.5% Ascorbic acid	PPO 95%	(El-Shimi, 1993)

PPO, polyphenol oxidase enzyme.

1.3.4. Recovery and isolation of polyphenols

As seen in **Table 2**, no single universal extraction process and solvent mixture can be employed for the extraction of polyphenols from cocoa beans and related products, in general, for plant sources (Chew et al., 2011). Many factors are involved in the solid-liquid extraction process that affect the final content of polyphenols recovered, such as the type of solvent and concentration, contact time, temperature, solid to solvent ratio, structure of the solid matrix and pH (Pinelo, Sineiro, & Núñez, 2006). Indeed, the extraction of polyphenols is a difficult task because:

- They occur in plant tissues bound with sugars, proteins, and different interactions with the food components are found (Gadkari & Balaraman, 2015).
- They are highly susceptible to many factors, including oxygen concentration, pH changes, and high temperature (Z. Fang & Bhandari, 2010).

- They readily undergo oxidation and epimerization reactions (Dube, Ng, Nicolazzo, & Larson, 2010).

These factors negatively impact the use of polyphenols as a natural food additive; moreover, reducing its antioxidant activity and *in vivo* bioavailability.

Before polyphenols' extraction, it is necessary to prepare the sample. It is very common to reduce the water content using oven-drying (Hii, Law, Cloke, & Suzannah, 2009; Santhanam Menon, Hii, Law, Shariff, & Djaeni, 2017; Tomas-Barberán et al., 2007) at mild temperature until uniform moisture is reached or preferably employing vacuum freeze-drying (Larrauri, Rupérez, & Saura-Calixto, 1997; Ratti, 2001; Santhanam Menon et al., 2017). Although freeze-drying preserves polyphenols and avoids their degradation, it is the most expensive process for manufacturing a dehydrated product (Ratti, 2001). The reduction of particle size from millimetric to micrometric size is often carried out by milling processes with either knife and balls mills (Pons-Andreu et al., 2008). To preserves the desired properties, cooling strategies such as cryogenic mills (Pons-Andreu et al., 2008) are suggested. Then, the obtained powder sample follows a degreasing process, which can be carried out by a) Soxhlet extraction using dichloromethane (Marco Arlorio et al., 2008) or petroleum ether (Noor-Soffalina, Jinap, Nazamid, & Nazimah, 2009), b) ultrasonic bath using n-hexane (Fayeulle et al., 2018), and c) supercritical fluids using CO_2 (Asep et al., 2008).

A number of recovery methods and analytical assays that describe the extraction and characterization of polyphenols from plant samples are summarized in **Table 10** and **Table 2**. Extraction methodologies can be divided into conventional and non-conventional methods. Conventional devices of extraction include Soxhlet (Cienfuegos-Jovellanos et al., 2009), solid-liquid extraction (S-L) (Pedan, Fischer, et al., 2017; Rodríguez-Carrasco et al., 2018; Toro-Uribe,

López-Giraldo, et al., 2018; Toro-Uribe, Montero, et al., 2018; Żyżelewicz et al., 2018), and liquid-liquid (L-L) extraction (Ćujić et al., 2016; Da Porto, Porretto, & Decorti, 2013; Mustafa & Turner, 2011). Non-conventional techniques consist of microwave extraction (Nsor-Atindana et al., 2012; Routray & Orsat, 2012), ultrasonic extraction (Ioannone, F. Mattia, C. D. di Gregorio, M. de Sergi, M. Serafini, M. Sacchetti, 2015; Todorovic et al., 2015; Vilkhu, Mawson, Simons, & Bates, 2008), and pressurized liquid extraction (PLE) (Hawthorne, Grabanski, Martin, & Miller, 2000; Herrero, Mendiola, Cifuentes, & ez, 2010; Kothe et al., 2013). In general, each methodology has advantages and disadvantages. For example, conventional methods are cheaper but time-consuming; and most of the case use toxic solvents (e.g., petroleum ether, n-hexane, acetone, among others) (Devgun, Nanda, & Ansari, 2012). The new extraction techniques have enabled automation, shortened extraction time, reduced organic solvent consumption, and use of green solvents (e.g., carbon dioxide, water), but are more expensive (Devgun et al., 2012; Pons-Andreu et al., 2008).

Based on a literature survey for cocoa polyphenols (**Table 2** and **Table 10**), the extraction of polar compounds employing solid-liquid extraction at temperature ranging from 30 – 100 ºC during several minutes is often used (Batista, de Andrade, Ramos, Dias, & Schwan, 2016; Cienfuegos-Jovellanos et al., 2007; Pons-Andreu et al., 2008; Pedan, Fischer, et al., 2017; Rodríguez-Carrasco et al., 2018; Tomas-Barberán et al., 2007; Toro-Uribe, López-Giraldo, et al., 2018; Żyżelewicz et al., 2018) as well as sonication bath (Belščak et al., 2009; Carrillo et al., 2014; Ioannone, F. Mattia, C. D. di Gregorio, M. de Sergi, M. Serafini, M. Sacchetti, 2015; Todorovic et al., 2015; Tomas-Barberán et al., 2007); although, supercritical CO_2 + ethanol (co-solvent) is preferred to extract a mixture of polar and mostly nonpolar compounds (M. Arlorio et al., 2005; Asep et al., 2008; Da Porto, Decorti, & Natolino, 2014; Da Porto, Natolino, & Decorti, 2014; Saldaña, Zetzl, Mohamed, & Brunner, 2002; Sarmento et al., 2008; Valadez-Carmona et al., 2018).

To the best of our knowledge even if there is extensive information regarding the extraction yield and comparing several plant extracts in terms of antioxidant activity and individual content of flavonoids, but not clear information can be found regarding the optimal solid-liquid extraction conditions for cocoa procyanidins.

In general, methanol and acetone allow higher extraction yields (Adamson et al., 1999; Pedan et al., 2016), but they have some limitations; for instance, Food and Drug Administration associates both methanol and acetone with toxic potential (Food and Drug Administration, 2017). To overcome this limitation, polar solvents such as water, ethanol or mixture of both are currently accepted. Additionally, new strategies for the recovery of flavonoids from plants include the combination of conventional and non-conventional techniques, for example, ultrasound-assisted extraction (Da Porto et al., 2013), microwave-assisted extraction (Liazid, Palma, Brigui, & Barroso, 2007), ultrasound-assisted soxhlet extraction (Luque-García & Luque De Castro, 2004), ultrasound assisted supercritical fluid extraction (A. jun Hu, Zhao, Liang, Qiu, & Chen, 2007), and ultrasound-assisted pressurized solvent extraction (Richter, Jiménez, Salazar, & Maricán, 2006). Thus, the application of ultrasound-assisted solid-liquid extraction is investigated in **Chapter 2**. This strategy was selected for allowing the recovery of hydrophilic compounds with different polarities and include some advantages for food applications since it is suitable, cheap, and reliable.

Once polyphenols are recovered from the plant sample, several analyses can be performed. In first stages, colorimetric assays are chosen for being easier and cheaper; thereby, total polyphenol (Singleton, Orthofer, & Lamuela-Raventós, 1998b), total flavonoid (Zhishen et al., 1999), total proanthocyanidins (Sun et al., 1998), and total antioxidant activity (Belščak et al., 2009; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006) are the most common assays

used for phenolic compounds (**Table 10**). Once again, normal and mostly reversed-phase-HPLC coupled to a diode array detector (DAD) and/or mass spectrometers (**Table 2** and **Table 10**) are the most widely used analytical tool for quantification and fine characterization of polyphenols (Tsao, 2010). Other methods include gas chromatography (GC) for the separation of isoflavones previously derivatized to methyl esters; and normal phase LC coupled to a fluorescence detector for the separation of procyanidins (Robbins et al., 2009).

Table 10. Summary of extraction, purification and analytic methodologies employed in the recovery of flavonoids.

Sample Pre-Treatment	Extraction	Purification Isolation	Conventional Analytical Methods	Advanced Analytical Methods
Drying • Drying Oven • Freeze Dryer Grinding • Knife • Hammer Milling • Ball mll • Hammermill • Knife mill • Cryogenic mill Hydrolysis • Acidic medium Degreasing • Hexane • Petroleum ether	Conventional • Maceration • SLE • LLE • Soxhlet No-Conventional • UAE • MAE • PLE • Combination	No-Preparative scale • SPE • LLE • TLC Preparative scale • Column chromatography • Prep. LC • CCC	Total polyphenols • Folin-ciocalteu Assay Total Flavonoids • $AlCl_3$ Assay Total Proanthocyanidin • Butanol-HCl assay • Vanillin assay • DMAC assay Antioxidant Activity… • DPPH • ORAC • ABTS	HPLC… • UV • FL GC… • FID MS… • QqQ • TOF • MALDI

SLE, solid-liquid extraction; LLE, liquid-liquid extraction; UAE, ultrasound-assisted extraction; MAE, microwave-assisted extraction; PLE, pressurized liquid extraction; SPE, solid-

phase extraction; TLC, thin-layer chromatography; CCC, countercurrent chromatography; DMAC, dimethylaminocinnamaldehyde; DPPH, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid); ORAC, oxygen radical absorbance capacity; HPLC, high performance liquid chromatography; FL, fluorescence detector; FID, flame ionization detector; GC, gas chromatography; MS, mass spectrometry, QqQ, triple quadrupole; TOF, time of flight; MALDI, matrix-assisted laser desorption/ionization.

Sometimes, the extract is submitted to a cleanup process and/or isolation process. Techniques for the cleanup process include solid-phase extraction (SPE) and liquid-liquid extraction (LLE). LLE using hexane or petroleum ether is employed to remove lipids and methanol-ethyl acetate mixtures or chloroform to recovery methylxanthines (X. Hu, Wan, Bal, & Yang, 2003; Hulbert, Biswal, Mehr, Walker, & Collins, 1998; Rao, 1975), but its selectivity is limited (Plaza, Domínguez-Rodríguez, Castro-Puyana, & Marina, 2018). SPE cartridge is widely used to remove interference compounds from extract, pre-concentration of the sample, and fractionation of the sample into different compounds (Berrueta, Gallo, & Vicente, 1995). Depending on the application, different stationary phases are loaded in the cartridge. Therefore, different solvents are employed. In this sense, Toyopearl HW-40/50 (J. He, Santos-Buelga, Mateus, & de Freitas, 2006; Sun, Leandro, de Freitas, & Spranger, 2006), Sephadex LH-20 (Kimura et al., 2011; Svedström, Vuorela, Kostiainen, Laakso, & Hiltunen, 2006), and Superdex 75 HR (McMurrough, Madigan, & Smyth, 1996) gel filtration eluting with aqueous acetone, aqueous alcohol, urea and their combinations have been commonly used for separation of proanthocyanidins.

Most selective techniques for separation and purification of polyphenols include preparative HPLC and recently high-speed countercurrent chromatography technique (HSCCC) (Esatbeyoglu et al., 2015; S. Zhang et al., 2015, 2017). The choice of these techniques will depend on the

equipment availability, nature of the analyte, and final application. In general, HSCCC allows high sample input, high recoveries, and is based on the partitioning of the solutes through the mixing of two immiscible phases (S. Zhang et al., 2015). Preparative column chromatography can be carried out employing either normal or reversed phase columns. For instance, apple and cocoa procyanidins were separated and isolated by normal phase fractionation using hexane-acetone (Yanagida et al., 2000) or acetonitrile with aqueous-methanol-acetic acid mixtures (Kelm et al., 2006) as mobile phases. Fractionation on preparative C_{18} column allowed the separation of dimers and trimers from grape extract (Sun, Belchior, Ricardo-Da-Silva, & Spranger, 1999). Nevertheless, up to date, the separation of procyanidins is limited by the degree of polymerization and deserve further study.

1.4 Encapsulation of flavonoids

Considering the physical and chemical instability of polyphenols, different strategies to protect the bioactive compound from adverse conditions, thus increase their shelf life and promote a controlled liberation of the encapsulated compounds have been developed (Shahidi & Han, 1993) The encapsulation is the process of incorporating the bioactive compound within some kind of carrier or matrix (McClements, 2014). The controlled delivery of bioactive compounds will maintain the active molecular form until the time of consumption and deliver this form to the physiological target within the organism (Lingyun Chen, Remondetto, & Subirade, 2006). Depending on the nature of the carrier (composed of single or multiple components) may have heterogeneous or homogeneous structure (Z. Fang & Bhandari, 2010). In particular, colloidal delivery systems can be constructed from food-grade ingredients such as proteins, lipid, surfactants, and polysaccharides (Acosta, 2009). According to McClements (2014) the choice of delivery system depends on a number of factors, for example:

- Nature of bioactive compound to be encapsulated
- The challenges that need to be overcome, e.g., for bioactive compounds, increase their bioactivity and bioavailability, their solubility characteristics and chemical instability, and protect them from acidic or alkaline environment.
- Nature of carrier system
- Field of application

In food applications, the encapsulation constitutes a major challenge because the carrier system should be recognized as safe, relatively inexpensive, easy to use, not impact the quality of the food product (e.g., appearance, texture, and flavor), preserve the bioactive compound during the different phases of food transformation and storage, and ensure high bioavailability upon ingestion. Moreover, the fabrication method should be economical, reproducible, and suitable for large-scale production (McClements, 2014; Munin & Edwards-Lévy, 2011; Sessa, Tsao, Liu, Ferrari, & Donsì, 2011; Shahidi & Han, 1993).

Table 5 summarizes the different systems assayed for incorporation of catechins or plant extracts into several delivery systems. As can been seen, there are several methodologies to generate nanoparticle systems and the major mechanical devices for fabrication can be further divided in homogenization, atomization, milling, extrusion, coating, and supercritical fluids (McClements, 2014). In addition, several physical and chemical characterization needs to be further researched, for instance, stability of bioactive into the carrier (e.g., particle size and distribution, ζ-potential, antioxidant or prooxidant activity), morphology, efficiency of encapsulation, measurement of their bioaccessibility (*in vitro* or *in vivo*) and absorption employing cell lines models or human studies.

Depending on the application to be addressed, each technique has advantages and disadvantages. For instance, spray drying encapsulation is widely used in the food industry but has limited available number of shell materials and produce particles with a mean size range of 10 – 100 μm (Z. Fang & Bhandari, 2010). On the other hand, particles with a diameter ranging from 1 to 1000 nm are reported for emulsions and liposomes employing homogenization methods (e.g., microfluidization and sonication) (Aditya, Aditya, Yang, Kim, Park, Lee, et al., 2015; Toro-Uribe, López-Giraldo, et al., 2018). In general, several parameters need to be optimized to achieve efficient formulations. These parameters depend on the type of delivery system. For emulsion and liposomes, concentration and type of lipids and/or surfactant type, pH of the system, concentration of the bioactive compound, ratio lipid/water, and method of homogenization should be studied (McClements, 2015).

In general, literature survey shows that only a small proportion of the polyphenols remains available after oral intake, due to insufficient gastric residence time, low permeability and solubility within the gut (Z. Fang & Bhandari, 2010; Mancini et al., 2018). For example, total polyphenols decreased by 77 %, and 97 % in gastric and intestinal phases, respectively (Ovando-Martínez, Gámez-Meza, Molina-Domínguez, Hayano-Kanashiro, & Medina-Juárez, 2018). Thereby, the encapsulation systems have gained greater attention to improve the bioaccessibility of polyphenols. This has been proved in several matrix systems such as curcumin from carrots (R. Zhang et al., 2015), tea catechins (Bhushani, Karthik, & Anandharamakrishnan, 2016; Bhushani, Kurrey, & Anandharamakrishnan, 2017), grape seed extract (Berendsen, Güell, & Ferrando, 2015), lycopene-enriched tomato extract (Ha et al., 2015), β-carotene (Gómez-Mascaraque, Perez-Masiá, González-Barrio, Periago, & López-Rubio, 2017; Salvia-Trujillo, Qian, Martín-Belloso, & McClements, 2013), resveratrol (Davidov-Pardo & McClements, 2014; Sessa et al., 2014),

catechins (Aditya, Aditya, Yang, Kim, Park, & Ko, 2015; Paximada, Echegoyen, Koutinas, Mandala, & Lagaron, 2017), quercetin (Pool, Mendoza, Xiao, & McClements, 2013), epigallocatechin gallate (Gómez-Mascaraque, Soler, & Lopez-Rubio, 2016), among others.

Studies on resveratrol showed that the ingredient is active only when encapsulated in a delivery system, which is capable of stabilizing and protecting it from degradation while preserving its biological activities and enhancing its bioavailability (Davidov-Pardo & McClements, 2014; Sessa et al., 2011). Also, Fang and co-workers (J. Y. Fang, Hung, Liao, & Chien, 2007) reinforced that nanometric size improved resveratrol cell uptake and was efficiently transported into the cardiovascular system when it was incorporated in liposphere. An improvement of curcumin and carotenoids bioaccessibility was appreciably higher with increasing lipid nanoparticle concentration, which was attributed to an increase in the solubilization capacity of the mixed micelle phase (Z. Zhang, Zhang, & McClements, 2016; L. Zou et al., 2016). In line with this trend, Ortega et al., (2009) compared the digestion of two formulations of cocoa with different fat content and proved that higher fat content favored the formation of fat-forming micellar structures and the incorporation of the cocoa phenols into the lipid phase, achieving a protective effect during duodenal digestion.

In the specific case of hydrophilic compounds, different delivery systems can be used (**Table 5**), for instance, single emulsions (Joyce, Gustafsson, & Prestidge, 2018; W. Liu, Wang, McClements, & Zou, 2018; R. Zhang et al., 2015) double-emulsions (Berendsen et al., 2015), polymers (Z. Li & Gu, 2014; Smith et al., 2010; Wisuitiprot, Somsiri, Ingkaninan, & Waranuch, 2011; T. Zou et al., 2012), liposomes (J.-Y. Fang, Lee, Shen, & Huang, 2006; Gibis, Thellmann, Thongkaew, & Weiss, 2014; Gibis, Vogt, & Weiss, 2012a; Toro-Uribe, López-Giraldo, et al., 2018), supercritical fluids (Sosa, Rodríguez-Rojo, Mattea, Cismondi, & Cocero, 2011),

carbohydrates (Ferreira, Rocha, & Coelho, 2007), and so forth. However, polyphenols create complex with polymers, thus reducing their bioaccessibility and antioxidant capacity (Ferruzzi & Green, 2006; Ozdal, Capanoglu, & Altay, 2013). Moreover, electrospray (Ferreira et al., 2007), supercritical fluids (Sosa et al., 2011), and polymer-based (B. Hu et al., 2012; B. Hu, Ting, Zeng, & Huang, 2013; Liyan Zhang & Kosaraju, 2007) technologies are promising encapsulation systems, but are expensive (high investment costs compared to conventional equipment), produce particles with large particle sizes (Berendsen et al., 2015; Ferreira et al., 2007; Sosa et al., 2011; Liyan Zhang & Kosaraju, 2007) and sometimes require food grade biopolymers (accepted by food safety agencies such as FDA and EFSA) which limit their use (Wandrey, Bartkowiak, & Harding, 2010).

On the other hand, by reducing the particle size below 500 nm, higher nanoparticles uptake and higher absorption of the encapsulated active compounds can be achieved (Acosta, 2009). In fact, Ha et al., (2015) reported high *in vitro* bioaccessibility and antioxidant activity for encapsulated lycopene at particle sizes between 100 – 200 nm. This phenomenon is explained for the larger surface area for the interaction with the biological substrates than the corresponding micrometric size carrier surface.

Among the different carrier systems, liposomes were selected for procyanidins' encapsulation; since these molecules possess a high number of hydroxyl groups and different distribution coefficients as a function of their molecular weight and chemical configuration (Toro-Uribe et al., 2018). More details of liposome delivery system can be found in **Chapter 5** and **6**.

Table 11. Summary of different systems evaluated for encapsulation of catechin and related compounds.

Compound	Based Delivery System	Carrier system/ Methodology	Particle size	Measurement of Bioavailability or absorption	Physicochemical Characterization	Ref.
C, EC PCs (2-7) Cocoa extract	Liposome	Lecithin Ultrasound	< 90 nm	-	%Efficiency Encapsulation Lipid Oxidation study Antioxidant activity	(Toro-Uribe, López-Giraldo, et al., 2018)
β-carotene	Emulsion	Caseinate/alginate/ Microfluidization	< 50 µm	*In vitro*	Particle size Charge Measurement Microstructure analysis Physicochemical stability	(W. Liu et al., 2018)
Cocoa Hull	Liposome	Lecithin Microfluidization	252 nm	*In vitro*	Particle size Charge Measurement Microstructure analysis %Efficiency Encapsulation Morphology analysis	(Altin, Gültekin-Özgüven, & Ozcelik, 2018)
Curcumin	Micelles	Sophorolipid pH driven method	61 nm	*In vitro* and *In vivo*	Particle size Charge Measurement X-ray analysis %Efficiency Encapsulation Physicochemical stability	(Peng et al., 2018)
Fish Oil	Emulsion	Silica solution and Phospholipid emulsifier Homogenization	313 ± 21 nm	*In vitro*	Particle size Charge Measurement Microstructure analysis %Loading drug	(Joyce et al., 2018)

EGCG	Protein	Ovalbumin-Dextran: Self-Assembled	173 - 347 nm	*In vitro*: Caco-2 cells	Morphology Charge Measurement %Efficiency Encapsulation	(Z. Li & Gu, 2014)
EC, C	Protein	BSA Desolvation	C: 45±5 nm EP: 48±5 nm	*In vitro*	%Efficiency Encapsulation Morphology	(R. Yadav, Kumar, Kumari, & Yadav, 2014)
Grape seed extract	Emulsions	$W_1/O/W_2$ emulsions: Membrane emulsification	10 µm	Release Kinetics at pH 3.5 and 7	Particle Size Thickness and density Charge Measurement Creaming stability Morphology Total Polyphenols	(Berendsen et al., 2015)

Compound	Based Delivery System	Carrier system/ Methodology	Particle size	Measurement of Bioavailability or absorption	Physicochemical Characterization	Ref.
EGCG	Polymer	Peptide/Chitosan Sonication and ionic gelation mechanism	143 ± 7 nm	*In vitro*: HepG2 cells	Morphology Charge Measurement %Efficiency Encapsulation	(B. Hu et al., 2013)
EGCG	Polymer	Chitosan Stirring and formed spontaneously	4 to 150 nm	*In vitro*: Caco-2 cell	Size Particle and Distribution Morphology %Efficiency Encapsulation Charge Measurement	(B. Hu et al., 2012)
Cocoa procyanidins	Protein, Polymer	Gelatin B, Chitosan Stirring and formed spontaneously	307 nm and 721 nm	*In vitro*: THP-1 cell line	Particle size %Efficiency Encapsulation Charge Measurement Morphology FTIR	(T. Zou et al., 2012)
Tea extract	SFE-CO_2	Polycaprolacton Supercritical Antisolvent	3-5 µm	-	DSC	(Sosa et al., 2011)
Catechins	Carbohydrates	Spray Drying	0.08±0.02 µm	-	Size Particle and Distribution Charge Measurement Morphology %Efficiency Encapsulation	(Ferreira et al., 2007)
Procyanidins from grape seed extract	Polymer	Gum arabic, Maltodextrin Homogenization	5-30 µm		%Efficiency Encapsulation DSC Morphology	(Lianfu Zhang, Mou, & Du, 2007)
Catechin	Polymer	Chitosan Gelling and coacervation-phase inversion	2 – 7 µm	*In vitro*: SIF and SGF conditions	Particle Size and Distribution Morphology Charge Measurement FTIR Spectroscopy DSC	(Liyan Zhang & Kosaraju, 2007)

1.4.1. Liposomes Liposomes are colloidal particles consisting of a membranous system formed by phospholipid bilayers encapsulating aqueous space(s) (Z. Fang & Bhandari, 2010). Phospholipids are major components of cell membranes of plants and animals and have essential functions in the growth and functioning of cells (Klang & Valenta, 2011). The phospholipid structure consists of two hydrophobic fatty acid "tails" (consisting of 2 long fatty acid chains) and a hydrophilic phosphate "head" (containing the negatively charged phosphate group), joined together by an alcohol or glycerol molecule (Singh, Gangadharappa, & Mruthunjaya, 2017) (**Figure 9**). Depending upon the chemical group on the phosphate, phospholipids can be classified into different classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), and phosphatidylinositol (PI) (Szuhaj, 1983). Soybean and egg lecithin are an important source of phospholipids with a commercial production of 200,000 tons/year and 300 tons/year, respectively (Burling & Graverholt, 2008). The phospholipid-based functional ingredients used as emulsifiers in commercial products are usually called lecithins (Klang & Valenta, 2011). Lecithin acts as an emulsifier (Afoakwa, Paterson, & Fowler, 2007) and can be classified as a mixture of phospholipids obtained from plant or animal sources (Szuhaj, 1983), therefore, it is composed 18% PC, 14% PE, 9% PI, 5% PA, 2% minor phospholipids, 11% glycolipids, 5% complex sugars, and 37% neutral oil (Y. Wu & Wang, 2003). Thereby, phospholipids have different susceptibility to oxidation because of the polar head groups and also the degree of unsaturation (Cui & Decker, 2016). For example, soybean lecithin contains high amounts of $C_{18:2}$ and $C_{18:3}$, therefore, it is susceptible to hydrolysis of the ester bonds to peroxidation of unsaturated acyl chains (A. V. Yadav, Murthy, Shete, & Sakhare, 2011), producing off-flavors and oxidization of the bioactive encapsulated within the liposomes (Toro-Uribe et al., 2018).

As shown in **Figure 9 ,** the water-soluble compound is entrapped in the water core and lipid-polar compounds in the lipid section. Liposomes formed single- or multiple-layer vesicles, separated by water compartments with size ranging from nanometers up to several micrometers (Panya et al., 2010; A. V. Yadav et al., 2011).They are often distinguished according to the number of lamellae and size: small unilamellar vesicles (SUV, 20 -100 nm), large unilamellar vesicles (LUV, > 100 nm), and large multilamellar vesicles (MLV, > 0.5 µm), oligolamellar vesicle (0.1 – 1.0 µm) or multivesicular vesicles (> 1 µm) are the main classes (Munin & Edwards-Lévy, 2011). When a phospholipid, such as lecithin, is dispersed in an aqueous phase, the liposomes form spontaneously through hydrophilic-hydrophobic interactions between phospholipids and water molecules (Gadkari & Balaraman, 2015).

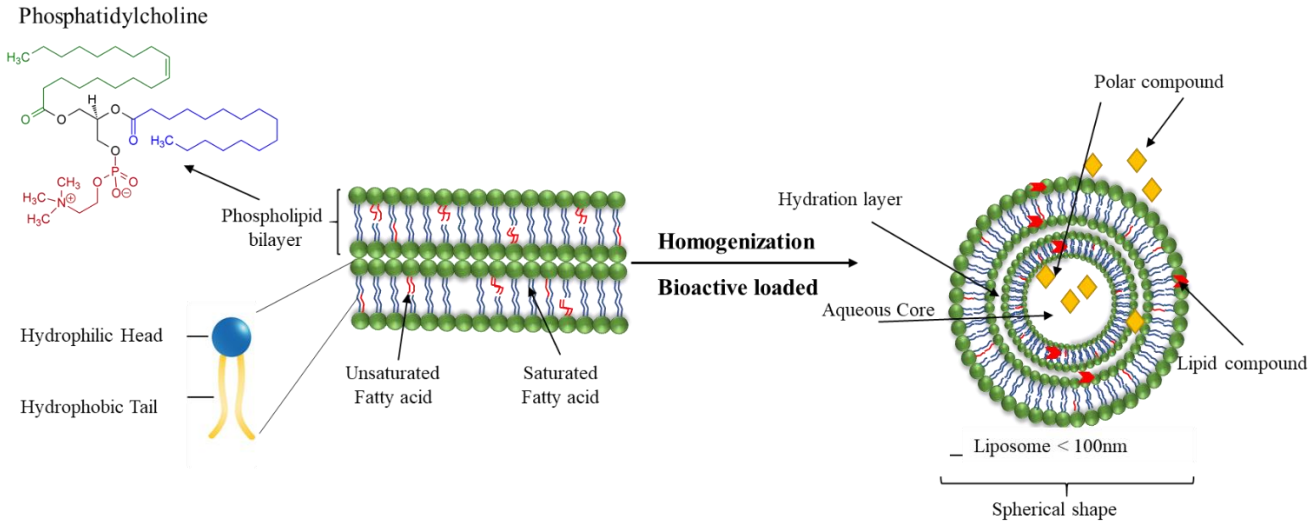


Figure 9. Schematic structure of a phospholipid and liposome (by the author).

Due to their chemical composition, liposomes are versatile carrier systems for both hydrophilic and hydrophobic bioactive materials (Kosaraju, Tran, & Lawrence, 2006). Thereby, pharmaceutical, cosmetic and food industry have utilized the liposomal system for wider applications. A significant advantage of their use is the target delivery and the ability to control

the release rate, thus increasing the bioavailability of the incorporated materials (Gadkari & Balaraman, 2015). In food industry, liposomes have gained increasing attention since they have the unique distinction of being natural, biodegradable, and nontoxic (Kosaraju et al., 2006) Liposomes can be produced by different methods such as a) hydration film (Bangham, Standish, & Watkins, 1965), b) ether injection/vaporization (Deamer, 1978) c) membrane extrusion (Olson, Hunt, Szoka, Vail, & Papahadjopoulos, 1979), d) micro-emulsification (Mayhew, Lazo, Vail, King, & Green, 1984), e) double emulsion vesicle (Zheng, Zheng, Beissinger, & Fresco, 1994), f) reverse-phase evaporation (Cortesi et al., 1999), g) homogenization (J.-Y. Fang et al., 2006), h) supercritical fluids (Trucillo, Campardelli, & Reverchon, 2018), and so on.

Since the spontaneous formation of MLVs is easily attained, techniques such as sonication, homogenization and membrane extrusion are often used for conversion of MLVs into LUVs and SUVs (Patil & Jadhav, 2014). In particular, sonication is perhaps the most extensively used method for the preparation of SUV from MLVs (Akbarzadeh et al., 2013). In general, sonicator tip is directly submerged into the liposome suspension and subjected to intense high pulse. As a consequence of high energy input, the sample is overheated, therefore, possible degradation of phospholipids and encapsulated compounds can arise. To avoid this phenomenon, control temperature of the vessel with icy water is needed (Akbarzadeh et al., 2013; Dua, Rana, & Bhandari, 2012).

To be effective, liposomal drug delivery system is dependent on several parameters, such as lipid composition, size and distribution, charge, bioactive ingredient and lipid ratio, and method of fabrication (Samad, Sultana, & Aqil, 2007). Exemplification of encapsulation of polyphenols employing liposome system is highlighted by Mancini et al., (2018) who demonstrated that phospholipid nanoformulations are interesting candidates to deliver antioxidants and monoamine

oxidase A inhibitors into the brain. Moreover, extended shelf life together with improved antioxidant activity against lipid oxidation was observed with the inclusion of procyanidins from grape seed and cocoa extract (Gibis, Vogt, & Weiss, 2012b; Toro-Uribe, López-Giraldo, et al., 2018). However, up to date, few studies have addressed the stability of encapsulated procyanidins-loaded liposomes under *in vitro* gastrointestinal conditions, which is the subject of the present Ph.D. thesis (**Chapter 6 and 7**).

1.5. Bioavailability of flavonoids

As mentioned previously, recent advancements have allowed the incorporation of bioactive products into several delivery systems to design new pharmaceutical and food products with new functionalities and applications. Optimization of new products and knowledge of its bioaccessibility is of great interest to ascertain the nutritional quality of a nutrient or bioactive compound not only in terms of quantities required to achieve dietary requirements but also to fine-tune the development of functional foods (Cilla, Bosch, Barberá, & Alegría, 2018).

In vivo and *in vitro* methodologies can be used to measure the bioavailability of the bioactive compound. *In vivo* methods are considered the "gold standard" but have some restrictions, like limitations in experimental design, the high cost of equipment and labor and ethical constraints (Cilla et al., 2018; Minekus et al., 2014a). On the contrary, *in vitro* models are faster, cheaper, safer, do not have ethical restrictions and can be used to study the bioaccessibility of different food sources (José Jara-Palacios, Gonçalves, Hernanz, Heredia, & Romano, 2018).

Depending on the focus of the research, two types of *in vitro* models can be used: static and dynamic. Static *in vitro* models are feasible and inexpensive to assess multiple samples in parallel, considering several experimental conditions (Alminger et al., 2014). Overall, this model allows

simulating the oral, gastric and small intestine phases. Although several models can be found in the literature to carry out static *in vitro* digestion, international consensus can be found in (Minekus et al., 2014a)(Minekus et al., 2014a)Minekus et al., (2014b) Dynamic models have the advantages to simulate the series of events during the gastrointestinal tract (e.g., food and water intake, release of digestive enzymes and variation of pH) together with the peristaltic contractions and transit through the stomach and intestines (Alminger et al., 2014). This methodology is more accurate due to closer resemblance to *in vivo* conditions and is used to confirm results obtained employing static models (Alminger et al., 2014). Up to date, humans gastrointestinal simulators (e.g., TNO and HGS) can be used for this purpose, but are expensive and not always available.

Concerning static *in vitro* digestion process, this comprises various steps (**Figure 10**): the food in solid or liquid state is partially digested in the oral phase through mastication and salivation. This process is a result of salivary secretion among other factors, which results in a food bolus (Minekus et al., 2014a). The bolus follows a dissolution process and placed under gastric conditions during a period that ranges from 1 – 3 h (Acosta, 2009). Gastric conditions are characterized by acidic pH (pH ca. 1 to 3), to favor the proteolytic activity of some enzymes (e.g., pepsin) (Rick & Fritsch, 1974). This process is required to optimize the intestinal digestion from food matrices: break down proteins, carbohydrates, lipids, delivery of the meal to the intestine phase, and help to increase emulsification in the stomach (Alminger et al., 2014; Minekus et al., 2014a). Once the food finishes the gastric phase, it is in the form of suspension and mixed with the intestinal fluid at pH ca. 7 containing bile salts and other enzymes such as pancreatin (mixture of amylases, lipases, and other digestive enzymes) (McClements & Li, 2010). During this stage (2 h or more), lipolysis takes place as well as the formation of micelles, vesicles, solubilization, and transport of the available compounds (R. Zhang et al., 2015). Thereby, the terms bioavailability

and bioaccessibility refer to the fraction that is available at the site of action in the body (Acosta, 2009), and the fraction that is released from the food matrix during the digestion process and then becomes accessible for intestinal absorption (McClements, 2014), respectively. Moreover, the bioactivity includes events linked to how the bioactive compound reached systemic circulation, are transported and reached the target tissue, and all the cascade of physiological effects it generates (Cilla et al., 2018).

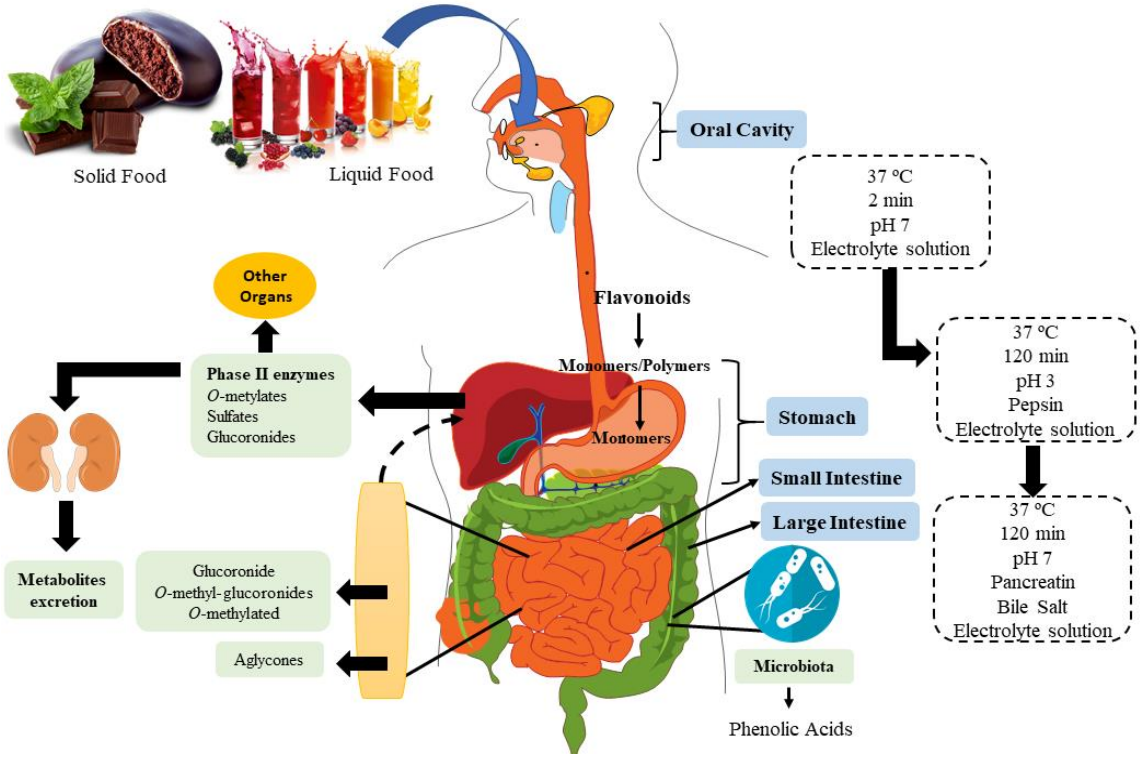


Figure 10. Overview of simulated digestion of flavonoids (by the author).

The bioavailability of polyphenols is a complex issue including multistep processes: exposition of wide pH variations, digestive release of the bioactive compound from the food product, solubilization of the stable compound in the gut lumen, uptake and transport through the gut, and metabolization (Alminger et al., 2014; Neilson & Ferruzzi, 2011). Furthermore, the bioavailability of polyphenols in the cell culture medium is much worse than in organic solvents or water (Lei

Chen, Cao, & Xiao, 2018), which leads to poor lipid solubility and limited ability to modulate pro-oxidant and pro-inflammatory pathways, thus reducing their biological activity (Lei Chen et al., 2018; Rosillo, Alarcón-De-La-Lastra, & Sánchez-Hidalgo, 2016).

In general, absorption of flavan-3-ols highly depends on the chemical structure (e.g., degree of glycosylation, conjugation, polymerization, and stereochemistry configuration) (Depeint, Gee, Williamson, & Johnson, 2002; Motilva et al., 2016; Watson et al., 2012), and physical and biological properties, which determine the rate of passive diffusion across cell membranes (Shoji, Masumoto, Moriichi, Akiyama, et al., 2006). Moreover, the results need to be examined with care since the magnitude of the analysis is a function of the loading dose, sample treatment, nature, and concentration of polyphenols. **Table 6** summarizes some recent studies focusing on the bioaccessibility and absorption of procyanidins using different food matrices or model systems.

Literature clearly shows that plasma concentration of phenolic compounds, monomeric flavonoids, quercetin and derivates, and procyanidins rarely exceeds 1 µM (Bouayed, Deußer, Hoffmann, & Bohn, 2012; Castello et al., 2018; Mendoza-Wilson et al., 2016; Motilva et al., 2016; Pereira-Caro et al., 2018; Rein et al., 2000; Richelle, Tavazzi, Enslen, & Offord, 1999; Wang et al., 2000). Competitive absorptions of epicatechin and catechin has been reported, being the epicatechin absorbed more efficiently (Baba et al., 2001; Fogliano et al., 2011; Motilva et al., 2016; Serra et al., 2009). Furthermore, aglycan flavonoids can be easily absorbed (Kumar & Pandey, 2013; Pereira-Caro et al., 2018) whereas conjugated flavonoids need to be hydrolyzed to reach the colon, thus undergoing an intense phase II metabolism (Fogliano et al., 2011; Motilva et al., 2016; Watson et al., 2012) (formation of sulphate and glucuronide conjugates), then sulfate conjugates and methyl derivatives in the liver (Sasot et al., 2017) are formed. For example, during the intake

of chocolate and cocoa samples, free (epi)catechin together with its metabolites (Baba et al., 2001) were detected, which reached significantly content in plasma concentration.

A series of experiments examining the stability of epicatechin and catechin under *in vitro* or *in vivo* digestion demonstrated that they are highly stable in gastric phase, and are completely lost during the intestinal phase (Zhu et al., 2002), that is, a pH-dependence was observed (Bermúdez-Soto, Tomás-Barberán, & García-Conesa, 2007; Bouayed et al., 2012; Neilson & Ferruzzi, 2011; Zhu et al., 2002). Indeed, the stability of the monomers and dimers at a pH between 5.0 and 9.0 can be ranked in the following order: (+)-catechin > (-)- epicatechin > Dimer B2 > Dimer B5 (Zhu et al., 2002). The vulnerability of catechin in alkaline conditions, specifically in the duodenum, can be due to auto-oxidation of 3', 4' and 5' hydroxyl groups (Aditya, Aditya, Yang, Kim, Park, Lee, et al., 2015). Interestingly, the degradation of catechins under digestive conditions appears to be most directly correlated to pH rather than to digestive enzyme activity (Bouayed et al., 2012). Besides, some authors have reported that (-)-epicatechin and (+)-catechin undergo epimerization to yield (-)-catechin and (+)-epicatechin (Zhu et al., 2002).

In the light of above mentioned, it is expected that after the intake and digestion of procyanidins, they would be hydrolyzed into minor compounds (Kumar & Pandey, 2013), metabolized by microbiota and absorbed in the form of small molecules exerting an antioxidant action (Watson et al., 2012). However, the literature is not completely clear in this case. For instance, Shoji, et al., (2006) reported that monomers and procyanidins dimer to pentamer were detected in the plasma ranging from 14.1 – 3.1 % of initial concentration, together with degradation of trimers and tetramer into catechin and dimer (José Jara-Palacios et al., 2018), and transformation of glycosides to aglycone forms under acidic conditions (Ortega et al., 2009). But other studies reported that the

efficiency of absorption of glycosides polyphenols in the small intestine was higher than that of the aglycone itself (Lei Chen et al., 2018; Petersen et al., 2016).

Table 12. Summary of *in vivo* and *in vitro* bioavailability and bioaccessibility of catechins and procyanidins.

Compound	Food Matrix	Digestion type	Dose	Plasma C_{max} or Bioaccessibility	Main plasma analytes	Ref.
EC Among others	Red grape pomance drink	*In vivo*	625 $mg_{ext}mL^{-1}$	6.8 nM 135.5 nM 87.0 – 94.9 nM 12.6 nM	EC-glucuronide-sulphate EC-glucuronide EC-sulphate Methyl-EC-sulphate	(Castello et al., 2018)
EC Dimer B1 Dimer B2…	Grape seed extract	*In vivo* (rats)	60 $mgkg_{bw}^{-1}$	14.3 nM 4.2 nM 3.6 nM	Nonconjugated epicatechin metabolites	(Pereira-Caro et al., 2018)
Gallic acid Quercetin	Triplaris gardneriana seeds	*In vivo* (rats)	25/200 $mLkg_{bw}^{-1}$	32.6% 50.9%	Gallic acid derivate Catechin Monogalloylated procyanidin dimer B Digalloylated procyanidin B 5-*O*-galloylquinic acid Monogalloylglucose Methyl gallate	(Neto et al., 2017)
EC C Dimer Trimer Tetramer	Standards	*In vitro* (IEC-18 cells)	50 – 300 μgmL^{-1}	1.7 nM 0.1 nM 34.5 – 69.1 pM 5.8 – 346.0 pM 0.1 – 0.13 pM	-	(Mendoza-Wilson et al., 2016)
Gallic acid C EC EGC EGCG Dimer Trimer Quercetin	Wine	*In vivo* (humans)	100/82.3 $mLkg_{bw}^{-1}$	76.8 nM n.d. 62.5 nM 63.4 nM n.d. 45.9 nM 17.9 nM 24.1 nM 16.7 nM	Gallic acid sulphate Gallic acid glucoronide C-sulphate EC-sulphate C-glucuronide EC-glucuronide	(Motilva et al., 2016)

Among others				8.3 nM	Methyl-C-sulphate Methyl-EC-sulphate Methyl-C-glucuronide Methyl-EC-glucuronide	
C EC EGC EGCG Dimer Trimer Quercetin Among others	Nano-encapsulated Wine phenol-enriched*	*In vivo* (humans)	100/82.3 mLkg_{bw}^{-1}	68.0 nM 64.4 nM 4.6 nM 63.4 nM 10.8 nM 15.2 nM 24.4 nM 23.3 nM	C-sulphate EC-sulphate C-glucuronide EC-glucuronide Methyl-C-sulphate Methyl-EC-sulphate Methyl-C-glucuronide Methyl-EC-glucuronide	(Motilva et al., 2016)

Compound	**Food Matrix**	**Digestion type**	**Dose**	**Plasma C_{max} or Bioaccessibility**	**Main plasma analytes**	**Ref.**
Catechin-curcumin	W/O/W	*In vitro*	2.5 mLW/O/W	58-70 %	-	(Aditya, Aditya, Yang, Kim, Park, Lee, et al., 2015)
GC EGC C EGCG EC GCG ECG CG	Green Tea	*In vitro* (Caco-2 cells)	50 mg_{ext}	6.2% 43.6 % 9.1 % 6.1 % 8.2 % 8.7 % 8.4 % 14.2 %	-	(Shim et al., 2012)
C EC Dimer B1 Dimer B2	Cocoa extract	*In vitro* (pig fecal microflora)	1/60 gmL^{-1}	0.02 mgg^{-1} 0.03 mgg^{-1} n.d. 0.01 mgg^{-1}	3-Hydroxyphenylpropionic acid 3-hydroxyphenylacetic acid	(Foglian o et al., 2011)

Catechin Epicatechin Dimer Trimer	Standards	*In vivo* (rats)	-	0.85µM 1.28 µM 2.40 µM 8.55 µM	3,4-dihydroxybenzoic acid Conjugated-EC C- and EC-glucuronide C- and EC-methyl-glucuronide C- and EC-methyl-sulphate	(Serra et al., 2009)
EC Dimer Trimer Tetramer Pentamer	Cocoa Liquor	*In vitro*	1/10 gmL^{-1}	397.1 % 417.8 % 441.5 % 96.6 % 7.8 % n.d.	Chlorogenic acid Vanillic acid Kaempferol-glycoside Quercetin-arabinoside Dihydroxykaempferol Epigallocatechin	(Ortega et al., 2009)
C EC Dimer Trimer Tetramer Pentamer	Cocoa Powder	*In vitro*	1/10 gmL^{-1}	88.2 % 92.5 % 254.7 % 295.2 % n.d.	Hydroxybenzoic acid Vanillic acid Kaempferol-glycoside Quercetin-arabinoside Quercetin-glycoside Epigallocatechin	(Ortega et al., 2009)
C EC Dimer B2 Dime B5	Cocoa	*In vitro*	1 mM	15.8%. n.d. n.d. n.d.	-	(Zhu et al., 2002)
Epicatechin	Chocolate	*In vivo* (humans)	80 /67 *g* per kg_{bw}	355 nM	-	(Wang et al., 2000)
Epicatechin Theobromine	Chocolate	*In vivo* (human)	40-80 / 75 *g* per kg_{bw}	0.4 – 0.7 µM 34 – 63 µM	-	(Richelle et al., 1999)

Moreover, procyanidins were no hydrolyzed into bioavailable monomers (Donovan et al., 2007) and conjugated or methylated forms of procyanidins were not evident (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009). Indeed, proanthocyanidins *per se* had very limited bioavailability as only trace amounts of dimers could be accumulated in plasma due to low apical and basolateral permeation values of procyanidins (DP 2 to 6), which are between $0.6 – 6\times10^{-7}$ cm/s, confirming their low absorption (Serra et al., 2009; Zumdick, Deters, & Hensel, 2012). In summary, better understanding of bioaccessibility, bioavailability, and absorption is indispensable for investigating the biological health effect of polyphenols, whatever the approach used (Lei Chen et al., 2018). In this regard, evaluation of *in vitro* bioaccessibility of procyanidins and cocoa extract can be found in **Chapter 6 and 7**.

1.5.1. Antioxidant activity of the bioaccessible compound Substances able to inhibit or retard oxidation are referred to as antioxidants (Wasowicz et al., 2004). For plant extracts, due to the high diversity of polyphenol sub-groups, it is suggested to evaluate the antioxidant activity as well as antiradical activity employing different assays such as 2,2-diphenylpicrylhydrazyl (DPPH), ABTS (2,2-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid), ferric reducing antioxidant power (FRAP), and oxygen radical absorbance capacity (ORAC), which are inexpensive, fast and straightforward (Alam, Bristi, & Rafiquzzaman, 2013; Z. Wu, Teng, Huang, Xia, & Wei, 2015).

As state above, recent applications of delivery systems for polyphenols improve bioaccessibility, bioavailability and biological activity of those compounds. Therefore, knowledge concerning the antioxidant activity of digested compound or transformed products is of high interest. However, studies focusing on the antioxidant activity of phenolic compounds before and during the gastrointestinal tract are scared and need further research. Indeed, data should be treated carefully due to the possible effect of many factors: nature of sample, food matrix effect,

interaction protein-polyphenols, antioxidant method tested, among others. For instance, José Jara-Palacios et al., (2018) showed that after the intake of seed pomace extract, the DPPH$^{\bullet}$ scavenging activity was significantly reduced during the transit time, while by using ORAC the opposite was observed. Variability for ABTS was also confirmed by Z. Wu et al., (2015) who investigated different tea products and found higher scavenging activity at the end of digestion for Liubao tea and black tea than for green tea. Comparison between gallic acid and quercetin after digestion indicated that gallic acid maintained their DPPH and FRAP values after digestion whereas quercetin was 3.4- and 15.5-fold lower for DPPH and FRAP (Neto et al., 2017).

Differences among antioxidant magnitude could be produced as a consequence of the formation of unknown compounds that impact the plasma antioxidant or the activity within the intestine in different levels (Donovan et al., 2007). Other studies show that when plasma is subjected to an *in vitro* oxidation, epicatechin and related catechins can prevent α-tocopherol depletion, acting as antioxidants of intermediate reactivity between ascorbate and α-tocopherol (Rein et al., 2000). Indeed, consumption of procyanidin-rich chocolate increased TBARS in plasma in a dose-dependent manner (Wang et al., 2000). Overall, polyphenols have demonstrated in *in vitro* and *in vivo* models antioxidant defense of the body (Belščak-Cvitanović et al., 2018), which is supported by epidemiological evidence such as the protective effect of polyphenols on cardiovascular diseases (Kim, Sung, & Kim, 2003; Jara Pérez-Jiménez et al., 2008; Tangney & Rasmussen, 2013; Zern et al., 2005) and remarkable increase in the resistance of blood plasma against oxidative stress markers (Birben et al., 2012; Koga et al., 1999; Zern et al., 2005).

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CHAPTER 1

INSIGHT OF POLYPHENOL OXIDASE ENZYME INHIBITION AND TOTAL POLYPHENOL RECOVERY FROM COCOA BEANS

ABSTRACT

A full factorial design (ascorbic acid/ L-cysteine inhibitors, temperature, and time as factors) has been studied to enhance inhibition of polyphenol oxidase (PPO) activity without decreasing cocoa polyphenols concentration. Data obtained have been modelled through a new equation, represented by Γ, which correlates both high polyphenol content with reduced specific PPO activity. At optimized values (70 mM inhibitory solution at 96 ºC for 6.4 min, Γ=11.6), 93.3% PPO inhibition and total polyphenol of 94.9 mgGAE/g were obtained. In addition, microscopy images confirmed the changes in cell morphology measured as the fractal dimension and explained the possible cell lysis and denaturation as a result of heat treatment and chemical inhibitors. Results also showed that PPO enzyme was most suitable (higher v_{max}/K_m ratio) for catechol with a reduction of its affinity of 13.7–fold after the inhibition heat treatment. Overall, this work proposed a suitable and food-safe procedure for obtaining enriched polyphenol extract with low enzyme activity.

Keywords: Polyphenol oxidase; cocoa; polyphenols; heat treatment; enzyme inactivation.

2. INSIGHT OF POLYPHENOL OXIDASE ENZYME INHIBITION AND TOTAL POLYPHENOL RECOVERY FROM COCOA BEANS

2.1. INTRODUCTION

Precursors of chocolate flavor are usually obtained through enzymatic and non-enzymatic reactions, in which polyphenol oxidase, invertase, and protease are the most important enzymes (Macedo, Rocha, Ribeiro, Soares, & Bispo, 2016). Polyphenol oxidase (PPO) is a major copper enzyme (Doğru & Erat, 2012), also known as catechol oxidase, tyrosinase, and so forth (de Oliveira Carvalho & Orlanda, 2017), and is the most important deteriorative enzyme that accelerate oxidation and degradation of polyphenols and their derivatives (Li, Chen, Zhang, & Fu, 2017). PPO is located in the chloroplasts (Araji et al., 2014; Osuga, Van Der Schaaf, & Whitaker, 1994) and its activation takes places during cell-damaging treatment (e.g. slicing, cutting or pulping) (Misnawi, Selamat, Bakar, & Saari, 2002) where oxygen is available, and the local pH is not too acidic (Cheema & Sommerhalter, 2015), thus causing the formation of brown pigments (Doğru & Erat, 2012; Jesus, Leite, & Cristianini, 2018). In fact, the oxygen catalyzes the enzyme reaction where the monophenols forming *o*-diphenols (monophenolase activity), and then the oxidized substrate, reacts producing *o*-quinones (diphenolase activity) (De la Rosa, Alvarez-Parrilla, & González-Aguilar, 2009; He, Luo, & Chen, 2008).

The rate of enzymatic browning on food is governed by PPO action, which depends of its concentration, pH, temperature, amount of phenolic compounds, and oxygen availability (de Oliveira Carvalho & Orlanda, 2017; Mishra, Gautam, & Sharma, 2013), so having a different level of influence in the development of flavors, color and softening, which in turn is reflected in the loss of nutritional and quality value (De Leonardis, Angelico, Macciola, & Ceglie, 2013). For instance, Misnawi et al., (2002) reported that PPO of dried unfermented beans increases the

polyphenol oxidation rate of (–)-epicatechin, total polyphenols, and total anthocyanidins. PPO is also susceptible during fermentation stage, therefore, total and specific activities remaining in unfermented beans are reduced up to 1% and 9% of the original, respectively (Misnawi et al., 2002). Despite the strong inactivation of PPO during fermentation, it can be regenerated during the drying process –pH increase and uptake of O_2–, and the remaining PPO activity is sufficient to catalyze oxidation of phenolic compounds (Brito, García, & Amâncio, 2002; Mishra et al., 2013). Thereby, phenolic compounds content in cocoa is affected by several factors, not only by the genetic origin, geographical and environmental conditions but also by enzyme attack and processing conditions for chocolate production.

Polyphenolic compounds have been widely studied since they possess an array of nutraceutical properties for human health related to cardiovascular effects (Kruger, Davies, Myburgh, & Lecour, 2014), antioxidant activity (Schinella et al., 2010), anti-inflammatory response (Nakajima et al., 2017), antibactericidal effect (Karar, Pletzer, Jaiswal, Weingart, & Kuhnert, 2014), and biological applications (Marchese et al., 2014). As a result of all these functional bioactivities, enriched polyphenol extracts have gained greater attention. For instance, new products such as exGrape®SEED, Vitaflavan from grape seeds, enriched capsules with high amount of cocoa procyanidins (PCs), enriched dark chocolates bars (e.g. CocoaVia from Mars, and FlavaBars® by Flavanaturals), and Fulyzaq™ for antiretroviral-induced diarrhea (PCs consisting of polymers up to 30 from *Croton lechleri*; FDA-approval) are used as a food supplement. In this sense, research focusing on inactivation of enzymes without affecting the total polyphenol content deserves further investigation.

Furthermore, cocoa flavonoids are characterized to include OHs groups in *ortho* position which makes an excellent substrate for PPO. Indeed, secondary metabolites from cocoa beans

are alkaloids (e.g. caffeine, theobromine, and theophylline) and flavan-3-ols, which comprises between 0.05- 1.7 wt% (Alañón, Castle, Siswanto, Cifuentes-Gómez, & Spencer, 2016), and 12-18 wt.% (Lamuela-Raventós, Romero-Pérez, Andrés-Lacueva, & Tornero, 2005), respectively. The (–)-epicatechin constitutes the major monomeric flavanol, which also forms oligomeric and polymeric procyanidins of (epi)catechin units up to tridecamers (Pedan, Fischer, Bernath, Hühn, & Rohn, 2017).

Regarding inactivation of PPO and its relationship with polyphenols, it has been studied on apricots, apples, grapes, tea leaves, potatoes, lettuce, coffee, black raisins, anthocyanidins from strawberries, catechins, quercetin, shrimps, and others (De la Rosa et al., 2009; He et al., 2008). Several inactivation strategies such as reacting enzymes (Guerrero-Beltrán, Swanson, & Barbosa-Cánovas, 2005), reducing agents (e.g. removal of oxygen using chemical agents) (Doğru & Erat, 2012), changes on pH (Pizzocaro, Torreggiani, & Gilardi, 1993), and increasing temperature (Menon, Hii, Law, Suzannah, & Djaeni, 2015) have been tested. Reducing agents such as sulfites have been widely employed, but currently, their use have been restricted because of their adverse effects on human health (Ali, El-Gizawy, El-Bassiouny, & Saleh, 2015) Other antibrowning agents can be used, but only a limited number are considered acceptable in terms of safety and costs to control the enzymatic browning in foods or food products (C. Y. Lee & Whitaker, 1995).

L-cysteine and ascorbic acid are the most widely used inhibitor agents; ascorbic acid acts as an antioxidant reducing *o*-quinone back to the original phenol compound (Pizzocaro et al., 1993) while L-cysteine acts forming complexes with *o*-quinones, thereby, inhibiting secondary oxidation and polymerization reactions (Doğru & Erat, 2012). In addition, commission

regulation EU No. 1129/2011 approves the use of ascorbic acid (food additive E-300) and L-cysteine (food additive E-920) in foods.

Regarding the inhibition of PPO in cocoa beans, heat inactivation at temperatures ranging from 60 – 98 ºC, for a period ranging from 3 – 45 min have been previously assayed (Menon et al., 2015; Pons-Andreu, 2008). For instance, Pons-Andreu (2008) proposed an enzymatic treatment for cocoa nibs using a blanching process. However, heating is not a valid treatment to enhance long-term PPO inhibition, since the enzyme is high thermostable (Manzocco, Anese, & Nicoli, 2008). In addition, several works evaluate the change of enzyme activity by qualitative color assays (melanosis index scale) instead to measure the specific enzymatic activity (Pons-Andreu, 2008; Yuan, Lv, Tang, Zhang, & Sun, 2016).

Furthermore, many questions remain unsolved concerning the inactivation process. None of the above-mentioned studies investigated the optimal temperature, time of heat treatment, type and concentration of chemical inhibitors to enhance lower enzyme activity in cocoa beans. To our knowledge, the relationship between the total polyphenol content during the PPO denaturation and how this affects the antioxidant capacity and the bioactive properties of cocoa beans has not been studied yet.

Therefore, the aims of this work were to: (a) determine the conditions to inhibit the PPO in cocoa beans while maintaining a high level of polyphenols; to do so, concentration of inhibitor (ascorbic acid and L-cysteine), temperature, and time were evaluated; (b) develop an equation showing the relationship between PPO inactivation and polyphenols content, and (c) investigate the enzyme kinetic parameters and their affinity to PPO using catechol, (+)-catechin, and (–)-epicatechin as substrates.

2.2. MATERIALS AND METHODS

2.2.1. Reagents All the chemicals used were analytical reagent grade and were not purified further. Folin-Ciocalteu reagent, gallic acid, sodium carbonate, catechol, bovine serum albumin, ascorbic acid, L-cysteine, sodium phosphate dibasic, citric acid, poly(vinylpyrrolidone) (PVP), and Coomassie brilliant blue G-250 dye were obtained from Sigma Aldrich (St. Louis, MO, USA). (+)-Catechin (≥ 99%), and (–)-epicatechin (≥ 99%) were purchased from ChromaDex Inc. (Irvine, CA, USA). Acetonitrile (HPLC-grade), ethanol (analytical-grade), and formic acid were acquired from Merck (Merck, Germany). Deionized water (18 $M\Omega cm^{-1}$) from Aqua Solution system (Aqua solution, Inc. Jasper, Georgia, USA) was used for the preparation of all solutions.

2.2.2. Recovery of cocoa polyphenol extract Fresh cocoa pods (Trinitary, clone ICS 39) were collected at Villa Santa Monica (San Vicente de Chucurí, Santander, Colombia) and immediately protected from light and transported on ice to CICTA Lab for processing. Cocoa pods are mainly composed of cocoa husk, cocoa beans, and mucilage. Thus, the cocoa beans were removed manually using a knife and immediately the beans surrounded by mucilage were removed using a mucilage remover (homemade). After that, the beans were immediately inactivated and used for further analysis.

2.2.3. Enzyme inhibition The inhibition of PPO enzyme in cocoa beans was enhanced by heat treatment. The samples were dipped in an aqueous inhibitory solution consisting of ascorbic acid and L-cysteine (1:1 *v/v* ratio) at same equimolar concentration. The assays were carried out as follow: 10 beans (ca. 25 g wet weight at ca. 4 ºC) were immersed into 200 mL of inhibitory solution at different concentrations (0 – 50 mM), time (1 – 5 min) and temperature (70 – 90 ºC) in accordance with the combinations of surface design 2^3 that includes four replicates central point and start points (Table 1). Immediately after that, the samples were cooled in ice water for 30 min,

and then rinsed (x3) again with deionized water (4ºC) to remove traces of ascorbic acid, and L-cysteine. Non-treated sample (fresh unfermented cocoa bean) was kept as control.

2.2.4. PPO enzyme extraction The treated and non-treated beans were chopped in small pieces and homogenized. The enzyme extraction was according to Babu et al., (2008) with few modifications. Briefly, the chopped pieces were homogenized in cold extraction buffer (ratio 1:1.5 w/v, 0.01 M McIIvaine citric phosphate, pH 6.5, containing 1% PVP) during 2 min at max speed (Vortex reax control, Heidolph, Germany) and filtered by Whatman Nº1 filter paper (Whatman, Inc., New Jersey, USA). Homogenates were centrifuged (Heraeus, Megafuge 16R, Thermo Scientific, Waltham, MA, USA) at 10000 *g*, 4 ºC for 20 min. Resulting supernatant, called crude enzyme extract, was filtered again and used for further experiments.

2.2.5. PPO enzyme activity measurement The enzyme activity (U_{PPO}) was determined spectrophotometrically according to Pizzocaro et al., (1993) The reaction mixture containing 1.0 mL of catechol solution (0.175 M) and 2.0 mL of Mcllvaine buffer pH 6.5 were added to 0.5 mL crude enzyme extract. The increase in absorbance at 420 nm (Genesys 20; Thermo Scientific-Fisher, Waltham, MA, USA) was recorded at intervals of 15 s up to 5 min at room temperature. The PPO activity was calculated by the slope of the linear portion of the curve absorbance vs. time. The enzyme activity corresponding to one unit of PPO activity was defined as the 0.001 unit change in absorbance per minute at 420 nm per mL of enzyme assay solution mixture.

The protein content of specific activity was measured according to the method described by Bradford (1976) Bovine serum albumin (BSA) was used as a standard (0 – 0.125 mg/mL) (r^2= 0.999). The specific activity was expressed as one unit of enzyme activity per one-unit mg^{-1} of BSA protein (U_{PPO}/mg).

The percent of total inhibition was calculated as follow (Eq. 1):

$$\text{Inhibition (\%)} = \frac{\text{Control-Test}_i}{\text{Control}} * 100 \quad (1)$$

where *i* is the number of the treatment according to the design. Control and $test_i$ were expressed as the amount of enzyme specific activity (U_{PPO}/mg).

2.2.6. Substrate kinetic constants of PPO The evaluation of inhibition constant was assayed using catechol, (+)-catechin and (–)-epicatechin (main catechins in cocoa) as substrate (5 – 200 mM) at optimal temperature for PPO activity, that is, 35˚C as previously reported in the literature (de Oliveira Carvalho & Orlanda, 2017; Siddiq & Dolan, 2017). The reaction was modeled using the Michaelis-Menten equation (Eq. 2). The K_m value and maximum velocity v_{max} were determined using a nonlinear regression by GraphPad Prism v. 6.0 (GraphPad Soft. Inc., La Jolla, California, US).

$$v = \frac{v_{max} * [s]}{K_m + [s]} \quad (2)$$

2.2.7. Recovery of total phenol content Recovery of phenolic compounds from non-treated cocoa beans (control sample) and beans remaining after the PPO inhibition treatment was determined as follows: beans were freeze-dried (Labconco Corp., Kansas City, MO, USA) for a final humidity < 4 % (AOAC method 931.04, 1990), milled and homogenized (Grindomix GM 200, Retsch GmbH & Co., Germany). The extraction was carried out as follows: 1 g of sample was added to 60 mL of a mixture of 50 % ethanol/water (w/w) at 50 ºC, 300 rpm for 30 min using a magnetic stirrer hotplate (IKA C-MAG HS7. Germany) and thermocouple (IKA ETS-D5, Germany). The resulting extract was centrifuged (5000 *g*, 4 ºC, 20 min); then the supernatant was filtered through 0.45 µm hydrophilic PTFE filter (Millipore, Milford, MA, USA), and immediately analyzed.

2.2.8. Determination of total polyphenol content The total polyphenol content of the sample was assayed using Folin-Ciocalteu reagent according to Singleton et al., (1998) with modifications as follows: the reaction was initiated by the addition of 50 µL of the sample with 1.5 mL of 10-fold diluted Folin-Ciocalteu reagent. After 5 min, 1.5 mL of 7.5% (w/v) sodium carbonate was added and vortexed for 10 s. The reaction medium was stored in the dark for 1 h at 25 ºC. Absorbance was measured at 765 nm (Genesys 20; Thermo Scientific-Fisher, Waltham, MA, USA) against a blank sample. A gallic acid calibration curve was prepared with 0.05-1.0 mg/mL (r^2= 0.999). Results of total polyphenols amount were expressed as mg gallic acid equivalents by gram of dried cocoa beans (mg GAE/g).

2.2.9. Chromatographic analysis by HPLC-DAD The reverse phase conditions and stationary phase were optimized to detect both catechins and procyanidins into the cocoa extract. Briefly, LC was assayed on a Shimadzu (LC-2030 LT Series-i, USA) and the separation was achieved using a C_{18}-phenyl column (4.6 × 50 mm, 2.5µm) (Xbridge, Waters, US) protected with a security guard from Phenomenex (AJ0-8788, Phenomenex, Torrance, CA). The procedure consisted of water/formic acid (99.99/0.01 v/v) (solvent A), and acetonitrile/formic acid (99.99/0.01 v/v) (solvent B). The linear gradient was as follows: 0-8 min, 2% B; 8 - 37 min, 10% B; 37- 40 min, 0% B and re-equilibrium for 10 min. The flow rate, column temperature, and diode array were 0.8 mL/min, 60 ºC, and 280 nm respectively.

Identification of both catechins and procyanidins were carried out by Ion Trap LC/MS (model 6320, Agilent Technologies, Waldbronn, Germany) equipped with an ESI source and ion trap mass analyzer, which was controlled by the 6300 series trap control software (Bruker Daltonik GmbH, V. 6.2). The mass spectrometer was operated under negative ESI mode with the following conditions: mass spectra recorded from 90 - 2200 *m/z*, nebulizer 40 psi, dry gas 12

L/min and dry temperature 350 ºC. Target compounds consisted of $[M-H]^-$ ions at *m/z* 289, 577, 865, 1153, 1442, and 1730 that correspond to monomers, dimers, trimers, tetramers, pentamers, and hexamers procyanidins structures, respectively.

2.2.10. Scanning electron microscopy and image analysis Scanning electron microscopy (SEM) was additionally used to evaluate the microstructure of a) non-treated cocoa beans, and b) cocoa beans with reduced PPO activity. Beans were cut into longitudinal and transversal sections with the objective to observe their microstructure. Sections were mounted on aluminum stubs with double-sided carbon adhesive tape and observed using the XL-30 Environmental Scanning Electron Microscope (Philips, USA) at 25 kV accelerating voltage with the BSE (backscattered electron) detector. The images were acquired in grayscale and stored in TIFF format at 712 x 484 pixels with brightness values between 0 and 255 for each pixel constituting the image.

A generalization of the box-counting method to evaluate the fractal dimension of the images (FDt) for four different images at the same magnification (1000x) was used. Therefore, the shifting differential box-counting method (SDBC) (Hernández-Carrión, Hernando, Sotelo-Díaz, Quintanilla-Carvajal, & Quiles, 2015) to evaluate the fractal dimension of texture of SEM images using the ImageJ 1.34 software, was carried out.

2.2.11. Statistical Analysis All measurements were repeated at least three times. Statistical analysis was done using Statistica v. 7.1 (Stat-Soft Inc., USA). The analysis of variance (ANOVA) and p-test were used to evaluate the influence of the factors and their interactions on the experimental design. ANOVA one-way and Tukey´s multiple range test at a 5% level of significance was also evaluated. The response surface methodology consisting of full factorial central composite rotatable design with four replicates at the central point was conducted according

to a completely randomized model. A second-order polynomial equation was used to fit the experimental data, as follow (Eq.3):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j \quad (3)$$

where *Y* is the predicted factor, β_0 is the value of the fitted response to the design, β_i, β_{ii}, and β_{ij} are the coefficients of linear, quadratic, and cross product terms, respectively.

2.2.12. Verification of the model R-squared coefficient of full factorial central composite design was measured. In addition, experimental runs were randomized to evaluate the concordance of experimental data and predicted values, therefore, the root mean squared error (RMSE, Eq. 4) was used, as follows:

$$RMSE = \sqrt[2]{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n}} \qquad (4)$$

where y_i and $\hat{y}_i$ is the measured value and predicted value by the model, respectively. And *n* is the number of the set data.

2.3. RESULTS

2.3.1. Preliminary inhibition assays Prior to optimizing the inhibition of PPO enzyme from cocoa beans, the following parameters were evaluated: (a) the nature of inhibitor, and (b) the size of the cocoa beans. Thus, the PPO activity as a function of different inhibitory agents was determined with a solution containing 1% (w/w) ascorbic acid, 1% (w/w) L-cysteine, and mixture of ascorbic acid/L-cysteine (1:1 ratio, 1% w/w,) using heat treatment at 90 ºC for 5 min as previously reported by Menon et al., (2015). At concentration >1% inhibitors behave as quinone

reducer similar to sulfides (Ali et al., 2015). The results showed that highest denaturation of the enzyme was enhanced by a mixture of ascorbic acid/L-cysteine (79.3 %) followed by ascorbic acid (72.8 %) and L-cysteine (67.5 %).

In addition, two sizes of cocoa beans consisting of (S_1) chopped cocoa bean (cross section of 1×0.5 cm^2), and (S_2) whole cocoa beans, were also evaluated. Results showed that S_1 treatment inhibited the PPO in 1.2 –fold higher than S_2. Interestingly, inhibition solution color after heat treatment (control system) was translucid-yellow, which was quite similar to S_2 treatment. However, a violet color in the waste solution of S_1 treatment was observed and could be as a consequence to the greater surface contact, thus facilitating the release of polyphenols to the waste solution. Indeed, analysis by HPLC-DAD-ESI-MS/MS showed that loss of catechins and procyanidins (up to hexamers) on S_1 and S_2 treatments were 0.5 and 1.2 wt.%, and 8 and 22 wt.%, respectively.

Hence, the maximization of the inactivation of PPO was carried out using whole cocoa beans, together with combination of ascorbic acid/L-cysteine at same equimolar concentration.

2.3.2. Influence of inhibition parameters on PPO activity The extent of PPO inhibition as a function of treatment time, temperature and inhibitor concentration is summarized in Table 1. In addition, the recovery of total polyphenol content for each assay was evaluated. As can be seen in Fig. S1, a non-linear relationship could be observed between values of enzyme inactivation and concentration of polyphenols (r^2 = 0.60). To better understand the relationship between the two response variables, several models such as quadratic (r^2 = 0.61), exponential (r^2 = 0.56), and logarithmic (r^2 = 0.59) equations were evaluated; however, none of them was able to describe the data adequately. Furthermore, a new equation (Eq. 5) which correlates high polyphenol content

with reduced PPO activity –expressed in percentage– in an inverse relationship, was established as follows:

$$\Gamma_i = \frac{\text{Total Polyphenol}_i\ (\%)}{\text{100-PPO Inhibition}_i\ (\%)} \quad (5)$$

where Γ –represented by the Greek uppercase letter– means the high polyphenol content with low enzyme activity as a function of % Total polyphenol recovered and % PPO inhibition; *i* is the number of the treatment according to the design.

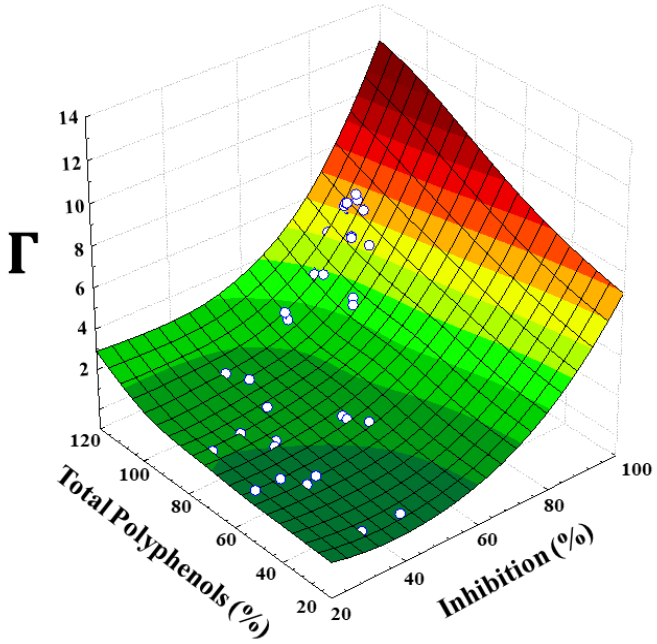

Figure 1. 3D scheme for the correlation Γ as a function of PPO inhibition (%) and total polyphenol content (%) from cocoa beans. See equation 5.

Figure 1 shows the experimental data adjusted according to our proposed model (Eq. 5). Plotting of Γ as a function of total polyphenol (%) or inactivation (%) had a good adjustment of r^2 equal to 0.91, and 0.92, respectively (Figure S2). Based on the new response variable, Γ, typical exponential profile was observed, in other words, Γ increased by increasing the enzyme

inhibition and polyphenol content until saturation value was reached. We hypothesized that heat treatment not only allowed to break down the enzyme-substrate complex but also to cause softening of the cell, thereby, increased the extraction yield of polyphenols.

2.3.3 Effect of temperature, time and concentration of inhibitor on PPO activity Analysis of variance shows that the selected quadratic model adequately represented the data obtained for Γ with a good coefficient of multiple determination of r^2adj = 0.85. (Table 2) and lower residual values (MS residuals equal to 0.748). Model's ability to accurately predict the data

Table 1. 2^3 full factorial surface design and experimental results for the inhibition of PPO enzyme and higher polyphenol content from cocoa beans

Run	T (ºC)	Time (min)	Inhibitor [mM]	Specific Activity [U_{PPO}/mg]	Total Polyphenol [mg GAE/g]	Γ*
1	90	1	50	8.97 ± 0.08	86.58 ± 2.53	3.74
2	70	5	0	18.48 ± 0.49	83.37 ± 1.45	1.68
3	63	3	25	8.40 ± 0.45	80.52 ± 3.21	3.45
4	90	1	0	20.42 ± 0.05	70.48 ± 0.90	1.05
5	90	5	0	12.30 ± 0.52	84.74 ± 4.65	2.61
6	80	3	67	6.86 ± 0.99	88.20 ± 3.43	5.07
7	80	3	25	15.05 ± 0.49	69.24 ± 1.22	1.36
8	80	3	25	18.42 ± 0.66	75.84 ± 1.34	1.38
9	80	1	25	20.08 ± 0.52	63.62 ± 4.50	0.81
10	70	1	0	21.11 ± 0.06	53.65 ± 2.32	0.41
11	70	1	50	21.15 ± 0.19	63.11 ± 1.01	0.75
12	80	6	25	5.60 ± 0.47	87.68 ± 3.21	6.14
13	70	5	50	5.17 ± 0.82	85.08 ± 2.21	6.27
14	80	3	25	14.03 ± 0.70	66.52 ±2.56	1.31
15	90	5	0	12.04 ± 0.41	85.58 ± 6.65	2.72
16	97	3	25	5.09 ± 0.36	86.32 ± 3.27	6.55
17	90	5	50	6.22 ± 0.90	85.21 ± 4.05	5.23
18	97	3	25	5.00 ± 0.31	86.73 ± 2.41	6.74
19	80	3	25	14.94 ± 0.07	68.57 ± 4.10	1.34
20	70	5	50	5.50 ± 1.14	87.67 ± 1.43	6.25

21	80	3	0	20.01 ± 0.23	71.01 ± 1.32	1.09
22	80	1	25	22.16 ± 1.14	65.75 ± 0.53	0.81
23	63	3	25	8.69 ± 0.60	79.99 ± 4.32	3.29
24	90	5	50	6.30 ± 0.41	85.15 ± 4.21	5.15
25	80	6	25	5.43 ± 0.16	87.50 ± 5.01	6.30
26	70	1	0	18.09 ± 0.04	53.24 ± 2.45	0.46
27	80	3	0	23.99 ± 0.79	74.21 ± 1.23	1.01
28	90	1	50	8.62 ± 0.86	85.60 ± 3.02	3.81
29	90	1	0	21.36 ± 0.43	74.44 ± 2.03	1.14
30	70	1	50	24.22 ± 0.26	65.94 ± 2.01	0.74
31	80	3	67	5.93 ± 0.33	82.54 ± 2.98	5.14
32	70	5	0	17.54 ± 0.78	80.84 ± 3.89	1.67

* Γ calculated according to equation 5.

based on randomly selected experiments (n = 15) by comparing how close predictions are to the actual outcomes, was assessed. Therefore, RMSE was 0.388, which reinforced the good performance of the model.

Table 2. ANOVA for PPO inactivation through 2^3 surface design + central points+ start points. r^2= 0.8083; r^2adj = 0.8462.

Factor	SS	df	MS	p
T	8.3298	1	8.32985	0.003001
T^2	19.4339	1	19.43392	0.000042
t	49.5296	1	49.52962	0.000000
t^2	2.2011	1	2.20111	0.100515
Inh	35.4793	1	35.47934	0.000001
Inh^2	0.0185	1	0.01846	0.876667
T x t	3.5258	1	3.52580	0.041099
T x Inh	0.0242	1	0.02417	0.859066
t x Inh	4.2416	1	4.24158	0.026414
Error	16.4760	22	0.74891	

Total SS	150.9190	31		

where SS is the sum of square, df the degree of freedom, MS the mean square, p is the value of confidence interval, T is temperature, t is time, and Inh is Inhibitor.

In general, ANOVA and the analysis of surface response (Table 2, Figure 2) confirmed the dependence of higher PPO denaturation as a function of the linear and quadratic effect of temperature and the linear effect of time of treatment, and concentration of inhibitor (Eq. 6). Besides, interactions between temperature with time and time with inhibitor concentration were also significant ($p<0.05$). These can happen because (*i*) heat treatment affects the conformational change of the enzyme and protein-enzyme dissociation(P. M. Lee, Lee, & Karim, 1991) and (*ii*) the dose-dependent inhibitory effect.(Doğru & Erat, 2012; Siddiq & Dolan, 2017) A similar trend was also observed by De Oliveira and Orlanda(de Oliveira Carvalho & Orlanda, 2017) who found that the PPO enzyme was stable at temperature < 67 ºC, but greater denaturation of 90% could be enhanced at temperature > 85 ºC after 20 min (Siddiq & Dolan, 2017).

$$Y = 54.649\text{-}1.496T + 0.010\,T^2 + 1.758\,t + 0.009\text{Inh}\text{-}0.023\,T{*}t + 0.010\,t{*}\text{Inh} \quad (6)$$

Maximum temperature (35 ºC) for PPO activity (de Oliveira Carvalho & Orlanda, 2017; Siddiq & Dolan, 2017) non-treated cocoa sample had a total polyphenol content of 42.1 $mgGAEg^{-1}$, maximun PPO specific activity of 32.0 ± 0.2 U_{PPO} mg^{-1}, and protein amount of 68.5 ± 3.6 mg mL^{-1}, which was consistent with P. M. Lee et al., (1991) and Misnawi et al., (2002) with PPO specific activity of 52 and 75 U_{PPO} mg^{-1}, and total protein of 70 and 73 $mgmL^{-1}$, respectively.

Thereby, significative effect of temperature ($p<0.05$; Figure 2A and 2C) on the reduction of enzyme activity was obtained. For instance, heat treatment between 70 - 90 ºC reduced by 42.3 -

61.6 % of initial PPO specific activity. Regarding the length of treatment time (Figure 2B and 2C; Eq. 6), showed the largest positive linear regression coefficient, suggesting that this factor is critical to enhancing the reduction of PPO activity. Also, the effect of concentration of inhibitor (Figure 2A and 2B) was significant ($p<0.05$), indicating the role of ascorbic acid to act as an antioxidant reducing the initial *o*-quinone, and L-cysteine acting as a reducing agent interfering with PPO activity before they can polymerize to melanin, which is in line with de Oliveira Carvalho & Orlanda, (2017).

These findings reinforce the synergic effect of ascorbic acid and L-cysteine as an efficient solution to prevent enzymatic browning reactions. In fact, effective use of ascorbic acid and/or L-cysteine in combination with other compounds has been previously confirmed (Dudley & Hotchkiss, 1989; Siddiq & Dolan, 2017).

Based on our results, the optimum conditions to obtain the lowest enzymatic browning and highest total polyphenol content were achieved with 70 mM inhibitory solution at 96 ºC, 6.4 min for a predicted $\Gamma = 11.8$, which agreed with the experimental results obtained under these conditions that provided a $\Gamma = 11.6 \pm 2.7$, that is, 93.3 ± 2.1% PPO inhibition and total polyphenol of 94.9 ± 4.09 mgGAE/g (55.6% higher than non-treated samples). At these conditions, a long-term study showed that cocoa beans inactivated (stored at 4 ºC) maintain over 2 years their total phenol content (ca. 92 ± 3.2 mg GAE/g) and PPO activity (ca. 89 ± 3.8 %) with no significant change over time ($p<0.05$).

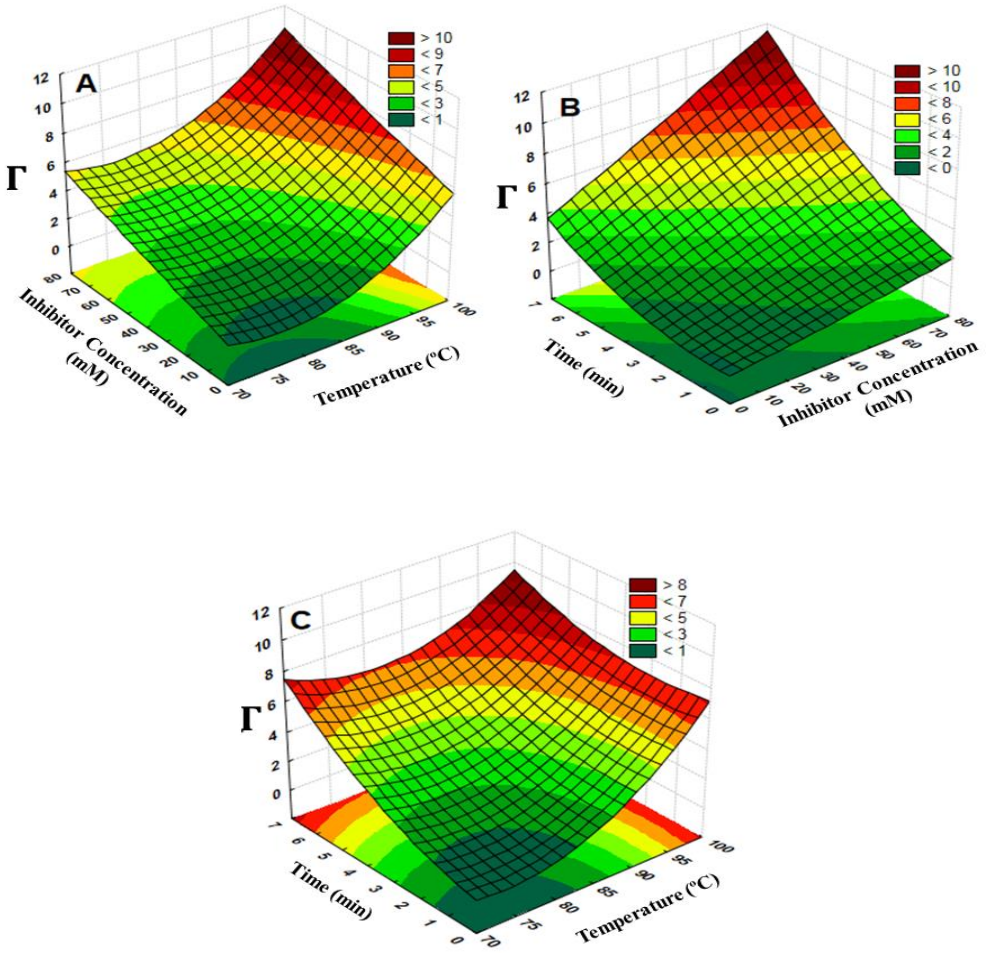

Figure 2. Surface response for the correlation of Γ with a) temperature (T) and concentration of inhibitor (Inh), b) time and Inh, c) t and T.

2.3.4. Kinetic parameters of PPO inhibition in cocoa beans The differences in PPO activity observed varying the substrate (catechol, (+)-catechin, and (–)-epicatechin) were determined in the enzyme kinetic parameters. As expected, all the substrates were oxidized displaying Michaelis-Menten kinetics (Cheema & Sommerhalter, 2015). The kinetic parameters calculated by nonlinear regression are summarized in Table 3. Regarding the catalytic power (v_{max}/K_m ratio), taken as an evaluation criterion, the enzyme seemed to be most suitable for small *o*-diphenol such as catechol (4440.98 U $mM^{-1}mL^{-1}min^{-1}$), followed by (–)-epicatechin (727.38 U $mM^{-1}mL^{-1}min^{-1}$), and (+)-

catechin (637.79 U $mM^{-1}mL^{-1}min^{-1}$). Indeed, higher affinity for catechol followed by catechin was also true by Doğru & Erat (2012).

Table 3. Michaelis-Menten kinetic parameters on different substrates as action of cocoa bean PPO.

Substrate	K_m **[mM]**	v_{max} **(U_{PPO} mL^{-1} min^{-1})**	v_{max}/K_m **(U_{PPO}/mM mL min)**
Catechol	0.6 ± 0.1^a	2709 ± 21.9^a	4440.9
Catechol*	8.4 ± 1.3^b	106.7 ± 3.7^b	12.9
Epicatechin	1.3 ± 0.4^a	916.5 ± 20.6^c	727.4
Catechin	1.5 ± 0.4^a	924.8 ± 19.0^c	637.8

*Substrate evaluated using PPO enzyme from inactivated cocoa beans at optimum conditions (70mM inhibitory solution at 96ºC for 6.4 min). Means within column sharing the same letter are not significantly different by Tukey ($p > 0.05$).

Affinity of PPO for catechins obtained in this study were quite similar to that reported by Wuyts et al., (2006) and Ho (1999) with K_m of 1.2 and 2.1 mM, respectively; as well as for (–)-epicatechin with K_m equal to 0.65 and 1.18 mM according to Liu et al., (2007) and Martinez-Cayuela et al., (1988) respectively. Kinetic constant for catechol was 0.61 mM, similar to the value ranged from 0.18 to 0.97 mM from cocoa pulp and beans (Macedo et al., 2016), respectively, but different from other plant samples with values ranged from 7.9×10^{-4} to 203.8 mM (Dincer, Colak, Aydin, Kadioglu, & Güner, 2002; Doğru & Erat, 2012; Siddiq & Dolan, 2017), which can be due to method of extraction, nature of the substrate, and method of measurement.

Additionally, denatured PPO enzyme recovered using the optimized variables (70 mM inhibitory solution at 96 ºC for 6.4 min) for the oxidation of catechol was also studied. Results showed that catechol had the lowest catalytic power with 12.76 U $mM^{-1}mL^{-1}min^{-1}$, therefore, its affinity for the substrate (K_m) was reduced by 92.8%. The lowest affinity of the enzyme could be as a consequence of the degradation of the protein site of the enzyme and/or morphological changes of the enzyme, which can be related to its low protein content (16.04 ± 3.96 mg mL^{-1}) and low specific activity (2.12 ± 0.65 U_{PPO} mg^{-1}) after the heat treatment, respectively.

Overall, these findings reinforced the high affinity of PPO for small *o*-diphenols, thermal enzyme denaturation, and also highlights the importance of inhibiting PPO activity for controlling the dramatical loss of polyphenols by the enzymatic action.

2.3.5. Microscopy analysis Evaluation of the effect of PPO inhibition on morphology and cell wall by scanning electron microscopy were carried out. As can be seen in Figure 3A, fresh sample (non-treated sample) maintains the cell walls, consisting of well oval- shaped, not fractured, solid and denser in appearance, and with the cellular content embedded in it (FDt= 2.542). Figure 3B clearly showed evident changes after the PPO inhibition which consisted of faster changes in temperature between 4 – 97 ºC for short times (1 – 6 min) with significant differences ($p < 0.05$) observed in the FDt values. Moreover, the cell walls become larger, smooth, fibrous, unfolded, and evidence of more interspace and holes in the microstructure (Figure 3B) as a result of the enzyme thermal denaturation or by the effect of heat shock proteins (FDt =2.872), which were consistent with Terefe et al., (2015) This observation reinforced that the heat treatment is a faster and robust method to change the conformational structure of PPO enzyme, thus reduce its activity in cocoa beans and increase the extraction yield of polyphenols.

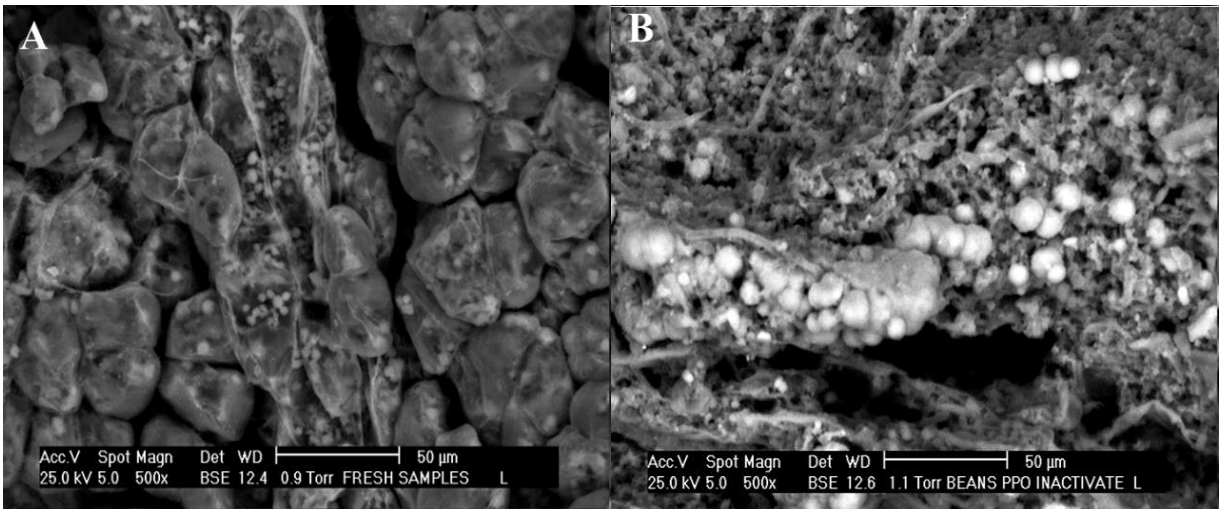

Figure 3. Microscopy images for the microstructure of A) non-treated sample (fresh cocoa bean), b) sample after optimal condition for the inhibition of PPO enzyme.

2.4. CONCLUSIONS

In this study, experimental conditions to inhibit the action of PPO enzyme in terms of the specific activity of PPO and total polyphenol content were optimized. Our study has reported, for the first time, an equation that correlates both high recovery of total polyphenolic and high inhibition of PPO enzyme from cocoa beans. Confirmation of heat-denaturation during inaactivation process by SEM images and high affinity of PPO for small *o*-diphenols, especially for catechol, followed by (–)-epicatechin have also been studied. This works provides a promising, robust, easier, and food-safe procedure for obtaining enriched polyphenol extract with longer oxidative enzyme stability.

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2.6. SUPPLEMENTARY MATERIAL

Figure S1: Relationship between specific activity of PPO and total polyphenol content on cocoa beans.

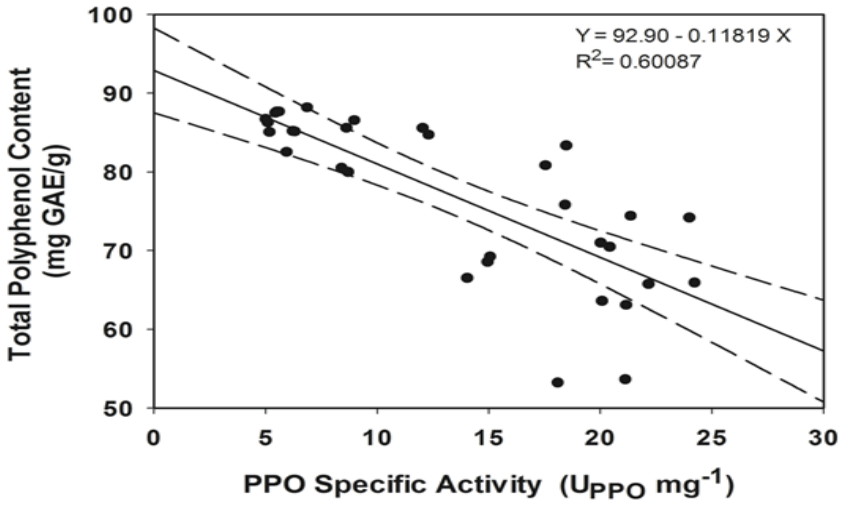

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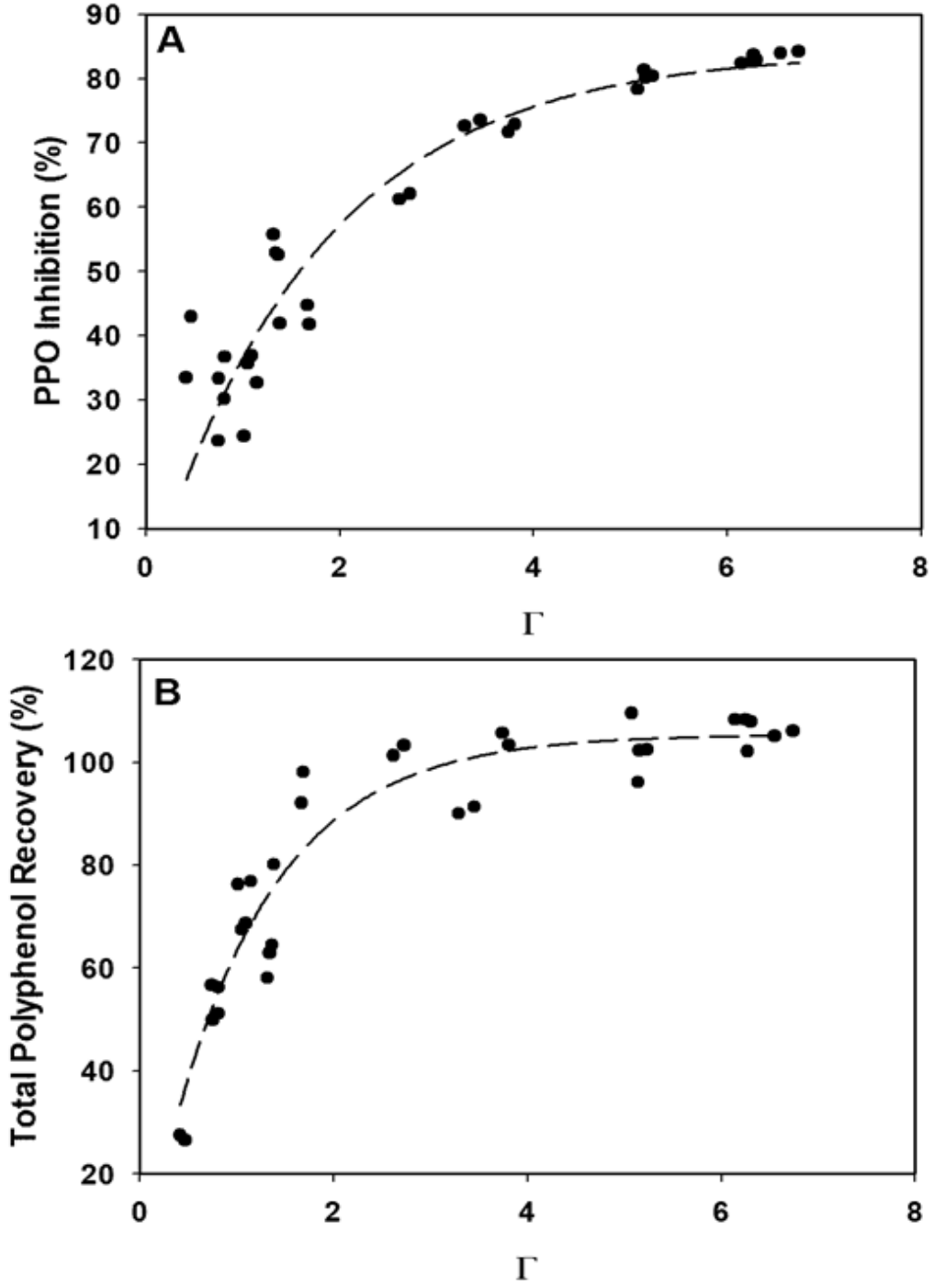
Figure S2: Correlation Γ (See Eq. 5) as a function a) PPO inhibition (%), and b) Total polyphenol (%) on cocoa beans.


A
PPO Inhibition (%)
90
80
70
60
50
40
30
20
10
0
2
4
6
8
Γ
B
Total Polyphenol Recovery (%)
120
100
80
60
40
20
0
2
4
6
8
Γ

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CHAPTER 2

FOOD-SAFE PROCESS FOR HIGH RECOVERY OF FLAVONOIDS FROM COCOA BEANS: ANTIOXIDANT AND HPLC-DAD-ESI-MS/MS ANALYSIS

ABSTRACT

Considering the increasing interest in the incorporation of natural antioxidants to enriched foods, this work aimed to establish a cheap, food-grade and suitable procedure for the extraction of polyphenols from cocoa beans avoiding the degreasing process. Results showed that ultrasound for 30 min with particle sample size < 0.18 mm impacts the microstructure of the cell, thus increasing the diffusion pathway of polyphenols and avoiding the degreasing process. Moreover, the effect of temperature, pH, and concentration of ethanol and solute on a solid-liquid extraction of polyphenols was evaluated. Through a 2^4 full factorial design, a maximum recovery of 122.34 ± 2.35 mg GAE/g, 88.87 ± 0.78 mg ECE/g, and 62.57 ± 3.37 mg ECE/g, for total content of polyphenols (TP), flavonoids (TF), and flavan-3-ols (TF3), respectively, was obtained. Analysis by HPLC-DAD-ESI-MS/MS showed that our process allowed a high concentration of methylxanthines (10.43 mg/g), catechins (7.92 mg/g), and procyanidins (34.0 mg/g) with a degree of polymerization > 7, as well as high antioxidant activity determined by Oxygen Radical Absorbance Capacity (1149.85 ± 25.10 uM Trolox/g) and 2,2-diphenyl-1-picrylhydrazyl (DPPH radicals, 120.60 ± 0.50 uM Trolox/g). Evaluation of several mathematical models (nth order, Page, Peleg, Weibull-type, Mincher and Minkov, and pseudo first order) to describe the kinetics of the solid-liquid extraction process was also carried out. Results showed a good agreement ($r \geq 0.98$) with the experimental results for the sorption/desorption model indicating an equilibrium time for recovery of TP, TF, and TF3 equal to 45, 39, and 34 min, respectively.

Keywords: Cocoa, polyphenols, solid-liquid kinetic extraction, antioxidants

3. FOOD-SAFE PROCESS FOR HIGH RECOVERY OF FLAVONOIDS FROM COCOA BEANS: ANTIOXIDANT AND HPLC-DAD-ESI-MS/MS ANALYSIS

3.1. INTRODUCTION

Flavanols are the most abundant compounds of polyphenols found in cocoa, with a degree of polymerization ranging from monomers to polymeric proanthocyanidins (Crozier & Hurst, 2013; Pedan, Fischer, & Rohn, 2016). Unfermented cocoa beans are composed of 1.3 - 3.3% methylxanthines (Batista, de Andrade, Ramos, Dias, & Schwan, 2016) and about 6% of condensed flavan-3-ols (Carrillo, Londoño-Londoño, & Gil, 2014; Pedan et al., 2016; Toro-Uribe, López-Giraldo, & Decker, 2018), therefore, they have been listed as the 4th richest dietary source of polyphenols (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). Cocoa is one of the top crops in Colombia in terms of economic impact, with a national production record of 60,535 tons in 2017 (FEDECACAO, 2018). There is also a government initiative to switch from cocaine to cocoa and to join forces with the private sector to enhance Colombia's competitiveness at international level (comprehensive national program for the replacement of illegal crops) (Presidencia de la República de Colombia, 2017).

Due to its nutraceutical properties and beneficial health properties, polyphenols have gained increasing attention as supplements and additives in functional foods. For instance, Vitaflavan from grape seeds and Crofelemer Mytesi^{TM} (oligomeric polymers up to 30) from *Croton lechleri* are used as food supplements. Moreover, polyphenol-enriched dark chocolate bars are offered by Flavanaturals and MARS Inc.

Before polyphenols' extraction, it is necessary to prepare the sample. Generally, samples prepared at a laboratory scale, by reducing sample from millimetric to micrometric particle size using milling processes (Pons-Andreu, 2008). To preserve the desired properties, cooling

strategies such as cryogenic mills (Pons Andreu, 2008) are suggested. Moreover, the total fat content in whole cocoa beans is over 50% (Servent et al., 2018) (on a dry basis), which constitutes a barrier for the release of polyphenols from the cells. Therefore, pressing of the beans, or using a solvent extraction such as hexane (Rodríguez-Carrasco, Gaspari, Graziani, Sandini, & Ritieni, 2018), petroleum ether (Tomas-Barberán et al., 2007) or other non-polar solvents to achieve a final fat content lower than <5% is commonly employed. When defatting using solvents, an additional step for residual solvent removal is thus needed. Supercritical fluid CO_2 can be used, which leaves no residue in the final product, but it is more expensive (Pons-Andreu, 2008).

Then, the sample follows an extraction process. This stage depends on many factors such as type of solvent, contact time, sample particle size, pH, solid to solvent ratio, and methodology of extraction, which impact the extraction yield (Pinelo, Sineiro, & Núñez, 2006). Recovery of polyphenols from cocoa and related products through several technologies such as maceration (Quiroz-Reyes, Aguilar-Mendez, Ramírez-Ortíz, & Ronquillo-De Jesus, 2013), microwave (Routray & Orsat, 2012), and pressurized liquid extraction (Okiyama et al., 2018) has been previously reported. In general, recovery of polyphenols is achieved at temperatures ranging from 25 – 100 ºC for some time interval (0.15 - 24 h) (Cienfuegos et al., 2007; Kealey et al., 2007; Rodríguez-Carrasco et al., 2018). Furthermore, methanol, acetone, isopropanol, and their mixtures allow higher extraction yield (Carrillo et al., 2014; Ioannone et al., 2015; Patras, Milev, Vrancken, & Kuhnert, 2014), but they have some limitations; for instance, Food and Drug Administration associates them with toxic potential (Food and Drug Administration, 2017). Therefore, polar solvents approved by food regulations agencies for human consumption (that is, water, ethanol or a mixture of both) are preferred. These are good examples showing that no single universal extraction processes can be employed for the extraction of natural polyphenols, therefore, the

analysis of polyphenol-rich samples, such as cocoa green beans extracts, always involves an in-depth study of all the parameters that affect the recovery of antioxidants. Moreover, from an industrial point of view, the optimization of such processes is a great of interest.

Thereby, the goal of the present work was to establish both the experimental conditions and kinetic parameters to enhance higher recovery of polyphenols from cocoa beans avoiding the fat removal process. Thus, a suitable, cheap, and reliable method for food applications at both laboratory scale (50 mL) and large-scale production (up to 10 L) was developed. The operational conditions for the pre-treatment process (drying and particle size reduction to avoid the degreasing) was also studied, as well as the chemical characterization of raw cocoa extract.

3.2. MATERIAL AND METHODS

3.2.1. Reagents All the chemicals used were analytical or reagent grade and were not purified further. Folin-Ciocalteu reagent, gallic acid, sodium carbonate, ascorbic acid, L-cysteine, sodium phosphate monobasic monohydrate ($NaH_2PO_4 \cdot H_2O$), disodium hydrogen phosphate (Na_2HPO_4), 2,2′-azobis(2-methylpropionamidine) dihydro chloride (AAPH), caffeine, and theobromine were obtained from Sigma Aldrich (St. Louis, MO, USA). (+)-Catechin hydrate, (–)-epicatechin, procyanidin B2 were purchased from ChromaDex Inc. (Irvine, CA, USA). Acetonitrile, methanol, (HPLC-grade), ethanol, n-hexane, citric acid and formic acid were acquired from Merck (Merck, Germany). Deionized water (18 $M\Omega cm^{-1}$) from an Aqua Solution system (Aqua solution, Inc. Jasper, Georgia, USA) was used for the preparation of all solutions.

3.2.2. Pre-treatment of cocoa beans Fresh cocoa pods (Trinitary, clone ICS 39) were collected at Villa Santa Monica (San Vicente de Chucurí, Santander, Colombia) and immediately protected from light and transported on ice to CICTA Lab for processing. Beans were removed manually from the pods using a knife, cleaned and separated from the pulp (mucilage) utilizing a mucilage

remover (Penagos Ltda, Colombia). After that, beans were heat treated (96 ºC for 6.4 min) with an enzyme inhibition solution (70 mM L-ascorbic acid and L-cysteine, ratio 1:1 *v/v*) until reach a polyphenol oxidase inhibition up to 93% (*see chapter 1-Part A*). The inactivated beans were frozen at -20ºC and used for further analysis.

3.2.3. Drying and milling process. Drying and milling processes were evaluated as follows:

a) *Drying*: Beans after PPO inactivation were used immediately to evaluate the effect of drying technology on the total flavonoid content. To do so, beans were chopped (cross section of 50×30 mm^2), oven dried (FD 23, Binder, Germany) at both 50ºC and 70ºC, and freeze-dried (Labconco Corp., Kansas City, USA) until to obtain final humidity < 4 %. The moisture content was evaluated by AOAC method 931.04 (AOAC, 1990).

b) *Milling*: As a strategy to avoid the use of a non-GRAS solvent for removing the fat from cocoa beans, different particle size distributions and ultrasound time were evaluated as a function of total flavonoid content. Dried beans were milled at max speed at -20ºC in N_2 environment for 30s during three cycles (Grindomix GM 200, Retsch Germany). The milled samples were sieved through steel mesh (W.S., Tyler, USA) with a sieve shaker (Gilson, Screen Co., USA) and fractionated in three groups: sieved and retained on 20 to 40 mesh (sample 1); 40 to 80 mesh (sample 2) and 80 to 200 mesh (sample 3). After that, the powdered samples were immersed in 50% aqueous ethanol and ultrasonicated (20 Hz, ice bath at 4ºC, Elma, Ultrasonic LC20H, Germany) for several intervals of time.

Control sample consisted of fresh cocoa beans that followed a drying, milling and degreasing process. Drying and milling were as described above. For fat removal, 1 g of cocoa bean powder was three times defatted with n-hexane (10 mL, extraction in an ultrasonic bath at 25 ºC for 15

min). The resulting powder was separated by decantation and then dried overnight at room temperature.

In all experiments, a standard polyphenol extraction step was carried out as follows: 1 g of sample was added to 60 mL of 50% aqueous ethanol at 50ºC for 30 min using a magnetic stirrer hotplate (IKA C-MAG HS7. Germany) with temperature being monitored with a thermocouple (IKA ETS-D5, Germany). The resulting extract was centrifuged (5000 *g*, 4ºC, 20 min), then the supernatant was filtered through 0.45µm hydrophilic PTFE filter (Millipore, Milford, USA), and the total polyphenol content was measured immediately as detailed in Section 2.4.1.

3.2.4. Scanning electron microscopy Scanning electron microscopy (SEM) was used to evaluate the microstructure of (a) raw cocoa beans, (b) cocoa beans with reduced PPO activity, and (c) cocoa beans with reduced PPO and treated by ultrasound. Bean samples cut into longitudinal and transversal sections were mounted on aluminum stubs with double-sided carbon adhesive tape and observed using the XL-30 Environmental SEM (Philips, USA) at 25 kV accelerating voltage with the BSE (backscattered electron) detector. The images were stored in TIFF format at 712 × 484 pixel in grayscale with brightness values between 0 and 255 for each pixel constituting the image.

3.2.5. Solid-liquid extraction of polyphenols. Extraction temperature, pH, solute/solvent ratio, and ethanol/water ratio were evaluated as the major factors that can affect the extraction yield. pH was adjusted with 3 M citric acid. The combination of these factors was modeled through a surface design consisting of 2^4 + four replicates at the central point + triplicates at the start point. Low and high levels for the different factors were, as follows: Temperature (40, 60 °C); pH (3, 5); solute/solvent ratio (w/v) (1/60, 1/30); ethanol/water ratio (*v/v*) (25/75, 75/25). The resulting

extracts were centrifuged at 5000*g*, 4ºC for 15 min and the supernatant was collected and filtered using Whatman Nº1 filter paper (Whatman, Inc., USA), and used for further experiments.

Once the optimum solid-liquid extraction level-factors were selected, large-scale study (up to 10 L) for the polyphenol recovery was carried out using a Bioflow-110 bioreactor (New Brunswick Scientific, USA).

3.2.6. Determination of total polyphenol, total flavonoids, and total flavan-3-ols content

3.2.6.1. Total polyphenol content by Folin-ciocalteu The total polyphenol (TP) content was assayed using the Folin-Ciocalteu reagent according to Singleton, Orthofer, & Lamuela-Raventós (1998) with modifications as follows. The reaction was initiated by the addition of 50 µL of the sample with 1.5 mL of 10-fold diluted Folin-Ciocalteu reagent. After 5 min, 1.5 mL of 7.5% (w/v) sodium carbonate was added and vortexed for 10 s. The reaction medium was stored in the dark for 1 h at 25 ºC. Absorbance was measured at 765 nm (Genesys 20; Thermo Scientific-Fisher, Waltham, MA, USA) against a blank sample. A calibration curve of gallic acid (ranging from 0.01- 0.8 $mgmL^{-1}$, r^2= 0.999) was prepared, and the results were expressed as mg gallic acid equivalents (GAE) per gram of dried cocoa beans.

3.2.6.2 Total flavonoid assay. Total flavonoid (TF) was measured according to Zhishen, Mengcheng, & Jianming (1999) Sample (500 µL) was added to 5 mL volumetric flask containing 2 mL H_2O followed by addition of 0.15 mL 5 % $NaNO_2$. After 5 min, 0.15 mL 10 % $AlCl_3$ was added and 1 min after, 1 mL 1M NaOH was added. The total volume was made up to 5 mL with H_2O. The reactants were vortexed and stored in the dark for 15 min at 25 ºC. Absorbance was measured at 510 nm (Genesys 20; Thermo Scientific-Fisher,

Waltham, MA, USA) against a blank sample. Total flavonoid content was expressed as mg (–)-epicatechin equivalents (ECE) per gram of dried cocoa beans.

3.2.6.3. Total flavan-3-ol assay. Total flavan-3-ol (TF3) content was determined by the vanillin-H_2SO_4 assay as described by B. Sun et al., (1998) The reaction consisted of 1 mL sample in methanol with 2.5 mL of 1 % vanillin in methanol and 2.5 mL of 9 N H_2SO_4. The reaction medium was well mixed at 30 ºC and allowed to react for 15 min. Absorbance was measured at 500 nm (Genesys 20; Thermo Scientific-Fisher, Waltham, MA, USA) against a blank sample. Total flavan-3-ol content was expressed as mg (–)-epicatechin equivalents (ECE) per gram of dried cocoa beans.

3.2.7. Kinetic of solid-liquid (S-L) extraction of polyphenols Once the optimum S-L extraction conditions were selected, the extraction kinetics were evaluated by plotting the concentration of the isolated target analyte versus time. Aliquots (100 µL) of each sample were taken out at various times to measure the total concentration of polyphenol, flavonoid, and flavan-3-ol. The extraction curves were adjusted by several kinetic models previously documented in the literature (Amendola, De Faveri, & Spigno, 2010; Doymaz & İsmail, 2011; Peleg, 1988; Sant'Anna, Brandelli, Marczak, & Tessaro, 2012; Spiro & Jago, 1982).

3.2.8. Characterization by HPLC-DAD-ESI-MS/MS. HPLC analysis was performed on a Shimadzu (LC-2030 LT Series-i, US) equipped with a photodiode detector, solvent degasser, quaternary pump, autosampler with temperature control, and thermostat column compartment. The separation was achieved using a C_{18}-phenyl column (4.6 × 50mm, 2.5µm particle size) (Xbridge®, Waters, USA) protected with a security guard obtained from Phenomenex (AJ0-8788, Phenomenex, Torrance, CA, USA). The procedure consisted of acidified water (water/formic acid, 99.9:0.01% *v/v*) (solvent A) and acidified acetonitrile (acetonitrile/formic acid, 99.9:0.01*v/v*)

(solvent B). The optimized linear gradient was as follows: 0-8 min, 2% B; 8 - 37 min, 10% B; 37-40 min, 0% B and 2% B for 10 min. The flow rate was 0.8 mL/min, and the temperature was 60 ºC. The detector acquisition was 190-800 nm. The calibration curves for caffeine, theobromine, catechins, and dimer B2 were made from commercially available analytical standards (r^2 = 0.99). Oligomeric procyanidin calibration curve was performed from isolated fractions (r^2 = 0.98) according to our previous work. Toro-Uribe et al., (2018) All the results are expressed as mg of sample per g of cocoa beans (dry matter basis).

The mass detector was an Agilent 6320 Ion Trap LC/MS (Agilent Technology, Waldbronn, Germany) equipped with an ESI source and ion trap mass analyzer which was controlled by the 6300-series trap control software (Bruker Daltonik GmbH). MS/MS analyses were carried out to obtain the structural information of the separated compounds. Mass spectrometer was operated under positive and negative ESI mode, nebulizer pressure, 40 psi; dry gas, 12 L min^{-1}; dry temperature, 350 ºC, and mass spectra recorded from 90 to 2200 *m/z*. MS characterization features were analyzed using extraction ion compound tool and commercial standards. It was also consulted METLIN and HMBD databases for matching exact mass.

3.2.9. Antioxidant assays Oxygen radical absorbance capacity (ORAC) assay described by Huang et al., (2002) was carried out as follow: ninety-six well microplates were filled with 50 µL of the daily-working fluorescein solution (4×10^{-3} mM in 75 mM phosphate buffer, pH 7.4), 50 µL of samples at a known concentration, and incubated at 37 ºC for 10 min in a microplate reader (Synergy HT Multi-Detection, Biotek Instruments, Inc. Winooski, VT, USA). The reaction was initiated by the addition of 50 µL of AAPH solution (221 mM in 75 mM phosphate buffer, pH 7.4) and the fluorescence decay was monitored kinetically for 2 h, using emission and excitation wavelength of 485 nm and 528 nm, respectively. A calibration curve was prepared with 12.5 – 375

µM Trolox (r^2 = 0.99). Results are expressed as µmol Trolox per g of cocoa beans (dry matter basis) as follows:

$$ORAC = \frac{AUC_{sample} - AUC_{blank}}{AUC_{Trolox} - AUC_{blank}} * k * \frac{molarity\ of\ trolox}{weight\ of\ sample}$$

where *k* is the sample dilution factor, and AUC is the area below the fluorescence decay curve of the sample, blank, and Trolox, respectively. The area under the curve of normalized data was calculated using GraphPad Prism v. 6.0 (GraphPad Soft. Inc., La Jolla, California, USA).

The reducing ability of antioxidants toward the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical was measured according to Brand-Williams et al., (1995) DPPH methanolic solution (68.5 µM) was adjusted with methanol until an absorbance of 0.550 ± 0.01 units at 517 nm (Genesys 20, Thermo Scientific, Waltham, MA, USA) was obtained. Samples (100 µL) were incubated with 1.45 mL of this DPPH solution for 60 min in the dark. A calibration curve was prepared from 1.95 – 150 µM Trolox (r^2 = 0.99). Results are expressed as µmol Trolox per g of cocoa beans (dry matter basis).

3.2.10. Statistical Analysis All measurements were repeated at least three times, and data were expressed as the mean ± standard deviation. Statistical analysis was done using Statistica 7.1 (Stat-Soft Inc., USA). The analysis of variance (ANOVA) and *F*-test were used to evaluate the influence of the factors and their interactions on the experimental designs. ANOVA one-way and Tukey´s multiple range test was also conducted at a 5% level of significance. The kinetic constants in this study were determined from experimental data using non-linear regression employing Quasi-Newton method and least squares as custom loss function (0.0001 for both start value and initial step size). The response surface methodology consisting of full factorial central composite rotatable design with four replicates at the central point was conducted according to a completely

randomized model. A second-order polynomial equation was used to fit the experimental data as follow:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{k-1} \sum_{j=2}^{k} \beta_{ij} X_i X_j$$

where *Y* is the predicted factor, β_0 is the value of the fitted response to the design, β_i, β_{ii}, and β_{ij} are the coefficients of linear, quadratic, and cross product terms, respectively.

3.2.10.1. Verification of the model The performance of full factorial central composite design method was measured by r and R^2. Experimental runs were also randomized to evaluate the concordance of experimental data and predicted values, therefore, the root mean squared error (RMSE) was calculated as follows:

$$RMSE = \sqrt[2]{\frac{\sum_{i=1}^{n} (y_i - \hat{y}_i)^2}{n}}$$

where y_i and $\hat{y}_i$ is the measured value and predicted value by the model, respectively. And *n* is the number of the set data.

3.3 RESULTS

3.3.1. Effect of drying temperature, particle size and non-degreasing process on the concentration of total phenols

3.3.1.1 Drying Technology Beans with reduce enzyme activity were manually chopped and the total flavonoid (**TF**) content was evaluated after (a) oven-air drying at 50ºC and 70ºC, and (b) freeze-drying. **Table 1** summarizes the effect of drying process on TF content. It is worth mentioning that all the drying processes were efficient to reduce the moisture content < 4%, so, higher dehydration levels at higher drying-air temperatures. Visual evidence showed that dried beans were slightly grey/purple color, which is

characteristic of dried unfermented cocoa beans with high procyanidin content (Schinella et al., 2010). However, drying at 50ºC for 20 h resulted in TF content 10% lower than drying at 70ºC for 3 h ($p < 0.05$), which showed that flavonoids were more sensitive to drying time than temperature. No significant differences in TF amount was observed between freeze drying and oven-dried at 70ºC treatments. These phenomenon could be explained by the formation of new flavan-3-ols products with higher phenol activity, configuration rearrangement of flavan-3-ols, or epimerization reactions that take place during drying at high temperature (100 – 160 ºC) for short time as reported by Kothe et al., (2013). Thereby, oven-air drying at 70ºC was chosen as drying technology for further experiments. Therefore, it was more economical and faster than freeze-drying.

Drying	T	Time	Total Flavonoids (mgECE/g)
Freeze	-80	48	43.99 ± 0.25^{a}
Oven-air	50	20	38.96 ± 1.47^{b}
Oven-air	70	3	43.20 ± 0.73^{a}

Table 1. Effect of drying process on total flavonoid amount in cocoa beans. Mean value ± standard deviation (n = 5) with similar letters mean are not significantly different by ANOVA One-way Tukey ($p > 0.05$).

3.3.1.2. Impact of particle size on extraction yield After the PPO enzymatic inhibition and drying steps, the samples were milled at low temperature for a short time, thus avoiding the fat melting during the reduction of particle size. In an attempt to discriminate which sizes led to better performance on yield extraction, three distinct samples varying on particles size were evaluated as follow: (S_1) >0.42 mm, (S_2) 0.42-0.18 mm, and (S_3) <0.18 mm. The results showed that the best treatment was S_3 with a TF recovery of 55.8 mg ECE/g being 2x and 1.2x fold higher than S_1 and S_2, respectively. This study reinforces the idea that there is an inverse relationship between the

particle size and the extraction yield. This behavior was also observed by Y. Sun et al., (2011) who found that extraction yields were 6–fold higher when particle size was decreased from 2 to 0.074 mm.

3.3.1.3 Conditions to avoid the degreasing process Non-defatted samples and defatted samples with a total fat content of 58.5% and 2.5%, respectively, were used for these experiments. In prior assays, results showed that flavonoid yield from the defatted samples was 60% higher than in non-defatted samples, which confirms that fat is a barrier for the diffusion of the bioactive compounds from the solid phase to the extraction medium. Since one of the aims of the present work was to avoid the use of toxic organic solvents, ultrasonic treatment was selected as an alternative clean extraction technology. Thus, non-defatted cocoa samples were placed in a test tube and mixed with 50% aqueous ethanol (1/60 w/v ratio solute/solvent) and ultrasonicated (20 kHz, 4 ºC). The effect of ultrasonic time (0 - 60 min) was tested as a function of TF amount. As can be seen in **Figure 1**, ultrasound increases the flavonoid extraction efficiency up to 30 min. This is because of electrical acoustic intensity in the liquid/solid extraction generate cavitation bubbles improving the polarity of the system; moreover, the bubbles collapsed violently causing localized pressure, plant tissue rupture, and increase the diffusion rate (Y. Sun et al., 2011). **Figure 1** also showed that sample ultrasonicated for 30 min had no significant difference with the defatted control ($p<0.05$). Thereby, the ultrasonic bath for 30 min was chosen as optimal value for further experiments.

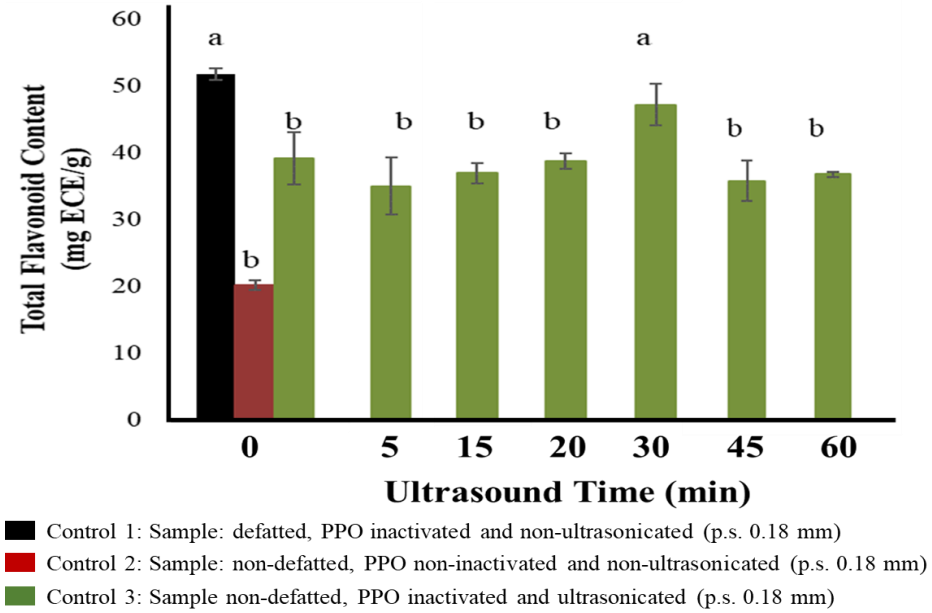

Figure 1. Impact of time of ultrasonic treatment on total flavonoid amount. Means with different letters were significantly different by Tukey ($p<0.05$).

3.3.2. Microscopy analysis Figure 2 shows SEM photographs confirming the mechanical effect of ultrasound on cell wall structure. As can be observed, non-treated samples had intact cell walls which were oval-shaped and not fractured as well as appearing more solid and denser due to the cellular contents remaining embedded in the cell (**Figure 2A**). In general, the cocoa bean is formed of parenchyma cells (containing cocoa butter and proteins) which represent over 80% of the mass, and about 12-18% of polyphenols and alkaloids that are placed into the vacuoles (Lopez, Dimick, & Walsh, 1987; Pedan et al., 2016). Physical changes were observed in the structure of cocoa starch granules that become larger, smooth and fibrous, quite likely as produced by heat treatment (**Figure 2B**). This behavior could be the result of enzyme heat treatment, which opens up the cell and favors diffusion of bioactive compounds from the matrix into the extraction medium, as well as, to the mobilization of proteins and polyphenols, and redisposition of fat within the cell (Lopez et al., 1987).

The morphological differences are more evident in Figure 2C. Smaller fragments were dispersed within the cell, and the microstructure was more porous, which could be the result of cell disruption and changes in the intercellular spaces. Hence, changes in the cell might increase the permeability and thus diffusion of polyphenols out of the cells. In accordance to our results, Rostagno, Palma, & Barroso, (2003) found that disruption of tissue and cell walls are most efficient when heat treatment together with ultrasound are used, which resulted in a greater penetration of solvent into the sample matrix, increasing the contact surface between solid and liquid phase, and as a result, the solute quickly diffuses from the solid phase to the solvent. The observation of this study confirms that the enzymatic inhibition by heat treatment, milling process, and ultrasounds play a significant role in the change of the internal structure of cocoa beans, thus enhancing a high concentration of polyphenols while avoiding the defatting process.

3.4. Solid-Liquid Extraction of Polyphenols from Cocoa Beans

Temperature, solute/solvent ratio, ethanol/water ratio, and pH were evaluated through a surface experimental design. As can be seen in **Table 2**, the concentration of ethanol had a strong impact on response factors, for instance, TP, TF and TF3 varied from 42.4 – 96.3 mgGAE/g, 15.2 – 86.0 mgECE/g, and 21.7 – 59.8 mgECE/g throughout the ethanol concentration range (0 – 100 %), respectively. Moreover, TP yield increased with increasing extraction temperature, e.g., extraction at 60 ºC (25% ethanol, pH 5, 1/60 solvent/solute ratio) was 1.12– fold higher than 40 ºC. Maximum values for TP (107.595 mgGAE/g), TF (86.045 mg ECE/g), and TF3 (59.783 mg ECE/g) were observed at different levels of factors, which indicate that combination of factors plays a key role in the extraction process. Results were fitted to a quadratic model (**supplementary Eq.1-3**) and the 3D surface graphs for dependent variables were plotted (**Figure 3**). Thus, analysis of variance summarized the observations and differences among the studied factors (**Table S1**). ANOVA

showed that the recovery of bioactive compounds mainly depended on the linear effect of temperature (T) and pH, linear and quadratic effect of ethanol/water (EW) ratio, and solute/solvent (SS) ratio.

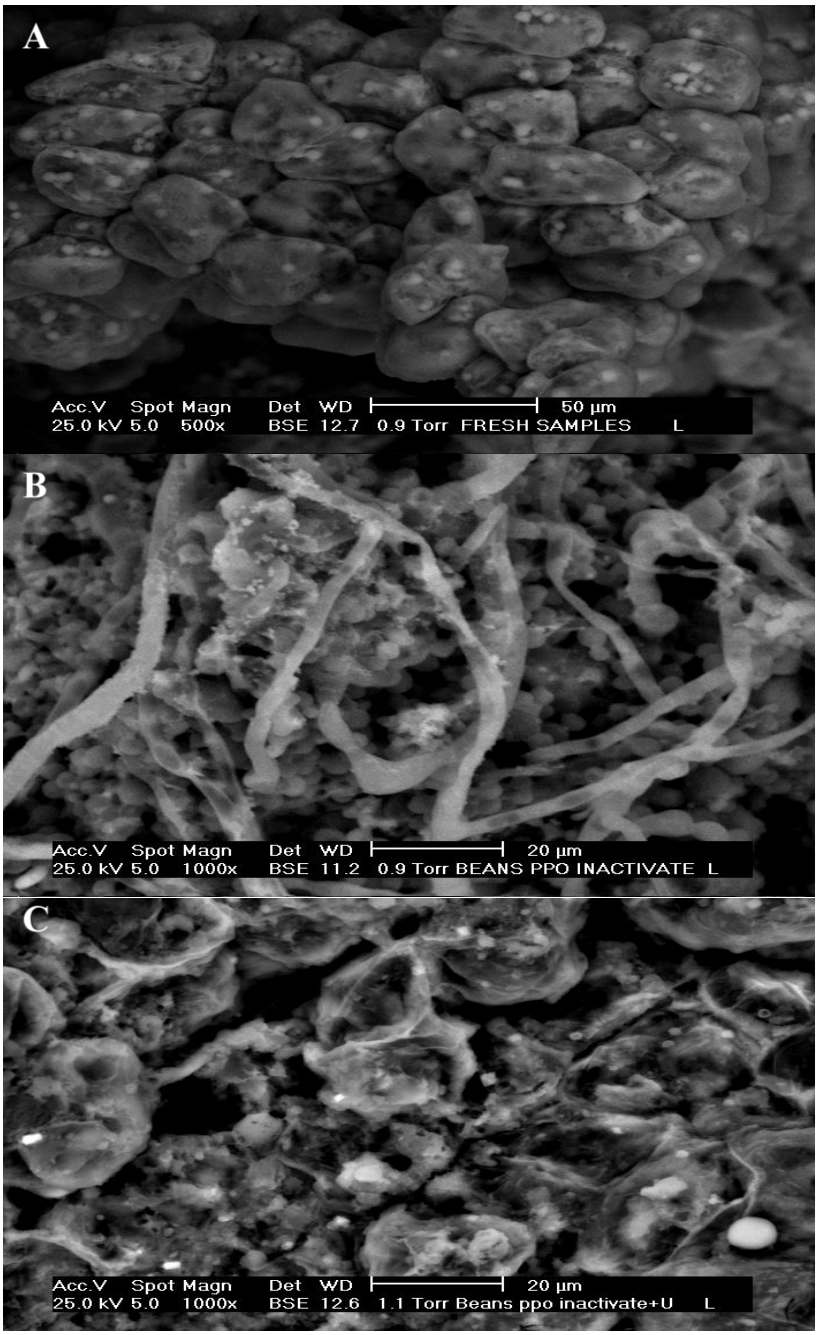

Figure 2. Microscopy images for the microstructure of a) non-treated cocoa bean, b) beans after PPO inhibition and c) beans after PPO inhibition and ultrasound treatment.

Table 2. 2^4 full factorial central composite rotatable design and experimental results for total polyphenol, total flavonoids, and total flavan-3-ols recovery from cocoa beans.

T (ºC)	SS (w/v)	EW(*v/v*)	pH	TP (mgGAE/g)	TF (mgECE/g)	TF3 (mgECE/g)
60	1/30	25	5	78.22 ± 1.17	62.30 ± 1.75	35.07 ± 0.14
50	1/24	50	4	94.15 ± 0.50	79.64 ± 1.88	48.62 ± 0.59
50	1/40	50	2	98.86 ± 0.52	55.87 ± 2.60	39.46 ± 0.25
60	1/60	75	3	103.57 ± 1.25	57.48 ± 0.39	43.78 ± 0.90
40	1/30	75	3	92.23 ± 1.79	77.30 ± 4.10	42.89 ± 0.27
60	1/60	25	3	91.59 ± 1.30	52.36 ± 0.39	40.74 ± 0.98
50	1/40	50	2	98.17 ± 1.10	53.84 ± 2.34	38.01 ± 0.13
40	1/30	75	3	95.81 ± 0.07	68.77 ± 3.51	40.75 ± 0.24
50	1/40	50	6	98.85 ± 0.65	66.45 ± 2.08	59.28 ± 0.98
50	1/40	50	4	95.96 ± 0.82	67.97 ± 4.16	51.26 ± 0.09
50	1/120	50	4	107.59 ± 0.52	53.74 ± 0.77	55.21 ± 1.39
50	1/40	50	6	97.53 ± 0.26	64.31 ± 0.26	59.78 ± 1.54
60	1/30	75	3	97.90 ± 0.93	73.11 ± 2.35	47.46 ± 0.04
50	1/40	50	4	95.52 ± 0.61	63.01 ± 3.38	47.38 ± 0.93
40	1/30	75	5	81.23 ± 0.23	76.18 ± 4.30	50.28 ± 1.40
40	1/30	25	5	76.97 ± 1.29	61.21 ± 0.96	42.77 ± 0.28
40	1/60	75	5	91.56 ± 0.19	49.34 ± 2.72	42.19 ± 0.63
60	1/30	25	3	82.64 ± 0.13	59.53 ± 0.59	39.84 ± 0.07
60	1/60	75	5	89.26 ± 0.45	57.78 ± 0.39	58.68 ± 1.12
50	1/40	100	4	46.79 ± 0.44	15.18 ± 1.30	22.72 ± 0.37
40	1/60	25	3	84.58 ± 0.19	50.93 ± 3.12	38.83 ± 0.99
40	1/30	25	3	76.06 ± 0.69	55.74 ± 2.91	43.87 ± 0.21
40	1/60	25	3	84.45 ± 0.99	51.83 ± 3.52	40.37 ± 1.28
30	1/40	50	4	88.59 ± 0.13	74.73 ± 1.56	42.52 ± 0.14
70	1/40	50	4	100.04 ± 0.69	79.73 ± 4.14	58.04 ± 0.51
60	1/60	25	3	82.98 ± 2.23	55.26 ± 1.16	39.85 ± 0.85
40	1/30	25	5	78.95 ± 1.03	59.04 ± 3.69	46.46 ± 0.21
40	1/60	75	3	102.28 ± 2.25	46.02 ± 3.91	29.49 ± 0.61
60	1/30	25	5	81.21 ± 0.45	64.10 ± 3.88	38.59 ± 0.36
50	1/120	50	4	104.96 ± 0.91	55.91 ± 1.56	55.57 ± 0.56
60	1/30	75	5	83.48 ± 0.58	74.43 ± 4.27	55.98 ± 0.52
50	1/40	0	4	43.45 ± 1.22	22.40 ± 1.56	21.73 ± 0.09
40	1/60	25	5	76.43 ± 1.17	56.35 ± 2.34	41.86 ± 0.42
50	1/40	0	4	41.32 ± 0.22	17.09 ± 0.26	25.81 ± 0.65

30	1/40	50	4	89.97 ± 0.91	77.91 ± 2.60	41.31 ± 0.23
40	1/30	25	3	79.12 ± 0.67	58.51 ± 0.39	39.37 ± 0.25
60	1/30	75	5	84.29 ± 0.39	75.47 ± 4.26	52.63 ± 1.39
60	1/30	25	3	82.19 ± 0.39	59.47 ± 3.50	36.21 ± 0.39
60	1/60	75	5	89.01 ± 1.36	57.96 ± 1.16	54.35 ± 1.74
50	1/40	50	4	90.36 ± 0.48	69.19 ± 4.17	44.92 ± 0.84
60	1/60	25	5	85.11 ± 0.33	59.55 ± 1.95	44.08 ± 0.35
50	1/24	50	4	100.27 ± 0.05	86.05 ± 4.53	53.69 ± 0.25
70	1/40	50	4	97.97 ± 0.26	82.05 ± 2.08	58.80 ± 0.84
60	1/60	75	3	95.49 ± 2.51	53.52 ± 0.78	46.89 ± 1.50
60	1/30	75	3	98.69 ± 0.57	72.95 ± 3.50	49.99 ± 1.22
50	1/40	50	4	93.54 ± 1.13	64.19 ± 1.30	45.78 ± 0.77
40	1/30	75	5	81.22 ± 0.49	70.55 ± 2.15	47.68 ± 0.77
50	1/40	50	4	90.52 ± 0.30	65.81 ± 4.16	50.50 ± 0.79
50	1/40	50	4	91.27 ± 0.17	64.09 ± 3.90	50.16 ± 0.19
40	1/60	25	5	77.75 ± 0.91	51.53 ± 0.39	41.35 ± 1.01
40	1/60	75	3	99.96 ± 1.39	45.00 ± 1.55	32.91 ± 1.12
60	1/60	25	5	87.44 ± 1.25	56.65 ± 2.73	41.67 ± 0.85
50	1/40	100	4	47.40 ± 0.35	15.19 ± 0.78	23.69 ± 0.282
40	1/60	75	5	92.33 ± 0.32	48.27 ± 3.50	46.59 ± 0.139
Control by S-L extraction (Yield = 14.9%)				89.35 ± 2.06[a]	55.81 ± 0.19[a]	33.41 ± 1.88[a]
Optimum by S-L Extract. (Yield = 16.8%)				122.34 ± 2.35[b]	88.86 ± 0.78[b]	62.57 ± 3.37[b]

where TP is total polyphenol TF is total flavonoid and TF3 is total flavan-3-ol. S-L is the solid-liquid extraction. Means (n = 3) with different letters were significantly different by ANOVA One-way Tukey ($p<0.05$). For more information see the methodology section.

Besides, the interactions of T×EW, and EW×pH for total polyphenol content; SS×T, and SS×EW for total flavonoid concentration; and T×SS, T×EW, SS×pH, and EW×pH for total flavan-3-ol amount were also significant ($p<0.05$). ANOVA analysis also showed that the selected quadratic model adequately represented the extraction process.

Therefore, the model has a good coefficient of multiple determination of $r^2 = 0.9567$, $r_{adj} = 0.9411$; $r^2 = 0.9541$, $r_{adj} = 0.9376$; and $r^2 = 0.9054$, $r_{adj} = 0.8715$ for total polyphenol,

flavonoid and flavan-3-ols content, respectively. Overall, higher accordance regression fit values mean that the model explained most of the variability in the responses. Thereby, the maximum experimental conditions were enhanced with 50/50 *v/v* ethanol/water ratio, 1/120 w/v solute/solvent ratio, pH 6 at 70ºC for a predicted recovery of 117.87 ± 16.68 mgGAE/g, 85.22 ± 18.51 mgECE/g, and 76.86 ± 15.98 mgECE/g, which were in agreement with the experimental data with 122.34 ± 2.35 mg GAE/g, 88.87 ± 0.78 mgECE/g, and 62.57 ± 3.37 mgECE/g, for TP, TF, and TF3, respectively.

3.4.1. Effect of independent factors on the recovery of total polyphenols and total flavonoids. As can be seen in **Figure 3**, the significant factors evaluated had a parabolic behavior on the extraction of TP, TF, and TF3. For instance, it was confirmed that temperature decreases the viscosity of the solvent, increases the solubility, diffusion rate and mass transfer of polyphenols (Fig 3 A, C, E), which is in agreement with Cacace & Mazza (2003) Indeed this factor had a linear effect until it reached a maximum after which the extraction yield decreased. This phenomenon is explained by the softening of plant tissue at high temperature, while at the same time, it weakens phenol-protein and phenol-polysaccharide interactions (Mokrani & Madani, 2016).

Moreover, results showed that higher diffusion rate is enhanced at lower ratio solute/solvent, which is confirmed by the significance of linear and quadratic effect for both total phenol and total flavonoid content. Indeed, Fick´s second law of diffusion predicts the influence of solute/solvent, that is, the amount of solute which has diffused reach a final equilibrium with the bulk solution. The pH impacts the independent factors at different levels (**Fig. 3 B, D, F**), thus influencing the stability of polyphenols and controlling the diffusion process. This is because the pH changes the surface charge of the molecules and the degree of ionization (Datta, Dutta, Dutta, & Chaudhuri, 2011). The influence of ethanol concentration (**Fig. 3 A, C, E**) suggested that the extract contains

polyphenols with different polarities, which also confirms the principle “like dissolve like” The lowest concentration of water or ethanol were not efficient for the extraction of flavonoids from cocoa beans.

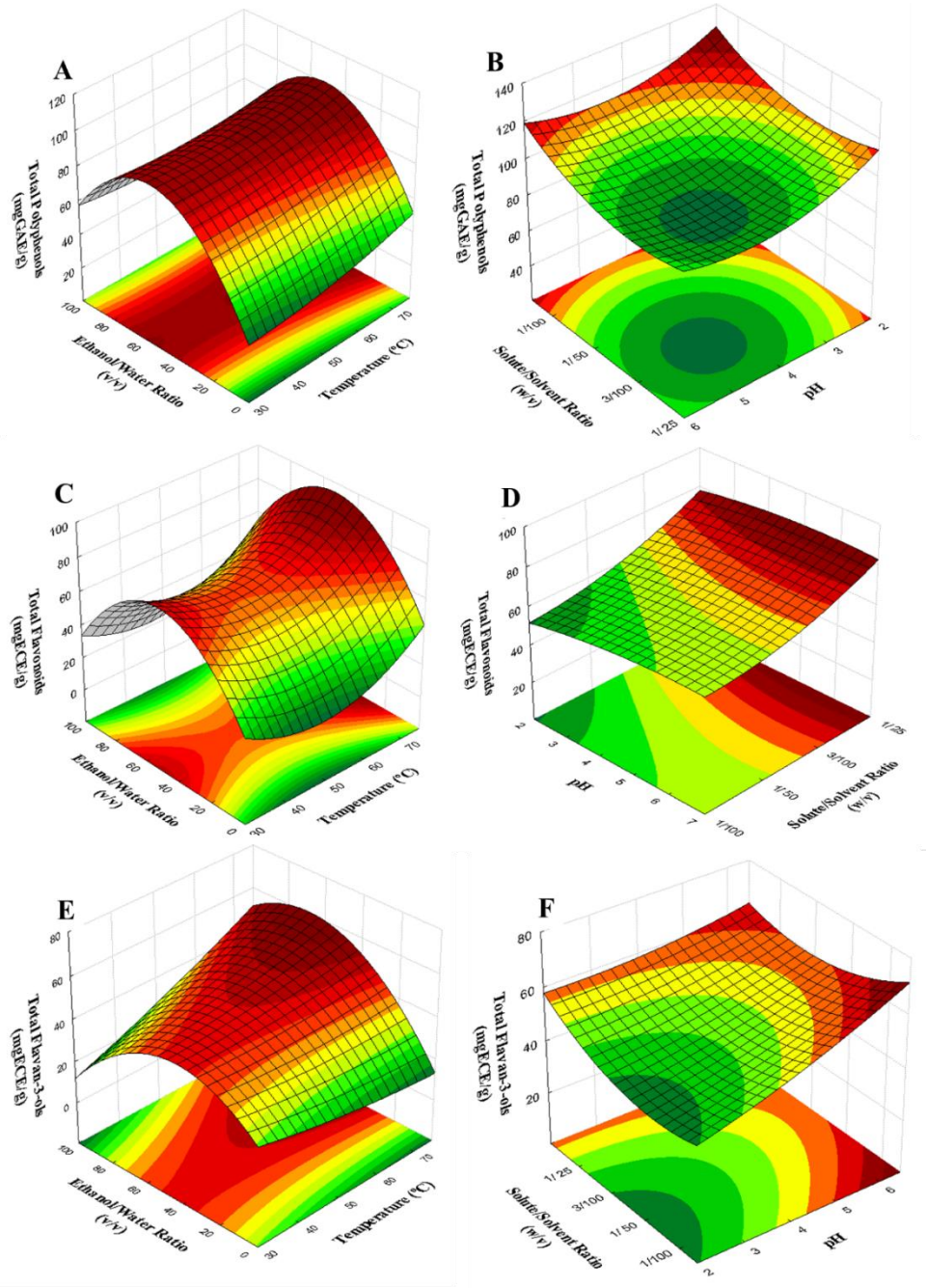

Figure 3. Surface response as function for Temperature vs Ethanol concentration, and pH vs Solute concentration for the recovery of total polyphenols (A, B), total flavonoids (C, D) and total flavan-3-ols (E, F).

Maximum extraction yield of phenolic compounds was enhanced using 50% ethanol due to different polarities of polyphenols affecting the kinetic and thermodynamics of the process. Previous works are consistent with our results, which reported a maximum extraction yield of polyphenols with an ethanol/water ratio of 40 - 50% (Chew et al., 2011; Rakotondramasy-Rabesiaka & Havet, 2010). Overall, at optimal conditions, the recovery of polyphenols, flavonoids, and flavan-3-ols were 27.0, 37.2, and 46.6% higher employing the optimized S-L extraction method than the control sample, respectively

3.5 Extraction kinetics parameters

Given the maximum conditions for the recovery of polyphenols, different extractions were carried out to evaluate the equilibrium time and kinetic parameters as a function of TP, TF, and TF3 content. The study of equilibrium time plays an essential role in economizing energy and cost of the industrial process,(Mokrani & Madani, 2016) thus improving the accuracy of the procedure and the quality of the final product. **Table 3** summarized the kinetic parameters, RMSE and the accordance of the model (*r*) for all the mathematical models selected, which have been previously used to model the solid-to-liquid extraction of bioactive compounds.

Overall, TP, TF, and TF3 content had the lowest *r* and highest RMSE for those equations that consider the extraction is occurring in one continuous step, e.g., Eqs. (1), (2), (4), and (5). Goodness of fit of the model and lower standard deviation of residuals were obtained for the two models that represent the recovery of polyphenols on two different rates (sorption/desorption), for instance, Eq. (3) and Eq. (6). These equations represented the kinetic process of a liquid/solid

system based on the solid capacity. As can be seen in **Figure 4**, the extraction curve shape had a faster extraction rate followed by a slower extraction rate and asymptotically approaching the equilibrium concentration.

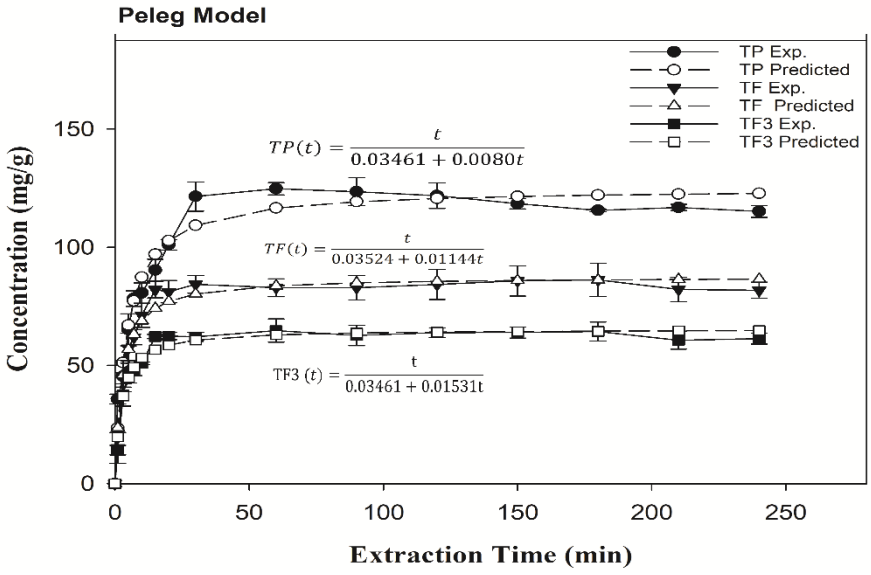

Figure 4. Experimental and calculated extraction curves for a total polyphenol (TP), total flavonoids (TF), and flavan-3-ols content. E (TF3). Extraction rate constant based on Peleg model and solid-liquid extraction at optimal conditions.

Results suggested that the Peleg model (Eq. 3) proved to be most suitable to model the solid-liquid extraction kinetics for the dependent variables ($r \geq 0.98$ and $RMSE \leq 0.71$). In fact, k_1 and k_2 represent the extraction rate constant and constant of extraction extent, respectively (Bucić-Kojić, Planinić, Tomas, Bilić, & Velić, 2007). **Table 3** showed that k_1 was similar in all the cases, but k_2 increased by TF3 ~ TF > TP, which is related to its maximum equilibrium concentration ($C_{t\to\infty}$). Thereby, the equilibrium extraction time were 45 min, 39 min and 34 min for TP, TF, and TF3 content respectively. Comparison of these data with previous authors showed that our

optimized extraction method was 2.7x, 4.4x, 6.0x, and 2.7x faster than the polyphenol recovery of cocoa beans (Tomas-Barberán et al., 2007), grape seed (Bucić-Kojić et al., 2007), and mango kernel (Maisuthisakul, 2009), respectively.

Table 3. Kinetic models used for the fitting for total polyphenol, total flavonoids, and total flavan-3-ols content from cocoa beans.

Model		Parameters	Parameters	Parameters	Ref.
nth order (1)	$C(t) = kt^n$	$k = 55.64$ $n = 0.16$	$k = 46.63$ $n = 0.13$	$k = 37.03$ $n = 0.11$	(Sant'Anna et al., 2012)
	r	0.93	0.90	0.90	
	RMSE	8.23	5.46	4.19	
Page (2)	$C(t) = e^{kt^n}$	$k = 4.06$ $n = 0.03$	$k = 3.87$ $n = 0.03$	$k = 3.64$ $n = 0.02$	(Doymaz & İsmail, 2011)
	r	0.927	0.89	0.898	
	RMSE	8.52	5.55	4.22	
Peleg (3)	$C(t) = \frac{t}{k_1 + k_2 t}$	$k_1 = 0.03$ $k_2 = 8.1x10^{-3}$	$k_1 = 0.03$ $k_2 = 0.01$	$k_1 = 0.03$ $k_2 = 0.01$	(Peleg, 1988)
	r	0.98	0.989	0.99	
	RMSE	0.71	0.62	0.67	
Weibull-type (4)	$C(t) = C_0 e^{kt^n}$	$C_0 = 1.7x10^{-8}$ $k = 21.92$ $n = 6.9x10^{-3}$	$C_0 = 2.8x10^{-7}$ $k = 13.17$ $n = 6.4x10^{-3}$	$C_0 = 2.0x10^{-7}$ $k = 19.03$ $n = 5.8x10^{-3}$	(Amendola et al., 2010)
	r	0.93	0.898	0.90	
	RMSE	8.33	5.51	4.32	
Mincher and Minkov (5)	$C(t) = A - Be^{-kt}$	$A = 96.94$ $B = 96.94$ $k = 289.60$	$A = 72.29$ $B = 72.29$ $k = 103.69$	$A = 55.07$ $B = 55.07$ $k = 117.03$	(Sant'Anna et al., 2012)
	r	0.64	0.68	0.71	
	RMSE	22.06	12.47	8.35	

Pseudo first order (6)	$C(t) = C_\infty - \frac{C_\infty}{e^{kt+a}}$	$C_\infty = 122.34$ $k = 0.11$ α = 0.124	$C_\infty = 88.87$ $k = 0.18$ α =0.024	$C_\infty = 62.57$ $k = 0.25$ α = 0.020	(Spiro & Jago, 1982)
	r	0.98	0.984	0.991	
	RMSE	6.49	5.59	2.29	

where TP is total polyphenol (mgGAE/g) TF is total flavonoid (mgECE/g), and TF3 is total flavan-3-ol (mgECE/g).

Large-scale results showed that recovery from 0.05 to 5 L did not significative ($p < 0.05$) impact of extraction yield, but the extraction at 10 L was 25.7 ± 0.9 % lower for TP content while was 23.0 ± 6.9 and 5.3 ± 2.6 % higher for TF and TF3 amount, respectively. These differences can be due to diffusional changes as results of the scaling up the process. Based on optimum processing conditions, a long-term study for inactivated cocoa beans (stored at 4 ºC) was carried out. Results showed that the beans maintain over 2 years their total phenol content (ca. 115 ± 5.4 mg GAE/g) and PPO activity (ca. 89 ± 3.8 %) with no significant change over time ($p<0.05$).

In general, this work confirmed the importance of studying the impact of extraction parameters on both secondary plant metabolites, therefore, increase the extraction yield. In terms of going beyond the highlights, the optimized ultrasound-assisted solid-liquid extraction not only allowed a high concentration of both total polyphenols and flavonoids but also employed food-grade solvents, reduced the number of stages (e.g., avoiding the degreasing), the extraction time (< 45 min), thus the energy consumption. Thereby, the extraction process could be suitable for large-scale applications. For example, cocoa polyphenol extract can be used to enriched products in very high demand in the food and cosmetic industries.

3.6. Chromatographic and antioxidant analysis

Identification of alkaloids, catechins and procyanidins in cocoa, were achieved by HPLC-DAD-ESI-MS/MS. The chromatographic method allowed not only the detection of main alkaloids but

also the procyanidins with different degree of polymerization up to 7 (**Figure 5**). As can be seen in **Table 4**, the cocoa extract obtained at maximum conditions contained 65% (w/w) procyanidins followed by methylxanthines (20 % w/w) and catechins (15 % w/w). Theobromine/caffeine ratio was 2.93 which can be used to classify hybrid genotypes (Hasing, 2004). Therefore, it was confirmed that cocoa sample is Trinitarian variety. (–)-Epicatechin (7.30 mg/g) was the main catechin being 11.8- fold higher than (+)-catechin. The major procyanidin in cocoa polyphenol extract was the Trimer C1 (11.9 mg/g) being 2.9x, 1.3x, 1.7x, 6.6x -fold higher than the dimer B2, tetramer, pentamer, and hexamer, respectively.

Our results are in agreement with previous works which reported that the main alkaloid and flavanol are the theobromine and (–)-epicatechin, but their concentrations vary considerably depending on the methodology of extraction and cocoa bean variety (Niemenak, Rohsius, Elwers, Ndoumou, & Liebereri, 2006). Theobromine and caffeine concentration were in agreement with previous reports (Carrillo et al., 2014; Okiyama et al., 2018), but epicatechin and catechin amount were 1.5 and 1.9, and 2.0 and 2.3-fold higher than those reported by Kothe et al.(Kothe et al., 2013) and Carrillo et al.(Carrillo et al., 2014). In general, procyanidin content was much higher than previous authors, e.g., dimer B2 was 2.0 and 1.5-fold greater than procyanidin B2 previously reported by Kothe et al.(Kothe et al., 2013) and Tomas-Barberán et al.(Tomas-Barberán et al., 2007).

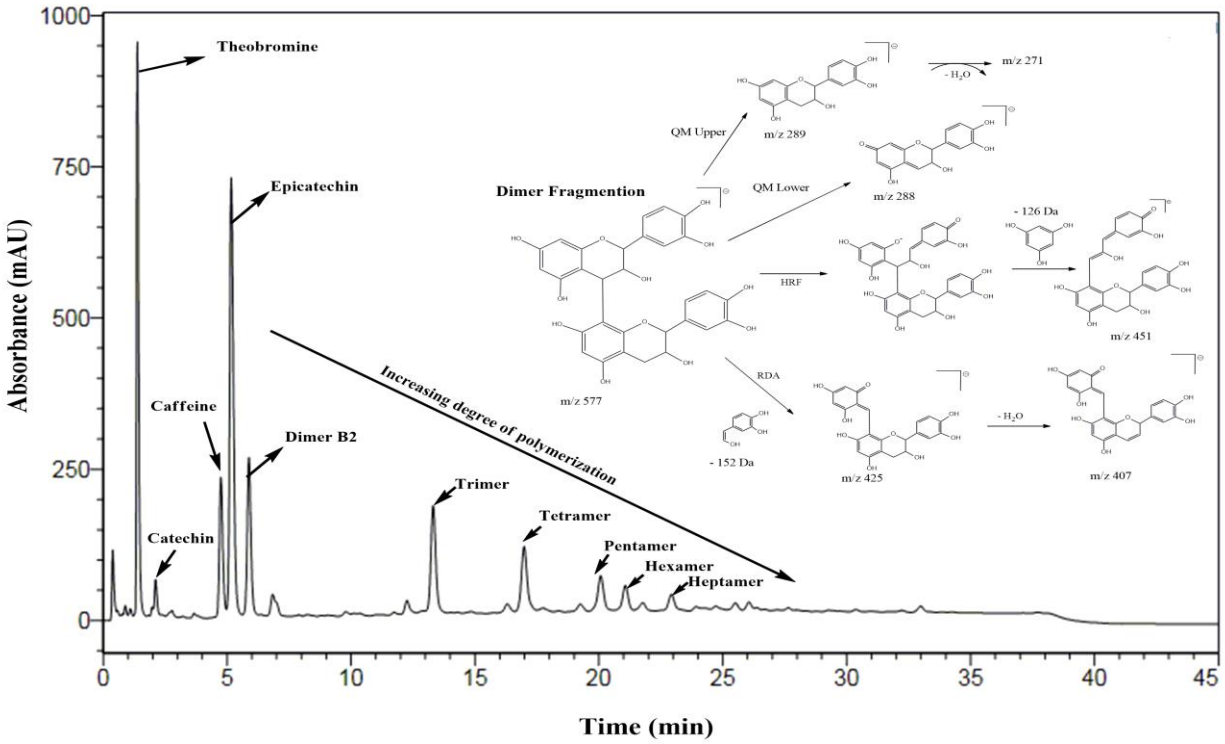

Figure 5. Chromatogram of cocoa extract and hypothetical fragmentation pathway (negative ionization mode) for procyanidin dimer. RDA, retro-Diels-Alder fission; QM, quinone methide cleavage; HRF, heterocyclic ring fission.

Table 4. Methylxanthine and procyanidin concentration, and characterization of cocoa beans using a HPLC-DAD-ESI-MS/MS method.

Compound	Ret. Time (min)	Concentration (mg/g)*	Ionization Mode	Error (mau)	MS^2 Fragment (*m/z*)
Theobromine	1.42	7.78 ± 0.01	$[M+H]^+$	0.73	137.5, 110.5
Caffeine	5.01	2.65 ± 0.02	$[M+H]^+$	0.42	158.4, 138.7
Catechin	2.16	0.62 ± 0.01	$[M-H]^-$	0.33	245.2, 195.0, 125.0
Epicatechin	5.46	7.30 ± 0.1	$[M-H]^-$	0.23	245.3, 195.1, 125.0
Dimer B2	6.22	4.06 ± 0.03	$[M-H]^-$	0.26	451.2, 425.1, 289.1, 271.1
Trimer C1	12.91	11.99 ± 0.25	$[M-H]^-$	0.21	695.2, 577.2, 451.0, 289.0
Tetramer D	16.81	9.33 ± 0.4	$[M-H]^-$	0.34	1027.3, 865.3, 739.2, 577.1
Pentamer	19.91	6.81 ± 0.52	$[M-H]^-$	0.03	1153.3, 865.2, 691.6, 574.3
Hexamer	20.85	1.81 ± 0.01	$[M-H]^-$	0.08	1535.0, 1153.3, 865.2, 574.2
Heptamer	22.51	-	$[M-H]^-$	1.85	1153.4, 995.3,851.3, 574.3

Data expressed as means of triplicate experiments. *Concentration expressed as mg polyphenol per g cocoa beans (dry weight basis)

In addition, the identification of oligomeric procyanidins was carried out by mass spectrometry in comparison with commercial standards and published literature. For instance, dimer B2 (B-type, EC-4β→8-EC-4β), trimer C1 (B-type, EC-4β→8-EC-4β→8-EC), tetramer, pentamer, hexamer and heptamer with a molecular ion $[M-H]^-$ m/z 577, 865, 1153, 1441, 1729, 2019, respectively were identified. Characterization of larger polymers was not possible due to their low

concentration, low ionization, peak signal dispersion, the formation of multiple ions, and limitations of the ion trap MS analyzer. Fragment patterns also suggested that various fragmentation mechanisms are involved in ESI such as quinone methide (QM), retro-Diels-Alder (RDA) as well as heterocyclic ring fission (HRF), which could take place on the extension unit or the terminal unit of the molecule (Rockenbach et al., 2012). For instance, the loss of a fragment with m/z 152, 170 and additional loss of water corresponding to RDA fission and loss of 288 Da corresponds to QM cleavage.

Among the antioxidant assays, our results showed that the cocoa extract had an ORAC of 1149.85 ± 25.1 µM Trolox/g. These values are higher than those reported by Hurst et al., (2009) and Carrillo et al., (2014) with TP between 58.0 – 61.7 and 45.3 – 70.0 mg GAE/g, and ORAC ranging from 797.0 – 947.0 and 387.3 – 618.1 µM Trolox/g, respectively. Moreover, DPPH radical scavenging activity was 120.6 ± 0.5 uM Trolox/g (equal to 0.72 uM Trolox/ mg cocoa extract), which was 2.4-fold higher than previously reported by Summa et al., (2006) These differences could be associated to the improved methodology of extraction at optimal conditions, thus enhancing both high composition and concentration of catechins (7.92 mg) and procyanidins (34.0 mg) with a DP ≥ 7.

3.7. CONCLUSIONS

Operating conditions to avoid degreasing and lyophilization were established, thus leading to a more cost-effective strategy. The optimized ultrasound-assisted solid-liquid process extraction for cocoa polyphenols allowed to increase on 27 and 37 % polyphenol and flavonoid yield, respectively. The process was suitable for food applications. Moreover, the extraction process was reproducible for scaling up to 5L, maintaining reduced PPO activity (89 ± 3.8 %) and total phenolic amount (115 ± 5.4 mgGAE/g) over 2 years.

3.8. ACKNOWLEDGMENTS

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3.9. SUPPLEMENTARY MATERIAL

Table S1. ANOVA for TP, TF, and TF3 through 24 surface design + central points+ start points.

Factors	Total Polyphenols (mgGAE/g)					Total Flavonoids (mgECE/g)				
	SS	df	MS	F	p	SS	df	MS	F	p
T	121.89	1	121.892	25.054	0.000025	145.61	1	145.612	25.0688	0.000025
T^2	24.53	1	24.534	5.043	0.032519	724.60	1	724.600	124.7481	0.000000
SS	279.34	1	279.342	57.416	0.000000	2281.04	1	2281.037	392.7065	0.000000
SS^2	283.53	1	283.529	58.277	0.000000	113.79	1	113.786	19.5896	0.000125
EW	765.38	1	765.383	157.318	0.000000	106.59	1	106.594	18.3514	0.000184
EW^2	5940.53	1	5940.527	1221.02	0.000000	5591.41	1	5591.412	962.6252	0.000000
pH	382.70	1	382.701	78.661	0.000000	134.49	1	134.493	23.1546	0.000043
pH^2	227.97	1	227.967	46.857	0.000000	5.92	1	5.920	1.0193	0.321044
T×SS	3.89	1	3.894	0.800	0.378321	43.38	1	43.376	7.4676	0.010587
T×EW	28.04	1	28.045	5.764	0.022992	9.23	1	9.226	1.5883	0.217613
T×pH	0.03	1	0.026	0.005	0.942165	1.21	1	1.210	0.2084	0.651431
	4.75	1	4.751	0.977	0.331216	511.66	1	511.657	88.0875	0.000000

Supplementary Equations: Predicted mathematical for TP, TF, and TF3 on cocoa beans, as follows:

Equation 1. Predicted model for total polyphenols content

$$Y_{TPC} = 104.36 + 0.01T - 2063.89\,SS + 36443.83\,SS^2 + 2.62\,EW - 0.02EW^2 - 19.01pH + 2.72\,pH^2 - 0.09EW * pH$$

Equation 2. Predicted model for total flavonoids content

$$Y_{TF} = 99.20 - 3.71T + 0.04\,T^2 + 23087.14\,SS^2 + 1.33\,EW - 0.02EW^2 + 19.19\,SS * pH$$

Equation 3. Predicted model for a total flavan-3-ol content

$$Y_{TF3} = 16320.24\,SS^2 - 0.01\,EW^2 - 21.08\,T * SS + 0.01\,T * EW + 5.81\,SS * pH + 0.08\,EW * pH$$

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CHAPTER 3

CHARACTERIZATION OF SECONDARY METABOLITES FROM GREEN COCOA BEANS USING FOCUSING-MODULATED COMPREHENSIVE TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

ABSTRACT

Proanthocyanidins as well as other secondary metabolites present in green cocoa beans were studied thanks to a new method involving the use of on-line comprehensive two-dimensional liquid chromatography coupled to tandem mass spectrometry (LC × LC-MS/MS). In order to enhance the performance of previously developed methodologies, the use of different modulation strategies were explored. Focusing modulation clearly allowed the attainment of higher resolving power and peak capacity compared to non-focusing modulation set-ups. Moreover, the use of active modulation by the addition of a make-up flow efficiently helped to compensate for the solvent strength mismatch produced between dimensions. The optimized method was useful to successfully describe the secondary metabolite composition of green cocoa that was characterized by the presence of 30 main compounds, including 3 xanthines, 2 flavan-3-ols, and 24 oligomeric procyanidins with a degree of polymerization up to 12. The obtained results showed that the proanthocyanidins found in the cocoa beans were exclusively B-type procyanidins. The existence of (epi)catechin subunits linked to sugar or galloyl moieties was not observed. The developed method produced a good separation of secondary metabolites allowing an improvement with respect to the available methodologies for the analysis of a complex food sample such as cocoa metabolites in terms of speed of analysis, resolution and peak capacity.

Keywords: Cocoa; Focusing modulation; LC×LC; Proanthocyanidins; Xanthines

4. CHARACTERIZATION OF SECONDARY METABOLITES FROM GREEN COCOA BEANS USING FOCUSING-MODULATED COMPREHENSIVE TWO-DIMENSIONAL LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

4.1. INTRODUCTION

Proanthocyanidins (PAs) are mixtures of oligomers and polymers of flavan-3-ol units linked mainly through $C_4 \rightarrow C_8$ or $C_4 \rightarrow C_6$ bonds. According to the linkage of interflavan bonds, the PAs can be classified into A or B-type proanthocyanidins (Xu & Howard, 2012). Many foods including cocoa-based products, red wine, coffee, apple and tea exclusively contain B-type procyanidins (Grosso, Stepaniak, Topor-Mądry, Szafraniec, & Pająk, 2014), but occasionally some matrices may present an additional $C_2 \rightarrow O_7$ or $C_2 \rightarrow O_5$ linkage, leading to doubly bonded A-type proanthocyanidins. For example, this kind of bonds can be present in avocados, peanuts and cranberries (Gu, Kelm, Hammerstone, Beecher, et al., 2003; Hellström, Törrönen, & Mattila, 2009; Prior et al., 2012).

Flavan-3-ols are the most complex subclass of flavonoids ranging from monomers to oligomeric and polymeric proanthocyanidins (Xu & Howard, 2012) .The complexity of the flavan-3-ols is due to their nature as non-planar molecules (Liao, Cai, & Haslam, 1992) having two chiral centers at C_2 and C_3, producing four isomers for each level of B-ring hydroxylation ((–)-epicatechin, (+)-epicatechin, (–)-catechin, (+)-catechin), and for oligomers an additional chiral center at C_4. Moreover, these compounds might form more complex structures due to the

inclusion of gallic acid and sugar moieties. As can be seen in Fig. 1, the main procyanidin monomeric compounds are the (+)-catechin and (–)-epicatechin units.

Some of the main antioxidant-rich food products are those derived from cocoa. Since 2010, cocoa is highlighted as the fourth richest dietary source of polyphenols, just behind cloves, pepper-mint and star anise (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). The main polyphenols in cocoa are (epi)catechins, proanthocyanidins, flavanol glycosides, and anthocyanins (Cienfuegos-Jovellanos et al., 2009; Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006; Tomas-Barberán et al., 2007). Procyanidins are, by far, the major flavonoids in cocoa. These compounds are exclusively composed of (epi)catechin units and are characterized for their high degree of polymerization (DP) (Kelm et al., 2006). In fact, procyanidins with DP up to 14 have been already described in cocoa (Robbins et al., 2009).

Cocoa procyanidins play an important role in the formation of color, solubility and astringency (Camu et al., 2008). For example, the enzymatic browning reaction is responsible for the brown color of cocoa beans (Vámos-Vigyázó & Haard, 1981). Moreover, the amount of total oligomeric procyanidins directly affects astringency and bitterness (Bonvehi & Coll, 1997), whereas the interaction of these polyphenols with proteins forming complexes affects the cocoa aromatic precursors (Lee, Lee, & Karim, 1991). Many studies have evaluated the biological properties of PAs suggesting that these polyphenols may confer positive effects including antioxidant activity at different levels (Heim, Tagliaferro, & Bobilya, 2002; Steinberg, Holt, Schmitz, & Keen, 2002), anti-carcinogenic activity reducing the oxidative DNA damage (Orozco, Wang, & Keen, 2003), chemopreventive action (Yamagishi et al., 2003), positive effects on cardiovascular health (Schramm et al., 2001), and anti-inflammatory and immune-modulatory activities (Terra et al., 2007). However, the bioavailability, bioactivity and potential

nutraceutical properties largely depend on the composition and concentration of the particular procyanidins found (Margalef et al., 2014). Thereby, better elucidation of the B-type isomers in cocoa as well as the molecular size of the procyanidins is of great interest in order to evaluate the activity, absorption, permeability, the physicochemical properties, and the affinity of PAs with proteins during the digestive process (Xu & Howard, 2012).

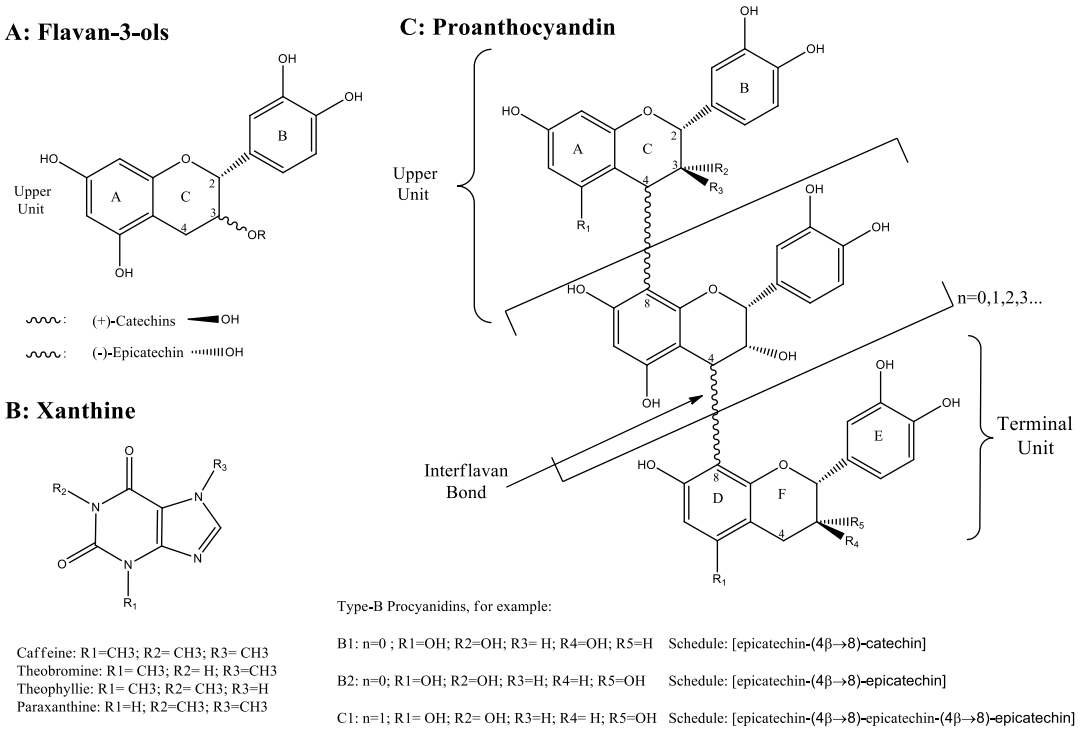

Figure 1. Chemical structure of methylxanthines and procyanidins. Skeleton of: A) monomeric catechins, B) xanthines, and C) condensed procyanidins.

Many strategies such as colorimetric assays, or chromatography coupled to mass spectrometry can be used to determine polyphenols from natural sources. In the particular case of the PAs, colorimetric assays are not specific methods due to severe interferences from phenolic acids, sugars, ascorbic acid or cysteine.(Singleton, Orthofer, & Lamuela-Raventós, 1998) Regarding the chromatographic methods, in general, separations from monomers through decamers of A-

and B- type procyanidins have been achieved using normal phase liquid separations with diol stationary phases coupled to fluorescence detectors;(Robbins et al., 2009) in this case, compounds can only be separated according to their molecular weight while isomers separation cannot be achieved. On the other hand, smaller compounds with DP ≤ 6 have been classically elucidated using reverse phase through C_{18} columns (Cooper et al., 2007; Kalili & de Villiers, 2009; Tomas-Barberán et al., 2007); through this approach, many of the isomers co-elute and the higher oligomers cannot be separated and elute as an unresolved hump at the end of the chromatogram. For this reason, the application of analytical techniques with higher resolving power is essential.

Two-dimensional liquid chromatography (2DLC) is a promising analytical tool since it combines two different separation procedures (dimensions) for the analysis of one sample, allowing the separation of the compounds present in the sample by two different retention mechanisms, greatly increasing peak capacity (Pirok, Gargano, & Schoenmakers, 2017). 2DLC can be run in heart-cutting (LC×LC) or comprehensive LC (LC × LC) approaches (François, Sandra, & Sandra, 2009), both in off-line, on-line or stop-flow modes (Guiochon, Marchetti, Mriziq, & Shalliker, 2008). Two-dimensional liquid chromatography has been widely employed for the analysis of phenolic compounds-rich food samples (F Cacciola, Jandera, Hajdú, Česla, & Mondello, 2007) including the analysis of cocoa procyanidins both using off-line 2DLC and stop-flow comprehensive LC (Kalili & De Villiers, 2013) while on-line LC × LC methods have been developed for the characterization of proanthocyanidins in grape seeds and apples (Montero, Herrero, Ibáñez, & Cifuentes, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013), and anthocyanidins in red wine (Willemse, Stander, Vestner, Tredoux, & de Villiers, 2015).

In particular, on-line LC × LC is considered the fastest and more reproducible 2DLC mode, although these separations present several important challenges. The main difficulty of this coupling is to solve the incompatibility of solvents that occurs when two different separation modes are coupled on-line, due to solvent mismatch between dimensions. This issue is responsible for lack of retention in the second dimension (^{2}D) column and results in broad and/or distorted peaks, producing a loss on total peak capacity. Many efforts have been made to solve this problem (Filgueira, Huang, Witt, Castells, & Carr, 2011). Recently, a new strategy named active modulation has been reported (Gargano, Duffin, Navarro, & Schoenmakers, 2015). This strategy consists on the use of a make-up flow installed between the output of the ^{1}D column and the input of the interface useful to adjust solvent strength. The use of this make-up flow rate produce an increment on the focusing effect in the ^{2}D column allowing a better compatibility between the injection solvent and the ^{2}D stationary phase and therefore, narrower peaks and higher peak capacity (Gargano et al., 2015). This strategy has also been successfully employed for the separation of complex plant secondary metabolites (Montero et al., 2017).

Considering the analytical challenges related to the study of the cocoa beans phenolic compounds together with the difficulties that the on-line HILIC × RP coupling involve, the aim of the present work was to optimize a new LC × LC method for the separation and identification of secondary metabolites from unfermented cocoa beans, in order to enhance the performance of the existing methodologies.

4.2. MATERIALS AND METHODS

4.2.1. Samples and reagents. Cocoa pods (*Theobroma Cacao L.*, genotype ICS 39) were collected from Villa Santa Monica (San Vicente de Chucurí, Colombia). All chemicals used were of analytical reagent grade with no further purification. (+)-Catechin hydrated (≥99%; ASB-

000003310), (–)-epigallocatechin gallate (EGCG) (≥99%, ASB 00005152), (–)-epicatechin (≥99%; ASB-00005127), procyanidin B1 (≥90%; ASB-00016230) and procyanidin B2 (≥90%; ASB- 00016231) were purchased from ChromaDex Inc. (Irvine, CA, USA). Theobromine, theophylline, caffeine, gallic acid, L-cysteine, ascorbic acid, formic acid, trifluoroacetic acid, acetic acid and citric acid were obtained from Sigma Aldrich (St. Louis, MO, USA). All the solvents employed (acetonitrile, ethanol, acetone and methanol) were of HPLC-grade and acquired from VWR Chemicals (Barcelona, Spain). Milli-Q water (Millipore system, Billerica, MA, USA) was used for the preparation of all solutions.

4.2.2. Preparation of cocoa polyphenols extract. Unfermented green cocoa beans were manually separated from cocoa husk and the mucilage coating around each bean was removed using a home-made mucilage remover. Beans were immediately stored at –20 °C until further use. With the aim of protecting polyphenols, the action of polyphenol oxidase enzymes (*i.e.*, POD, and PPO) was controlled by placing the frozen cocoa beans in a solution of 70 mM ascorbic acid/L-cysteine (1:1 *v/v*) and thermally processing the beans at 96 °C for 6.4 min followed by immediate cooling in ice for 30 min. Afterwards, the sample was re-washed with distilled water (4 °C) to remove traces of ascorbic acid and L-cysteine. After that, the sample was chopped by hand using a knife and it was directly oven dried (Binder model FD 23, Tuttlingen, Germany) at 70 °C for 3 h (final humidity <4% water w/w). The dried sample was milled (Grindomix GM 200, Retsch GmbH & Co., Haan, Germany) at –20 °C and sieved through a 120 mesh (W.S., Tyler, OH, USA).

The recovery of polyphenol extract consisted on the optimization of a solid-liquid extraction assisted by ultrasounds, as follows: 1 g of sample was mixed with 120 mL of 50% ethanolic solution and submitted to ultrasounds-assisted extraction for 30 min at 20 kHz (Ultrasonic LC 30H, Elma, Singen, Germany), followed by acidification at pH 6 (using 3 M citric acid solution)

and incubation at 70 °C for 45 min under constant stirring. The resulting extract was centrifuged (5000 g, 4 °C, 20 min) (Heraeus, Megafuge 16 R, Thermo Scientific, Waltham, MA, USA), and the supernatant was collected and filtered with Whatman filter paper Nº1. The ethanol was removed by vacuum evaporation (R-100, Büchi, Flawil, Switzerland). The aqueous crude extract was then freeze-dried (Labconco, Kansas City, MO, USA) and the resulting violet-powder crude cocoa extract was stored at –80 °C until further use. The studied cocoa extract was characterized by a total phenol content of 115.4 ± 5.4 mg gallic acid equivalents/g dried cocoa beans, measured according to Singleton & Rossi (1965) and an antioxidant capacity value of 1201.5 ± 41.3 µmol Trolox/g dried cocoa beans determined by using the ORAC assay (Huang, Ou, Hampsch-Woodill, Flanagan, & Prior, 2002).

For the two-dimensional LC analyses, 100 mg of extract were dissolved in 1 mL of acetone/water/acetic acid (78:20:2 *v/v/v*), and then centrifuged at 5000 g for 5 min. Solutions were filtered through hydrophilic Durapore PVDF membrane 0.22 µm (Millipore, USA) prior injection.

4.2.3. LC × LC instrumentation. Comprehensive two-dimensional liquid chromatography instrumentation consisted on a first dimension (^{1}D) composed by an Agilent 1200 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) equipped with an autosampler and a diode array detector which was connected at the exit of the second dimension. Additionally, a Protecol flow-splitter (SGE Analytical Science, Milton Keynes, UK) was placed between the ^{1}D pump and the autosampler with the aim of obtaining reproducible low flow rates. The second dimension (^{2}D) was carried out using an additional LC pump (Agilent 1290 Infinity). An electronically-controlled two-position ten-port switching valve (Rheodyne, Rohnert Park, CA, USA) was used to connect both dimensions. Sampling loops (50 µL) or C_{18} trap columns (10 × 3

mm, 2.6 µm, Accuore, Thermo Scientific, Waltham, MA, USA) were connected to the switching valve to collect the fractions from the ^{1}D and to inject them into the ^{2}D. Separations were recorded at 280, 320 and 370 nm although, in addition, UV-Vis spectra were collected from 190 to 550 nm at a sampling rate of 20 Hz in the DAD. For the active modulation configuration, an additional make-up flow was provided by a third LC pump (Agilent 1200 series) connected through a T-piece between the outlet of ^{1}D and the switching valve. LC image software (Zoex Corp., Houston, TX, USA) was used to plot the results as 2D and 3D images.

An Agilent 6320 ion trap mass spectrometer (Agilent Technologies, Waldbronn, Germany) was used for the identification of the separated compounds interfaced by an ESI ion source. MS/MS analyses were carried out to obtain the structural information of the separated compounds. The ion trap mass analyzer was controlled by the 6300 series trap control software (Bruker Daltonik GmbH, V. 6.2). The flow eluting from second dimension was split via a T-piece in order to allow the introduction of a flow of ca. 700 µL min-1 in the MS. The instrument was operated under negative and positive ESI modes using the following conditions: mass spectra recorded from *m/z* 50 to 2200; nebulizer pressure, 40 psi; dry gas, 12 L min-1, and; dry temperature, 350 ºC.

4.2.4. LC × LC separation conditions. A HILIC separation in the ^{1}D was coupled to a reversed phase separation in the ^{2}D. After optimization (see Sections 3.1 and 3.2), the following conditions were employed:

^{1}D separation: a Lichrospher Diol-5 (150 × 1.0 mm, 5 µm d.p., HiChrom, Reading, UK) column was employed protected with a security guard column containing the same stationary phase. Acidified acetonitrile (2% acetic acid, *v/v,* A) and methanol/water/acetic acid (95:3:2 *v/v,* B) were used as mobile phases. Under optimum conditions, the gradient was: 0-1 min, 0% B; 5

min, 20% B; 18 min, 35% B; 50 min, 42% B; 75 min, 100% B; 77 min, 100% B, 80 min, 0%B. The flow rate was set at 20 µL min^{-1}, the column temperature at 30 ºC and the injection volume was 3 µL.

^{2}D separation: two partially-porous columns with different length (Ascentis Express C_{18} 30 × 4.6 mm, 2.7 µm, and 50 × 4.6 mm, 2.7 µm, Supelco, Bellefonte, CA, USA) were tested. Gradient, flow rate, temperature, type of acid and solvents used were optimized separately. The optimal conditions were achieved using the 30 mm column with acidified H_2O (0.05% TFA, solvent A) and ACN (0.05%TFA, solvent B) as mobile phases. The optimum gradient was as follow: 0 min, 2% B; 0.05 min, 8% B; 0.5 min, 20% B; 0.9 min, 25% B; 0.91 min, 2% B and re-equilibrium at starting conditions until 1 min. Unless stated otherwise, the flow rate was set at 3.5 mL min^{-1} and the column temperature was 30 ºC maintained using a temperature control module (Cecil Instrument Ltd, Cambridge, England).

Once these parameters were optimized, the coupling between both dimensions was achieved employing 1 min repetitive second dimension separations; thus, the modulation time was 1 min. The third pump used for achieving the active modulation was set at 100 µL min^{-1}, delivering solvent with the same composition than the initial ^{2}D gradient conditions (2% B).

4.3 RESULTS

Plant extracts are well known for the complexity of their chemical composition. In general, this is due to the diversity of different families of compounds that can form their metabolic profile. That is the case of the cocoa extract, mainly composed by phenolic compounds, in particular proanthocyanidins. Due to the importance of these compounds as potential beneficial health ingredients, the knowledge about its chemical composition is essential. As mentioned, the

chemical complexity of these com-pounds makes their elucidation a big challenge and the use of conventional separation techniques does not provide enough separation power to obtain the comprehensive map of compounds. For this reason, in this work a LC × LC method has been developed for the exhaustive characterization of this complex extract. An initial individual optimization for both, ^{1}D and ^{2}D was carried out. Once the preliminary conditions were set, their coupling was also studied and fine-tuned.

4.3.1. Optimization of the HILIC × RP-DAD-ESI-MS/MS method for the chemical characterization of proanthocyanidins from cocoa beans. The use of 2DLC is only justified when the complexity of the sample is complex enough that 1DLC is not able to guarantee the complete resolution of the analytes. In other words, samples that are analyzed by 2DLC present more than one chemical variable defined as the sample dimensionality (Giddings, 1995). This fact means that a new sample that is considered for a 2DLC analysis is different and presents its own particular characteristics. Some of the characteristics that greatly affect the separation are the matrix effect of the sample and the composition of the analytes of interest. Therefore, a complete evaluation of the method developed for its ^{2}D analysis is required. Although proanthocyanidins have been previously analyzed using different 2DLC approaches (Kalili & de Villiers, 2009; Kalili & De Villiers, 2013; Montero, Herrero, Ibáñez, et al., 2013; Montero, Herrero, Prodanov, et al., 2013), the analysis of a new sample, such as cocoa green beans extracts, always involves an in-depth study of all the parameters that affect the ^{2}D separation.

4.3.2. Operational conditions for HILIC-based ^{1}D separation. HILIC has been successfully employed for the separation of procyanidins before, involving conventional LC. In particular, Robbins et al., (2009) achieved the separation of proanthocyanidins from a cocoa extract according to their degree of polymerization, getting the separation of proanthocyanidins up to 12 units of

(epi)catechin with a Develosil Diol column. In the present work, HILIC has also been used as ^{1}D separation mechanism in order to obtain the separation of phenolic acids, alkaloids as well as monomeric and polymeric procyanidins eluted as a function of their DP. Separation optimization is challenging as LC × LC conditions imply to run ^{1}D under suboptimal chromatographic conditions (Francesco Cacciola et al., 2011; Dugo, Kumm, Chiofalo, Cotroneo, & Mondello, 2006; Jandera, Hájek, Staňková, Vyňuchalová, & Česla, 2012). The injection solvent composition is a critical parameter in a microbore column; solvents similar to the initial mobile phase should be used to promote the sorption of the analytes into the column in a narrow band. For this reason, the solvent used to inject the sample in the microbore ^{1}D column was carefully studied and optimized; three different solvents were tested to dissolve the crude cocoa extract: methanol, acetonitrile/methanol (70:30 *v/v*) acetone/water/acetic acid (78:20:2 *v/v/v*). Considering the initial conditions of the analysis, acetonitrile should be selected as solvent to dissolve the sample since it is the weak organic solvent for the ^{1}D. However, proanthocyanidins are not well dissolved in acetonitrile and precipitated. Instead, the most favorable conditions included the use of acetone/water/acetic acid (78:20:2 *v/v/v*) as sample solvent.

Fig. 2 shows the ^{1}D separation of the crude cocoa extract at optimum conditions. As can be observed, with a run time of ca. 75 min and a slow gradient the elution of all the components present in the sample using a low flow rate was achieved. The first chromatographic peaks in the chromatogram correspond to alkaloids, mainly theobromine and caffeine, immediately followed by monomeric (epi)catechin. Between 30 and 50 min the oligomeric proanthocyanidins up to DP 8 were separated and at the end of the analysis (~65 min) the higher proanthocyanidin polymers were eluted.

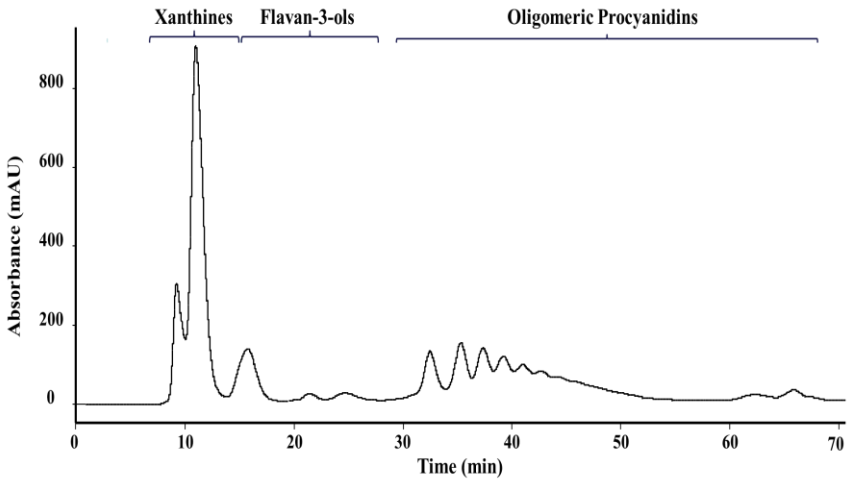

Figure 2. ^{1}D Chromatogram (280 nm) for cocoa extract under the optimum conditions using a Lichrospher Diol-5 column. The mobile phase consisted of ACN/AcOH (98:2 *v/v*) and MeOH/H2O/AcOH (95:3:2 *v/v/v*) at flow rate of 20 µL min^{-1}. For more details, see text.

4.3.3. Operational conditions for ^{2}D separation. The appropriate optimization of individual ^{2}D separation conditions is critical to achieve a successful LC × LC separation. In LC × LC, the ^{2}D available analysis time and re-equilibration time is equal to the modulation time, and this time has to be as short as possible in order to get the maximum number of ^{2}D analyses per ^{1}D peak. However, this short time implies the need to carry out very fast ^{2}D analyses. Besides, in the ^{2}D is where the solvent mismatch between dimensions becomes apparent, hampering the retention and proper elution of peaks, and having a critical influence on the total peak capacity attainable.

With the aim to optimize the ^{2}D separation in the shortest possible analysis time, two C_{18} partially porous columns of different length (30 and 50 mm) were tested. In this regard, different total ^{2}D separation available times (60 and 90 s), gradients, flow rates (from 2 to 4 mL min^{-1}), mobile phases (water, acetonitrile, methanol and their combinations) and acidic modifiers (formic acid and trifluoroacetic acid) were studied. Slightly better peak shapes were obtained

using TFA, probably due to its pKa value and interaction with ionizable groups of flavonoids, whereas the presence of methanol was always detrimental for the elution of the extract components. In Figure S1, the 2D chromatograms of the analysis employing acetonitrile (Figure S1A) and methanol (Figure S1B) as organic mobile phase under preliminary gradient conditions are showed. Comparing both analyses, a great fraction of the compounds eluted in the void volume without any retention when methanol was employed, whereas using acetonitrile the retention of the compounds is improved and, besides, a better peak resolution is obtained. Once the mentioned separation conditions were studied, the influence of the separation temperature was also tested. It is well known that high separation temperatures may favor resolution and reduce analysis times due to a decrease on the solvent viscosity and to an improvement in mass transfer kinetics.(Tanaka, Zhou, & Masayoshi, 2003) Separation temperatures between 30 and 55 °C were evaluated. However, a significant difference in the separation of oligomeric compounds was not observed and, thus, the minimum temperature (30 °C) was maintained.

Moreover, the gradient in the ^{2}D was deeply studied to enhance the separation of dimer, trimer and tetramer isomers and to achieve a good peak resolution for oligomeric and polymeric proanthocyanidins previously separated in the ^{1}D. In this sense, the finally selected gradient consisted on a progressive increment of the organic phase up to a 25%. With this optimum gradient, the oligomeric proanthocyanidins (DP 2–6) and polymers eluted in the middle and at the end of the ^{2}D analysis, respectively, as can be observed in Fig. 3A, where peaks in the ^{2}D separation occur between 25 and 45 s.

In spite of this optimization of the separation conditions, the complexity of cocoa proanthocyanidins, implying a mixture of very similar chemical structures, meant that the

separation of the different isomers was not completely achieved. For this reason, other modulation strategies including a focusing effect were studied in a second step.

4.3.4.Focusing modulation to improve the ^{2}D separation of the proanthocyanidins isomers.

4.3.4.1. Focusing configuration using trapping columns. The use of trapping columns instead of loops in the interface has been reported as a good alternative to produce a focusing effect for other food samples (F Cacciola et al., 2007; Egeness, Egeness, Breadmore, Hilder, & Shellie, 2016; Montero et al., 2017). These trapping columns must possess the same or similar selectivity than the ^{2}D stationary phase in order to produce a pre-concentration and retention step of the analytes eluted from ^{1}D in the trapping columns during the collection position, which will be then eluted from the interface with the ^{2}D solvents once the valve is actuated to the injection position. Therefore, to produce this trapping effect, two C_{18} trapping columns (to match the selectivity of the ^{2}D column) with a void volume of 42 µL were installed in the switching valve on a forward elution mode using the same separation conditions previously optimized for the LC × LC method without focusing. In this work, forward elution mode from the traps was selected in opposite to backflush elution based on previous experience in which a slight but noticeable improvement in separation was observed when forward elution was employed (Montero et al., 2017); since the trapping column is not actually filled with ^{1}D effluent between modulations, longer interaction is allowed in forward elution mode. Comparing loops vs trapping columns, using the latter a slightly better retention of the compounds was achieved (Fig. 3B) since in each ^{2}D separation, peaks appear more defined than in the non-focusing configuration (Fig. 3A). Indeed, as can be observed, when trapping columns are used peaks are slightly more

separated as well as more intense, probably due to the minimization of the solvent strength mismatch produced and an improvement of ^{2}D peak shapes. As a result of the better separation, the use of trapping columns allowed the separation of several isomers from each degree of polymerization that were previously not resolved using the non-focusing approach (Fig. 3A and B).

4.3.4.2. Focusing configuration using active modulation. Active modulation consists on the use of the same trapping columns configuration to which an additional make-up flow composed by a weak solvent for the ^{2}D (ideally the same composition of the initial ^{2}D gradient) is added between the ^{1}D exit and the interface (Gargano et al., 2015). The role of this additional flow is to reduce or completely remove the ^{1}D solvent by the dilution of the transferred fraction with a ^{2}D compatible solvent combined with the use of trapping columns. This fact produces an increment of the retention of the analytes in the interface and, therefore, an effective pre-concentration of the analytes in the trapping columns (Montero et al., 2017). Thereby, in this work, an additional make-up flow pump was connected through a 3-way PEEK connection in which one way was connected to the ^{1}D effluent, whereas the others corresponded to the flow from the additional pump and to the exit to the switching valve. The composition of the make-up flow employed was 98% acidified H_2O (0.05% TFA) and 2% ACN (0.05% TFA) to match the initial conditions found at the start of the ^{2}D optimum gradient. The influence of the make-up flow rate on the separation of the cocoa extract was also evaluated; flow rates ranging from 50 to 160 μL min^{-1} were studied. As can be seen in Fig. 4, the lowest flow rate did not improve the separation of compounds (procyanidins window), as this additional flow would not be enough to completely eliminate the solvent strength mismatch between ^{1}D and ^{2}D solvents, as can be seen observed from Fig. 4A. On the other hand, flow rates higher than 100 μL min^{-1} positively affected the retention of the sample components, achieving better separation and resolution. From a comparison between Fig. 4B and C and Figure S2, it can be inferred that no significant differences on separation were obtained. Thus, the lowest make-up flow rate (100 μL min^{-1}) was retained as optimum conditions.

Moreover, the performance of the different LC × LC studied set ups was evaluated by determination of the attainable peak capacity. Using the optimized method for both ^{1}D and ^{2}D

separations, the peak capacity was calculated according to Davis, Stoll, & Carr (2008) as follows:

$$n_c = 1 + \frac{t_g}{(\frac{1}{n})\sum_1^n w}$$

where, n_c represents the peak capacity, n is the number of compounds, $t_g$is the gradient time and, w is the average peak width. The total theoretical peak capacity of two-dimensional LC is the product of the peak capacities of the first and second dimension, $n_{c,2D} = {}^1n_c \times {}^2n_c$. Recently, Li, Stoll, & Carr (2008) defined the effective ^{2}D peak capacity taking into account the modulation time, the influence of the under-sampling of first dimension and peak broadening, as follows:

$${}^{2D}n_c' = \frac{{}^1n_c \times {}^2n_c}{\sqrt{1 + 3.35 \times \left(\frac{{}^2t_c \ {}^1n_c}{{}^1t_g}\right)^2}}$$

where $n_{c,2D}^{'}$ is the effective ^{2}D peak capacity, ${}^2t_c$ is the second-dimension cycle time and ${}^1t_g$ is the first-dimension gradient time. To more realistically describe the method performance, orthogonality (A_0 was also estimated in agreement with the asterisk equations method (Camenzuli & Schoenmakers, 2014). This value was subsequently employed to correct the effective peak capacity values as follows:

$${}^{2D}n_{c,corrected} = {}^{2D}n_c' \times A_0$$

Moreover, dilution factor (DF) produced during the analysis was also calculated. In agreement with the equations proposed by Vivó-Truyols, Van Der Wal, & Schoenmakers (2010) considering that dilution factor produced in each dimension is

$$DF = \sqrt{2\pi}\,\frac{\sigma F}{V_i}$$

Where F and V^i are the flow rate and injection volume, respectively. The dilution of the complete ^{2}D process will be:

$$^{2D}DF = {}^{1}DF \times {}^{2}DF$$

As summarized in Table 1, compared to the effective peak capacity of the non-focusing configuration of this work (712) the peak capacity was higher when active modulation was used, reaching a maximum effective peak capacity equal to 879. This value was also higher than the data provided in other works (551 and 771) (Kalili & De Villiers, 2013), although in this case it is important to remark that the comparison is not completely fair as the samples analyzed are also different. These data confirmed that the additional make-up flow allows improving the separation and the resolution of complex compounds as procyanidin isomers. An inspection of the theoretical (1184) and effective (879) peak capacities obtained for the optimized method demonstrates that a good modulation performance was obtained, as the two values are very close. In any case, the orthogonality values obtained for all three set-ups were relatively low, reducing the values of corrected peak capacity (Table 1). Nevertheless, as can be deduced from the values shown in Table 1, the improvement that active modulation produces in the ^{2}D separation was evident; 40% higher orthogonality values were obtained using active modulation compared to non-focusing modulation. Besides, the positive effect of focusing due to active modulation is directly related to the sensitivity that is related to the dilution factor (DF). This value was reduced by 20% using the active modulation approach, and therefore a gain in sensitivity was potentially achieved.

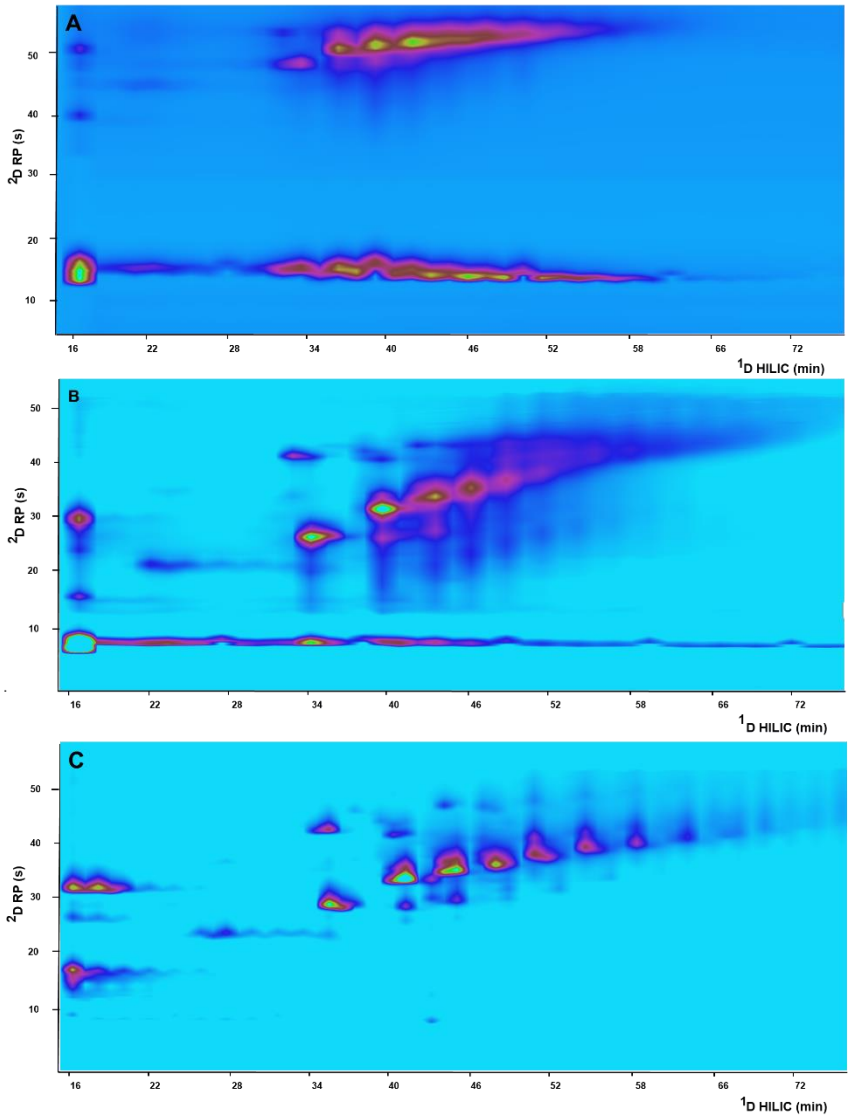

Figure 3. Two-dimensional plots (280 nm) of cocoa secondary metabolites using optimum separation conditions for each modulation set-up: A) sampling loops, B) C_{18} trapping columns and, C) C_{18} trapping columns þ active modulation. ^{1}D separation was carried out on Lichrospher Diol-5 column, and mobile phase consisted of ACN/AcOH (98:2 *v/v*) and MeOH/H_2O/AcOH (95:3:2 *v/v/v*) at flow rate of 20 µL/min ^{2}D separation was carried out Ascentis Express C_{18} partially-porous column, using H_2O (0.05% TFA) and ACN (0.05% TFA) as mobile phases at flow rate of 3.5 mL/min. For the rest of separation conditions, see text.

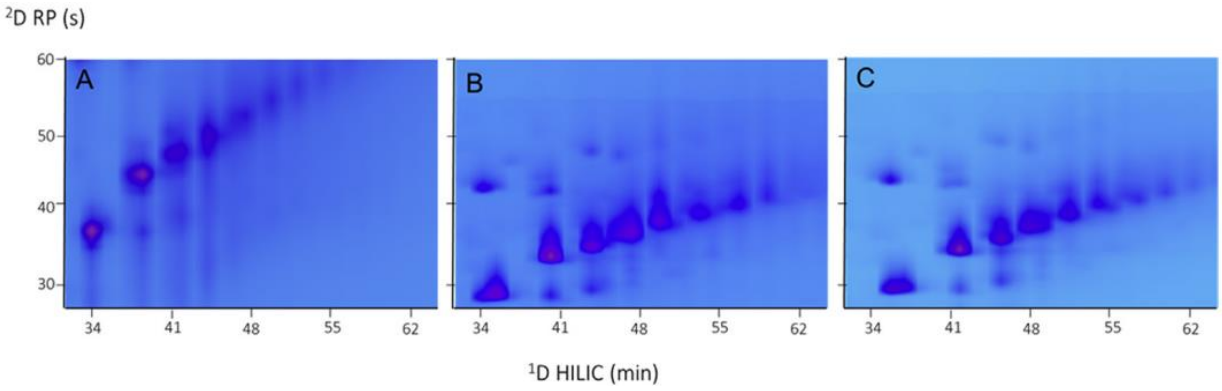

Figure 4. Two-dimensional plots (280 nm) of cocoa secondary metabolites obtained under optimum separation conditions using active modulation with different make-up flow rates: A) 50 µL min^{-1}, B) 100 µL min^{-1}, C) 160 µL min^{-1}.

Table 1. Peak capacity values calculated for the LC × LC focusing modulation set-ups studied.

Parameter	Non-focusing modulation	Focusing Modulation	
	Loops	Trapping Columns	Trapping columns + active modulation
^{1}D peak capacity, ^1n_c	37	37	37
^{2}D Peak capacity, ^2n_c	26	28	32
Theoretical Peak Capacity, $^{2D}n_c$	962	1036	1184
Effective Peak Capacity, $^{2D}n'_c$	712	767	879
Orthogonality, A_0	47%	56%	66%
Corrected Peak capacity, $^{2D}n'_{c,corr}$	335	430	580
Dilution Factor, ^{2D}DF	36.7	33.9	29.3

A_0 orthogonality; $^{2D}n_c = {}^1n_c \times {}^2n_c$; $^{2D}n'_c$: calculated to Li et al., (2008) $^{2D}n'_{c,corr} = {}^{2D}n_c \times A_0$; $^{2D}\text{DF} = {}^1\text{DF} \times {}^2\text{DF}$.

In summary, after studying and optimizing all possible configurations and conditions, the final selected conditions to carry out the in-depth characterization of cocoa extracts were as follows (Fig. 3C): ^{1}D separation was carried out on Lichrospher Diol-5 column, using ACN/AcOH (98:2 *v/v*) and MeOH/H_2O/AcOH (95:3:2 *v/v/v*) as mobile phases at a flow rate of 20 µL min^{-1}. In the ^{2}D, the short C_{18} partially porous column (30 × 4.6 mm, 2.7 mm) H_2O (0.05% TFA, solvent A) and ACN (0.05% TFA, solvent B) were used as mobile phases using 1 min gradients at flow rate of 3.5 mL/min, whereas 98% H_2O (0.05% TFA) and 2%ACN (0.05% TFA) was used as make-up flow for active modulation at 100µL min^{-1}.

4.3.5. Characterization of the cocoa extract. As can be seen in Table 2, the optimized focusing modulated LC × LC method was able to separate 30 compounds as a function of two different mechanisms; in the ^{1}D the proanthocyanidins were separated as function of their DP while in the ^{2}D the separation of different isomers within the same DP was possible. Table 2 summarizes the tentative identifications of each separated peak as well as their main MS and MS/MS features. In addition of flavan-3-ols, three xanthines also present in the metabolic profile of cacao were similarly assigned. The identification of these three alkaloids was carried out in positive ionization mode. On the other hand, procyanidins were detected under negative ionization due to the acidity of the phenolic protons which means that these are better detected under that mode. Besides, there are more available charges as the chain length increases (Es-Safi, Guyot, & Ducrot, 2006). Fig. 5 shows the 3D plot of the studied crude cocoa extract separated under the optimized LC × LC conditions. As can be observed, good separation in the complex mixture of flavan-3-ols units and their isomers was attained. As expected, the compounds can be grouped in two main areas, corresponding to xanthine family and monomeric (epi)catechin at the beginning of the analysis, and, on the other hand, oligomers of (epi)catechin units according to their

increasing degree of polymerization (up to DP 12). Oligomers higher than DP ≥ 12 were not completely resolved mainly due to their low concentration in the studied extract.

The first eluted compound corresponds to caffeine that presents a molecular ion ($[M+H]^+$) at *m/z* 195.1 (peak 1), followed by theophylline and theobromine (peaks 2 and 3) with *m/z* 181.3 and 181.1 ($[M+H]^+$), respectively, which were assigned by comparison with commercial standards. The presence of others common xanthines in cocoa such as paraxanthine or 7-methylxanthine was not detected.

Regarding flava-3-ols, the cocoa extract sample contained (+)-catechin and (–)-epicatechin, in agreement with other reports (Kelm et al., 2006; Tomas-Barberán et al., 2007). Indeed, (+)-catechin and its isomer (–)-epicatechin eluted at 17.3 min and 17.6 min, respectively (peaks 4 and 5) and were precisely assigned by comparison with commercial standards. No evidence of galloylated catechin was found. Among the observed peak signals detected through the rest of the analysis, these show molecular ion species consistent with those of procyanidins oligomers (Es-Safi et al., 2006). For example, by comparing with commercial standards, the identification of procyanidin dimers B2 [EC-4β→8-EC] at 35.5 min (peak 8) and B1 [EC-4β→8-C] at 35.7 (peak 9) min was confirmed; both showed ions at *m/z* 577.8 and 577.4, respectively, whose fragmentation generated ions at *m/z* 425, 289 and 245. Thanks to the combination of HILIC and RP separations, the present development allowed the separation of different isomers for a given polymerization degree, as can be observed from the information collected in Table 2.

Regarding high DP procyanidins, these compounds are more difficult to assign due to the lack of commercial standards. In this regard, their tentative identification was based on mass spectra data, correlation of retention time for both ^{1}D and ^{2}D, UV-Vis spectra and careful correlation of LC × LC analysis (see Figure S3 as example). Moreover, the detected molecular ions followed a

Table 2. Characterization of cocoa secondary metabolites separated using the optimized LC×LC-ESI-MS/MS method developed.

Peak	Compound	Ret. Time (min)	*m/z*	Main Fragments
Xanthines ($[M+H]^+$)				
1	Caffeine	15.3	195.1	175; 158; 121
2	Theophylline	15.5	181.3	137; 117; 99
3	Theobromine	15.6	181.1	158; 138; 104
Monomeric flavan-3-ols ($[M-H]^-$)				
4	Catechin	17.3	289.3	245; 195
5	Epicatechin	17.6	289.4	245; 195
6	Unknown compound	27.6	589.0	589; 441; 295; 113
Oligomeric proanthocyanidins ($[M-H]^-$)				
7	Procyanidin Dimer	35.4	578.1	425; 290; 245;205
8	Procyanidin Dimer	35.5	577.8	577; 425; 245;289
9	Procyanidin Dimer	35.7	577.4	577; 425; 245;289
10	Procyanidin Trimer	40.6	865.6	577; 455; 384; 289
11	Procyanidin Trimer	41.5	865.4	578; 441; 384; 289
12	Procyanidin Trimer	41.8	865.7	739; 577; 425; 289
13	Procyanidin Trimer	43.5	865.4	664; 580; 440; 113
14	Procyanidin Tetramer	44.7	1153.3	865; 531; 384; 289
15	Procyanidin Tetramer	45.5	1153.5	865; 577; 455;289
16	Procyanidin Tetramer	45.6	1153.8	866; 575; 395; 289
17	Procyanidin Tetramer	45.8	1153.7	865; 575; 395; 289
18	Procyanidin Pentamer	47.8	1441.3	1153; 865; 384; 289
19	Procyanidin Pentamer	48.8	1441.5	1153; 865; 775;384
20	Procyanidin Pentamer	49.6	1441.5	1154; 863; 720; 384
21	Procyanidin Hexamer	51.6	1730.2	1146; 865; 384; 289
22	Procyanidin Hexamer	51.7	1729.2	1151; 865; 384; 289
23	Procyanidin Hexamer	53.6	1729.3	1151; 865; 384; 289
Doubly-charged proanthocyanidins ($[M-2H]^{2-}$)				
24	Procyanidin Heptamer	55.7	1008.6	865; 515; 384; 289
25	Procyanidin Heptamer	57.7	1008.7	779; 531; 384; 289
26	Procyanidin Octamer	59.7	1153.3	779; 515; 384; 289
27	Procyanidin Nonamer	63.8	1297.4	1175; 865; 739; 515
28	Procyanidin Decamer	65.9	1442.5	1152; 865; 384; 289
29	Procyanidin Undecamer	68.0	1599.2	865; 515; 384; 289
30	Procyanidin Dodecamer	69.8	1733.9	1440; 865; 383; 290

particular pattern described by the following formula: [290 + 288 (*n*-1) +152*g* + 162*h*]$^{-}$, where 290 represents the molecular weight of terminal catechin/epicatechin unit, *n* is the degree of polymerization, *g* is the number of galloyl esters and *h* the number of hexosides. This formula may indicate the presence of glycosidic and galloylated procyanidin structures (Es-Safi et al., 2006; Hellström et al., 2009).

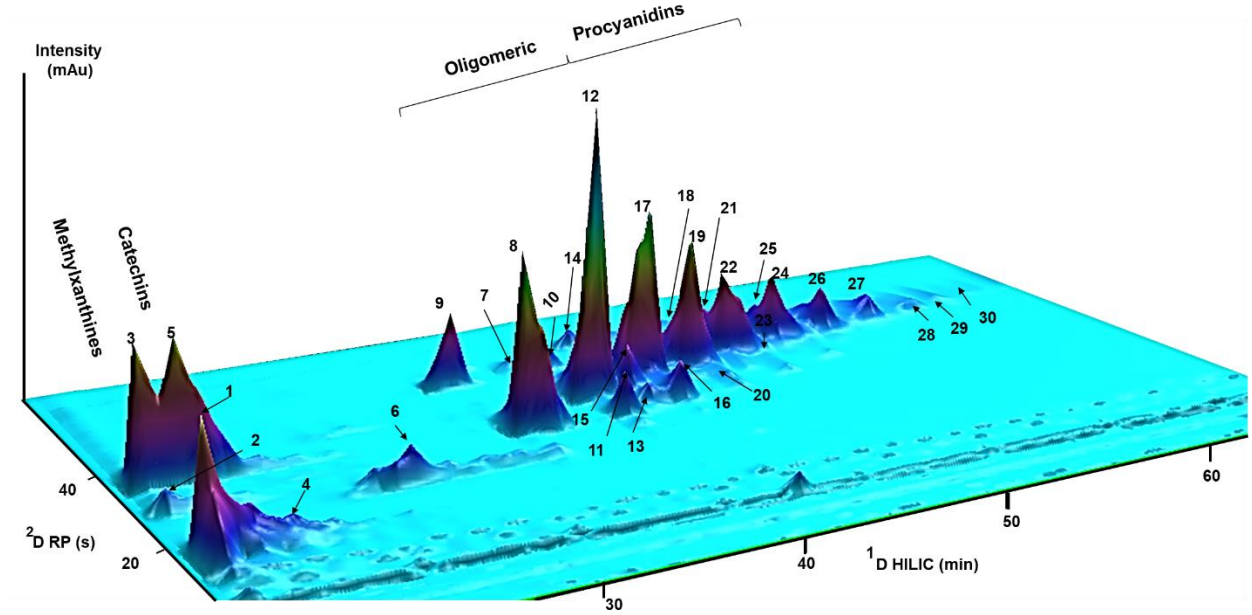

Figure 5. Three-dimensional chromatogram of cocoa secondary metabolites obtained under the optimized conditions using the novel LC × LC method developed. For detailed separation conditions, see text. Peak identification as in Table 2.

The results showed that different series of ions (peaks 13-23) separated by 288 Da from *m/z* 865 to 1730, were consistent with procyanidins with DP 3-6. For example, ions at *m/z* 865, 1153, 1441, and 1729 correspond to molecular species of mono-charged trimers, tetramers, pentamers and hexamers, respectively. The fragmentation pattern also followed a retro-Diels-Alder fission

mechanism (RDA) as well as quinone methide mechanism (QM) which is in agreement with previous reports (Gu, Kelm, Hammerstone, Zhang, et al., 2003). For example, the ion at *m/z* 577 presents a loss of 288 Da (final *m/z* 289) and corresponds to QM cleavage whereas the fragments with *m/z* 425 can be assigned to RDA ($[M-H-152]^-$) re-arrangements.

As mentioned before, each DP of proanthocyanidins presents numerous isomers, and the composition and number of isomer depends on several factors such as the plant variety or the geographical area where the plant is harvested. Thanks to the separation obtained by the developed LC × LC method, the identification of the particular isomer composition of these oligomers present in cocoa beans was possible. In particular, three dimeric-, three trimeric-, four tetrameric-, three pentameric- and three hexameric procyanidins isomers that could not be separated employing 1DLC were identified.

Doubly-charged ions ($[M-2H]^{2-}$) species with fragmentation patterns consistent with procyanidins oligomers were detected for higher oligomers starting from DP 7. Indeed, ions with *m/z* 1008, 1153, 1297, 1442, 1599, and 1733 (peaks 24-30) were tentatively attributed to procyanidin heptamers, octamer, nonamer, decamer, undecamer, and dodecameric respectively. The formation of multiply charged species beyond the doubly-charged ions was not detected. The identification of larger tannins was difficult due to the lower polymer concentration, peak signal dispersion, formation of multiple ions, and limitations of the ion trap MS analyzer. In addition, presence of prodelphinidins, and galloylated or glycosylated (epi)catechin were not detect.

The obtained results are in agreement with previous reports by Kalili & de Villiers (2009) and Kalili & De Villiers (2013) Continue in the same line, off-line and on-line comprehensive two-dimensional LC methods were employed for the analysis of procyanidins from cocoa. The off-

line method was able to separate A- and B- type procyanidins among other compounds, at the cost of very long analysis times. On the other hand, using the on-line method, authors obtained completely resolved peaks of procyanidins up to DP = 8 using a most sensible fluoresce detector in 100 min. In the present work, the application of a focusing modulation approach through an active modulation strategy coupled to a DAD allowed obtaining an effective separation of different procyanidin isomers up to DP = 12, reducing analysis time to 75 min. Moreover, although comparatively, off-line two-dimensional LC analyses offer less restriction on ^{2}D analysis time compared to on-line LC × LC, the on-line method developed in the present contribution provides advantages such as full automation, faster analyses and less risk of analyte loss.

In summary, the application of the developed on-line LC×LC-DAD-MS/MS method for the analysis of procyanidins from green cocoa allowed a good separation of main monomeric and oligomeric (epi)catechins. Thirty compounds including 3 xanthines, 2 monomeric catechins and 24 oligomeric procyanidins with a degree of polymerization up to 12 were separated and tentatively identified. It was possible to separate no only procyanidins in agreement to their increasing DP, but also their major isomers. Consequently, the present developed method implies a step forward the existing methodologies for the analysis of procyanidins in cocoa.

4.4. CONCLUSIONS

The use of a new LC × LC coupled to ESI-MS/MS allowed the attainment of significant gains for the separation and elucidation of secondary metabolites present in cocoa, mainly flavan-3-ol monomers and procyanidin oligomers. After careful optimization of the separation conditions, including flow rate, temperature, type of solvent and gradients employed in both dimensions, the implementation of focusing modulation approaches based on the use of trapping columns and

active modulation was also studied. The results obtained showed that the combination of trapping columns and active modulation allowed better separation of proanthocyanidins and their isomers with a DP up to 12. This is the first time that active modulation is used for the separation of proanthocyanidins. Using this approach, solvent mismatch effects between dimensions are minimized producing better retention, peak shapes and sensibility of procyanidins. This novel method reinforced the applicability of two-dimensional LC × LC for the analysis of food complex samples, such as procyanidins from cocoa

4.5. ACKNOWLEDGEMENTS

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4.6. SUPPLEMENTARY MATERIAL

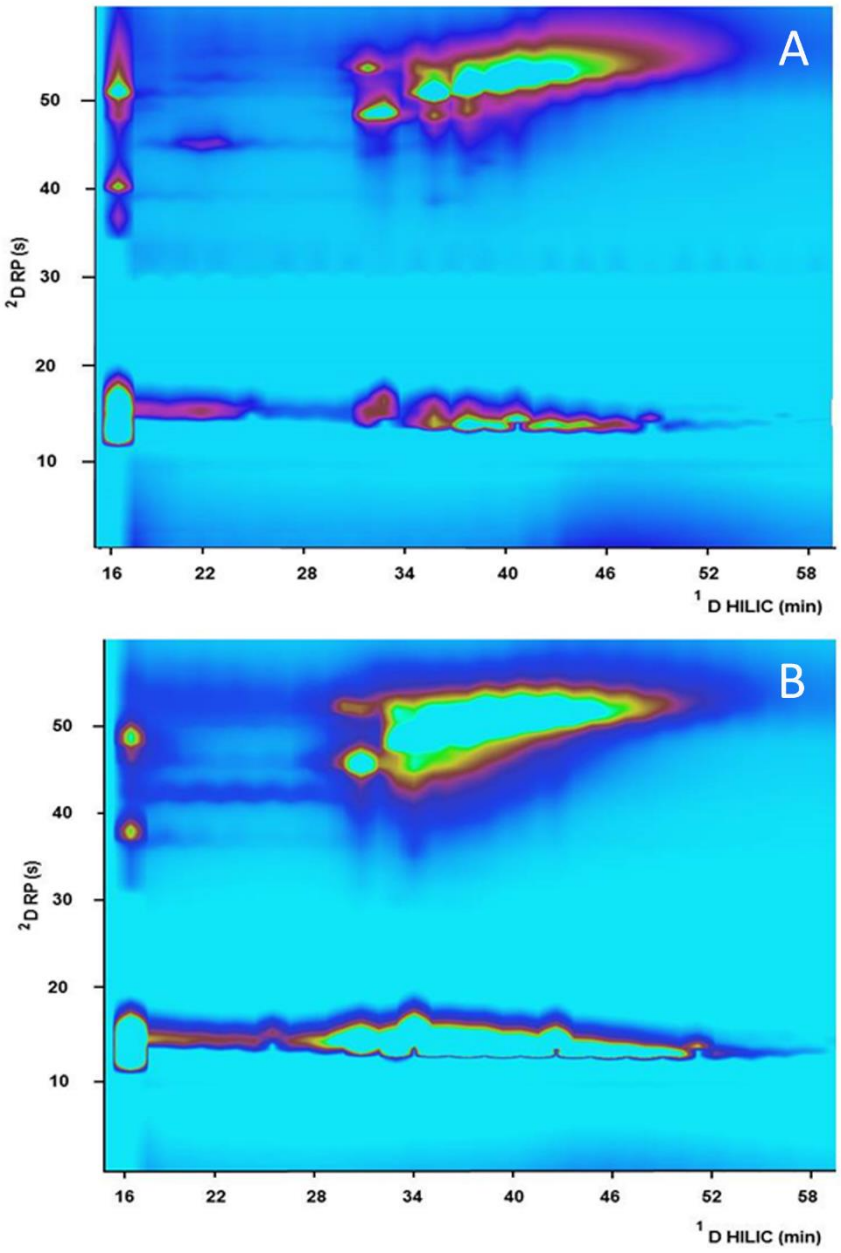

Figure S1. Comparison of the two dimensional LC × LC chromatograms (280 nm) of the cocoa secondary metabolites using A) acetonitrile or B) methanol as organic modifier in the ^{2}D mobile phase. For the rest of separation conditions, see text.

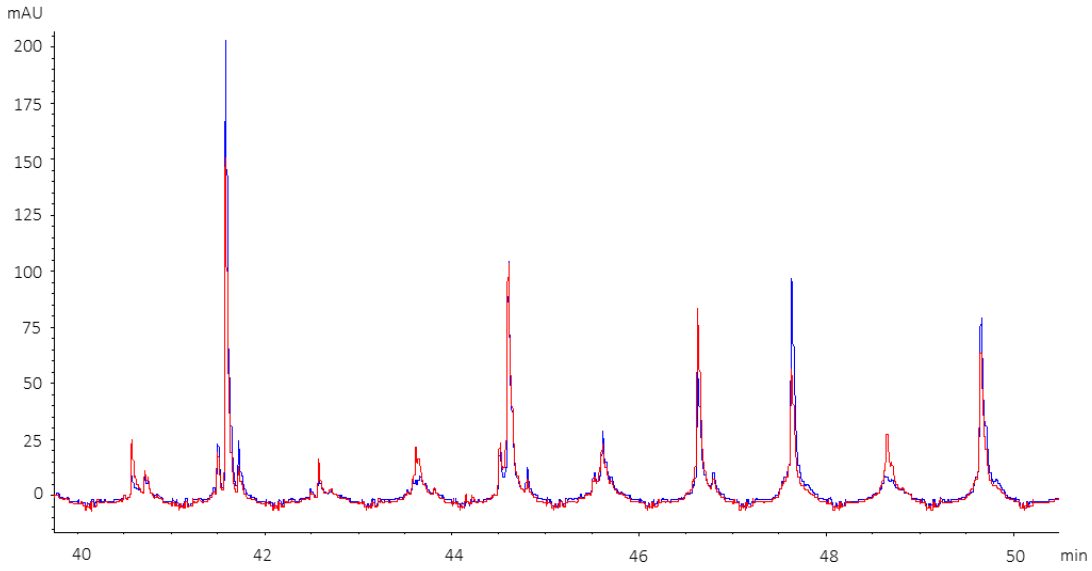

Figure S2. Linear chromatograms (280 nm) of the cocoa secondary metabolites obtained after the analysis by LC × LC using active modulation with a make-up flow composed by 98% acidified H_2O (0.05% TFA) and 2% ACN (0.05% TFA) at two different flow rates: 100 µl min^{-1} (blue line) and 160 µl min^{-1} (red line).

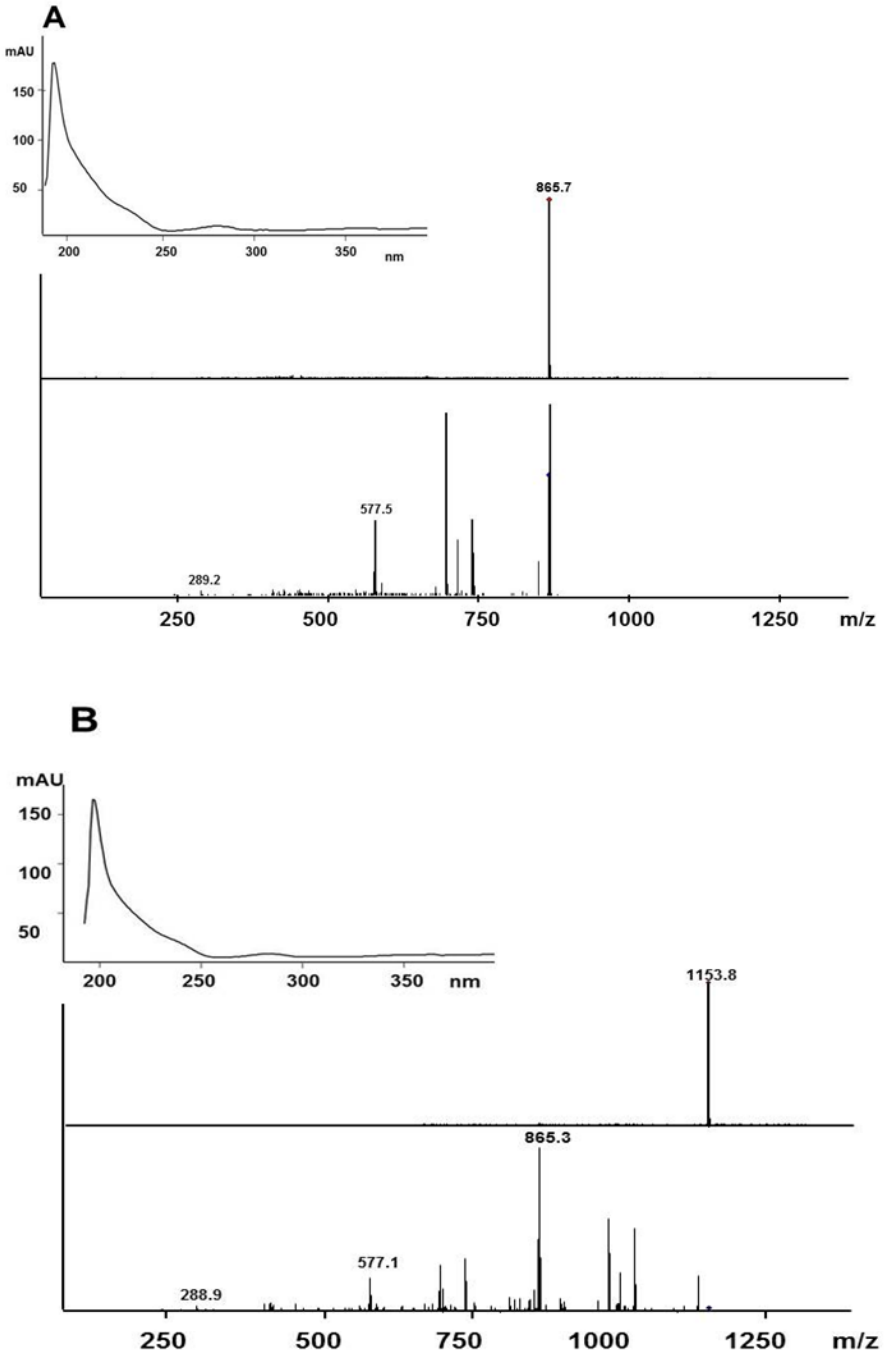

Figure S3. UV-Vis and MS spectra of procyanidin a) trimer and b) tetramer. Main detected fragments at *m/z* 865.7 ($[M-H]^-$): 577.5 ([M-H-epicatechin]), 289 (epicatechin). Main detected fragments at *m/z* 1153.8 ($[M-H]^-$): 865.3 (trimer); 577 (dimer); 288.9 (epicatechin)

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CHAPTER 4

PREPARATIVE SEPARATION OF PROCYANIDINS: COMPARATIVE STUDY OF DIFFERENT FRACTIONATION TECHNIQUES

ABSTRACT

Cocoa procyanidins were fractionated by several methodologies, including solid phase extraction, Sephadex LH-20 gel permeation, and preparative HPLC using C_{18} and diol stationary phases. In addition, all the isolated fractions were analyzed by UHPLC-Q-TOF-MS to determine their purity and final composition. According to our results, classical techniques allowed good separation of alkaloids and small procyanidins (PCs), e.g. catechins, dimers, and trimers, but were inefficient for oligomeric PCs. Preparative C_{18}-HPLC method allowed the attainment of high purity fractions of alkaloids, catechins, PCs with DP < 4, and one enriched fraction containing a mixture of PCs. However, the best results were obtained by preparative diol-HPLC, providing a separation according to the increasing degree of polymerization. According to the mass spectrometry fragmentation pattern, the 9 isolated fractions (fractions II to X) consisted of exclusively individual PCs and their corresponding isomers (same degree of polymerization). In summary, an efficient, robust, and fast method using a preparative diol column for the isolation of PCs is proposed.

Keywords: Cocoa; fractionation; preparative separation

5. PREPARATIVE SEPARATION OF PROCYANIDINS: COMPARATIVE STUDY OF DIFFERENT FRACTIONATION TECHNIQUES

5.1. INTRODUCTION

Procyanidins (PCs) are one of the subclass of flavonoids which consist of flavan-3-ols units such as (+)-catechin and/or (–)-epicatechin linked through $C_4 \rightarrow C_8$ bonds but also extent through a $C_4 \rightarrow C_6$ linkages, both are referred to as B–type PCs (Domínguez-Rodríguez, Marina, & Plaza, 2017; Kimura, Ogawa, Akihiro, & Yokota, 2011; H. J. Li & Deinzer, 2008). Additional ether bond between position 2 and 7 ($C_2 \rightarrow O \rightarrow C_7$) can be found and is denoted as A-type PCs, which is less common and previously reported (Kimura et al., 2011; H. J. Li & Deinzer, 2008). These flavan-3-ols compounds can be also esterified with gallic acid and glucose moieties (Glavnik, Vovk, & Albreht, 2017), therefore increasing the structural diversity of PCs.

These compounds are relevant to human diet since they are present in different foodstuffs, such as tea, grape, apple, fruit juices, and cocoa-based products (de Pascual-Teresa, Santos-Buelga, & Rivas-Gonzalo, 2000; Winterhalter, Esatbeyoglu, & Wilkens, 2010) among others. In general, the molecular weight of PCs is expressed as degree of polymerization (DP). According to their DP, PCs are classified in monomers (DP = 1), oligomers (DP = 2-10) and polymers (DP >10) (Winterhalter et al., 2010). Previous works have reported a DP up to 12, 17 and 20 for cocoa, cider apple skin and litchi pericarp, respectively (Guyot, Doco, Souquet, Moutounet, & Drilleau, 1997; Le Roux, Doco, Sarni-Manchado, Lozano, & Cheynier, 1998; Robbins et al., 2009).

The importance of these compounds is related to their functional bioactivities. For instance, PCs have a protective and nutritional role in plants (Treutter, 2006), they also influence the development of flavors and colors of the fruits or food product (Afoakwa, Paterson, Fowler, & Ryan, 2008; Alonso García, Cancho Grande, & Simal Gándara, 2004), and show *in vivo* and *in vitro* bioactivities, acting as protective agents for cancer and increasing the antioxidant status of human organism, which is widely discussed by Dillard & German (2000).

However, studies focusing on functional activities, dietary effects, interaction with proteins, changes occurring under gastrointestinal conditions, metabolic fate and cytotoxic actions of individual oligomeric procyanidins are poorly developed. In fact, numerous works include the use of monomeric catechins, but few of them include higher molecular weight procyanidins (e.g., procyanidin B1, B2, and C1) mainly due to high production costs and lack of commercial standards.

A variety of classical approaches have been used to fractionate procyanidins. For example, catechin and alkaloids have been fractionated by liquid-liquid separation using a solvent system consisting on different polarity solvents (e.g., methanol-ethyl acetate mixtures), moderately polar (e.g methylene chloride), non-polar solvents (e.g., chloroform) and polar protic solvents (e.g., methanol, n-butanol, water) (Hu, Wan, Bal, & Yang, 2003; Hulbert, Biswal, Mehr, Walker, & Collins, 1998; Rao, 1975). Size exclusion chromatography using Toyopearl HW-40/50 or Sephadex LH-20 eluting with aqueous acetone, aqueous alcohol, urea and their combinations have been commonly used (Kimura et al., 2011; Sun, Leandro, de Freitas, & Spranger, 2006; Svedström, Vuorela, Kostiainen, Laakso, & Hiltunen, 2006); for instance, A Yanagida et al., (2003) used Toyopearl HW-40F in the presence of aqueous acetone and 8 M urea, achieving good separation of catechin monomers, procyanidin B2, and procyanidin C1, but the remaining

fractions consisted of enriched oligomeric compounds. Similar results using polyethylene glycol resin column were obtained by Sasaki et al., (2009) whom obtained fractions of mixed procyanidins. Other classical techniques such as thin-layer chromatography, and column chromatography have been also reported but several disadvantages have been identified, such as: time-consuming, secondary pollution, complex process, irreversible adsorption on the solid-phase materials, low yield, and high-cost (Luo et al., 2016).

Recently, centrifugal partitioning chromatography (CPC), based on the partitioning of the solutes through the mixing of two immiscible phases, has been suggested since it allows higher sample input, high recoveries, and high repeatability (Zhang et al., 2015). Overall, there are several reports using CPC and its related techniques (e.g. counter-current chromatography, high-speed countercurrent chromatography, and flash counter current chromatography) for separation of alkaloids, catechins, dimers, and enriched oligomeric procyanidin fractions (e.g. trimers and tetramers) (Phansalkar et al., 2018; Valls, Millán, Martí, Borràs, & Arola, 2009). Main drawbacks included an incomplete separation of flavonoids because of their similar partition coefficient (K), and the need for several separations in series, or combination with preparative HPLC, to achieve a high degree of purification. In addition, longer run time, up to 9 h (L. Li et al., 2016) and no complete elution of flavonoids with K greater than 2, that can remain on the coil of the apparatus, are observed (Köhler, Wray, & Winterhalter, 2008). As a consequence, fractionation of procyanidins from plant extracts is still a challenge. Furthermore, the selection of a method is a critical factor since each plant source has its particular chemical profile, and its stereochemical diversity affects the degree of separation achieved (Phansalkar et al., 2018).

Normal-phase HPLC separation of procyanidins occurs according to the increase of molecular weight and has been previously assayed with dichloromethane-methanol-formic acid-water as

eluent (Rigaud J Prieur C, Souquet JM, Cheynier V., 1993). In this particular case, the separation of procyanidins is limited to medium DP values; moreover, the use of chlorinated solvents is considered hazardous and considerably limits the fractionation scale-up (Akio Yanagida et al., 2000). Better resolution and separation was obtained by Robbins et al., (2009) for cocoa beans in an analytical diol-phase column; these authors clearly separated alkaloids and procyanidins from monomer to dodecamers which were eluted using a gradient of acidified acetonitrile and aqueous acidified methanol. But large-scale fractionation of oligomeric procyanidins using both reverse and normal phase, together with structural characterization of the fractions, deserves further research.

Thereby, this work is aimed to establish a robust and easy procedure for the fractionation of procyanidins (with different degree of polymerization) from a cocoa extract, and to carry out their chemical characterization using UHPLC-QTOF-MS. For this purpose, various separation conditions such as temperature, solid-liquid ratio, gradient and run time were optimized for both semi-preparative C_{18}-reverse phase and diol columns. Moreover, the purification grade obtained was compared to classical separation methodologies involving the use of solid-phase separation and gel permeation chromatography on a Sephadex LH-20 column.

5.2. MATERIALS AND METHODS

5.2.1Samples and reagents. All the chemicals used were of analytical -reagent -grade and were not purified further. (+)-Catechin hydrate (≥ 99%; ASB-000003310), (–)-epicatechin (≥ 99%; ASB-00005127), procyanidin B1 (≥ 90%; ASB-00016230) and procyanidin B2 (≥ 90%; ASB-00016231) were purchased from ChromaDex Inc. (Irvine, CA, USA). Theobromine, caffeine, formic acid, sodium hydroxide, and glacial acetic acid were obtained from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, acetone were of HPLC-grade and acquired from VWR

Chemicals (Barcelona, Spain). Ethanol HPLC-grade were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Sephadex LH-20 was obtained from GE Healtcare (GE Healthcare GmbH, Freiburg, Germany). Milli-Q water (Millipore system, Billerica, MA, USA) was used for the preparation of all solutions. Polyphenolic extract from unfermented cocoa beans with low polyphenol oxidase activity was donated by CICTA lab (Bucaramanga, Colombia) according to the procedure by Toro-Uribe, López-Giraldo, & Decker (2018).

5.2.2. Fractionation of procyanidins.

5.2.2.1. Solid phase separation using diol-C_{18} cartridges connected in series. Solid phase extraction was performed with SPE-Cartridge C_{18} (300 mg; Lida, Kenosha, WI, USA) and SPE-Cartridge diol (500 mg, Discovery DSC-Diol, 70-Å pore diameter, Supelco, Bellefonte, USA). The diol – C_{18} cartridges were connected in series. Cartridges were preconditioned with 20 mL water (pH 7.0). Then, the sample was allowed to get adsorbed into the matrix by gravity. After that, the different solvent mixtures were sequentially passed through by negative pressure (vacuum manifold instrument operated at 0.03 MPa) as follows: 10 mL water (pH 7, eluent I) to flush out the phenolic acids and alkaloids, 15 mL ethyl acetate (eluent II) to elute the catechin and low molecular weight PCs, 15 mL of acetonitrile (eluent III), and then 15 mL of acetonitrile-methanol (60/40 *v/v*). The preparation of the sample (2.0 mg/mL) consisted of dissolution in water with 0.01% formic. The concentration of the fraction was under a gentle flow of nitrogen gas at 30ºC (TurboVap LV, Caliper Life Science, Hopkinton, MA). Then, the dried fractions were stored at -80 ºC until further analysis. All the fractions were analyzed by UHPLC-QTOF-MS method described in section 2.2.5 to determine its final composition.

5.2.2.2. Column chromatography by Sephadex LH-20. Ten grams of Sephadex LH-20 were swelled in excess of methanol, and stirred (Thermomixer, Eppendorf, Hamburg, Germany) at room temperature for 6 h. Then, a glass column (3 × 50 cm), plugged with cotton wool and a layer of sand (0.6 cm), to prevent the leaking of the stationary phase, was first washed with methanol and then carefully packed with a slurry containing Sephadex LH-20 in methanol. To ensure complete packing, vacuum (0.08 MPa) was applied by attaching the vacuum tubing to the bottom of the column without deforming the bed. After that, the column was equilibrated with five-bed volumes of 60% (*v/v*) aqueous methanol at a flow rate of ~ 6 cm/h. The polyphenolic sample (100 mg) was dissolved in methanol-water-formic acid (60/39.9/0.1 *v/v/v*), filtered by hydrophilic Durapore PVDF membrane 0.45 µm (Millipore, USA), and applied to the Sephadex LH-20 column. The solvent system for the separation of PCs was based on Kennedy & Taylor (2003) with few modifications as follows: 60% (*v/v*) aqueous methanol (eluent I), 75% (*v/v*) aqueous methanol (eluent II), 90% (v/v) aqueous methanol (eluent III), methanol-water-acetone (80/10/10 *v/v/v*) (eluent IV), methanol-water-acetone (65/15:20 *v/v/v*) (eluent V), methanol-water-acetone (40/30/30 *v/v/v*) (eluent VI), 60 % (*v/v*) aqueous acetone (eluent VII), and methanol-water-acetone (30/50/20 *v/v/v*) (eluent VIII). Each eluent (consisting on 200 mL) was applied sequentially, and the corresponding pooled fractions were immediately concentrated as above mentioned (section 2.2.1). All the fractions were analyzed by UHPLC-QTOF-MS method described in section 2.2.5 to determine its final composition.

5.2.2.3. Preparative isolation by reverse phase HPLC As we previously reported Toro-Uribe et al., (2018) the preparative HPLC (Prominence LC, Shimadzu, USA) system consisting of a degasser, a quaternary pump, UV-Vis detector, an autosampler, and automatic fraction collector was operated at 12 mL/min using as solvents acidified water (0.1% acetic acid, solvent A), and

pure ethanol (solvent B). The optimal linear gradient was: 0 min, 6% B; 20 min, 10% B; 30 min, 15% B; 40 min, 30% B. After each injection, the column reached 80% B for 5 min followed by 15 min re-equilibrations of the column to initial conditions. The preparative HPLC column was a C_{18} (250 × 10 mm, 10µm, Alltech, USA) connected to guard column refill C_{18} (Part # 28551, Alltech, USA) and was operated at 55ºC using a temperature control module (TCM, Millipore, Waters, USA).

The preparation of the sample consisted of dissolution in solvent A and then filtering through 10 µm and then 2 µm pore size filters (nylon membrane filter, diameter 47 mm, GVS Maine Magna, USA). Each injection volume was equivalent to 80 mg/load. Isolated fractions were initially stored at -20ºC and concentrated using a vacuum evaporator connected to a dry-ice cold trap (R-114, Büchi, USA) followed by freeze-drying in a VirTi Genesis freeze dryer (SP Inc, Gardiner, NY, USA) for 72 h and finally stored at -80 ºC under nitrogen atmosphere until further analysis. The purity of each fraction was determined by the analytical UHPLC-QTOF-MS method described in section 2.2.5.

5.2.2.4. Preparative isolation by HPLC using a Diol column. A semi-preparative HPLC instrument (1290 infinity series II, Agilent Tech., Germany) consisting of a degasser, a quaternary pump, a UV-Vis detector, a thermostated autosampler, an oven and equipped with an automatic fraction collector was used. The separation was performed on a Develosil Diol column (250 × 10 mm, 5 µm) at 50 ºC. The flow rate was set at 5 mL/min, and the injection volume was equivalent to 70 mg/load. The mobile phase was based on a previous work by Robbins et al., (2009) using acetonitrile/acetic acid (98/2 *v/v,* A) and methanol/water/acetic acid (95/3/2 *v/v*, B). The optimal linear gradient applied was: 0 min, 10% B; 0.5 min, 12%B; 1.5 min, 12%B; 6.0 min, 18% B; 12.5 min, 35%B; 12.6 min, 100 %B; 13.6 min, 100 %B. Then, returned to initial conditions (10% B)

and re-equilibrated for 10 min. The sample was dissolved in acetone/water/acetonitrile/acetic acid (60/29.5/10/0.5 *v/v/v/v*) and filtered through a 0.45 μm (Durapore PVDF, Millipore, USA) membrane. The thawed fractions were pooled and stored at -80 ºC for less than 5 days. After that, collected samples were dried using a N_2 stream at 30ºC (TurboVap LV, Caliper Life Science, Hopkinton, MA). The dried fractions were stored at -80 ºC until further analysis. The purity of every fraction was determined by the analytical UHPLC-QTOF-MS method described in section 2.2.5.

5.2.3. Characterization by UHPLC-QTOF-MS Analyses were performed with an Agilent UHPLC (1290 Infinity series I, Agilent, USA), consisting of a degasser, binary pump, column oven, UV-Vis detector and a thermostated autosampler. The data acquisition was carried out using the Mass-Hunter LC/MS software (v. 5.01, Agilent). All the samples were dissolved in water with 0.01% formic acid, and 4 μL was injected into a Zorbax Eclipse Plus C_{18} column (50 × 2.1 mm, 1.8 μm) maintained at 55ºC. The flow rate was 0.7 $mLmin^{-1}$ with solvent A composed of water with 0.01% formic acid, and organic solvent B composed of acetonitrile with 0.01% formic acid in negative ionization mode. For positive ionization mode, the concentration of formic acid was 0.1% in both solvents A and B. Gradient elution was applied as follows: 0 min, 0% B, 3.9 min, 1.5% B; 4.0 min, 4% B; 11.0 min, 10 %B, 14.0 min, 35% B; 14.2 min, 100% B; 16.5 min, 100% B; 17.0 min, 0% B; 23 min, 0% B. The resulting separation was recorded at 280 nm. All the samples were injected by triplicate. Additionally, a blank sample was injected between every sample.

The data were collected in negative ESI mode and selected samples were also analyzed in positive ESI mode on a QTOF-MS instrument (model 6540, Agilent) operated in full scan mode from *m/z* 25-3200 using the following settings: capillary voltage, 4000 V; acquisition rate, 2

spectra per second; nebulizer pressure, 40 psi; drying gas, 10 L/min; temperature, 350 ºC. During the analysis, two reference masses ($C_5H_4N_4$ and $C_{18}H_{19}O_6N_3P_3F_{24}$) were used. Thus, *m/z* 119.0363 and *m/z* 966.0007, and *m/z* 121.0508 and *m/z* 922.0098 for negative and positive mode were employed, respectively. These masses were continuously infused to the system to allow constant mass correction. Data treatment was performed using Mass-Hunter Qualitative Analysis (Agilent, B.07.00). MS characterization features were analyzed using commercial standards reagents, extraction ion compound tool, and exact mass databases searched against the METLIN and HMBD databases.

5.3. RESULTS

In this study, different techniques to fractionate procyanidins from cocoa extract were evaluated and compared. As mentioned, classical fractionation techniques based on solid phase extraction and gel permeation column were chosen for being rapid and straightforward. In addition, preparative HPLC with two different polarity columns was also studied.

5.3.1. Separation using solid phase extraction. Optimization of this procedure consisted on an individual evaluation of C_{18} and diol cartridges (data not shown). Better results were obtained when diol and C_{18} cartridges were coupled in series (Figure 1). Thus, four fractions were collected (as described in Section 2.2.1) and analyzed by UHPLC-QTOF-MS. As can be seen in Figure 1, fraction I consisted mainly of theobromine, caffeine, (–)-epicatechin, and a small amount of (+)-catechin. Interestingly, within fractions II-IV the oligomeric procyanidins were eluted sequentially, e.g., fraction II, III and IV were enriched with DP of 2-5, 5-8, and 4-9, respectively.

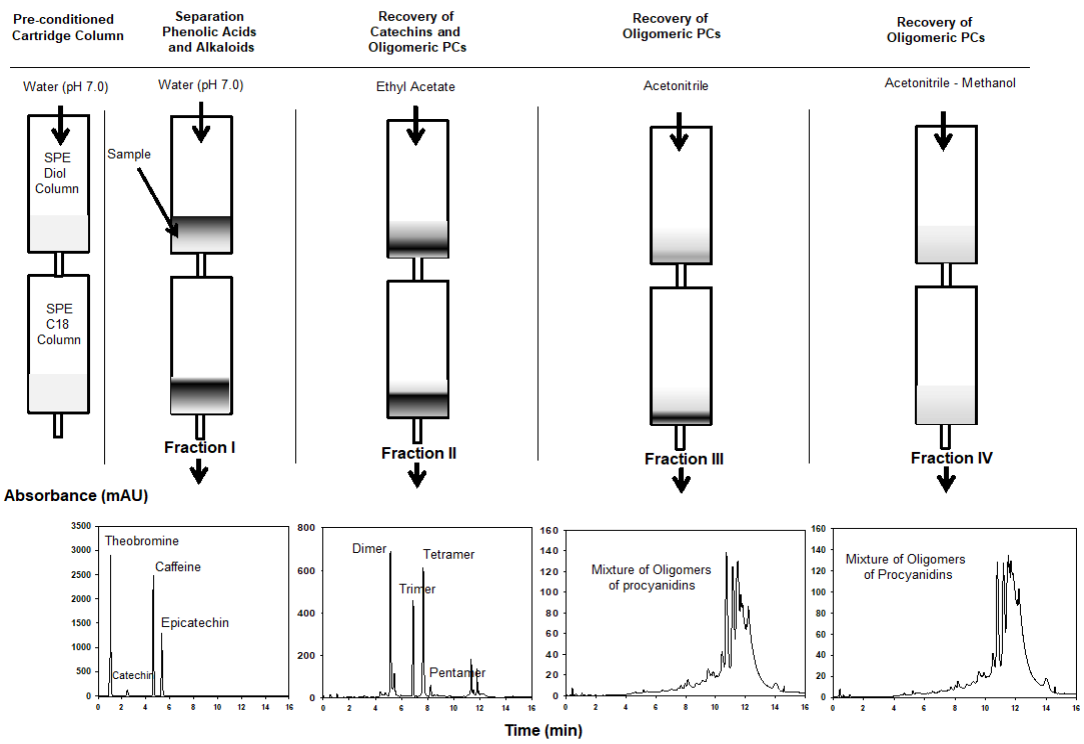

Figure 1. Scheme for the fractionation of procyanidins using SPE cartridges Diol-C_{18} coupled in series.

5.3.2. Separation on Sephadex LH-20. Sephadex LH-20 was chosen as a second classical fractionation technique for being simple and widely reported for the separation of non-polymeric and polymeric phenols (Amarowicz & Shahidi, 1996; Kimura et al., 2011; Sun et al., 2006; Svedström et al., 2006). Following the procedure by Kennedy & Taylor (2003) eight fractions were collected (see Section 2.2.2. for fractions' elution); their main constituents are detailed in Figure 2. The chromatographic profiling at 280 nm showed that fraction I consisted mainly of theobromine and caffeine, whereas fraction II-III contained catechins, traces of oligomeric PCs (DP 2-4) and alkaloids. In fact, these fractions were obtained using aqueous methanol which enables the recovery of non-phenolic compounds and PCs with DP≤4. A subsequent elution by increasing the concentration of acetone in methanol allowed the recovery of oligomeric PCs

(fractions IV-VII), but all the fractions consisted on a mixture of PCs with different DP and a hump approximately at 12.5 min of retention time because of the co-eluted polymeric polyphenols (Figure 2). The last fraction, corresponding to the elution with acetone-water-methanol, showed a lower DP than previous fractions since washing phase could dissolve low molecular procyanidins adsorbed on the stationary phase. In agreement to our results, Neto et al., (2006) could obtain the fractionation of proanthocyanidins from cranberry, being able to separate eight fractions consisting of a mixture of oligomeric proanthocyanidins (DP 2-11). These results suggest that individual PCs compounds cannot be separated using classical gel permeation column.

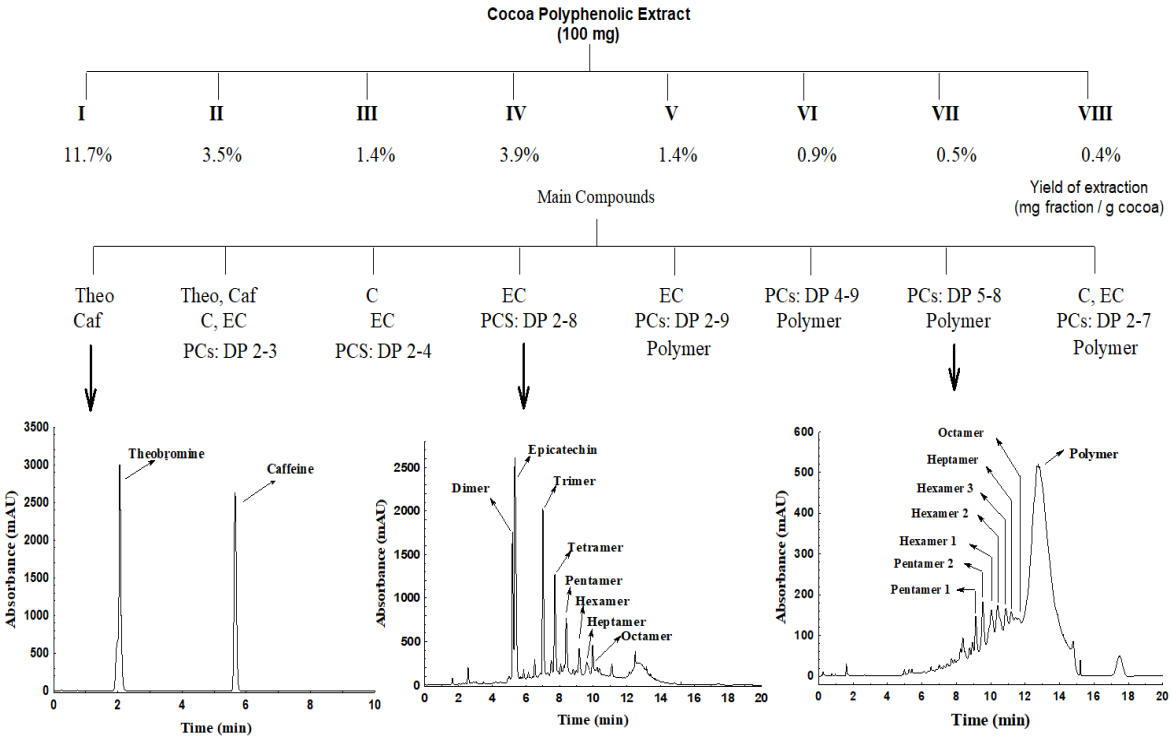

Figure 2. Scheme for the separation of cocoa procyanidins using Sephadex LH-20. Theobromine ,Theo; Caffeine, Caf; (+)-Catechin, C; (-)-Epicatechin, EC; PCs, procyanidins; DP, degree of polymerization

5.3.3. Separation by preparative HPLC under reversed phase conditions. In order to achieve successful separation of procyanidins using a preparative C_{18} column, run time, solvent gradient, and flow rate were optimized. In fact, analysis of procyanidins at analytical scale employing C_{18} columns have been previously assayed (Tomas-Barberán et al., 2007). However, the scale up from analytical scale to preparative scale is still a challenge. In this study, different factors influencing the final separation were tested: column temperature between 25-55 ºC, flow rate from 8 to 20 mL/min, and gradient was modified by changing % B (increasing 5% B in each test) and by changing the concentration of acetic acid (0.1 – 2.0 % *v/v*) in phase A. Besides, acetonitrile and ethanol were evaluated in previous assays as organic phase, but not significant improvement was observed when acetonitrile was used (data not shown). Thereby, the solvent B consisted of pure ethanol.

In general, lower flow rates increased analysis time, and neither improved the separation nor procyanidins peak shape. On the other hand, at higher flow rates, procyanidins with higher molecular weights eluted as an unresolved peak. No significant changes on procyanidin separation were observed with the increase on the concentration of acid present in solvent A.

Therefore, the lowest concentration (0.1% acetic acid, *v/v*) was chosen. Regarding temperature, better resolution between dimers, trimers and tetramers were evidenced at separation temperature higher than 50 ºC. Overall, the best separation conditions consisted on acidified water (0.1 % acetic acid, Solvent A) and ethanol (Solvent B) at a flow rate of 12 mL/min (more details in methodology section 2.2.3). As can be seen in Figure 3A and Table 1, the elution order of cocoa extract consisted on a first elution of theobromine (6.8 min), followed by dimer (10.8 min), epicatechin (11.8 min), trimer (20.4 min), and mixtures of higher oligomers

(DP 4-7) from 22.2 to 42.8 min. Table 1 summarizes the tentative identification of the main compounds collected using reversed phase, as

Table 1. Yield, purity, characterization and structural mass pattern for each procyanidin fraction obtained by preparative reversed phase.

F	Yield (%)*	Main Compound	Other Compounds	RT (min)	Purity	Collision Energy (eV)	Mode	MS**	MS/MS fragments of main compounds**
0	1.10	Theobromine	ND	6.8	>95	20	$[M+H]^+$	181.0721	138.0661, 104.1065, 77.0385
I	1.21	Dimer	Traces of EC	10.8	>97	20	$[M-H]^-$	577.1350	407.0770, 289.0719, 125.0243
II	3.2	Epicatechin	Traces of Dimer	11.8	>95	20	$[M-H]^-$	289.0717	245.0809, 137.0232, 96.9593
III	0.4	Caffeine	Traces of EC	15.2	>95	20	$[M+H]^+$	195.0880	158.0267, 138.0664, 77.0388
IV	0.50	Trimer	Traces of dimer, tetramer and pentamer	20.4	>80	20	$[M-H]^-$	865.1972	577.1336, 425.0860, 289.0716
V	0.12	Tetramer	Traces of dimer. Trimer, and pentamer	22.2	>50	20	$[M-H]^-$	1153.2636	865.1994, 575.1237, 287.0563
VI	0.21	Hexamer	Tetramer, and heptamer	27.4	≤ 60	20	$[M-2H]^{2-}$	865.1962	577.1331, 417.0807, 289.0713
VII	0.07	Heptamer	Mixture of PCs with DP 3-7	32.7	-	20 20	$[M-2H]^2$ $[M-3H]^3$	1008.7238 672.1475	864.1825; 577.1337; 289.0719 575.1143; 289.0700; 125.0245
VIII	0.06	Octamer	Mixture of PCs with DP 3-9	36.7	-	20	$[M-3H]^3$	768.1671	575.1192; 413.0882; 289.0723

IX	0.75	Trimer to Pentamer	Mixture of PCs with DP 3-9	42.8	-	-	-	-	-

Table 2. Yield, characterization and structural mass pattern for each procyanidin fraction separated by preparative diol column.

Fraction	Yield (%)*	Main Compound	No. Isomers	Other Compounds	Collision Energy (eV)	Mode	MS	MS/MS fragments of main compounds
Alkaloids								
0	1.30	Theobromine	-	Traces of catechins	20	$[M+H]^+$	181.0724 195.0881	158.0260, 122.9975, 104.0602 138.0663, 110.0668, 77.0365
Catechins								
I	1.41	(+)-Catechin (-)-Epicatechin	-	Traces of dimer	20	$[M-H]^-$	289.0727	212.0750, 137.0241, 96.9602
B-type Procyanidins								
II	0.64	Dimer	5	Traces of EC, and trimer	20	$[M-H]^-$	577.1358	407.0781, 289.0727, 125.0246
III	0.61	Trimer	8	Traces of EC, and dimer	20	$[M-H]^-$	865.1993	713.1509, 575.1200, 287.0564
IV	0.39	Tetramer	14	Traces of EC	40	$[M-H]^-$	1153.2624	865.1971, 575.1197, 287.0564
V	0.63	Pentamer	17	Traces of EC and dimer	40	$[M-H]^-$	1442.3301	1153.2597, 863.1834, 287.0551
VI	0.26	Hexamer	18	Traces of EC	40	$[M-H]^-$	1730.3923	1151.2449, 863.1826, 287.0576
VII	0.60	Heptamer	19	Traces of EC	20	$[M-2H]^{2-}$	1008.7250	863.1819, 577.1364, 289.0723
VIII	0.16	Octamer	17	Traces of EC	40	$[M-2H]^{2-}$	1152.7555	865.2007, 575.1195, 287.0562
IX	0.29	Nonamer	15	Traces of EC	40	$[M-2H]^{2-}$	1296.7858	1152.2475, 863.1834, 287.0568
X	0.23	Decamer	14	Traces of EC	40	$[M-2H]^{2-}$	1440.8102	1151.2432, 863.1846, 287.0578

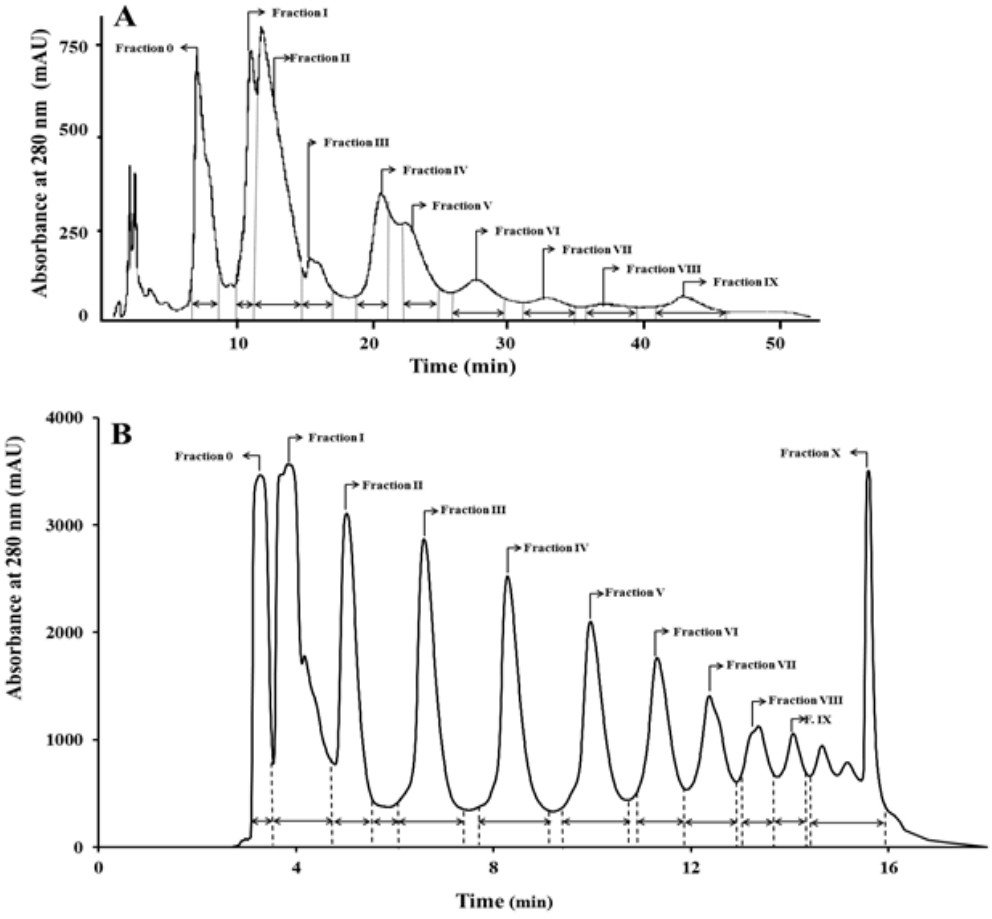

Figure 3. Preparative chromatogram obtained at optimum conditions using a) C_{18} stationary phase and b) diol stationary phase columns.

well as their main MS and MS/MS features. For instance, several fractions were collected showing purities > 95% for theobromine (fraction 0), caffeine (fraction III), and (–)-epicatechin (fraction II). As we previously reported, good separation was observed for fraction I (ca. 98 % dimer B2, 2% (–)-epicatechin), and fraction IV (ca. 2% dimer B2, 81 % trimer C1, 10% tetramer and 7% pentamer) (Toro-Uribe et al., 2018). For other fractions it was difficult to obtain purities around 80% and, as can be observed in Table 1, they were composed of mixtures of different oligomers, e.g. fraction V consisted of 3% dimer, 10 % trimer, 54% tetramer, and 33% pentamer.

Despite the careful optimization, the complexity of the sample and the number of isomers of procyanidins, that imply a mixture of very similar chemical structures closely eluting, did not allow a complete separation of oligomers (Figure 3A).

5.3.4. Separation by preparative HPLC using a diol column. The selection of mobile phase and column was made according to Robbins et al., (2009) The optimization of the separation of procyanidins was aimed at reducing solvent consumption whereas improving method reproducibility. As the separation occurs by increasing degrees of polymerization, the concentration of solvent B plays a key role on the complete separation of each procyanidin. Thereby, several experiments were performed to obtain the optimum gradient. For instance, it was observed that starting at 8 – 10% solvent B (methanol/water/acetic acid, 95/3/2 *v/v/v*) allowed a fast separation of alkaloids and monomeric catechins; however, when higher initial amount of solvent B was tested, a co-elution of oligomers and loss of baseline separation was obtained. Finally, a good separation of oligomers was achieved with a slow gradient of solvent B. Moreover, complete elution of polymeric PCs was reached using 100% of solvent B in the mobile phase.

Once the gradient was optimized, the target was to reduce analysis time, minimize pressure changes and achieve better resolution among high molecular weight procyanidins. The first factor to optimize was the column temperature from 30 – 50 ºC. As expected, faster and better separations were obtained at higher temperature (50 ºC); following, the effect of sample solvent was tested with the objective of dissolving PCs whereas, at the same time, removing non-phenolic compounds.

Thus, raw procyanidin extract was dissolved in solvent A (acetone/water/acetic acid, 70/29.5/0.5, *v/v/v*), solvent B (methanol/acetonitrile/acetic acid, 38/60/2, *v/v/v*), and solvent C (acetone/water/acetonitrile/acetic acid, 60/29.5/10/0.5, *v/v/v/v*). After dissolution, the sample was

centrifuged (5000 rpm, 5 min) and the supernatant was collected, filtered and injected in the system. Results showed better resolution of procyanidins with solvent A but caused an increase on the column backpressure as a result of the progressive precipitation of sample with the mobile phase and higher contamination of the column. Using solvent B higher shift on retention times and higher distortion of the peaks was observed. Therefore, solvent C was selected since it allowed elimination of interfering compounds without compromising the separation and the performance of the column and the chromatographic system.

Sample loading was also investigated considering from 30 to 150 mg of raw cocoa extract per injection. As expected, higher amounts (> 100 mg) led to loss of baseline, co-elution of PCs, low resolution and system overpressure, whereas a small amount decreased the overall yield of the process. As a result, 70 mg of sample was selected as the optimum amount for a good balance of yield-time-reproducibility of the process. Thus, in summary, fractionation of procyanidins using a preparative diol column was achieved at 5 mL/min, and 50 ºC of column temperature using the following linear gradient: 0 min, 10% B; 0.5 min, 12% B; 1.5 min, 12% B; 6.0 min, 18% B; 12.5 min, 35% B; 12.6 min, 100 % B; 13.6 min, 100 % B; 13.7 min, 10 % B; 23.7 min, 10 % B.

As can be in Figure 3B, good separation of the different procyanidins according to their increasing degree of polymerization was achieved. In fact, fractions labelled 0 to X were collected, pooled and used for further experiments. Pooled fractions were concentrated following three procedures: P_1) rotary evaporation at 30 ºC under vacuum plus drying with N_2 at 25 ºC; P_2) neutralization of each fraction using 0.1N NaOH plus drying with N_2 at 25 ºC; and P_3) drying using N_2. Results showed that during the rotary evaporation, the concentration of acid increased and led to degradation or oxidation of the sample. Regarding treatment P_2, the formation of salt as a result of the reaction between NaOH and CH_3COOH was observed and, as a consequence,

interfering compounds were detected during the analysis. Treatment P_3 was carried out successfully without affecting the chemical structure of the present compounds.

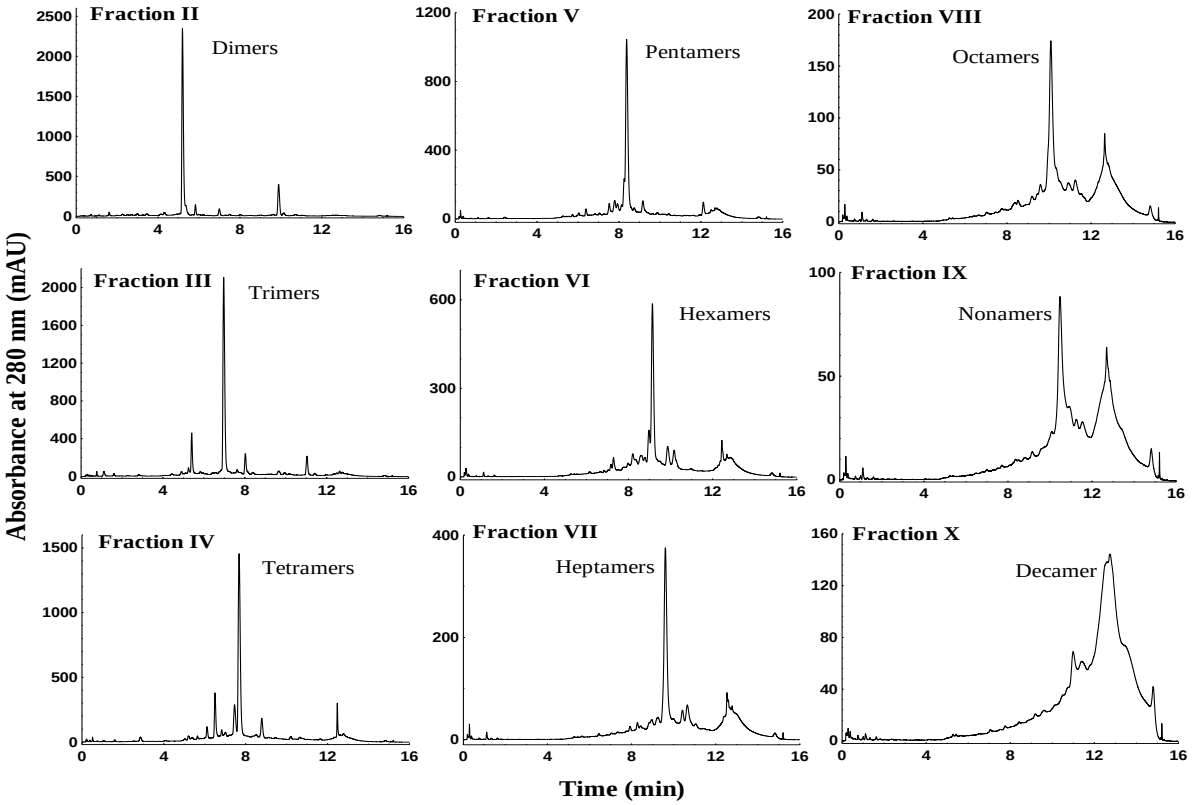

Figure 4. UHPLC profiles of oligomeric fractions obtained using a diol column

Chromatograms recorded at UV 280 nm for fractions II-X and average mass spectrum corresponding to those fractions are shown in Figures 4 and 5, respectively. As shown in Table 2, fraction II to VI showed $[M\text{-}H]^-$ ions at *m/z* 577.1358, 865.1993, 1153.2624, 1442.3301, 1730.3923, corresponding to dimer, trimer, tetramer, pentamer, and hexamer structures, respectively. Interestingly, all collected fractions consisted exclusively of PCs with the same DP; for instance, fraction III presented 4 main peaks, all of them identified as trimers, showing $[M\text{-}H]^-$ ions at *m/z* 865.1993 and MS/MS fragmentation patterns including ions at *m/z* 713.1509, 575.1200 and 287.0564. Also, doubly charged ions ($[M\text{-}2H]^{2-}$) detected at *m/z* 1008.7250,

1152.7555, 1296.7858, and 1440.8102 that corresponded to heptamer, octamer, nonamer, and decamer structures, respectively, were also identified in fractions VII – X.

It is worth to mention that lower concentrations of A-type procyanidins were also found, for example, trimer and tetramer with *m/z* 863.1868 (-2Da) and 1149.3679 (- 4Da), respectively, together with the presence of *m/z* 739.1888, 1025.2326, and 1313.2987 which can be attributed to A-type procyanidins (one or two type) linked to glucose moiety (e.g., β-galactopyranose, α-arabinopyranose and glucopyranosyl); as well as, the presence of novel configuration of dimer (*m/z* 575.1195 and 561.1358), trimer (*m/z* 849.2045), tetramer (*m/z* 1313.2987), pentamer (*m/z* 1425.3268), and hexamer (*m/z* 864.1901, $[M\text{-}2H]^{-2}$). These observations highlight the importance of high-resolution MS as useful tool for the characterization of cocoa proanthocyanidins as well as further studies involving their complete characterization, isolation, and metabolomic fate.

These finding confirmed the high diversity of chemical structures of proanthocyanidins (Spranger, Sun, Mateus, de Freitas, & Ricardo-da-Silva, 2008). Indeed, considering only molecules with catechin and epicatechin, the number of asymmetric carbon atoms, and the possible forms of rotation around the interflavan bonds (C_4–C_8 or C_4–C_6), theoretically, for higher oligomer and polymers the possible number of stereoisomers and rotational isomers is $2^{(5*DP-3)}$(Zhang et al., 2017). For example, 128, 4096 and 131072 combinations for dimers, trimers and tetramers could be found, respectively.

5.3.5. Overall discussion and comparison. Considering the available literature, this study reinforces the advantages of preparative HPLC compared to classical fractionation methods such as gel permeation or solid phase cartridges. In general, diol-C_{18} cartridges coupled in series and Sephadex LH-20 columns provide simple and fast methods for the separation of non-phenolic and phenolic compounds. However, no isolation of individual components can be achieved. In fact,

the purity of the individual procyanidins was poor; for instance, the contribution of each procyanidin was 33.3 % (dimer), 22.2% (trimer), 37.0% (tetramer), 0.3% (pentamer), and 7.2% (polymer) in fraction II obtained by diol-C_{18} cartridges, and 14.3%, 33.9%, 19.9%, 13.1%, 8.2%, 3.9%, 3.0%, and 3.8% from monomeric to octameric PCs in fraction IV obtained by Sephadex LH-20, respectively. These results are in agreement with those reported by Pedan et al., (2016) who found that complex groups, such as polymeric PCs, interacted irreversibly with Sephadex LH-20. This can be due to the separation mode, which is mainly based on adsorption phenomena rather than size-exclusion and, thus, polyphenols containing PCs are partly separated according to the different affinity for the gel matrices (A Yanagida et al., 2003). It can be concluded that classical techniques are mainly recommended for the isolation of alkaloids, a mixture of PCs with DP < 3 and a mixture of high molecular weight oligomeric compounds.

According to the mass spectrometry-based characterization, better separation and a limited number of isomers of procyanidins are obtained by preparative HPLC using a C_{18} column as a stationary phase. Results showed that better separation of individual alkaloids, catechins, dimer, and trimer could be achieved. However, the resulting fractions did not possess a purity of individual procyanidins higher than 80%.

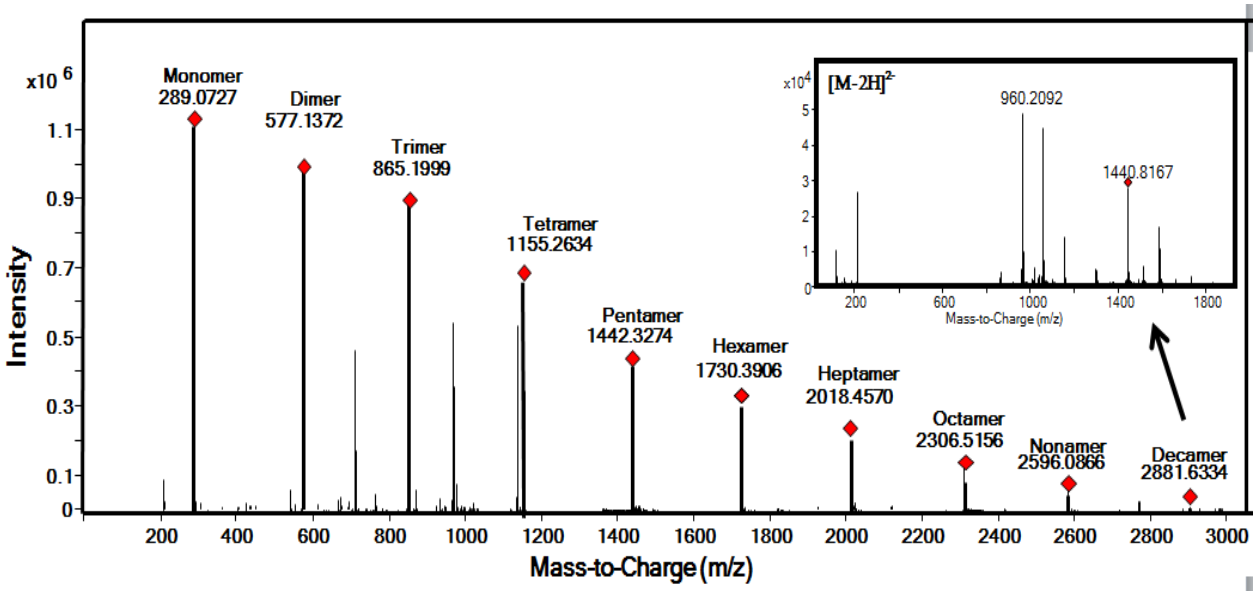

Figure 5. Negative ESI QTOF average mass spectrum obtained for all the cocoa procyanidins fractions.

For instance, fraction V and VI were composed by 54% tetramer (3% dimer, 10 % trimer, and 33% pentamer) and 60% hexamer (18% tetramer, and 22% heptamer) through reverse phase (Toro-Uribe et al., 2018) respectively. In addition, an increase of backpressure over time was also observed and, therefore, a clean-up procedure between batches was needed. This phenomenon could be a result of the adsorption of higher oligomers onto the stationary phase. One of the advantages of this procedure was the use of ethanol as mobile phase, which makes this process cheap and free of toxic solvents. Overall, the preparative reverse phase was effective for the isolation of theobromine, caffeine, catechins, and low molecular weight PCs with high purity. The main drawback was that it required a low injection volume in order to allow high reproducibility and, thus, provided low yields of the target compounds.

On the other hand, preparative diol-HPLC afforded higher reproducibility, higher yield and higher separation power of procyanidins as compared to the above-discussed procedures. All the collected fractions were characterized by a high degree of purity (> 95%) of isomers of procyanidins with the same DP.

5.4. CONCLUSIONS

A new and effective method for the large-scale separation of procyanidins has been developed using preparative HPLC using C_{18} and diol stationary phase columns. In addition, comparison of preparative HPLC vs. classical fractionation of procyanidins was also studied. Results confirmed that preparative HPLC has many advantages compared with solid phase separation and Sephadex LH-20 gel permeation. All the methodologies studied allowed good separation of alkaloids and catechin. Also, enriched oligomeric samples could be obtained using classical approaches and preparative reversed phase HPLC. Better separation of individual PCs according to the degree of

polymerization was only obtained using preparative-HPLC with a diol column. This procedure was demonstrated to be, by far, more efficient and robust than the use of C_{18} stationary phase.

5.5. ACKNOWLEDGEMENTS

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CHAPTER 5

RELATIONSHIP BETWEEN THE PHYSIOCHEMICAL PROPERTIES OF COCOA PROCYANIDINS AND THEIR ABILITY TO INHIBIT LIPID OXIDATION IN LIPOSOMES

ABSTRACT

The aim of this paper is to evaluate the effect of cocoa polyphenols and procyanidins with different degrees of polymerization to be encapsulated in a liposome delivery system and inhibit lipid oxidation at pH 3.0 and 5.0. In general, liposomes at pH 3.0 and 5.0 were physically stable in the presence of polyphenols and procyanidins with mean particle sizes of 56.56 ± 12.29 and 77.45 ± 8.67 nm and ζ-potential of -33.50 ± 3.16 and -20.44 ± 1.98 mV at pH 3.0 and 5.0, respectively. At both pH 3.0 and 5.0, all the polyphenols and procyanidins inhibited lipid hydroperoxide and hexanal formation, and antioxidant activity increased with increasing polymer chain size. The greater antioxidant activity of the isolated procyanidins was likely due to their increased metal chelating capacity as determined by FRAP, and the greater partitioning into the lipids as determined by Log Kow and encapsulation efficiency. The crude extract had the greatest antioxidant activity which could be due to the presence of other antioxidants or that combinations of the different polyphenol and procyanidin could inhibit lipid oxidation synergistically.

Keywords: Flavonoids, cocoa, liposome, antioxidant activity, lipid oxidation.

6. RELATIONSHIP BETWEEN THE PHYSIOCHEMICAL PROPERTIES OF COCOA PROCYANIDINS AND THEIR ABILITY TO INHIBIT LIPID OXIDATION IN LIPOSOMES

6.1. INTRODUCTION

Procyanidins are the main antioxidants present in cocoa, coffee, tea and grape seed based-products (Dreosti, 2000) that are widely known for properties such as scavenging reactive oxygen species by hydrogen atom donation, metal chelating, regeneration of α-tocopheryl radicals, and also displaying health-promoting activities such as anti-carcinogenic, anti-inflammatory and cardio-protection properties (Kris-Etherton & Keen, 2002; Steinberg, Bearden, & Keen, 2003; Yamagishi et al., 2003). The basic structure of procyanidins found in cocoa is shown in Figure 1. These compounds exhibit high antioxidant activity due to the presence of a) *o*-hydroxylation on the B-ring of the polyphenol molecule, b) the presence of additional free hydroxyl groups, and c) the C_2-C_3 double bond in the C-ring (Bors, Heller, Michel, & Saran, 1990).

Liposomes are microscopic vesicles consisting of membrane-like phospholipid bilayers surrounding an aqueous medium (Fang, Lee, Shen, & Huang, 2006). Several techniques are employed to produce liposomes from lecithin resulting in three different properties, namely, multilamellar vesicles (MLVs), small unilamellar vesicles (SUVs), and large unilamellar vesicles (LUVs) (Maherani, Arab-Tehrany, R Mozafari, Gaiani, & Linder, 2011). Liposomes have potential applications in both food and pharmaceutical industries as carriers of nutrients (McClements & Li, 2010; Torchilin, 2005). For example, liposomes can be used to target

drug/food component delivery to tissues, increase the bioavailability of drugs/food components and protect sensitive compounds from the environment (Alving, 1989; Iwanaga et al., 1999).

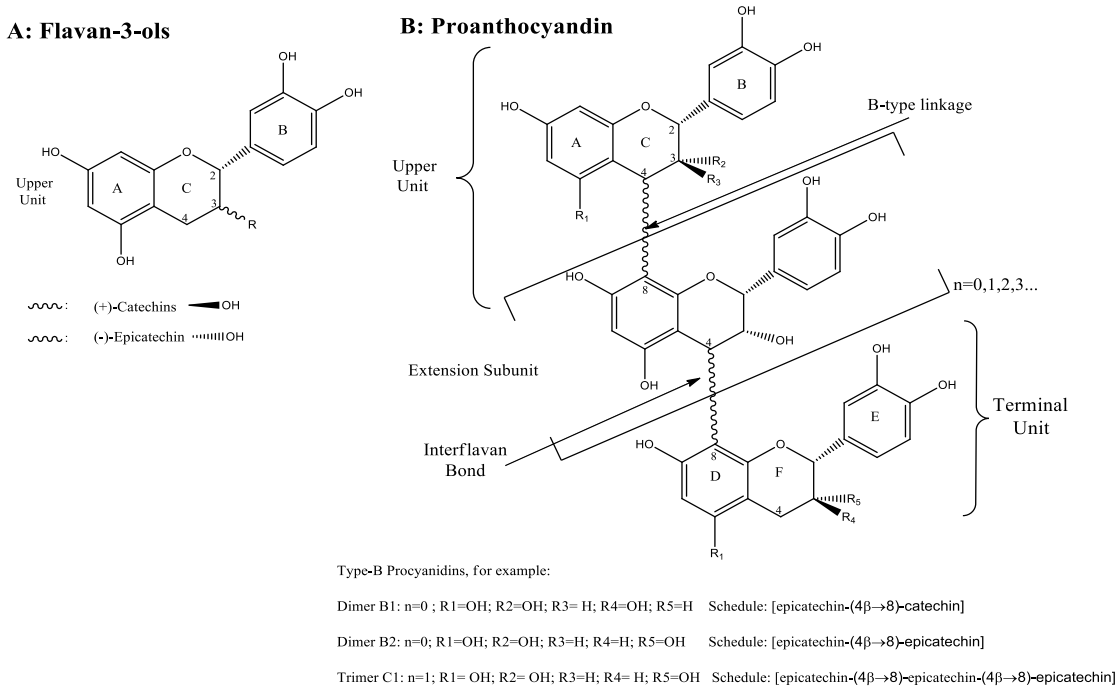

Figure 1. Procyanidins structure, A) Skeleton of monomeric catechins and B) Basic structure of condensed procyanidins.

Liposomes can encapsulate both hydrophilic and hydrophobic compounds (Akbarzadeh et al., 2013) making them versatile nutrient delivery systems. Liposomes can be produced from food grade lecithins which are biodegradable, biocompatible and nontoxic substances that are listed as generally recognized as safe (GRAS) food ingredients (Gibis, Zeeb, & Weiss, 2014). Soybean lecithin is one of the most common food-grade lecithins and typically consists of a mixture of phospholipids such as phosphatidylcholine and phosphatidylethanolamine which have a fatty acid profile consisting primarily of $C_{16:0}$; $C_{18:0}$, $C_{18:1}$; $C_{18:2}$ and $C_{18:3}$ as well as triacylglycerols and carbohydrates (Scholfield, 1981). Lecithin is mainly used in the food industry as an emulsifier, releasing agent and crystal modifier (Gibis, Rahn, & Weiss, 2013a). Since soybean

lecithin contains high amounts of $C_{18:2}$ and $C_{18:3}$, it is susceptible to lipid oxidation which can destabilize the liposome's highly flexible and fragile bilayer membrane system, produce off-flavors and co-oxidize nutrients encapsulated within the liposomes (Gibis et al., 2014). Furthermore, cocoa procyanidins could have dual roles with liposomes as either an agent to protect liposomes from oxidation or as an encapsulated nutraceutical.

Crude cocoa extract can be obtained using several techniques, e.g. solid-liquid extraction, liquid-liquid extraction, and supercritical fluids. Fresh cocoa beans contain 1.0-2.0 wt % theobromine, 0.1–0.9 wt % caffeine (Sotelo & Alvarez, 1991) and 12-18 wt % flavan-3-ols with a degree of polymerization ranging from 2 to 12 (Kim & Keeney, 1984; Robbins et al., 2009). Moreover, cocoa is the fourth richest dietary source of polyphenols with only cloves (1^{st}), peppermint (2^{nd}) and star anise (3^{rd}) having higher polyphenol contents (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). Catechins and their polymers can inhibit lipid oxidation by both free radical scavenging and metal chelation. The effectiveness of free radical scavenging antioxidants is controlled by both their ability to scavenge radicals as well as their ability to partition into lipids that are undergoing oxidation, both of which are influenced by their chemical structure (Kaneko, Baba, & Matsuo, 2001). The proposed mechanism for free radical scavenging by catechins is electron abstraction followed by free radical transfer to the antioxidant (Martinez, Valek, Petrović, Metikoš-Huković, & Piljac, 2005).

The ability of monomeric catechins to inhibit lipid oxidation in liposomes has been previously studied by several investigators (S.-W. Huang & Frankel, 1997; Kajiya, Kumazawa, & Nakayama, 2001; Van Acker et al., 1996). These studies reported that both catechin and epicatechin can inhibit lipid hydroperoxide and hexanal formation in a dose-depend manner and also the antioxidant action of catechins is dependent on pH (Muzolf, Szymusiak, Gliszczynska-

Swiglo, Rietjens, & Tyrakowska, 2008). In fact, the ability to decrease the prooxidant activity of free radicals by the catechol B-ring increases as the pH becomes more alkaline (Sotelo & Alvarez, 1991), which is probably due to the increased stability of the six-membered ring over that of the five-membered ring complex (Mira et al., 2002). The deprotonation of catechins is effected by their planarity, electron dislocation and torsion angle of the B–ring which all strongly influences free radical scavenging activity (Van Acker et al., 1996).

Cocoa polyphenols are unique in that they are exclusively formed by (epi)catechin units and can form oligomers and polymers of these units (Crozier, Clifford, & Ashihara, 2007). Polyphenols in tea and grape seed also have polymers but they consist of (epi)catechins units and esterified catechins with gallic acid moieties (Kalili & De Villiers, 2010, 2013; Montero, Herrero, Prodanov, Ibáñez, & Cifuentes, 2013). The effectiveness of antioxidants is strongly related to the ability of the antioxidants to partition at the location where lipid oxidation is most prevalent and the lack of gallic acid moieties in the cocoa polyphenols could provide unique partitioning properties (Choe & Min, 2009; Decker, Warner, Richards, & Shahidi, 2005). Several studies have encapsulated natural extracts such as green tea (S.-W. Huang & Frankel, 1997), rosemary (Arabi, Chabok, Mirzapour, & Saffari, 2017), black carrot (Guldiken, Gibis, Boyacioglu, Capanoglu, & Weiss, 2017), and grape seed (Gibis, Rahn, & Weiss, 2013b; Yi, Meyer, & Frankel, 1997) into liposome systems. However, the partitioning behavior of the antioxidants in the extracts are often not reported especially for the individual antioxidants and their polymers. In addition, how the partitioning behavior impacts antioxidant activity is not well understood.

Therefore, the aim of this study was to compare the free radical scavenging and antioxidant activity of monomeric catechin and epicatechin, isolated procyanidins and crude cocoa extract

when they are added to soybean lecithin liposomes. In addition, how the monomeric catechin and epicatechin and isolated procyanidins impact the physical properties of the liposomes and how they partition into the liposomes was also determined. By understanding the relationship between partitioning behavior and antioxidant activity, it could be possible to determine if the polyphenols from cocoa as well as crude cocoa extract has unique antioxidant properties that could make them effective food antioxidants.

6.2. MATERIALS AND METHODS

6.2.1. Reagents. All chemicals used were analytical reagent grade with no further purification. L-α-Phosphatidylcholine (PC) from soybean (Type IV-S ≥ 30% PC), iron (II) sulfate heptahydrate, barium chloride dehydrate, cumene hydroperoxide (80% purity), (+)- catechin hydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH), fluorescein sodium salt, sodium di-hydrogen phosphate (anhydrous) (NaH_2PO_4), di-potassium hydrogen phosphate (anhydrous) (K_2HPO_4), were obtained from Sigma Aldrich (St. Louis, MO, USA). (–)-Epicatechin (≥ 99%; ASB-00005127), and procyanidin B2 (≥ 90%; ASB-00016231) were purchased from ChromaDex Inc. (Irvine, CA, USA). Sodium acetate trihydrate, hydrochloric acid, glacial acetic acid, ethanol, iron (III) chloride hexahydrate, TPTZ (2,4,6-tripyridyl-s-triazine), acetonitrile, trifluoroacetic acid, 1-octanol, and methanol were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Double distilled and deionized water was used for the preparation of all solutions. To minimize the presence of metals contaminants, all the glassware was acid washed.

6.2.2. Preparation of cocoa polyphenols crude extract. Fresh cocoa pods, clone ICS-39, were collected at Villa Santa Monica (San Vicente de Chucurí, Colombia). Fresh cocoa beans were used to get the greatest possible levels of polyphenols, so they could be used in all subsequent studies.

Cocoa pods mainly contained the cocoa beans, mucilage, and cocoa husk. Unfermented cocoa beans were manually separated from cocoa husk, and the mucilage coating around each bean was removed and beans were immediately stored at -20ºC until further use. Cocoa polyphenol oxidase (PPO) activity was decreased by 93.3% by placing the frozen cocoa beans in a solution of 70 mM ascorbic acid/L-cysteine (1:1 v/v) and thermally processing at 96ºC for 6.4 min followed by immediately cooling in ice water for 30 min. After that, the beans were washed three times with distilled water (4ºC) to remove traces of ascorbic acid and L-cysteine. The cocoa beans with low PPO activity were chopped by hand (cross section of 50 × 30 mm^2) using a knife and immediately oven dried (Binder model FD 23, Germany) at 70ºC for 3h (final water content < 4% w/w). After drying, the samples were cooled to -20ºC and milled (Grindomix GM 200, Retsch GmbH & Co., Germany) 3 times for 30 s and then sieved through a 120-mesh screen (W.S., Tyler, USA). Preliminary studies showed that further particle size reduction did not increase the ability to extract the polyphenols (data not shown). The samples were stored at -20ºC until further analysis.

The crude cocoa extract was produced from PPO inactivated, dried and milled cocoa beans using ultrasound-assisted solid-liquid extraction. Briefly, a 50% ethanolic solution was mixed with the milled cocoa beans (pH 6, 1:120 cocoa beans to solvent ratio), and ultrasonicated (Elma, Ultrasonic bath LC 30H, Germany) at 20 kHz for 30 min followed by incubation at 70ºC for 45 min under constant stirring. The resulting extract was centrifuged (Heraeus, Megafuge 16R, Thermo Scientific, Germany) at 5000 *g*, 4ºC for 20 min, and then the supernatant was collected and filtered with Whatman filter paper Nº1. The ethanol was removed by vacuum evaporation (R-100, Büchi, Switzerland). The crude extract was then freeze-dried (Model 18, Labconco Corp., Kansas City, MO, USA), and the resulting violet-powder was stored at -80ºC until further analysis.

6.2.3. Fractionation of cocoa polyphenols and procyanidins. To obtain high enough concentrations of the procyanidins for antioxidant and partitioning experiments, preparative reverse phase HPLC was utilized. A Prominence LC system (Shimadzu, USA) consisted of a UV-Vis Detector (SPD-20AD, Shimadzu USA), quaternary pump (LC-20AP, Shimadzu, USA), autosampler (SIL-10AP, Shimadzu, USA) and automatic fraction collector (FRC-10A, Shimadzu, USA). The reverse phase column used was a C_{18} column (10 × 250 mm, 10µm particle size, Alltech, USA), connected to refill guard column packed with C_{18} supplied by Alltech (Alltech, USA). The binary linear gradient consisting of acidified water (Solvent A) (H_2O:HOAc 99.9:0.1 v/v) and ethanol (Solvent B) was at a flow rate of 12 mL/min (~1000 psi background pressure). The optimal linear gradient was as follows: starting at 6%B; 0-20 min, 6-10% B; 20-30 min, 10-15% B; 30-40 min, 15-30% B. After each injection, the column reached 80% B for 5 min followed by 15 min of re-equilibration of the column at initial conditions. The column temperature was held at 55 ºC using a temperature control module (TCM, Millipore, Waters, USA). The chromatogram was recorded at 280 nm.

The sample were dissolved in solvent A, followed by filtration through 10 µm and then 2 µm pore size filters (nylon membrane filter, diameter 47mm, GVS Maine Magna, USA). Thus, the injection volume was equivalent to 80 mg of crude cocoa extract. The fraction collector was set up to automatically collect the chromatographic peaks. Isolated fractions were initially stored at -20ºC and multiple thawed fractions were pooled and concentrated using a roto-evaporator connected to cold trap (dry ice) (R-114, Büchi, USA) followed by freeze-drying in a VirTis™ Genesis freeze dryer (SP Inc, Gardiner, NY, USA) for 72h and storage at -80 ºC under nitrogen atmosphere until further analysis. The purity of every fraction was determined by the analytical HPLC-MS/MS method described previously.

6.2.4. Determination of polyphenol and procyanidin composition by analytical liquid chromatography. Both crude cocoa extracts and fractionated procyanidins compositions were analyzed by analytical normal phase HPLC (Robbins et al., 2009) utilizing a Shimadzu LC equipped with an SCL-10Avp system controller, quaternary pump (LC-20AD), a solvent degasser (DGU-14A), manual injector, diode array detector and fluorescence detector (RF-20Axs). Separation was achieved using a Phenomenex Develosil Diol 100Å column (250 × 4.6 mm, 5 µm particle size) (Torrance, CA, USA) protected with a security guard AJ0-4348 containing the same stationary phase from Phenomenex (Torrance, CA, USA). Acidified acetonitrile (CH_3CN:HOAc, 98:2 v/v) (solvent A) and acidified aqueous methanol (CH_3OH:H_2O:HOAc, 95:3:2 v/v) (solvent B) were used as mobile phase. The gradient was as follows: 0-3 min, 7% B; 3-60 min, 7-37.6 % B; 60-63 min, 37.6 – 100% B; 63 -70 min, 100-7% B; and re-equilibrium for 10 min with 7% B. The flow rate was 1 mL/min, the diode array was set at 190-800 nm and the fluorescence detector was at 230/321 nm, excitation and emission, respectively. Other fluorescence detector conditions included ×4 gain and high sensitivity. The column temperature was maintained at 25ºC and the volume injection was 20 µL.

For identification of unknown compounds, LC-MS/MS analysis was performed on an Agilent 6320 Ion Trap LC/MS (Agilent Technologies, Waldbronn, Germany) equipped with an ESI source and ion trap mass analyzer which was controlled by the 6300 series trap control software (Bruker Daltonik GmbH, V. 6.2). Ammonium acetate (10mM in methanol, 0.1mL/min) was added using an additional LC pump (Agilent 1290 Infinity, Waldbronn, Germany) into the flow via three-way-splitter before the MS to overcome suppression of electrospray ionization by the acetic acid. The mass spectrometer was operated under negative and positive ESI mode with the following conditions: mass spectra recorded from 90 - 2200 *m/z*, nebulizer 40 psi, dry gas 12

L/min and dry temperature 350 ºC. Target compounds consisted of $[M+H]^+$ ions at *m/z* 181 and 191 for theobromine and caffeine, respectively, and $[M-H]^-$ ions at *m/z* 289, 577, 865, 1153, 1442, and 1730 that correspond to monomers, dimers, trimers, tetramers, pentamers, and hexamers procyanidins structures, respectively. The calibration curves for theobromine, caffeine, catechin, epicatechin, and dimer B2 were made from commercially available analytical standards (r^2 = 0.99). Oligomeric procyanidin calibration curve was performed from isolated fractions (r^2 = 0.98). All the results are expressed as mg of sample per g of cocoa beans (dry matter basis).

6.2.5. Antioxidant activity assays. The antioxidant activity of the different fractions was first determined by *in vitro* antioxidant assays. The Ferric Reducing Ability (FRAP) of the antioxidants was determined according to Benzie & Strain, (1999). Briefly, 1.4 mL of FRAP reagent [25 mL of acetate buffer (300 mM, pH 3.6), 2.5 mL of 10 mM 2, 4, 6- tripyridyl-s-triazine, 40 mL of HCl and 2.5 mL of 20 mM $FeCl_3 \cdot (6H_2O)$] was incubated with 75 µL of the samples for 30 min in the dark. The absorbance was measured at 593 nm (Genesys 20, Thermo Scientific, Waltham, MA, USA). A Trolox calibration curve (1.95 – 250 µM; r^2 = 0.99) was used to calculate iron reducing ability. Results are express as a mol Trolox / mol of polyphenol.

The ability of the antioxidant to interact with nonpolar DPPH (2,2-diphenyl-1-picrylhydrazyl) radicals was determined as described by Brand-Williams, Cuvelier, & Berset, (1995) with some modifications. Initial DPPH solution (6.75 mg / 25 mL methanol) was stored at -20 ºC until use. The working solution consisted of adjusted DPPH methanolic solution to obtain an absorbance of 0.550 ± 0.01 units at 517 nm (Genesys 20, Thermo Scientific, Waltham, MA, USA). Samples (100 µL) were incubated with 1.45 mL of this DPPH solution for 60 min in the dark. A calibration curve was prepared from 1.95 – 150 µM Trolox (r^2 = 0.99). Results are express as % radical scavenging. The DPPH activity was calculated as follows:

$$DPPH\ = 1 - \frac{Abs_{sample} - Abs_{blank}}{Abs_{ref} - Abs_{blank}} * 100$$

Free radical scavenging activity was also determined in the presence of water-soluble free radicals by the oxygen radical absorbance capacity (ORAC) assay as described by D. Huang et al., (2002) with minor modifications. A fluorescein solution (3 mM) was made in double distilled water. Then, 35 µL of fluorescein solution was added to 25 mL of 75 mM phosphate buffer (2.2 g $NaH_2PO_4 \cdot H_2O$, 8.4 g Na_2HPO_4; pH 7.4) to form a fluorescein stock solution (4×10^{-3} mM). The stock solution was wrapped in foil and stored at 4ºC. The daily working fluorescein solution (8.2×10^{-5} mM) consisted of stock solution diluted 1:1000 with 75 mM phosphate buffer (pH 7.4). A 221 mM solution of 2,2´-azobis (2-methylpropionamidine) dihydrochloride (AAPH) dissolved in phosphate buffer (pH 7.4) was also prepared.

Ninety-six well microplates were filled with 50 µL of the working fluorescein solution and 50 µL of samples at a concentration of 125 µM. The plate was incubated for 10 min in the microplate reader (Synergy HT Multi-Detection, Biotek Instrumets, Inc. Winooski, VT, USA) at 37 ºC. The reaction was initiated by the addition of 50 µL of AAPH. Fluorescence was monitored kinetically for 2 h, using emission and excitation wavelength of 485 nm and 528 nm, respectively. A calibration curve was prepared with 12.5 – 375 µM Trolox (r^2 = 0.99). Results are express as a µmol Trolox / mol of polyphenol.

$$ORAC = \frac{AUC_{sample} - AUC_{blank}}{AUC_{Trolox} - AUC_{blank}} * k * \frac{molarity\ of\ trolox}{molarity\ of\ sample}$$

where *k* is the sample dilution factor, and AUC is the area below the fluorescence decay curve of the sample, blank, and Trolox, respectively. The area under the curve of normalized data was calculated using GraphPad Prism V. 6.0 (GraphPad Soft. Inc., La Jolla, California, USA).

6.2.6. Liposome preparation. The liposomes were made by sonication. Soybean lecithin (5% w/w) was added to the sodium acetate-acetic acid buffer (0.1M; pH 3.0 ± 0.1 or pH 5.0 ± 0.1) and stirred at 25ºC until it was dispersed followed by homogenization with a high shear blender (T10 Ultra-Turraz, IKA, Staufen, Germany) to form a coarse liposome suspension. Multilayer liposomes were made by sonicating the coarse liposomes for 7 min (amplitude 75%, 10 s on/10 s off with the sonicator probe submerged 1 cm up from the bottom of the container, model 505, Fischer Scientific, USA) in an ice bath (4ºC). The final liposomal solution was filtered through 0.45 µm hydrophilic PTFE filter (Millipore, Milford, MA, USA). When testing antioxidant activity in the liposomes, the monomeric catechins, isolated procyanidins fractions or crude cocoa extract were dissolved in ethanol (2.5% v/v) and then added to a buffer solution (0.1M; pH 3.0 or pH 5.0). After completely dissolution, the soybean lecithin was added and homogenized as described above. No antioxidant control liposomes were prepared with the same ethanol and buffer in the absence of polyphenols or procyanidin fractions.

For the crude extract and isolated fractions, the concentrations of catechins and procyanidins were calculated from the HPLC profiles. For example, in the crude extract, the percentage of each polyphenol and procyanidin was determined, and then the molecular weight of each compound was used to calculate the molarity, and the sum of all compounds was used as the total molarity of the total catechins and procyanidins. The same procedure was used to determine the total catechins and procyanidins compounds in the isolated fractions.

6.2.7. Measurement of liposome particle size and ζ-potential. Prior to measuring particle size and ζ- potential, empty and antioxidant-loaded liposomes were diluted with an appropriate amount of the same buffer (sodium acetate-acetic acid buffer solution; 0.1M; pH 3.0 ± 0.1 or pH 5.0 ± 0.1) to prevent multiple scattering effects (Clogston & Patri, 2011). Droplet size distribution

was measured by dynamic light scattering (Nano-ZS, Malvern Instruments, Worcestershire, UK) and ζ-potential measurement (Nano-ZS, Malvern Instruments, Worcestershire, UK) was assayed according to Panya et al., (2010).

6.2.8. Antioxidant Partitioning. The physical location of compounds is important in their ability to inhibit lipid oxidation. The partitioning behavior of the polyphenols and procyanidins in cocoa was determined by water/octanol distribution (log Kow) and their ability to be associated with the liposomes. Octanol/water distribution was determined by dissolving the samples in water at 125 uM. Samples (750 µL) were then placed in Eppendorf tubes (2 mL), and 750 µL of 1-octanol was added. Samples were vigorously vortexed three times for 1 min and centrifuged (20ºC, 15 min, 8000 g) (Megafuge 16, Thermo Scientific, Waltham, MA, USA). The top fraction (octanol) and bottom fraction (aqueous) were carefully collected with a needle and syringe, and antioxidant concentrations were determined by analytical normal phase HPLC as described above. The partitioning coefficient was expressed as the polyphenol concentration in octanol by the polyphenol concentration of the aqueous phase.

Partitioning behavior was also determined by measuring the amount of free antioxidant in the aqueous phase of a liposome suspension. The aqueous phase was isolated from the liposomes by high-speed centrifugation (Sorvall WX Ultra 80, Thermo Scientific Inc., Asheville, USA) at 145000 *g*, 4 ºC for 2h. The supernatant (200 uL) was carefully collected and used to determine antioxidant concentrations by analytical normal phase HPLC as described above. Partitioning was expressed as encapsulation efficiency (EE) which was calculated as:

$$EE\ \% = \frac{m_1 - m_2}{m_1} * 100\%$$

m_1 is the initial concentration added to the liposomes and m_2 was the concentration in the aqueous phase.

6.2.9. Effect of polyphenols and procyanidins on oxidative stability of liposomes. Liposomes (1 mL) samples were placed into 10 mL headspace vials, sealed with poly(tetrafluoroethylene) butyl septa, and incubated at 55ºC in the dark. The formation of primary and secondary lipid oxidation products (lipid hydroperoxide and hexanal, respectively) were periodically determined during storage. Lipid hydroperoxides were measured according to Hu, McClements, & Decker (2003). Briefly, 0.3 mL of liposome was mixed with 1.5 mL of isooctane/2-propanol (3:1 v/v) by vortexing (10s, 3 times) in a test tube. The test tube was then centrifuged (1000*g*, 2 min) and the organic phase (200 µL) was carefully recollected and mixed with 2.8 mL of methanol/1-butanol (2:1 v/v), followed by 15 µL of the ferrous iron solution and 15 µL of 3.97 M ammonium thiocyanate solution. The mixture was incubated for 20 min in dark conditions and the absorbance was measured at 510 nm (Genesys 20, Thermo Scientific, Waltham, MA, USA). A calibration curve, prepared with 0 - 0.6 mM cumene hydroperoxide (r^2 = 0.99), was used to calculate lipid hydroperoxide concentrations.

Hexanal formation was measured using a gas chromatograph (model GC-2014, Shimadzu, Tokyo, Japan) equipped with a headspace autosampler and flame ionization detector (FID) using the method by Hu et al., (2003) Briefly, gas chromatography conditions were as follows: sample incubation at 55 ºC for 10 min, insertion of a DVB/carboxen/PDMS) solid-phase microextraction (SPME) fiber (50/30 µm, Supelco, Bellefonte, PA, USA) into headspace for 6 min, desorption in injector for 3 min at 250 °C at a split ratio of 1:7, pressurization 10 s; venting 10 s. Compounds were separated with an HP methyl silicone (DB.1) fuse silica capillary column (50m, 0.31 mm i.d., 1.03 µm fil thickness) (Equity-1, Supelco). The isothermal separation of the samples was

carried out at 65 ºC for 10 min and the flame ionization detector was 250 ºC. A standard curve with hexanal (0 – 50 mM) was used to calculate hexanal concentrations (r^2 = 0.99).

6.2.10. Statistical Analysis. All measurements were repeated at least three times and data are expressed as the mean ± standard deviation. Statistical analysis was done using GraphPad Prism V. 6.0 (GraphPad Soft. Inc., La Jolla, California, USA). The data were reported as the mean of triplicate with standard deviation. Anova one-way was tested and if it was significant ($p<0.05$), Tukey was also determined using a 5% level of significance.

6.3. RESULTS

6.3.1. Chromatographic analysis of cocoa procyanidins. Currently, there are several extraction processes to recover polyphenol from plant sources, e.g. solid-liquid, microwave, soxhlet, ultrasound-assisted, extrusion and supercritical fluid extraction. The majority of these techniques use hazardous solvents (e.g. hexane, methanol, acetone) to enhance the extraction yield of polyphenols. From a practical point of view, new extraction strategies need to be developed that are economical, reproducible, robust, suitable for large-scale production and safe for food applications (Fonoudi et al., 2016; Gerson & Mukherjee, 2005; McClements, 2014). In an attempt to meet these goals, a GRAS solvent (ethanol) was used for polyphenols and procyanidin extraction in this study. The extraction of unfermented cocoa beans after the PPO inactivation, followed by ultrasound-assisted solid-liquid extraction and removal of solvent resulted in a yield of 168 mg of crude cocoa extract / g cocoa beans (dry matter basis). The total theobromine and caffeine were 5.6 and 3.2 mg/g of cocoa beans, respectively. Procyanidins profile and quantification was performed by normal phase HPLC as described by previous authors (Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006).

Figure 2A shows that the crude cocoa extract had procyanidins with a degree of polymerization up to 14. These results are in agreement with previously published results (Robbins et al., 2009). In our extract, the main catechin consisted of 8.08mg of (–)-epicatechin /g of cocoa bean, which was 11.7-fold higher than (+)-catechin (Table 1). Several authors have reported that (–)-epicatechin is the main polyphenol in cocoa beans, but the concentration of this compound can vary considerably by the methodology of extraction and cocoa bean variety. For example, Kim & Keeney (1984) reported that the concentration of catechin can change according to the cocoa variety (e.g. Criollo, Forastero, Nacional), and recently Carrillo, Londoño-Londoño, & Gil (2014) evaluated 18 genotypes of cocoa and found that the concentration of (–)-epicatechin and (+)-catechin ranges from 1.4 -3.7 mg/g and 0.2 – 1.3 mg/g, respectively. The major procyanidin in the crude cocoa extract was the trimer (13.5 mg/g cocoa beans) which was 2.9x, 1.4x, 1.7x and 2.5x –fold greater than the dimer B2, tetramer, pentamer, and hexamer, respectively (Table 1).

6.3.2. Isolation of procyanidins from cocoa extract. Preparative reverse phase HPLC was utilized to isolate cocoa procyanidins in quantities sufficient for testing their antioxidant activity and partitioning in soybean liposomes.

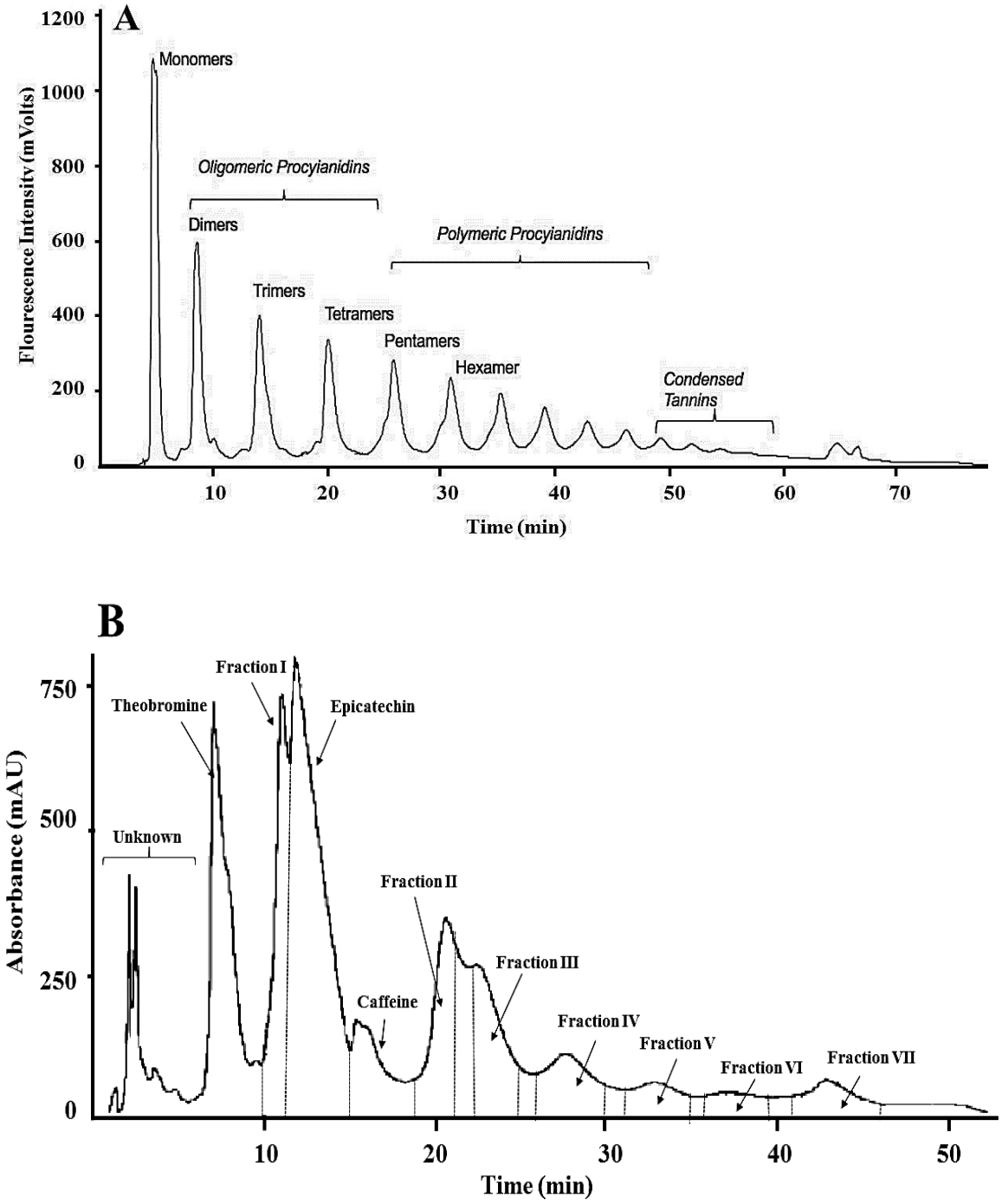

Figure 2. Chromatogram of cocoa polyphenolic crude extract in A) Analytical normal-phase HPLC-DAD-FL, B) Preparative LC-UV using reverse phase.

Table 1. Procyanidin concentration in raw cocoa beans.

Compound	Retention Time (min)	Concentration (mg/g)*
Catechin	5.800	0.69 ± 0.04
Epicatechin	5.810	8.08 ± 0.40
Dimer B2	6.784	4.68 ± 0.54
Trimer	14.766	13.46 ± 4.43
Tetramer	20.800	9.68 ± 2.96
Pentamer	26.516	8.07 ± 3.06
Hexamer	30.650	5.40 ± 0.42

Data expressed as means of triplicate experiments. *Concentration expressed as mg polyphenol per g cocoa beans (dry weight basis).

Figure 2B shows the preparative reverse phase HPLC chromatogram. We collected 7 fractions for further testing. The composition of all fractions was verified by analytical HPLC and mass spectrometry as described above. Several fractions were not completely separated so the collection was done at the beginning or end of the eluted peaks in an attempt to obtain the highest concentration possible of the individual compounds.

For example, fraction I contained 2% epicatechin and 98% dimer [EC-4β→8-EC], fraction II contained 2% dimer, 81% trimer [EC-4β→8-EC-4β→8-EC], 10% tetramer and 7% Pentamer. It was difficult to obtain > 80% of the remaining procyanidins with fraction III containing 13% trimer, 54% tetramer and 33% pentamer, fraction IV containing 18% tetramer, 60% hexamer and

22% heptamer (octamers and greater could be identified but verification of their exact structural composition was not possible by mass spectrometry due to their low ionization and the lack of standards). Fraction V to VII could not be collected at concentrations sufficient to test their antioxidant activity and partitioning in the soybean liposomes.

6.3.3. Impact of cocoa polyphenols and procyanidins on liposomes properties. Antioxidant capacity of monomeric catechins, isolated fractions, and crude cocoa extract were measured using ORAC, DPPH and FRAP assays (Table 2). FRAP values increased with increasing degree of polymerization, and crude cocoa extract had the largest FRAP value. As with the FRAP assay, DPPH radical scavenging increased with increasing degree of polymerization. No significant differences were found for ORAC values for monomeric catechins and between isolated procyanidins fractions (except for fraction I, $p < 0.05$). Similar ORAC trend was also reported for tea catechin and catechin-gallate moiety (Roy et al., 2010). In general, the crude cocoa extract in this study had greater antioxidant activity than previously reported. These differences could be associated with the concentration and differences in cocoa procyanidins composition in this study. For example, Miller et al., (2006) reported a maximum ORAC of 875 ± 93 µmol Trolox/g cocoa and total phenol content of 60.2 ± 4.5 mg gallic acid equivalent (GAE)/g cocoa , whereas in our results the crude cocoa extract contained 122.3 ± 2.4 mg GAE/g cocoa of total polyphenols and ORAC value of 1201.5 ± 41.3 µmol Trolox/g cocoa (or 50.2 ± 1.7 µmol Trolox/mol). In general, the longer the procyanidin polymer, the greater the antioxidant activity.

6.3.4. Impact of cocoa polyphenols and procyanidins on liposomes properties. Assays such as FRAP, DPPH, and ORAC often do not correlate well with the ability of compounds to inhibit lipid oxidation in food systems. This is often due to the inability of simple *in vitro* assays to account for how the physical location of antioxidants impacts their ability to scavenge lipid radicals

(Decker et al., 2005). Liposomes were used in this study as they can be used as nutrient delivery systems and they are a model for cell membranes which can oxidize in food systems such as meats. The physical stability of liposomes are important when they are used as delivery systems and their physical stability depends on factors such as temperature, pH, ionic strength, presence of other molecules that can affect their charge and size (Lasic, 1990). The liposomal size distributions, ζ-potentials, and procyanidin entrapment efficiencies (EE) were determined at both pH 3.0 and 5.0 in the presence of different concentrations of the monomeric catechins and procyanidin fractions (Table 3).

Table 2. Antioxidant activity of catechins, isolated fractions, and cocoa crude extract determined by ORAC, DPPH, and FRAP.

Sample	ORAC	FRAP	DPPH (%)
Catechin	$13.72 \pm 1.04^{a,b}$	2.00 ± 0.02^{a}	28.39 ± 0.38^{a}
Epicatechin	$12.98 \pm 0.52^{a,b}$	2.23 ± 0.01^{a}	23.07 ± 0.88^{a}
Frac. I	16.55 ± 0.16^{c}	3.97 ± 0.28^{b}	44.63 ± 0.50^{b}
Frac. II	$15.40 \pm 0.55^{b,c}$	6.99 ± 1.09^{c}	75.95 ± 3.26^{c}
Frac. III	$13.88 \pm 0.70^{a,b}$	7.31 ± 0.40^{c}	85.36 ± 3.01^{d}
Frac. IV	12.36 ± 0.97^{a}	7.42 ± 1.21^{c}	86.07 ± 5.52^{d}
Crude Cocoa Extract	50.16 ± 1.72^{d}	9.97 ± 0.36^{d}	141.97 ± 0.63^{e}

Data expressed as means of triplicate experiments. ORAC ($p < 0.0001$) is expressed as μmol Trolox equivalents per mol^{-1} of polyphenol, FRAP ($p < 0.0001$) is expressed as mol Trolox equivalents per mol^{-1} of polyphenol, and DPPH ($p < 0.0001$) as % radical scavenging. Means within column sharing the same letter are not significantly different by Tukey ($p > 0.05$). All the samples were tested at 125 μM.

The ζ-potential and size of the liposomes without procyanidins at pH 3.0 was –31.5 ± 0.3 mV and 56.7 ± 1.5 nm, respectively and at pH 5.0 was –18.7 ± 0.1 mV and 75.2 ± 1.5 nm, respectively. ζ-potential of liposomes has been shown to decrease with decreasing pH (Sabín, Prieto, Ruso, Hidalgo-Álvarez, & Sarmiento, 2006) due to the loss of protonation of the phospholipid amines. Decreasing pH can also decrease particle size distribution due to rearrangement of vesicle into more tightly packed structures via electrostatic and hydrophobic interactions (Taylor, Gaysinsky, Davidson, Bruce, & Weiss, 2007).

Table 3. Physical parameters for liposomes loaded with monomeric and isolated procyanidins fractions at pH 3.0 and pH 5.0

Type	Sample	uM	Soybean-liposomes pH 3.0				
			Size (nm)	ζ-potential (mV)	Log K_{OW}	EE (%)	Size (nm)
-	Control	-	56.71 ± 1.49	-31.50 ± 0.28	-	-	75.15 ± 1.48
Monomers	EC	125	52.32 ± 6.10	-32.45 ± 0.37	0.464 ± 0.027	41.3 ± 2.4	82.83 ± 0.62
	C	125	50.91 ± 6.68	-33.50 ± 0.28	0.406 ± 0.001	37.2 ± 0.7	82.10 ± 2.38
Isolated Fractions	Dimer in F. I	125	43.47 ± 0.11	-32.25 ± 2.19	0.725 ± 0.001	71.6 ± 0.6	60.43 ± 2.39
	Trimer in F. II	125	67.66 ± 10.95	-30.20 ± 1.27	0.817 ± 0.023	86.1 ± 2.6	76.38 ± 5.52
	Tetramer in F. III	125	58.65 ± 2.20	-32.80 ± 0.28	0.809 ± 0.005	90.8 ± 0.6	84.14 ± 1.18
	Hexamer in F. IV	125	43.19 ± 1.56	-34.70 ± 1.27	0.869 ± 0.008	91.4 ± 0.7	71.25 ± 2.31

Table 4. Physical parameters for liposomes loaded with crude cocoa extract at pH 3.0 and pH 5.0[a].

le	uM	Soybean-liposomes pH 3.0				Soybean-liposome pH 5.0		
		Size (nm)	ζ-potential (mV)	Log K_{OW}	EE (%)	Size (nm)	ζ-potential (mV)	Log K_{OW}
ner	125	79.56 ± 0.10	-40.60 ± 2.12	0.338 ± 0.006	54.8 ± 0.1	87.28 ± 0.77	-22.35 ± 2.05	0.428 ± 0.0
er				0.921 ± 0.010	86.4 ± 0.4			0.949 ± 0.0
er				1.051 ± 0.011	90.8 ± 1.6			1.095 ± 0.0
ner				1.219 ± 0.001	88.9 ± 0.7			1.439 ± 0.0
ner				1.263 ± 0.025	94.2 ± 1.2			1.388 ± 0.2
ner				1.767 ± 0.068	94.5 ± 4.2			1.929 ± 0.0

[a]Size and ζ-potential are for liposomes with crude extract. Log Kow and EE are for the individual compounds in the crude extract as determined by HPLC.

At pH 3.0, the addition of the monomeric catechins had little impact on either size, which ranged from 50.9±6.7 to 52.3 ± 6.1 nm, or ζ-potentials, which ranged from –32.5 ± 0.4 to –33.5 ± 0.3 mV. The procyanidins fractions resulted in a wider range of liposome size which ranged from 43.2 ± 1.6 to 67.6 ± 11.0 nm but there was no consistent trend in these size differences and polymer size. Procyanidins had little impact on ζ-potential which ranged from –30.2 ± 1.3 to –34.7 ± 1.3 mV. Crude cocoa extract significantly altered liposome charge decreasing ζ-potential to –40.6 ± 2.1 mV and producing larger vesicles at 79.6 ± 0.1 nm (Table 4).

At pH 5.0, the polyphenol monomers and procyanidins had a very little effect on ζ- potential which ranged from –17.9 ± 0.6 to –22.5 ± 0.6 mV and again no consistent effect on size which ranged from 60.4 ± 2.4 to 84.1 ± 1.2 nm (Table 3). The liposomes in the presence of the crude extract at pH 5.0 were slightly larger 87.3 ± 0.8 nm but had a similar ζ-potential, –22.4 ± 2.1 mV (Table 4).

The partitioning behavior of the polyphenols and procyanidins was first determined in water/octanol bilayers (log Kow) at a concentration of 125 μM. Log Kow was slightly greater for epicatechin than catechin. Although the isolated polymer fractions were not pure, utilization of HPLC-MS allowed for specific analysis of the partitioning of the individual polymers. Log Kow was greater for the polymers in fractions I to IV compared to the monomers at both pH 3.0 and 5.0. Overall, the longer the procyanidin polymer, the greater the partitioning into the liposomes. Similar results were found by Plumb et al., (1998) who observed a general trend of increasing partition coefficient with increasing degree of polymerization tetramer > trimer > dimer ≈ epicatechin for procyanidins isolated from grape seed.

In addition, using fluorescence quenching to measure the association of surface-active phenolic antioxidants (e.g. catechins, trolox, caffeic acid, and gallic acid) with liposomes found a dose-dependent behavior, and their interaction with the lipid bilayer was reported to be affected by their stereochemical structure, bonding capacity, and polarity (Gray, 1978; S.-W. Huang & Frankel, 1997; Kajiya et al., 2001). These findings support that the partitioning behavior of different phenolic antioxidants in liposomes were greatly affected by their pH dependence and polymer size.

The partitioning of the polyphenols and procyanidins was also determined by measuring liposomal encapsulation efficiency since the liposomes can encapsulate the samples both in the lipid phases as well as in the aqueous phase in between lipid layers. As was also seen in Log Kow, encapsulation efficiency was greater for epicatechin than for catechin at 125 µM, while catechin incorporation into the liposomes was slightly greater at pH 5.0 compared to pH 3.0. These results can be explained due to the stereochemistry differences between EC and C. Catechins incorporated into lipid bilayer have axially symmetric motion with slightly different wobbling amplitudes (Kajiya, Kumazawa, Naito, & Nakayama, 2008) which can impact encapsulation efficiencies.

In addition, complexes of β-cyclodextrin and EC exhibit different binding patterns in vibrational spectra than those of C, presumably due to different hydrogen bonding interactions with EC than C (Khedkar, Gobre, Pinjari, & Gejji, 2010). Also, based on molecular modeling and NMR observations, Yan, Xiu, Li, & Hao (2007) found that EC binds more strongly to β-cyclodextrin. Although these latter two examples are with a carbohydrate instead of a lipid, it does highlight that EC and C and have different binding properties despite their very similar structures. Also, in agreement with log Kow, the major polymers in fractions I to IV (individual

polymers were monitored by HPLC, Table 4) had higher encapsulation efficiencies then catechin and epicatechin. In general, the polymers were more highly associated with the liposomes at pH 5.0 than pH 3.0.

The addition of the crude cocoa extract (at a total polyphenol concentration of 125 μM) to the liposomes showed similar partitioning behavior as C and EC and the isolated polymer fractions (Table 3) when the partitioning of the specific compounds was determined by HPLC. The Log Kow and encapsulation efficiency were lower for the monomers than the polymers.

6.3.5. Impact of polyphenols and procyanidins on the oxidation of liposomes. The ability of the polyphenols and procyanidins to inhibit oxidation was evaluated in the liposome system. Initially, the ability of catechin and epicatechin, Figure 3 and 4 respectively, to inhibit lipid hydroperoxide and headspace hexanal formation at pH 3.0 was determined to establish concentrations that were antioxidative and estimate appropriate lag phases for future studies. Figure 3 and 4 show that both catechin and epicatechin were effective antioxidants at all concentrations tested (25-1000 μM).

The lag phases were generally longer for EC than C at concentrations > 250 □M which would have been anticipated since the encapsulation efficiency was greater for EC than C at pH 3.0 and antioxidants which are retained in lipids tend to be better antioxidants in lipid dispersions (Porter, Black, & Drolet, 1989). At 125 μM, the hydroperoxide and hexanal lag phases for both catechin and epicatechin were 8 days. The intermediate lag phase observed at 125 □M would allow for observation of both prooxidant (shorter lag phase) and antioxidant (longer lag phase) activity of the procyanidin fractions so this concentration was used for future studies.

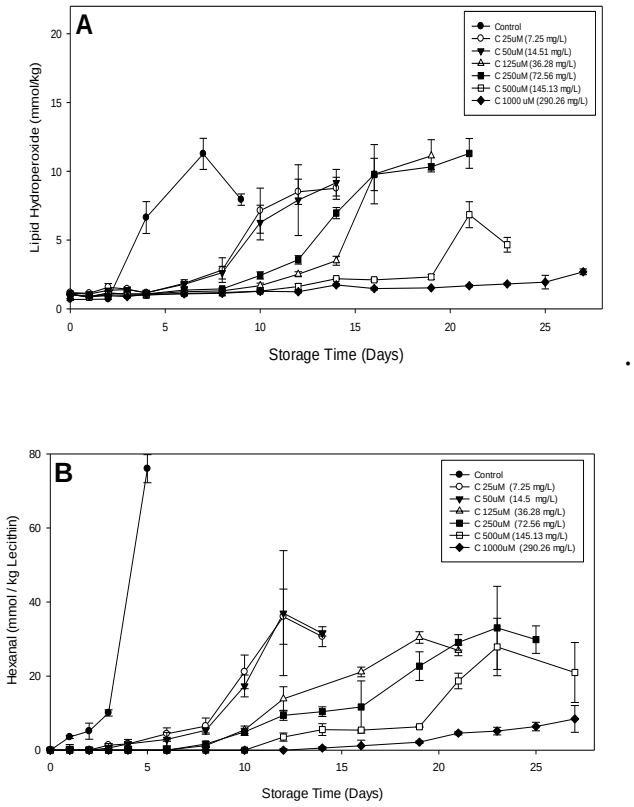

Figure 3. Oxidative stability of liposomes with (+)-catechin (pH 3.0 at 55ºC) A) Lipid hydroperoxide, B) Hexanal formation.

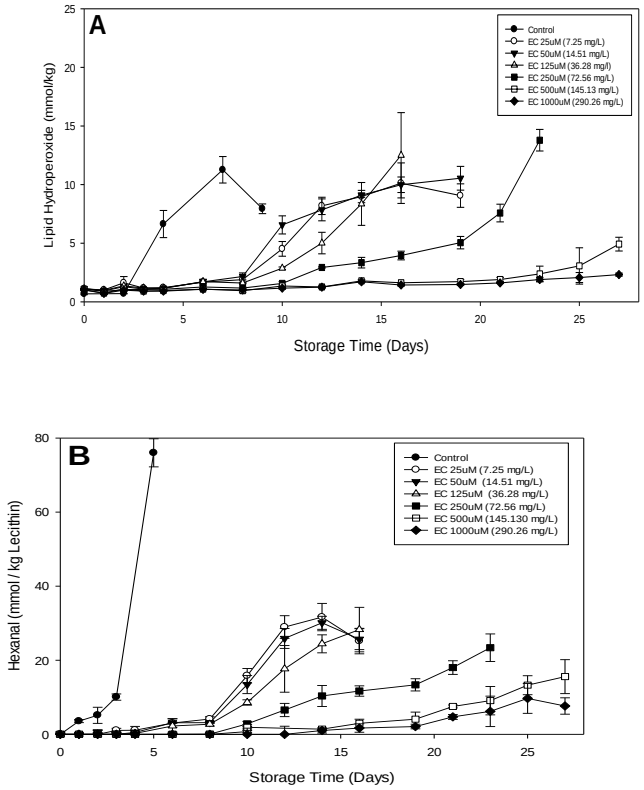

Figure 4. Oxidative stability of liposomes with (–)-epicatechin (pH 3.0 at 55ºC) A) Lipid hydroperoxide, B) Hexanal formation

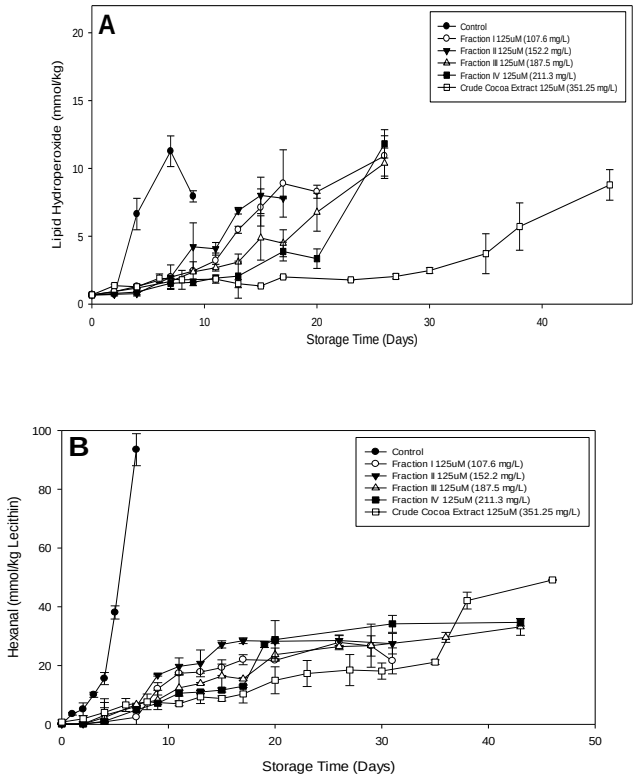

Figure 5. Oxidative stability of liposomes with procyanidins fractions and cocoa crude extract at 125µM (pH 3.0 and 55ºC) as determined by A) Lipid hydroperoxide and B) Hexanal formation.

Figure 5 shows the formation of lipid hydroperoxide and hexanal in the liposomes in the presence of 125 uM procyanidins and cocoa extract at pH 3.0 and 55 ºC. Lag phases for hydroperoxide formation for fractions I, II and III were similar at 7 to 9 days while fraction IV had a lag phase of 12 days and the highest antioxidant activity was observed in the presence of crude cocoa extract. Hexanal formation did not have as clear of lag phases as hexanal concentrations increased more gradually during storage. However, a trend was observed in increasing degree of polymerization resulted in less hexanal formation, and the crude cocoa

extract was again the strongest antioxidant. Overall, fractions I and II had the lowest encapsulation efficiencies, so this could explain why they had lower antioxidant activity than fractions III and IV.

The antioxidant activity of cocoa procyanidins in liposomes was also studied in liposomes at pH 5.0. As can be seen in Figure 6, all the samples displayed antioxidant activity. At pH 5.0, the ability of the procyanidin fractions to increase the lag phase of hydroperoxide formation was I < II < IV < III. Hexanal formation followed a similar trend. Interestingly, while antioxidant effectiveness was quite different between the procyanidin fractions, the encapsulation efficiency was quite similar. The greater effectiveness of the longer polymers at pH 5.0 could be due to their ability to inhibit lipid oxidation beyond just free radical scavenging, possibly through metal chelation (Roy et al., 2010). Researcher have found that catechin dimerization is higher at higher pH and these dimers have higher metal chelating and superoxide anion scavenging activity (Neilson et al., 2007; Sang, Lee, Hou, Ho, & Yang, 2005; Yoshino, Suzuki, Sasaki, Miyase, & Sano, 1999).

Overall, antioxidant activity by the procyanidins was greater at pH 5.0 than 3.0 which again could be due to their greater encapsulation efficiency at higher pH. The greater antioxidant activity of the crude extract than isolated fractions at the same total polyphenol and procyanidin concentrations also suggests that other antioxidants existed in the crude extract or those combinations of the different polyphenol and procyanidin could inhibit lipid oxidation synergistically. This study demonstrates for first time that both the catechins and procyanidins inhibited the oxidation of the liposomes with the crude extract being the most effective antioxidant. The ability of the polyphenols and procyanidins in cocoa to effectively inhibit lipid oxidation suggests that they could be used in products such as meats where lipid oxidation

occurs primarily in the cell membrane. This finding suggests that cocoa extracts could be processed in a manner where polyphenol polymerization was increased with polyphenol oxidase to increase their antioxidant activity.

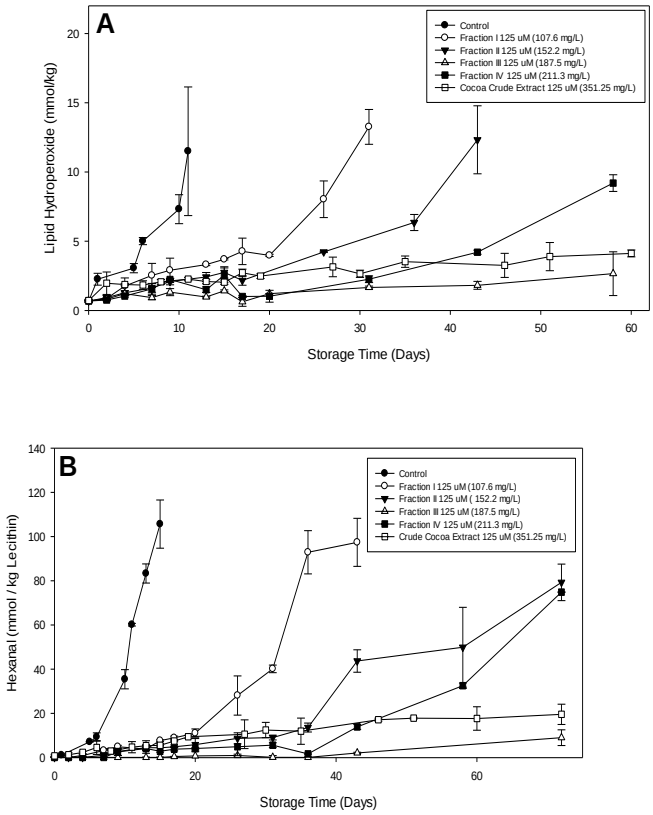

Figure 6. Oxidative stability of liposomes with procyanidins fractions and cocoa crude extract at 125µM (pH 5.0 and 55ºC) as determined by A) Lipid hydroperoxide and B) Hexanal formation.

6.4. CONCLUSIONS

Sonicated soybean lecithin liposomes were prepared. All the formulations were formed by anionic liposomes with particle size lower than 90 nm. Formulations at pH 5 showed better stability and higher antioxidant activity to inhibit the lipid oxidation than those samples at pH 3. In general, cocoa extract showed the highest antioxidant performance. The ability of the polyphenols and procyanidins in cocoa could be used in products such as meats where lipid oxidation occurs primarily in the cell membrane.

6.5. ACKNOWLEDGEMENTS

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CHAPTER 6

DESIGN, FABRICATION, CHARACTERIZATION AND *IN VITRO* DIGESTION OF ALKALOID-, CATECHIN-, AND COCOA EXTRACT-LOADED LIPOSOMES

ABSTRACT

Liposomes containing theobromine, caffeine, catechin, epicatechin, and a cocoa extract were fabricated using microfluidization and sonication. A high encapsulation efficiency and good physicochemical stability were obtained by sonication (75% amplitude, 7 min). Liposomes produced at pH 5.0 had mean particle diameter ranging from 73.9 to 84.3 nm. The structural and physicochemical properties of the liposomes were characterized by transmission electron microscopy, confocal fluorescence microscopy, and antioxidant activity assays. The release profile was measured by ultra-high performance liquid chromatography coupled to diode array detection. The bioaccessibility of the bioactive compounds encapsulated in liposomes was determined after exposure to a simulated in vitro digestion model. Higher bioaccessibilities were measured for all catechins-loaded liposome formulations as compared to nonencapsulated counterparts. These results demonstrated that liposomes are capable of increasing the bioaccessibility of flavan-3-ols, which may be important for the development of nutraceutical-enriched functional foods.

Keywords: Alkaloids, Catechins, Cocoa polyphenols, Liposomes, *In vitro* digestion.

7. DESIGN, FABRICATION, CHARACTERIZATION AND *IN VITRO* DIGESTION OF ALKALOID-, CATECHIN-, AND COCOA EXTRACT-LOADED LIPOSOMES

7.1. INTRODUCTION

Polyphenols are secondary metabolites found in plants that are considered to be the most abundant phytochemicals in our diet (Ignat, Volf, & Popa, 2011). The main dietary sources of polyphenols are fruits, beverages, vegetables, whole grains, and cereals (Ignat et al., 2011). In particular, coffee-, tea-, grape-, apple-, and cocoa-based products have become one of the most important and popularly consumed food and beverage sources of polyphenols globally. Catechins and alkaloids are the most notable secondary metabolites found in these sources with an average daily intake of 84 mg for caffeine and 17 - 39 mg for flavan-3-ols for a body weight of 70 kg (Frary, Johnson, & Wang, 2005; Gu et al., 2004). Catechins (such as (–)-epicatechin and (+)-catechin) and alkaloids (such as theobromine and caffeine) are also the major secondary metabolites in cocoa constituting up to 35 wt.% of the total phenolics and 3 wt.% on a fat-free basis, respectively (Watson, Preedy, & Zibadi, 2012). Alkaloids stimulate the nervous system and act as vasodilators, and are toxic to many animals (Eteng, Eyong, Akpanyung, Agiang, & Aremu, 1997). Catechins are chemically characterized by possessing several hydroxyl groups in their structure, and have been shown to exhibit several health benefits, which have been discussed in detail by Cirillo et al., (2016) and Kumar & Pandey (2013).

Despite their good antioxidant activities, several reports have highlighted that catechins are unstable during storage and processing, and are sensitive to oxidation, light, and pH (Fang & Bhandari, 2010). Poor stability under gastrointestinal conditions has also been reported for several groups of polyphenols. For instance, the plasma concentration of phenolic acids, monomeric

flavanols, procyanidins B1 and B2, and quercetin rarely exceeds 1 µM (Bouayed, Deußer, Hoffmann, & Bohn, 2012). Ovando-Martínez et al., (2018) reported that total flavonoids and polyphenols decreased by 83% and 77%, and 87 and 97 % in gastric and intestinal phases, respectively. Moreover, the DPPH radical scavenging activity was reduced by 62 % within the gastric phase, while it increased by 27% within the intestinal phase.

The oral bioavailability of polyphenols depends on a variety of factors, including the release from the food matrix during gastrointestinal digestion, solubilization in the intestinal fluids, transport across the mucus layer, cellular uptake, metabolism, and further transport in the circulatory system (Bouayed et al., 2012). Bioaccessibility is defined as the fraction of polyphenols released from the food matrix that are in a form that is suitable for intestinal absorption. Thus, the overall bioactivity of polyphenols depends on the amount present in the original plant, as well as the fraction that can actually be absorbed (Ovando-Martínez et al., 2018). For example, M. Lee et al., (2002) reported that when epigallocatechin gallate was administered, only 0.1% of the ingested dose appeared in the blood and the fraction absorbed is preferentially excreted through the bile to the colon. Meanwhile, epigallocatechin and epicatechin appear to be more bioavailable, but the fractions of these compounds that appeared in the plasma are also low, and only 3.3 and 8.9% of the ingested substances were excreted in the urine.

Encapsulating bioactive compounds into well-designed colloidal delivery systems could help to overcome some of the above limitations. Previous studies have investigated the factors that affect the bioaccessibility and bioavailability of commercial flavonoids and/or polyphenolic extracts from several plant sources (e.g., cocoa, tea, apple, pepper, and carrots) using *in vitro* or *in vivo* studies (Bouayed et al., 2012; M. Lee et al., 2002; Neilson & Ferruzzi, 2011; Ovando-Martínez et al., 2018; Zhang et al., 2015). In the case of cocoa polyphenols, several studies have

been carried out, based on microencapsulation in a carbohydrate matrix by spray drying (e.g., starches and maltodextrins) (Ferreira, Rocha, & Coelho, 2007), on electrostatic extrusion in alginate-chitosan microbeads (Belščak-Cvitanović et al., 2011), and on encapsulation through emulsion electrospraying (Paximada, Echegoyen, Koutinas, Mandala, & Lagaron, 2017). However, many of these technologies produced large particle sizes, irregular particle shapes, and do not allow the incorporation of polyphenols with different polarities (Fang & Bhandari, 2010), for example, with different degrees of galloylation or polymerization (Toro-Uribe, López-Giraldo, & Decker, 2018).

A variety of colloidal delivery systems have also been assessed for their potential to encapsulate these types of nutraceuticals, including: nanoemulsions (Bhushani, Karthik, & Anandharamakrishnan, 2016), W/O/W emulsion (Aditya et al., 2015), and uncoated (Toro-Uribe, López-Giraldo, et al., 2018), or coated liposomes (e.g., with chitosan, calcium pectinate, or hydroxypropyl methylcellulose) (Altin, Gültekin-Özgüven, & Ozcelik, 2018; J. S. Lee, Chung, & Lee, 2008). Liposome-based systems are considered to be particularly suitable for encapsulation and delivery for both water- and oil-soluble functional compounds (Altin et al., 2018). Liposomes are typically spherical, single- or multiple-layer vesicles, having an aqueous core enclosed by one or more membrane-like concentric bilayers with diameters ranging from tens of nanometers to several micrometers (Panya et al., 2010; Yadav, Murthy, Shete, & Sakhare, 2011). Due to their ability to encapsulate both hydrophilic and hydrophobic bioactive, liposomes have gained attention in the food and pharmaceutical industries as promising delivery systems for polyphenolic compounds. In particular, they can be designed to increase the dispersibility, protect from degradation, and increase the bioavailability of polyphenols. In general, the bioavailability of encapsulated components is higher in nanoliposomes (d = 10 to 100 nm) than in conventional

liposomes (d > 100 nm). However, preparation of nanoliposome-based delivery systems is challenging using traditional methods because of difficulties in generating small particle sizes and ensuring high entrapment efficiency (Yang et al., 2013).

Several factors impact the physicochemical performance of liposomes, including the nature of the phospholipids used to fabricate them. For example, soybean lecithin contains high amounts of $C_{18:2}$ and $C_{18:3}$, and is susceptible to hydrolysis of the ester bonds and peroxidation of the unsaturated acyl chains (Yadav et al., 2011), producing off-flavors and oxidization of the bioactive encapsulated within the liposomes (Toro-Uribe, López-Giraldo, et al., 2018). Moreover, the method of preparation can affect the shelf-life of liposomes (e.g., due to leakage, aggregation, or separation), and impact their encapsulation efficiency, thereby, affecting their efficacy as delivery systems.

The aim of this study was to develop a suitable food-grade method for liposome preparation using soybean lecithin, and to compare the bioaccessibility of catechins, alkaloids and whole cocoa extract under simulated gastrointestinal fluids. The process parameters were optimized to achieve a small particle size, narrow polydispersity, extended shelf life, and high encapsulation efficiency. The effect of sonication and microfluidization parameters on the formation and performance of the delivery systems was also evaluated so as to identify optimized conditions to produce them.

7.2. MATERIALS AND METHODS

7.2.1. Reagents and Samples. All chemicals used were of analytical grade and used with no further purification. L-α-phosphatidylcholine from soybean (Type IV-S ≥ 30% PC), (+)-catechin hydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), caffeine, theobromine, trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), hexanal, Nile red (72485), Triton X-100, cumene

hydroperoxide, iron (II) sulfate heptahydrate, barium chloride dehydrate, ammonium thiocyanate, potassium persulfate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride, α-amylase (type IX-A), porcine pepsin, porcine pancreatic lipase, and bile salts were obtained from Sigma Aldrich (Sigma–Aldrich, Steinheim, Germany). (–)-Epicatechin (purity ≥ 99%) was purchased from ChromaDex Inc. (Irvine, CA, USA). Sodium acetate trihydrate, hydrochloric acid, glacial acetic acid, ethanol, methanol, and 1-butanol were obtained from Fischer Scientific (Fair Lawn, NJ, USA). Decanox MTS-90G mixed with tocopherols were obtained by ADM (Archer Daniels Midland Company, Decatur, IL, USA). Milli-Q water (Millipore system, Billerica, MA, USA) and deionized water were used for the preparation of all solutions. To minimize the presence of metals contaminants, all the glassware was acid-washed. Polyphenolic extract recovered from unfermented cocoa beans with low polyphenol oxidase activity was obtained according to our previous published procedure (Toro-Uribe, López-Giraldo, et al., 2018; Toro-Uribe, Montero, López-Giraldo, Ibáñez, & Herrero, 2018).

7.2.2. Liposome preparation. Soy lecithin was added to sodium acetate-acetic acid buffer (0.1 M; pH 3.0 ± 0.1 or pH 5.0 ± 0.1) containing 2.5 % (v/v) ethanol, and final lecithin concentrations of 1, 3, 5, or 10% (w/v). Pre-homogenization was carried out by stirring the system at 25 ºC until complete dissolution, followed by homogenization with a high shear blender (T10 Ultra-Turrax, IKA, Staufen, Germany) at 20,000 rpm for 2 min. The resulting coarse liposome suspension was then further homogenized using two different approaches: (1) *microfluidization* (110T, Microfluidics, Newton, MA, USA) at 137.9 MPa (20,000 psi) for several passages (up to 8 cycles) in an ice bath (4 ºC); or (2) *sonication* (Model 505, Fischer Scientific, USA) with a sonicator probe set at 10 s on/off pulses and submerged 1 cm from the bottom of a container placed in an ice water

around 4 ºC. The amplitude (50 to 75 %) and time of sonication (2.5 to 15 min) were varied. The liposomal solutions prepared were then filtered through 0.45 µm hydrophilic PTFE filter (Millipore, Milford, MA, USA).

Bioactive compounds (1000 µM) were dissolved in ethanol (2.5% v/v) and then added to a buffer solution. Soy lecithin was then added and the mixture was homogenized as described above. Liposomes without active ingredient (control) were also prepared. The bioactive compounds tested were (+)-catechin (C), (–)-epicatechin (EC), theobromine (Theo), caffeine (Caf), and cocoa extract.

7.2.3. Characterization of liposomes

7.2.3.1. Particle size and ζ-potential measurements. The mean particle diameter, polydispersity index (PDI), and ζ-potential were measured using a combined dynamic light scattering/electrophoresis instrument (Nano-ZS, Malvern Instruments, Worcestershire, UK) according to Panya et al., (2010) The particle size distribution was calculated using the Stokes-Einstein equation while the ζ-potential was calculated using the Smoluchowski model. Liposome suspensions were diluted with an appropriate buffer (sodium acetate-acetic acid buffer solution; 0.1M; pH 3.0 ± 0.1 or pH 5.0 ± 0.1) prior to analysis to prevent multiple scattering effects.

7.2.3.2. Encapsulation efficiency. The level of bioactive encapsulated within the liposomes was determined by measuring the amount of free bioactive in the aqueous phase of a liposome suspension according to the method of Toro-Uribe et al., (2018) with few modifications. Briefly, the samples were transferred to Optiseal bell-top ultracentrifuge tubes (Beckman Coulter, USA) and high-speed centrifuged (Beckman L-70, 70 Ti rotor, Beckman Instruments Inc, CA, USA) at 50,000 rpm, 4 ºC for 2 h. Then, the supernatant (200 µL) was carefully collected and used to determine bioactive concentrations using analytical reverse phase UHPLC-DAD as described

later. Encapsulation efficiency (EE) was expressed on a weight percentage basis according to the following equation:

$$EE\ \% = \frac{C_i - C_{free}}{C_i} * 100$$

C_i is the initial concentration of bioactive added to the liposomes and C_{free} is the concentration of free bioactive remaining in the aqueous phase.

7.2.4. Physicochemical stability

7.2.4.1. Influence of ph. As liposomes are pH-sensitive, the impact of both pH 3.0 ± 0.1 and pH 5.0 ± 0.1 on the oxidative stability (lipid hydroperoxide and hexanal formation) and encapsulation efficiency of the liposomes was determined. To do so, 125 µM of unloaded and EC-loaded liposomes (5 wt. %) were incubated at 55 °C. Based on these results, pH 5.0 ± 0.1 was chosen and used for further studies.

7.2.4.2. Influence of temperature and storage time. The impact of temperature on the storage stability of the liposomes was determined by placing liposome suspension (1 mL, pH = 5.0) into 10 mL headspace vials, sealing them with poly(tetrafluoroethylene) butyl septa, and then incubating them at 4, 32, 37, and 55 °C in the dark. Samples were collected at several time points (0 to 20, 30, 35, 40, 45, 50, 60, 90, 120, 150, 180, 210, 240, 270, 300 days) and immediately analyzed. The changes in particle size and ζ-potential were determined as previously mentioned, and color and hexanal formation were also measured as described later.

Lipid oxidation was analyzed by monitoring lipid hydroperoxides (a primary oxidation product) and headspace hexanal (a secondary oxidation product) at various time points. Hydroperoxides were analyzed according to the spectrophotometric method described by Hu, McClements, & Decker (2003). Samples (300 µL) were mixed with 2.8 mL of methanol/butanol (2:1 v/v), then 15

μL of 3.94 M ammonium thiocyanate and 15 μL of 0.072 M Fe^{2+} (ferrous sulfate) were added. The ferrous sulfate solution was made by mixing 0.13 M $BaCl_2$ and 0.14 M $FeSO_4$. The reaction was incubated in the dark at room temperature for 20 min, and the absorbance was measured at 510 nm (Genesys 20, Thermo Scientific, Waltham, MA, USA). The calculation was made from a standard curve of cumene hydroperoxide (0 – 0.6 mM, r^2 = 0.99) and data is expressed as mmol hydroperoxide per kg lecithin.

Gas chromatography (GC) was utilized to quantify headspace hexanal. A GC instrument equipped with a headspace autosampler and flame ionization detector (FID) (Shimadzu GC 2014, Shimadzu, Tokyo, Japan) was used. The operating parameters used were selected according to Hu et al., (2003). Briefly, samples were incubated for 10 min at 55 ºC in their sealed containers. A 50/30 μm DVB/Carboxen/PDMS solid-phase microextraction (SPME, Supelco, Bellefonte, PA, USA) fiber needle pierced the silicone/PTFE septa to a depth of 22 mm and adsorbed volatiles for 6 min. Then, the fiber was placed in the injector (250 ºC) port at a split ratio of 1:7 and desorbed for 3 min. The GC separation was isothermal at 65 ºC for 10 min on a HP methyl silicone (DB-1) fused-silica capillary column (50 m, 0.31 mm diameter x 1 μm) (Supelco, Bellefonte, PA, USA). Other parameters include pressurization 10 s; venting 10 s and helium as a carrier gas. A standard curve using hexanal (0 – 50 mM) was prepared to calculate hexanal concentrations in the samples (r^2 = 0.99). Data are expressed as mmol hexanal kg^{-1} lecithin.

Based on both peroxide value and hexanal formation *versus* storage time plots, the lag phase was determined to quantify the time for lipid oxidation. The lag phase was defined as the last day before there was a statistically significant increase in the concentration of primary or secondary oxidation products before the exponential phase was entered.

7.2.5 Color measurement. The color of the liposomes was measured using an instrumental colorimeter (ColorFlex, HunterLab Reston, VA, USA) to evaluate changes in the appearance of the liposome suspensions due to lipid peroxidation as previously described (Qian, Decker, Xiao, & Mcclements, 2012). To do so, 10 mL of liposome suspensions were collected at different time points and placed into a Petri dish to perform the analysis. The total color difference (ΔE) was calculated from the CIE tristimulus color coordinates as follows:

$$\Delta E = \sqrt{(L^* - L_i^*)^2 + (a^* - a_i^*)^2 + (b^* - b_i^*)^2}$$

Here, the L*a*b* values are the CIE tristimulus color coordinates: *L** (black to white) represents the lightness; *a** represents red to green; and *b** represents yellow to blue. The subscript *i* represents the initial color values. Additionally, the color intensity of the samples was calculated through the difference in the chroma (ΔC*) values:

$$\Delta C^* = \sqrt{(a^* - a_i^*)^2 + (b^* - b_i^*)^2}$$

7.2.6. In vitro digestion stability of liposomes

7.2.6.1. Simulated digestion model. Three groups of samples were studied (i) bioactive-loaded liposomes; (ii) empty liposomes; (iii) bioactive compounds dispersed in aqueous solutions. The bioactive compounds consisted of (+)-catechin, (–)-epicatechin, theobromine, caffeine, and crude cocoa extract. The empty liposomes and aqueous systems were used as controls. All the samples were passed through a three-stage *in vitro* digestion model consisting of mouth, stomach, and small intestinal phases according to the standardized static method of Minekus et al., (2014) as follows:

A simulated saliva fluid (SSF) consisting of 1.25-fold concentrated stock solution was prepared that consisted of 15.1 mM KCl, 3.7 mM KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2(H_2O)_6$, 0.06 mM $(NH_4)_2CO_3$, and 1.1 mM HCl. To carry out the assay, 25 mL of sample were mixed with 17.5 mL of SSF electrolyte solution, and then, 2.5 mL of 1500 U/mL salivary α-amylase solution in electrolyte stock solution were added, followed by 125 µL of 0.3 M $CaCl_2$, adjusted to pH 7 with 1 M HCl. Finally, the volume was made up to 50 mL with deionized water and the sample was incubated for 2 min at 37 °C.

A simulated gastric fluid (SGF) electrolyte stock solution was prepared (1.25-fold concentrated) that consisted of 6.9 mM KCl, 0.9 mM KH_2PO_4, 25mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2(H_2O)_6$, 0.5 mM $(NH_4)_2CO_3$, and 15.6 HCl mM. After the oral phase, the sample was exposed to the simulated gastric phase. To do that, 5 parts of liquid sample were mixed with 4 parts of SGF stock electrolyte solution, and then, 4 mL of *ca.* 25000 U/mL porcine pepsin stock solution and 12.5 µL of 0.3 M $CaCl_2$ were added, followed by adjustment of the pH to 3 using 1 M HCl. Finally, the volume was made up to 50 mL with deionized water and flushed with nitrogen liquid. The gastric phase was carried out in a rotary shaker (Infors HT Multitron Standard, Switzerland) set at 100 rpm, for 2 h at 37 °C.

For the simulated *in vitro* digestion, simulated intestinal fluid (SIF) was prepared (1.25-fold concentrated) consisting of 6.8 mM KCl, 0.8 mM KH_2PO_4, 85mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, and 8.4 HCl mM. Then, 25 mL of gastric digest was mixed with 13.75 mL of SIF stock solution, 6.25 mL of 800 U/mL pancreatin, 3.125 mL of 160 mM fresh bile, and 50 µL of 0.3 M $CaCl_2$. Then, the pH was adjusted to 7.0 with 1 M NaOH, and finally, the volume was made up to 50 mL with deionized water and flushed with nitrogen liquid. The small intestine phase

was carried out in a rotary shaker (Infors HT Multitron Standard, Switzerland) set at 100 rpm, for 6 h at 37 °C.

7.2.7. Release kinetics of bioactive compounds. At predetermined time points (every 30 min during simulated gastric digestion, and every 1 h during duodenum simulated digestion), 200 µL of dissolution media was withdrawn for each digestion phase, and immediately snap frozen in liquid nitrogen and subsequently stored at -86 °C (ULT Ultralow freezer, Haier, Quingdao, China) until further analysis. For quantitative analysis, the samples were diluted and adjusted to pH 5. Then, the sample solution was centrifuged at 13400 rpm for 30 min at 4 °C. The supernatant was carefully collected, considered as the total amount of compound remaining, and analyzed by UHPLC-DAD as described later. The *in vitro* release behaviors were plotted as a function of time as follows:

$$Remaining\ (\%) = \frac{C_t}{C_i} * 100$$

where C_t is the sample concentration for each time point, and C_i is the initial concentration.

7.2.8. Determination of the bioaccessibility of the bioactive compounds. After *in vitro* digestion, 200 µL of raw digest was taken out and used for further analysis. To completely fracture the liposome membranes, samples were snap frozen in liquid nitrogen and conditioned at room temperature (repeated 5 times). Then, the sample was diluted with a solution containing 1% Triton X-100 and adjusted to pH 5. Subsequently, the sample was centrifuged (Heraeus Fresco 21, Thermo Scientific, Hamburg, Germany) at 13400 rpm, 4°C, for 30 min. The supernatant was carefully collected and considered to be the "micelle" fraction, in which the bioactive compounds were solubilized (Zhang et al., 2015). The amount of the bioactive present was quantified by UHPLC-DAD (see below), and the bioaccessibility was calculated as follows:

$$Bioaccessibility\ (\%) = \frac{C_{Digesta}}{C_i} * 100$$

where $C_{Digesta}$ and C_i are the concentrations of the bioactive compounds in the mixed micelle phase and the initial concentration, respectively.

7.2.9. Chromatographic analysis. The UHPLC system (Agilent 1290 Infinity series II, Agilent Tech. Santa Clara, CA, USA) consisted of a binary pump delivery system, an on-line degasser, a thermostated autosampler, and a diode array detector (DAD). System control and data analysis were processed using OpenLab CDS software (Agilent ChemStation). 12 µL of diluted samples were filtered through a 0.45 µm hydrophilic Durapore PVDF membrane (Millipore, USA) and then injected into a C_{18} reverse phase Zorbax Eclipse Plus column (50 × 2.1 mm, 1.8 µm) connected to a Zorbax SB-C8 guard column (5 × 2.1 mm, 1.8 µm). The column oven was set at 55 ºC. The flow rate was 0.7 mL min^{-1} eluting with water (0.01% formic acid, solvent A) and acetonitrile (0.01% formic acid, solvent B). A linear gradient program was performed as follows: 0 min, 0% B; 3.9 min, 1.5% B; 4.0 min, 4% B; 11.0 min, 10 %B, 14.0 min, 35% B; 14.2 min, 100% B; 16.5 min, 100% B; 17.0 min, 0% B; 23 min, 0% B. The resulting separation was recorded at 280 nm. All the samples were injected in triplicate. Additionally, a blank sample was injected between every sample. The content of (+)-catechin, (–)-epicatechin, theobromine, and caffeine in the tested samples were calculated from standard curves for each compound ($r^2 \geq 0.99$).

7.2.10. Microstructure and morphology analysis. Before and after *in vitro* digestion, 150 µL of either catechin-, epicatechin-, theobromine-, caffeine-, and cocoa extract-loaded liposome samples were taken for analysis using confocal optical scanning laser microscopy and transmission electron microscopy (TEM). Prior to analysis, samples were mixed with Nile Red solution (1 mg/mL) at a ratio 2/1 v/v, to dye the oil phase. Then, the dyed samples were placed on a microscope

slide, covered by a coverslip, and observed by confocal optical scanning laser microscopy with a 60× oil immersion objective lens (Nikon D-Eclipse C1 80i, Nikon, Melville, NY, USA). The excitation and emission spectrum were set at 534/605 nm, respectively. The images were acquired using image analysis software (NIS-Elements, Nikon, Melville, NY).

TEM images were used to examine the morphology and to confirm the mean particle size of the samples. A liposome suspension (ca. 50 µL) was absorbed onto a Formvar carbon-coated 200 mesh thick grid (Ted Pella Inc., USA) for fixation for 2 min. Then, the grid was stained with uranyl acetate aqueous solution (2 %) for 1 min and air-dried at room temperature, and the excess of liquid was removed with filter paper. The grid-sample was examined using a transmission electron microscope (JEOL JEM 1010, Tokyo, Japan) operating at an acceleration voltage of 100 keV, equipped with a Gatan ES1000W digital camera. The images were processed using Digital Micrograph software.

7.2.11. Antioxidant assays. The antioxidant activity of samples before and after the *in vitro* simulated digestion phases was measured using two *in vitro* assays: $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free radicals scavenging assays. The DPPH assay was carried according to the procedure described by Brand-Williams, Cuvelier, & Berset (1995) with the following modifications: 68.5 µM of DPPH methanolic solution was diluted with methanol to obtain an absorbance of 0.570 ± 0.010 units at 517 nm using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) controlled by Gen5 software (Gen5 v. 2.04 BioTek Inst. Inc). Then, 100 µL of sample was added to 745 µL of a methanolic solution of daily-working DPPH solution at room temperature for 1 h in the dark. Then, 300 µL were taken out and placed in ninety-six well microplates to measure their total absorbance. Methanol and DPPH• solution without test samples were used as blank solutions and controls, respectively. Results are expressed as % radical scavenging:

$$DPPH\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100$$

For the ABTS assay, the antioxidant activity was assessed by their ability to scavenge the $ABTS^{\bullet+}$ free-radical cation using the method proposed by Re et al., (1999). Briefly, $ABTS^{\bullet+}$ was produced by reacting 2.5 mL of 7 mM ABTS stock solution with 44 µL of 2.5 mM potassium persulfate, allowing the mixture to stand in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ solution (1 mL) was diluted with 70 mL of 5 mM phosphate buffered saline (pH 7.4) to an absorbance of 0.700 ± 0.002 at 734 nm. The reaction mixture was placed in ninety-six well microplates containing 10 µL sample/standard and 300 µL of reagent; then the reaction was incubated in the dark at room temperature for 45 min. The absorbance was measured at 734 nm using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) controlled by Gen5 software (Gen5 v. 2.04 BioTek Inst. Inc). Appropriate solvent blanks were run in each assay. A Trolox calibration curve (0.48 – 125 µM; r^2 = 0.99) was used to calculate the radical scavenging ability. Results are expressed as µmol Trolox equivalents / µmol of sample

7.2.12. Statistical Analysis. All determinations were carried out at least three times, and data were expressed as the mean ± standard deviation. Statistical analysis was done using GraphPad Prism V. 6.0 (GraphPad Soft. Inc., La Jolla, California, USA). One-way ANOVA was performed and Tukey´s significance difference *post hoc* test at 5% level of significance by IBM SPSS Statistics version 25 (IBM Corporation, New York).

7.3. RESULTS & DISCUSSION

The effectiveness of colloidal delivery systems depends on their ability to effectively encapsulate, retain, stabilize, and release bioactive compounds (McClements, 2014). The

performance of liposome-based delivery systems depends on their composition, size, surface charge, and stability, as well as the location of the bioactive compounds within their structure (Toro-Uribe, López-Giraldo, et al., 2018). For this reason, we examined the impact of the initial composition and preparation method on the stability and functionality of bioactive-loaded liposomes.

7.3.1. Optimization of liposome preparation. Initially, two liposome fabrication technologies that operate on different physicochemical principles were assessed: microfluidization and sonication. The main goals of this part of the study were to generate small liposomes, narrow particle size distributions (PSD), uniform dispersions, extended shelf life, and good oxidative stability. Liposomes were prepared using these two methods by dispersing 1 % lecithin in sodium acetate-acetic acid buffer at pH 3.0 ± 0.1 using: (a) microfluidization at 20,000 psi for different numbers of passes; (b) sonication at several amplitudes and treatment times.

7.3.2. Preparation of liposomes. Figure S1 compares the PSDs of the liposome suspensions obtained using the two fabrication technologies. Overall, both methods produced anionic liposomes with dimensions in the nano-scale. However, the PSDs of the liposome suspensions were very broad, exhibiting multiple peaks, whose size and location depended on the homogenization conditions used. None of the homogenization conditions used produced a monomodal PSD, which could be due to the nature of the lecithin and/or solution conditions used.

For microfluidization, even one pass through the homogenizer gave a large decrease in mean particle diameter, e.g., from 906 nm for the non-homogenized liposomes to 45.8 nm after 1 pass. The mean particle diameter then decreased slightly with increasing number of passes but remained relatively constant after 5 passes.

For sonication, pulse intensity and treatment time affected the efficiency of liposome formation. Initially, the impact of pulse amplitude (50, 60 and 75 %) was studied using 10 s on/off pulses applied for different treatment times. To avoid bubble formation and foaming, the sonicator tip had to be submerged 1 cm from the bottom of a container to allow proper circulation and mixing of the sample throughout homogenization. The results showed that overheating (T < 40 °C) of the samples could be avoided using a combination of high intensity and low treatment time. For instance, a mean droplet diameter of 42.5 nm was obtained applying a pulse intensity of 75 % for 4 minutes. A polydispersity index ≤ 0.4 was obtained for all formulations using both sonication and microfluidization. Our findings are in agreement with previous studies where microfluidization (Lajunen et al., 2014) or sonication (Silva, Ferreira, Little, & Cavaco-Paulo, 2010) were used to form liposomes.

7.3.3. Influence of preparation of liposomes on encapsulation efficiency. As above mentioned, effective preparation was achieved with: (a) microfluidizer at 20,000 psi for 5 cycles and (b) sonication at 75% for 7 min (10 s on /off pulses). After preparation using these conditions, liposomes (1.0 % lecithin) with and without a model compound (125 µM EC) were incubated at 55°C and the encapsulation efficiency and susceptibility to lipid oxidation were measured. The same lag phase for oxidation (4 days) was observed for the control liposomes in both systems (Table 1). However, when the bioactive compound was loaded into the liposomes, the lag phase was slightly longer for sonication than microfluidization. This may have been because of their slightly higher encapsulation efficiency. Both fabrication techniques produced fairly similar mean particle diameters (50.7 – 62.2 nm) and ζ-potential values (-32.4 to -37.3 mV). The absence or presence of antioxidant did not significantly affect the ζ-potential of the liposomes, which was also reported in previous studies by Toro-Uribe et al., (2018) and Gibis, Vogt, & Weiss, (2012).

Nevertheless, the mean particle diameter of EC-loaded liposomes was higher than that of unloaded liposomes, which can be explained by the fact that phenolic compounds might be absorbed onto the surface of the lipid bilayers and/or incorporated into the core. Moreover, phenolic compounds can participate in both hydrophilic and hydrophobic interactions with the carrier system, which may impact its dimensions (Gibis et al., 2012).

7.3.4. Impact of the concentration of lecithin on encapsulation efficiency. To determine the most suitable ratio of lecithin-to-core material to enhance the encapsulation efficiency (EE), four concentrations of soy lecithin (1, 3, 5 and 10 % wt.) were tested. In this study, the presence of cholesterol (widely used for the preparation of liposomes) was not considered. Although many studies use cholesterol as a stabilizer and to reduce bilayer permeability, other works showed that the presence of cholesterol cause limited space for the incorporation of compounds due to the steric hindrance provided by this steroid, thereby reducing the EE and affecting the release rate profile Briuglia, Rotella, McFarlane, & Lamprou, 2015). Our results showed that higher a EE was obtained by increasing the level of phospholipids present, but at the highest concentration assayed (10 %), the liposome suspension behaved as a prooxidant with a lag phase of only 2 days. The highest EE was achieved using 5% soy lecithin; for instance, their incorporation into liposomes increased from 27.0 to 44.7 % for sonication and from 18.0 to 38.6% for microfluidization (Table 1). In fact, Dag & Oztop (2017) reported than microfluidization was less effective in incorporating polyphenolic compounds, although the liposomes produced were relatively stable to aggregation and fusion during storage. Moreover, Chung et al., (2014) found that a higher ratio of material to lecithin affected the mean particle size and encapsulation efficiency of liposomes produced by microfluidization, being around 1.1% lower when the ratio increased from 1:4 to 1:5.

Based on these results, sonication at an amplitude of 75% was applied for 7 min for all further studies. Besides, sonication requires less sample (no loss during processing), a lower processing time, and therefore, lower energy and production costs.

7.3.5 Influence of pH on physical stability. The mean particle diameter and ζ-potential of the liposomes depended on pH (Figure S2). A high stability to aggregation and phase separation were achieved from pH 3 to 5, while highly unstable

Table 1. Physiochemical parameters and bioaccessibility of liposome samples for the designed process and during simulated *in vitro* GIT digestion.

	Lecithin (wt %)	pH	Concentration (μM)	Lag phase (day)	Mean size (nm)	ζ-potential (mV)	Encapsulation Efficiency (%)	Bioaccessibility (%)
Microfluidization (20 Kpsi, 5 passes) *								
- Control Liposome	1	3.0	-	4	59.07 ± 3.90^{a}	-36.80 ± 2.69^{a}	-	-
- Epicatechin Liposomal	1	3.0	125	5	62.24 ± 0.57^{a}	-32.40 ± 3.54^{a}	18.04 ± 0.03^{a}	-
- Epicatechin Liposomal*	5	3.0	125	6	59.07 ± 3.90^{a}	-36.80 ± 2.69^{a}	38.56 ± 0.20^{b}	-
Sonication (75% amplitude, 7 min) *								
- Control Liposome	1	3.0	-	4	50.75 ± 7.86^{a}	-37.30 ± 1.98^{a}	-	-
- Epicatechin Liposomal	1	3.0	125	6	53.09 ± 4.53^{a}	-35.35 ± 8.41^{a}	27.02 ± 0.02^{a}	-
- Control Liposome	5	3.0	-	3	56.71 ± 1.49^{a}	-31.50 ± 0.28^{a}	-	-
- Epicatechin Liposomal	5	3.0	125	8	52.32 ± 6.10^{a}	-32.45 ± 0.37^{a}	44.70 ± 0.02^{b}	-
Formulation at pH 5.0 loaded liposomes								
- Control Liposome	5	5.0	-	8	74.03 ± 0.01^{a}	-17.50 ± 0.08^{a,b,c}	-	-
- Theobromine Liposomal	5	5.0	1000	-	75.72 ± 1.48^{a}	-15.70 ± 1.70^{b,c}	0.03 ± 0.02^{a}	35.86 ± 2.48^{a}
- Caffeine Liposomal	5	5.0	1000	-	75.66 ± 0.18^{a}	-15.25 ± 0.78^{c}	0.04 ± 0.03^{a}	39.40 ± 1.61^{a}
- Catechin Liposomal	5	5.0	1000	-	79.46 ± 0.58^{b}	-18.70 ± 0.57^{a,b}	46.66 ± 3.89^{b}	57.73 ± 3.27^{b}
- Epicatechin Liposomal	5	5.0	1000	-	73.99 ± 1.27^{a}	-20.00 ± 1.27^{b,d}	48.28 ± 1.03^{b}	49.16 ± 2.25^{c}
Cocoa extract-loaded liposome**	5	5.0	1000	-	84.3 ± 0.07^{c}	-22.85 ± 2.54^{d}	-	-
- Theobromine							0.05 ± 0.04^{a}	8.44 ± 0.74^{a}
- Caffeine							0.07 ± 0.06^{a}	31.49 ± 2.68^{b}
- Catechin							48.99 ± 0.27^{b}	30.58 ± 3.31^{b}
- Epicatechin							61.10 ± 0.06^{c}	27.51 ± 1.86^{b}
Formulation-in-aqueous-system								
Free cocoa extract**	-	-	1000	-	-	-	-	-
- Theobromine								26.60 ± 1.21^{a}
- Caffeine								83.50 ± 10.80^{b}
- Catechin								13.07 ± 4.46^{a}
- Epicatechin								12.59 ± 3.56^{a}
Free compounds	-	-	-	-	-	-	-	-
- Theobromine			1000					79.37 ± 4.73^{a}
- Caffeine			1000					75.09 ± 5.96^{a}
- Catechin			1000					29.38 ± 2.51^{b}
- Epicatechin			1000					22.55 ± 3.63^{b}

liposomes were obtained at pH 2 and at pH ≥ 8. These phenomena can be explained by the fact that the acid/basic environment surrounding the liposomes impacts the electrostatic, hydrophilic, and hydrophobic interactions in the system, thereby influencing the interfacial rheology and permeability of the bilayer membrane, the aggregation state, and the encapsulation efficiency of the system (Sabín, Prieto, Ruso, Hidalgo-Álvarez, & Sarmiento, 2006; Sułkowski, Pentak, Nowak, & Sułkowska, 2005). Previously, Sabín et al., (2006) reported that at pH values ranging from 3 to 5 the osmotic balance across phospholipid membranes is enhanced. The largest vesicles were formed at pH 2, which is close to the measured isoelectric point (Figure S2). A highly acidic environment contributes to a larger particle size because the anions (Permeability coefficient, $P \approx 10^{-11}$-10^{-12}) are more permeable than water ($P \approx 10^{-3}$-10^{-4}) (Sabín et al., 2006). Moreover, the electrostatic repulsion between neighboring liposomes is reduced. No dependence between particle size and ζ-potential was observed at pH values ranging from 6 to 10, in which the surface potential of the liposomes was unchanged, but the particle size was variable.

Based on these results, liposome suspensions at pH 3.0 and 5.0 were selected for further study because of their good stability. As can be seen in Figures S3A and S3B, the oxidative stability of EC-loaded liposomes was much higher than non-loaded liposomes, thus demonstrating the antioxidant activity of the polyphenol-loaded liposomes. However, the formulations at pH 5.0 were more stable. For instance, the lag phase for hydroperoxide formation was 2 and 8 days the lag phase for hexanal formation was 8 and 11 days for EC-loaded liposomes at pH 3.0 and 5.0, respectively. In addition, the encapsulation efficiency was 2.8% higher at pH 5 than pH 3. Based on our results, better encapsulation efficiency and oxidative stability, a lower particle size, and high ζ-potential values were obtained at pH 5.0. Therefore, sodium acetate-acetic acid buffer at pH 5.0 was used for further studies.

7.3.6. Stability of liposomes. The long-term stability of empty and EC-loaded liposomes was determined by analyzing changes in their mean particle diameter, ζ-potential, pH, color, and hexanal formation over time (pH 5.0 ± 0.1). These parameters were chosen as good indicators of the physical and oxidative stability of the liposomes. The original color of the liposome suspensions was translucid-yellow but as soon as lipid oxidation occurred, the samples became turbid, which led to appreciable changes in their color coordinates (L*, a*, b*). For instance, the ΔE values equal to 22.4 and 17.1, and ΔC* values equal to 18.0 and 13.5 were observed for empty and EC-loaded liposomes, respectively. These phenomena can be explained because during lipid oxidation the amines interacted with the aldehyde products forming yellow-brown pigments as a result of non-enzymatic browning reactions (Thanonkaew, Benjakul, Visessanguan, & Decker, 2007).

For the samples stored at 55, 37, 32, and 4°C, the lag phases were 3, 4, 18, and 150 days for controls and 11, 13, 30 and 210 days for EC-loaded liposomes, respectively (Figure 1). Before lipid oxidation, no aggregation or sedimentation was observed during storage, which demonstrates the good physicochemical stability of the liposome formulations as well as adequate preparation. Furthermore, EC-loaded liposome samples were lysed and analyzed by UHPLC-DAD. The results showed that 50 % of the epicatechin (Figure 1C) remained after about 5 days, which confirmed that once the antioxidant was absent, lipid oxidation occurred, leading to the maximum formation of primary and secondary reaction products. Once the system was oxidized, there was a change in the mean particle diameter (74.0 ± 0.0 to 167 ± 2.8 nm, and 73.9 ± 1.3 to 177 ± 3.5), ζ-potential (-17.5 ± 0.1 to -37.3 ± 1.4, and -20.0 ± 1.33 to -38.9 ± 0.4), and pH (5.0 ± 0.1 to 5.34 ± 0.1, and 5.0 ± 0.1 to 5.26 ± 0.2) for the non-loaded and epicatechin-loaded liposomes (55°C), respectively. The remarkable increase in the particle size and significant reduction in ζ-potential are in agreement

with the results of Chung et al., (2014) who reported that this phenomenon is mainly due to the swelling of the liposomes and the formation of a complex macromolecular structure with changes in the surface properties under acidic conditions.

7.3.7. Simulated *in vitro* gastrointestinal digestion of liposomes. The main aim of these experiments was to investigate the bioaccessibility and kinetic release profiles of liposomes loaded with the studied compounds and to compare these parameters with those attainable for the bioactive compounds simply dispersed in aqueous solutions. Liposome samples were therefore prepared based on the optimum conditions established in previous sections: sonication at an intensity of 75% for 7 min; 5 wt.% soy lecithin; pH 5.0 ± 0.1; 0.1 M ionic strength. Free and liposome-loaded theobromine, caffeine, catechin, epicatechin, and cocoa extract were then incubated with simulated oral, gastric, and small intestine digestion fluids. The physicochemical properties and stability of the bioactive compounds and liposome formulations were then measured.

7.3.7.1. Electrical charge of liposomes. The ζ-potential of the initial liposomes were -15.7, -15.3, -18.7, -20.0, and -22.9 mV, for theobromine, caffeine, catechin, epicatechin, and cocoa extract, respectively (Table 1). The strong negative charge on the liposomes (Figure S2) can be attributed to the presence of charged phosphate groups (Sabín et al., 2006).

After the mouth phase, there was a slight change in the electrical charge on the liposomes being -11.8, -11.1, -15.7, -15.9, and -17.2 mV for theobromine-, caffeine-, catechin-, epicatechin-, and cocoa extract-loaded liposomes, respectively (Figure 2A). This may be due to the interaction of mucin with the liposome surfaces, which reduced the surface potential through electrostatic screening and binding effects (Zhang et al., 2015).

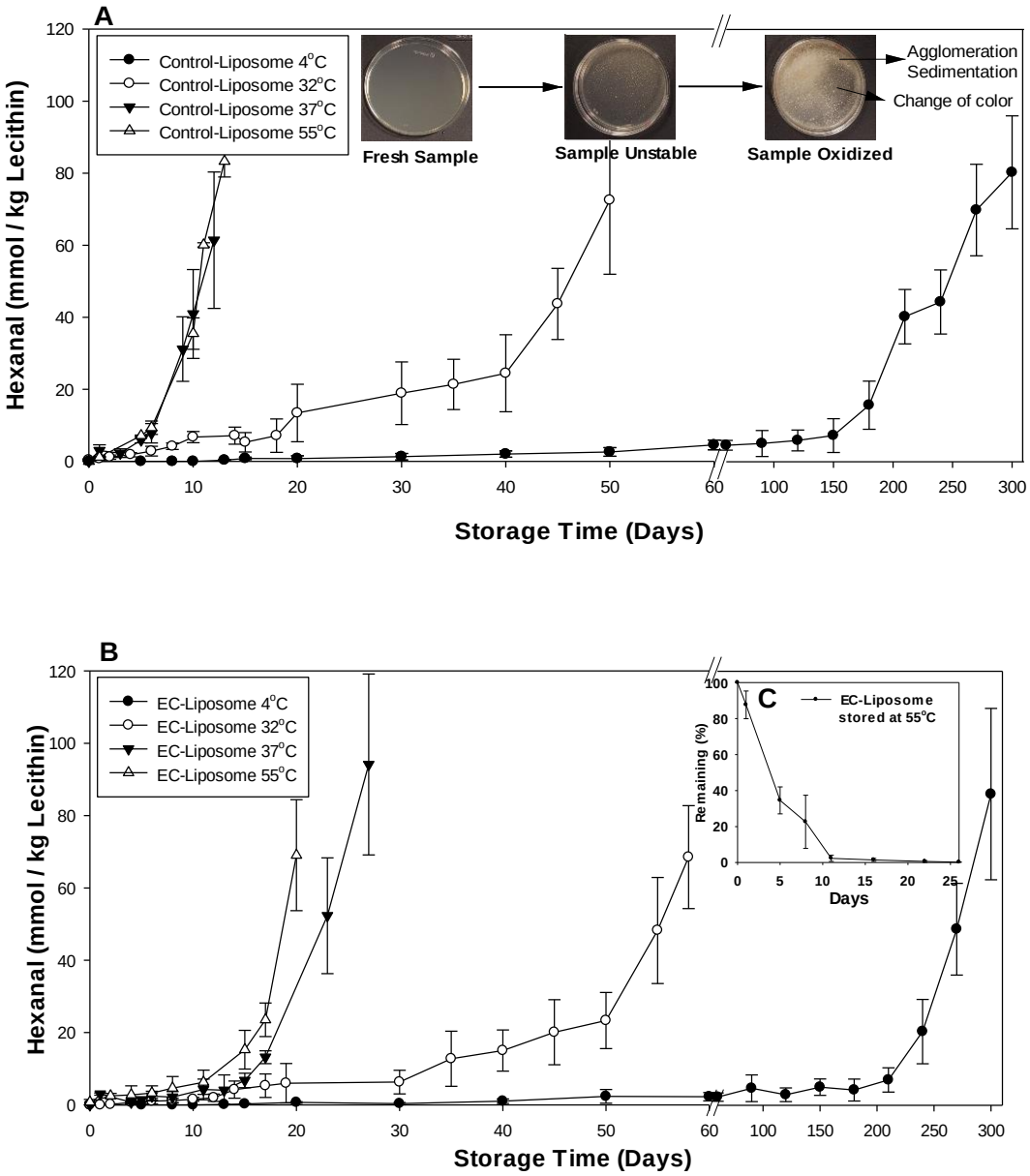

Figure 1. Time to lipid oxidation determined by hexanal formation for (A) non-loaded liposomes, (B) epicatechin-loaded (EC, 125 μM) liposomes, and (C) epicatechin-loaded liposome release over time (stored at 55 °C).

After the stomach phase, the magnitudes of the ζ-potentials on the liposomes became close to zero for all samples (Figure 2A). These changes can be attributed to the high ionic strength of the

in vitro gastric phase, as well as the highly acidic gastric fluids that impacts the ionization state and charge distribution of the phosphatidylcholine heads, which agreed with Sułkowski et al., (2005).

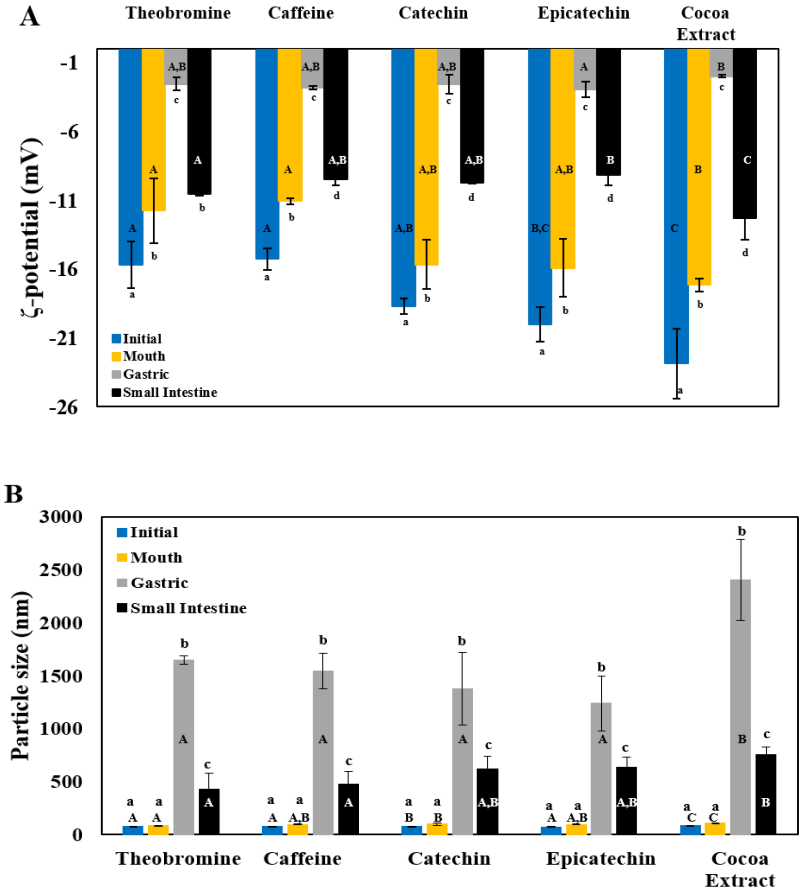

Figure 2. ζ-potentials (A) and mean particle size (B) of compounds-loaded liposomes before and after each simulated *in vitro* digestion step. Samples with different capital letters (A, B, C) indicate significant differences ($p<0.05$) between same digestion phases for the different bioactive compounds. Samples designated with different lower-case letters (a, b, c, d) were significantly different ($p< 0.05$) between different digestion phases for the same bioactive compounds.

After the small intestine phase, an increase in the magnitude of the negative charge on the particles in the digested liposome suspensions was observed. For instance, the ζ-potential was -

10.5, -9.5, -9.7, -9.2, and -12.3 mV for theobromine-, caffeine-, catechin-, epicatechin-, and cocoa extract-loaded liposomes, respectively. Previously, Zhang et al., (2015) reported that an increasing magnitude of the negative charge is due to the presence of various types of anionic particles in the digesta, including bile salts, micelles, vesicles, phospholipids, free fatty acids, and undigested lipid droplets. Interestingly, no noticeable difference in the electrical surface charges for all the liposome formulations ($p < 0.05$) were observed, which indicates that the differences during the *in vitro* gastrointestinal assays are related to the nature of the soy liposomes instead of the encapsulated compound.

7.3.7.2. Particle size and microstructure. To better understand how the liposome membrane was affected within the *in vitro* gastrointestinal tract, the particle size was determined by light scattering and the microstructure was determined by TEM and confocal microscopy. Figure 3 summarizes the proposed mechanism of release and transformation of liposomes within simulated digestive fluids. Morphological changes from spherical to oval shape, swelling, interaction with digestive components, and perturbation of the membrane are suggested to account for the observed effects based on the results obtained in the present study. Further details are given below.

Figure 2B shows that the lowest particle size was obtained for all the samples before digestion. More insight is provided by the TEM images (Figure 4 A-D), where it can clearly be observed that vesicles were semi-spherical with similar particle size distributions, which agrees with our dynamic light scattering study. Therefore, non-loaded liposomes together with theobromine-, epicatechin-, and cocoa extract-loaded liposomes with cross section vesicle lengths between 40-90, 80-130, 70-130, and 30-115 nm, respectively, were formed. Interestingly, honeycomb- or cluster-like structures consistent with the presence of circular interlayer contacts (Polozova et al., 2005) were observed. A slight increase of the hydration layer by around 1.0, 1.0, 1.2, and 1.5-2.0

nm for non-loaded, theobromine-, epicatechin- and cocoa extract-loaded liposomes respectively, were seen. These data confirmed the interaction of the compounds having different polarities with the lipid bilayer, in particular for the cocoa extract. Analysis of the surface morphology of the vesicles indicated that all the formulations contained bilayer hetero-junctions with similar interlayer thicknesses from 3 to 7 nm. Nevertheless, the average number of lamellae per liposome appeared to be affected by the nature of the encapsulated compound. For instance, it was observed that non-loaded and Theo-loaded liposomes preferably formed a unilamellar layer, while EC-loaded liposomes were multilayered. Overall, the bilayer surfaces had very smooth and thin appearances, with regular curved edges. Solubility-diffusion theory considers the bilayer membrane to be a homogeneous slab of bulk organic material through which the permeant must partition into and diffuse across (Maherani, Arab-Tehrany, Kheirolomoom, Geny, & Linder, 2013). Moreover, the capture volume was smaller for the empty liposomes, followed by Theo-loaded liposomes, and being highest for EC- and cocoa extract-loaded liposomes. These findings suggest that the level of entrapped compounds into the core (EE catechins >>> alkaloids) may play a role in the vesicle formation process. Thus, liposome size is a determining factor for permeability and may affect the release rate (Sabín et al., 2006). Electron and confocal microscopy images suggested no appreciable difference in the mean particle size of the liposomes within the mouth phase (Figure 2B, Figure S4 E-G, and Figure 4 E-G).

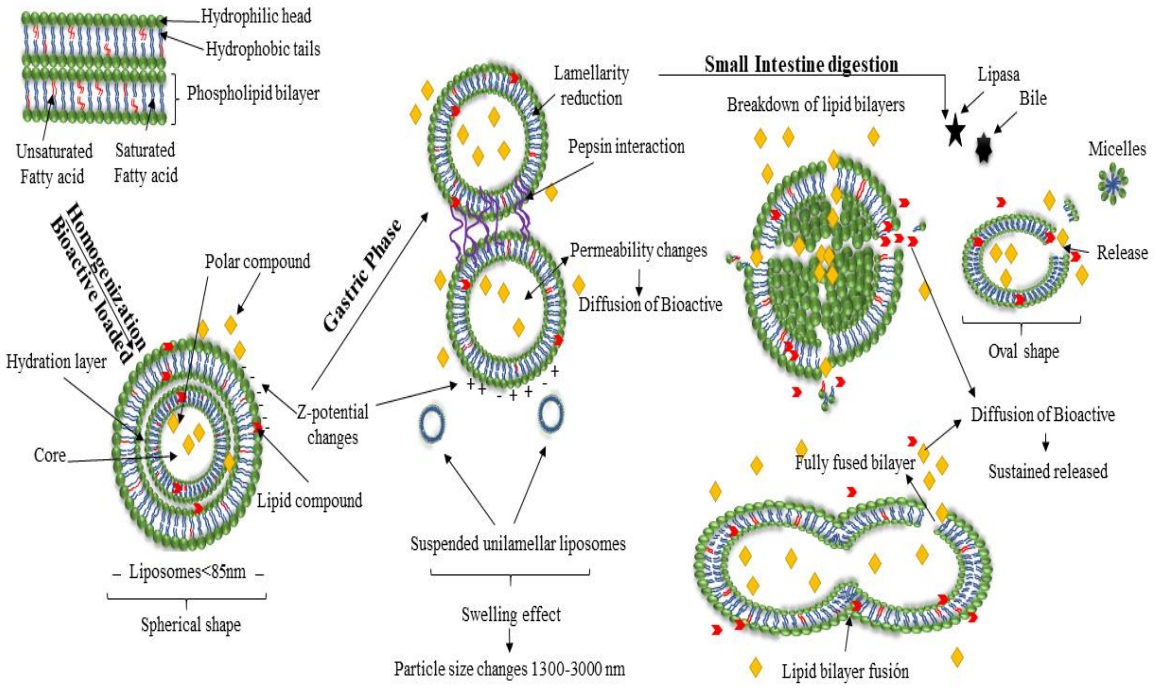

Figure 3. Suggested mechanisms of physicochemical changes of liposomes before and after simulated gastrointestinal digestion.

However, Figure S4F showed slight differences on particle size as a consequence of the interaction of polyphenol compounds with α-amylase, which have been previously explained by Xiao et al., 2011). Extensive agglomeration changes of morphological structure forming larger vesicles and greater core volume into the liposome were observed in Figure S4 I-L and Figure 4 I-L. This may be due to the addition of HCl to the medium, that can change phospholipid permeability and osmotic pressure (Sabín et al., 2006), leading to liposome swelling. In fact, Figure 2B shows that during the gastric phase, the increase of particle size diameter was 1650 ± 40, 1540 ± 170, 1380 ±340, 1240 ± 260, and 2400 ± 380 nm, for Theo-, Caf-, C-, EC-, and cocoa extract-loaded liposomes, respectively. Despite all these changes, lysis or membrane disruption were not observed suggesting that a controlled release profile could be achieved throughout the digestion transit time.

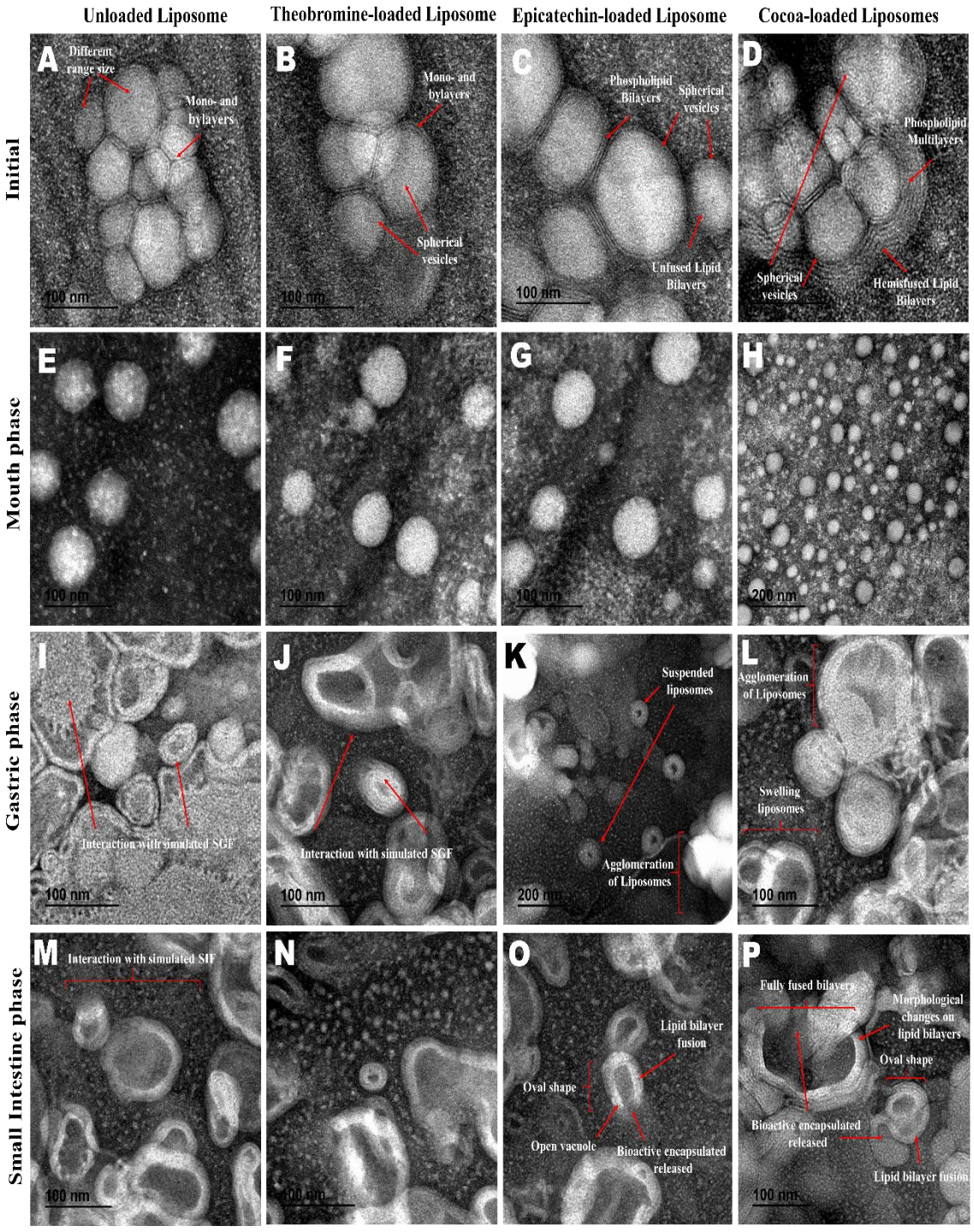

Figure 4. TEM images of (A) non-loaded and (B) theobromine-, (C), epicatechin-, and (D), cocoa extract-loaded liposomes, before and after physicochemical changes produced during simulated *in vitro* gastrointestinal digestion

Interestingly, at a later stage, when the sample moved from the stomach to the small intestine (Figure 4 M-P), a significant reduction in the number of bilayers was observed, which would be expected to impact the molecular diffusion process. Moreover, there was a breakage of the interlayer junctions, formation of unilamellar liposomes, and a heterogeneous morphological shape and size. All these factors favored the released of the encapsulated compounds. Moreover, at the end of the duodenal phase, the size of the liposomes decreased, which was confirmed by dynamic light scattering and confocal microscopy images (Figure 2B and Figure S4 M-P). Unfortunately, it proved to be difficult to obtain good images of these samples by TEM.

The observed reduction of particle size in the small intestine could have occurred for a number of reasons. Firstly, the added Na^+ ions modified the osmotic forces; liposomes react to this change by evacuating water from their insides to compensate for the excess of cations outside of them, thus causing them to decrease in diameter (Sabín et al., 2006). Secondly, cationic ions adsorbed to the bilayers (e.g., K^+, Na^+, and Ca^{2+}) and altered their interactions and optimum curvature. Thirdly, bile salts entered the phospholipid bilayers and disrupted the liposome structure. Fourthly, the fatty acids released from digestion of the phospholipids were solubilized in the mixed micelles. These findings are in line with those reported by Zhang et al., (2015) who observed that lipid droplets were digested by lipase molecules, resulting in the formation of free fatty acids, vesicles, monoacylglycerols, and mixed micelles. Indeed, a lower fluorescence signal was observed at the end of digestion, which indicates that the oil phase had been digested by lipase (Figure S4 M-P). These results are consistent with the free fatty acid profiles (data not shown), where the measurement of the volume of NaOH required to keep the pH equal to 7.0 was relatively constant for 6 h in simulated small intestine, that is, almost complete digestion of lipids were achieved.

7.3.7.3. Comparison of the simulated in vitro digestion of loaded liposomes (model system) vs. free bioactive compounds. The concentration of each bioactive compound during digestion was determined by collecting aliquots at several time points and then analyzing by UHPLC-DAD. Overall, all the formulations (free and liposome-loaded bioactives) were highly stable during the simulated oral phase, as a consequence of the short residence time (2 min). On the other hand, significant differences among free and encapsulated bioactives were observed, confirming that the nature of the compound, carrier, and encapsulation efficiency impacted bioactive release.

During the gastric phase (0 to 2 h), alkaloids were resistant to acid hydrolysis, with the Theo-loaded liposomes showing fairly similar degradation as the Caf-loaded liposomes (Figure 5 A-B). At the end of the gastric phase, 100.9 ± 2.7 and 100.1 ± 3.0 % free Theo and Caf still remained, which was 1.37 and 1.26-fold higher than Theo, and Caf-loaded liposomes, respectively (Figure 5 A-B). Regarding catechins, free C and EC were also highly stable at low pH, therefore, their concentrations were only reduced by 1.5 and 0.8%, respectively (Figure 5 C-D). In comparison, when C- and EC-loaded liposomes formulations were exposed to gastric phase, the lowest concentrations were reached being equal to 25.6 ± 1.6 and 35.3 ± 4.5 %, that is, 3.8– and 2.8– fold lower than the free bioactive, respectively. These findings suggest that lower amount detected could be as consequence of i) greater extent and/or transformation of catechins, ii) good performance of the delivery system to protect the active ingredient from simulated gastric environment or iii) the swelling effect of liposome allowing greater incorporation of bioactive into the membranes.

Free Theo and Caf exposed to intestinal digestion (2 to 8 h) were still stable, reducing their concentration only by 20.6 and 24.9 %, respectively. These results agree with Mogi et al., (2012)

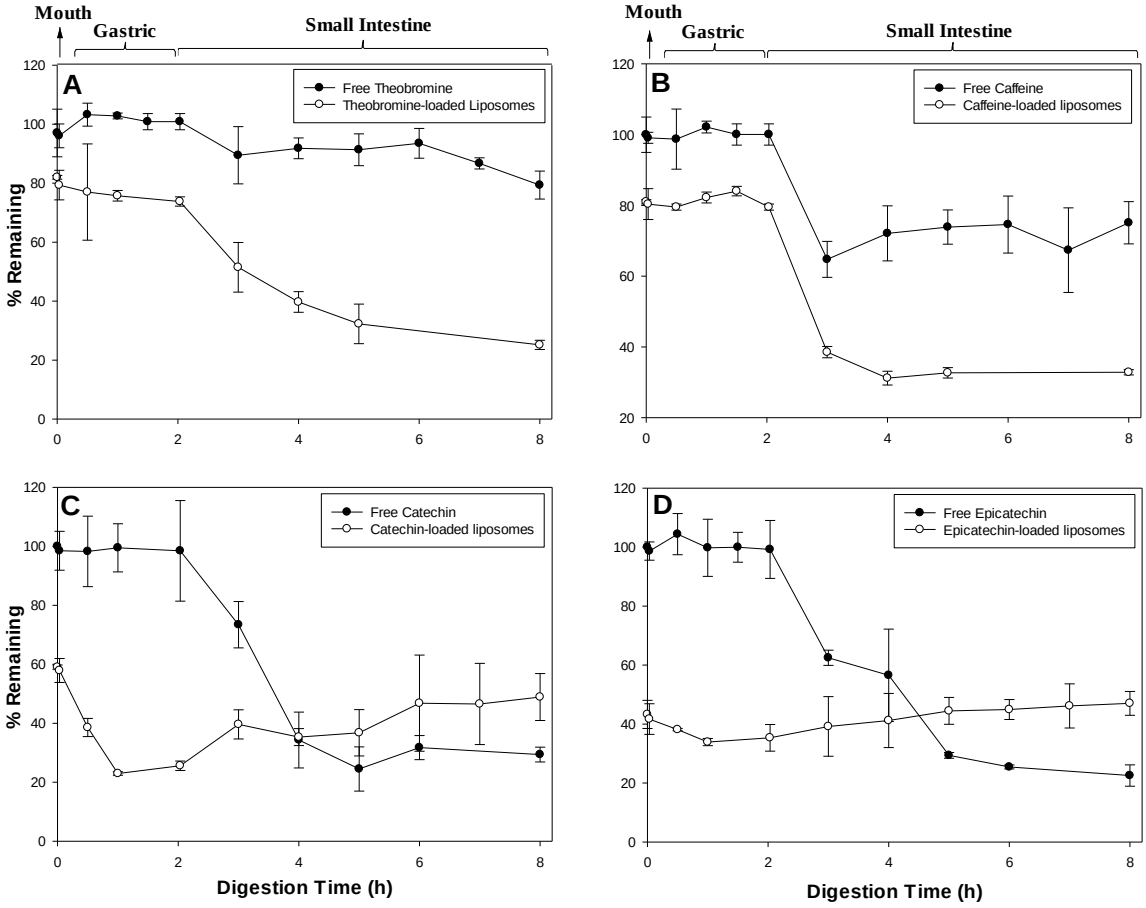

Figure 5. Stability profile under simulated *in vitro* conditions of free and liposome-loaded (A) theobromine, (B) caffeine, (C) catechin, and (D) epicatechin.

who reported that plasma bioavailability was approximately 80% for caffeine up to 24 h after dosing. Nevertheless, Theo- and Caf-loaded liposomes dramatically decreased to 25.2 ± 1.6 and 32.8 ± 0.7 % (Figure 5 A-B). As the only difference between the aqueous system and the liposome formulation is the phospholipid membrane, we hypothesized that the degradation of alkaloid-loaded liposomes appears to be directly correlated not only to the hydrolysis of lipids, triggered by pancreatic excretions, especially the phospholipase A2 and bile salts, but also by the poor EE of theobromine (0.03%) and caffeine (0.04%) rather than pH. These results might indicate that the

delivery system for alkaloids deserves further research, possibly by the addition of a coating layer of liposomes, and better formulation, leading to better stability in the gastrointestinal system.

The main loss of free catechins was due to intestinal phase degradation, with reductions of ca. 70.6 and 77.5 % for C and EC. Hence, the duodenal losses were about 70.2 and 77.3 % higher than the gastric phase losses, respectively (Figure 5 C-D). Therefore, the high stability under stomach conditions for catechin is comparable to previously reported results *in vitro* (Bouayed et al., 2012; Neilson & Ferruzzi, 2011). Moreover, epimerization of (+)-catechin → (–)-epicatechin, and (–)-epicatechin → (+)-catechin was also detected. According to the literature, degradation of catechins under digestive conditions appears to be directly correlated to pH rather than to digestive enzyme activity. For instance, Bouayed et al., (2012) evaluated the content of epicatechin from several apples varieties and reported losses during gastric digestion of 19.8 – 69.8 % and complete degradation in the small intestinal phase.

On the other hand, Figure 5 demonstrates a typical prolonged and sustained drug-release profile for C- and EC-loaded liposomes. As expected, as soon as the carrier comes into contact with the intestinal digestion medium, an initial burst release was observed, which could be related to the release of the active ingredient adsorbed on the lipid surface or encapsulated into the core of the liposomes. Thereafter, the release rate became slow and reached equilibrium during the transit time. As can be observed in Figure 5 C-D, and compared to free catechins, a peak concentration for C- and EC-loaded liposomes became apparent after ca. 4 and 4.6 h of digestion, respectively. Indeed, higher bioaccessibilities for C (57.7 ± 3.3 %)- and EC (49.2 ± 2.3 %)-loaded liposomes were 2.0 and 2.2- fold higher than free C and EC (Table 1), respectively. Overall, our data suggest that the formulation containing catechin-loaded liposomes improved its bioaccessibility and may lead to a higher bioavailability and intestinal uptake.

7.3.7.4. Comparison of the simulated in vitro digestion of cocoa extract-loaded liposomes vs. free cocoa extract. Cocoa extract is mainly composed of theobromine, caffeine, catechin, epicatechin, and procyanidins with a degree of polymerization up to 14 (Toro-Uribe, Montero, et al., 2018). In this study, the primary focus was on the *in vitro* digestion of the alkaloids and catechins present in this extract. To do so, the cocoa extract was dissolved in an aqueous system (free) as well as loaded into liposomes.

The alkaloids from the free cocoa extract were highly stable within the mouth and gastric phases. In this case, caffeine was more stable than theobromine during all three phases assayed (Figure 6 A-B). For example, the theobromine present in the free cocoa extract was 1.45 and 2.98 -fold lower than free theobromine alone in the gastric and small intestine phases, respectively. Similarly, caffeine from free cocoa extract was 1.24-fold lower than free caffeine alone in the gastric phase, and quite similar during the intestine phase, being around 75.1 and 83.5 % for caffeine from free cocoa extract and from free caffeine, respectively. On the other hand, the alkaloids from the cocoa extract-loaded liposomes had worse performance for theobromine and slightly better for caffeine. Bioaccessibility for caffeine (83.5 to 31.5 %) and theobromine (26.6 to 8.4 %) from free cocoa extract was also higher than the values obtained for these compounds in the cocoa extract-loaded liposomes (Table 1). These findings rule out the considerable decomposition of theobromine, thus formation of unknown compounds or the conversion of the latter into caffeine. In addition, the highest magnitude reported for free cocoa extract alkaloids could be explained as a result of the high dose assayed together with the effect of the food matrix which could impact their bioaccessibility and absorption (Neilson & Ferruzzi, 2011).

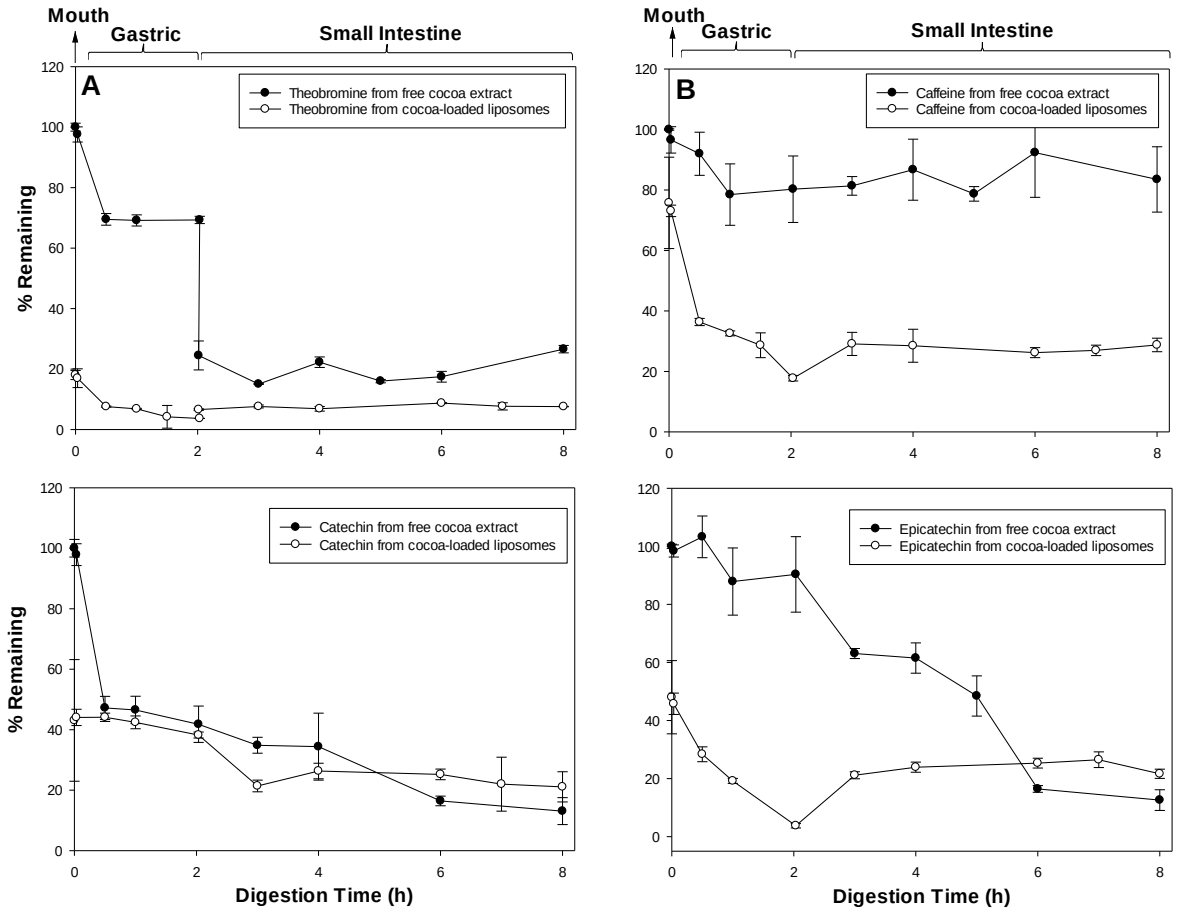

Figure 6. Concentration remaining under simulated *in vitro* digestion of (A) theobromine, (B) caffeine, (C) catechin, and (D) epicatechin from free extract and cocoa extract-loaded liposomes.

After the cocoa extract-loaded liposomes were exposed to the intestinal phase, an increasing concentration and then a sustained release profile of their bioactive compounds were detected (Figure 6 C-D). Indeed, the peak concentration of C and EC from cocoa extract-loaded liposomes become greater than those present on the free cocoa extract at about 5 and 6 h, respectively, which could be explained by lysis of the liposomes (Figure 6 C-D). Furthermore, the bioaccessibility of C and EC from cocoa extract-loaded liposomes was 2.3 and 2.2–fold higher than those from free cocoa extract (Table 1). Overall, this data highlights that liposomes may be a good carrier system to improve the *in vitro* controlled release of catechins.

7.3.7.5. DPPH· radical scavenging activity. DPPH radical scavenging activity was quantified in terms of the percentage inhibition after exposure to different phases of the gastrointestinal model (Table 2). As expected, Theo- and Caf-loaded liposomes were inefficient as antioxidants, with no significant changes due to the digestion

system (similar values for non-loaded liposomes). In addition, the initial DPPH scavenging activity of alkaloids can be attributed to the presence of other compounds in the lecithin such as tocopherols (soy lecithin composed of 16.5 ± 0.6, 102.0 ± 24.9, and 1.5 ± 0.3 $mg/kg_{Lecithin}$ of β, δ, and γ tocopherols, respectively).

Overall, both non-encapsulated catechins and encapsulated ones displayed a higher radical scavenging activity. Higher antioxidant activities in duodenal phase for free EC, and C were 1.27- and 1.40-fold higher for duodenal than gastric phases, respectively. These findings can be explained as a result of greater remaining concentration during the digestion and/or formation of autooxidation products from catechins (e.g., homodimers), which may contribute to higher antioxidant activity (Neilson & Ferruzzi, 2011). Catechin-loaded liposomes had the highest and lowest reduction of radical scavenging activity before digestion and during the gastric phase. The latter could be attributed to the lowest content of antioxidants released into the whole system (Figure 5C-D), the lower reaction between antioxidants and DPPH radical, and to the lower deprotonation at acidic pH. Significant differences were observed between cocoa formulations; in general, cocoa extract-loaded liposomes had higher antioxidant activity than the free extract ($p<0.05$), which could be attributed to the protection of liposomes against the degradation of polyphenols. Among the samples, cocoa extract had the highest antioxidant activity, which could be attributed to the presence of other non-polyphenolic compounds or highly solubilized micelles that impact the activity and rate of the DPPH radical scavenging; for instance, Chat et al., (2011)

Table 2. ABTS and DPPH antioxidant assays of tested compounds before, and after the gastric and duodenal phases of *in vitro* gastrointestinal conditions.

ABTS Assay	Free compounds			Compounds-loaded liposomes		
Compound	Initial	Gastric	Duodenal	Initial	Gastric	Duodenal
Theobromine	< LOQ	< LOQ	0.44 ± 0.00^{a}	0.05 ± 0.21^{a}	1.08 ± 0.01^{a}	0.91 ± 0.18^{a}
Caffeine	< LOQ	< LOQ	0.40 ± 0.00^{a}	0.05 ± 1.00^{a}	1.12 ± 0.01^{a}	0.94 ± 0.18^{a}
Catechin	0.90 ± 0.03^{c}	1.76 ± 0.08^{b}	7.06 ± 0.13^{b}	1.41 ± 6.01	4.78 ± 0.04^{b,c}	6.01 ± 0.15^{b}
Epicatechin	0.96 ± 0.02^{c}	2.27 ± 0.05^{b}	5.79 ± 0.13^{c}	1.48 ± 6.29	4.73 ± 0.05^{b}	6.29 ± 0.25^{b}
Cocoa extract	0.62 ± 0.03^{d}	3.70 ± 0.05^{c}	8.85 ± 0.07^{d}	1.66 ± 13.41	4.97 ± 0.02^{c}	13.41 ± 0.40^{c}

DPPH Assay						
Unloaded Liposome				14.88 ± 1.46^{a}	16.27 ± 1.45^{a}	32.95 ± 1.43^{a}
Theobromine	15.77 ± 0.73^{a}	15.77 ± 2.16^{a}	16.65 ± 1.63^{a}	16.94 ± 0.95^{a,b}	20.74 ± 0.44^{a}	39.18 ± 1.97^{b}
Caffeine	16.12 ± 1.18^{a}	16.68 ± 0.88^{a}	17.18 ± 0.83^{a}	17.41 ± 1.43^{b}	20.59 ± 0.94^{a}	41.18 ± 0.97^{b}
Catechin	> 100^{b}	66.01 ± 0.92^{b}	92.49 ± 0.35^{b}	> 100^{c}	43.95 ± 1.68^{b}	91.19 ± 0.54^{c}
Epicatechin	> 100^{b}	75.19 ± 0.77^{b,c}	95.24 ± 1.39^{b}	> 100^{c}	46.42 ± 1.86^{b}	92.96 ± 3.54^{c}
Cocoa extract	> 100^{b}	80.25 ± 10.63^{c}	81.54 ± 1.76^{c}	> 100^{c}	93.08 ± 3.04^{c}	> 100^{d}

For ABTS and DPPH data were expressed as µmol Trolox/ µmol sample, and scavenging activity in percentage (%), respectively. LOQ, lower limit of detection. Means within a column (comparison between same digestion phases for the different bioactive compounds) with different letter are significantly different by Tukey ($p < 0.05$). reported the solubilization capacity of various surfactant systems to scavenge radicals increasing following the order cationic > non-ionic > anionic.

In general, by increasing the pH of the surrounding medium, a maximum scavenging inhibition was reached. This behavior reflects that catechins are in deprotonated forms instead of neutral. Therefore, upon deprotonation, the radical scavenging capacity of the catechins increases because electron donation becomes much easier (Muzolf, Szymusiak, Gliszczynska-Swiglo, Rietjens, & Tyrakowska, 2008). These data also provide more insight into the mechanism (pH-sensitive liposome) underlying the ability for neutral pH and/or enzymatic action to affect the liposome membrane conformation, thus, favoring the release of the encapsulated compound from the inner membrane.

7.3.7.6. $ABTS^{\cdot+}$ radical cation analysis. Similarly, to DPPH data, alkaloids were inefficient for scavenging $ABTS^{\cdot+}$ free-radical cations. In fact, Brezová, Šlebodová, & Staško (2009) previously reported that caffeine is inert to $ABTS^{\cdot+}$ and $DPPH^{\cdot+}$ oxidants, but effective in the scavenging of $^{\bullet}OH$ radicals. According to our results, significant variations in ABTS radical scavenging activity were observed, which demonstrate the effect of pH on the radical scavenging capacities. Muzolf et al., (2008) verified the pH-dependent increase in the TEAC values, that is, upon deprotonation of catechin, for instance, C and EC had ABTS values between 0 - 3.5 mM Trolox per mM/sample for pH ranging of 0 – 9.5.

As can be seen in Table 2, the ability to scavenge $ABTS^{\cdot+}$ cation radicals for free compounds rose by increasing the digestion transit time. In fact, phenolic acids do not react with the $ABTS^{\cdot+}$ free-radical cation at low pH and exhibit the most pronounced increase of the antioxidant values at physiological pH. Regarding the small intestine, differences among samples with and without liposomes were observed which could be due to nature of the bioactive compound and/or the influence of carrier system. In general, high antioxidant activity was achieved even at lower concentration of residual bioactive compound, for instance, free C and EC were 29.4 and 22.5 bioaccessible at duodenal stage. A similar trend was also found by Wootton-Beard, Moran, & Ryan (2011) who reported that many polyphenols from several vegetable sources had a higher ABTS scavenging activity during the duodenal phase. Based on these results, it can be hypothesized that the highest ABTS radical activity was not only function of remaining compounds, but also suggested the presence of new products as a result of epimerization and oxidation of bioactive compounds.

Significant differences ($p < 0.05$) between free extract and cocoa extract-loaded liposomes were observed. As expected, the highest antioxidant activity was for cocoa extract-loaded liposomes.

This phenomenon can be attributed to a number of reasons: (a) the antioxidant activity can be masked by the interaction of free flavonoids with proteins, (b) adsorption of cocoa polyphenols into the lipid phase in different magnitude (c) charge of micelles and its role on the chemical behavior of the bioactive compounds that may affect their final antioxidant value, and (d) different rate of deprotonation of phenolic hydroxyl groups at alkaline pH. For instance, deprotonation of the 3'-OH group in the catechin (pKa = 4.6) (Jovanovic, Steenken, Tosic, Marjanovic, & Simic, 1994) can dissociate resulting in a mixture of neutral and anionic species, while other hydroxyls groups could be responsible for scavenging of free-radicals (e.g., 5- and 7-OH groups at A-ring, and 3-OH at C ring). It is worth to mention, the highest free radical scavenging for cocoa extract-loaded liposomes is comprehensive since the liposome system contribute at high amount of phospholipids, therefore, higher solubilized micelles. Thereby, solubilization of antioxidant compound within the Stern (hydrophilic), or Palisade (hydrophobic) layer of micelles together with electrostatic forces provides a more appropriate microenvironment to donate H atoms to reduce $ABTS^{\cdot+}$ into nonradical form easily (Wu & Wang, 2017).

In general, the present study showed that liposomes are capable of increasing the bioaccessibility of flavan-3-ols, which may be important for the development of nutraceutical-enriched functional foods. Our results highlight the importance of further studies (*in vitro* and *in vivo*) on the bioaccessibility, bioavailability and biological fate of polyphenols (e.g., polymeric catechins) in both aqueous solutions and incorporated into delivery systems, as well as evaluation of their releasing mechanism in real food systems.

7.4. CONCLUSIONS

Soybean lecithin liposomes were fabricated and optimized using food-grade solvents. All anionic liposomes displayed a particle size lower than 130 nm confirmed by TEM pictures.

Samples-loaded liposomes enhanced *in vitro* bioaccessibility than those in non-encapsulated form, but except for theobromine and caffeine. The latter could be as a consequence of lower encapsulation efficiency (< 0.5%). All liposome formulations showed sustained release, therefore, higher antioxidant activity at the end of digestion. Moreover, the mechanisms of physicochemical changes of liposomes before and after simulated gastrointestinal digestion was proposed.

7.5. ACKNOWLEDGEMENTS

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7.6. SUPPLEMENTARY INFORMATION

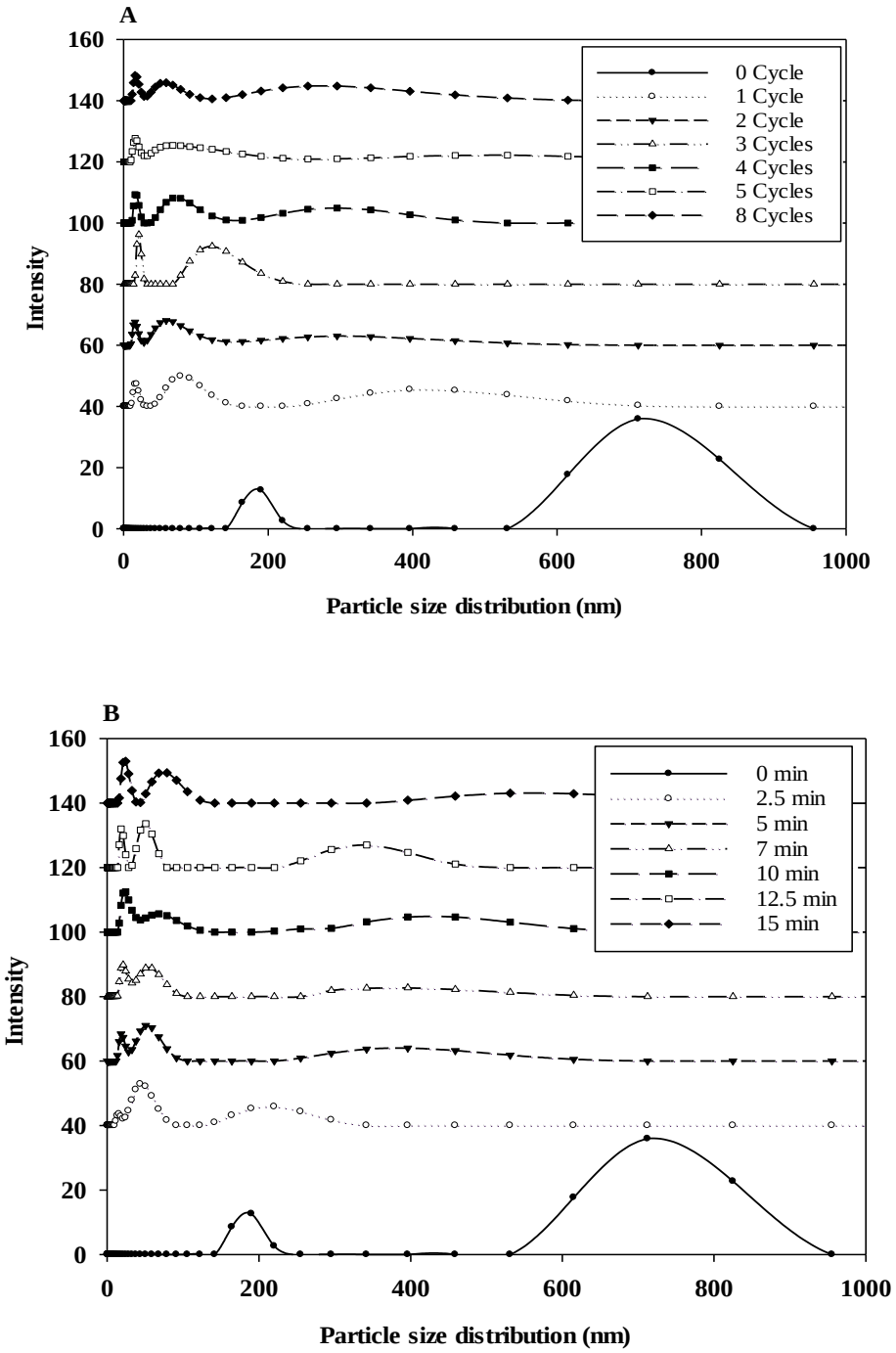

Figure S1. Particle size distribution of non-loaded liposomes for both (A) microfluidization, and (B) sonication technologies assayed at several passages and ultrasound time, respectively.

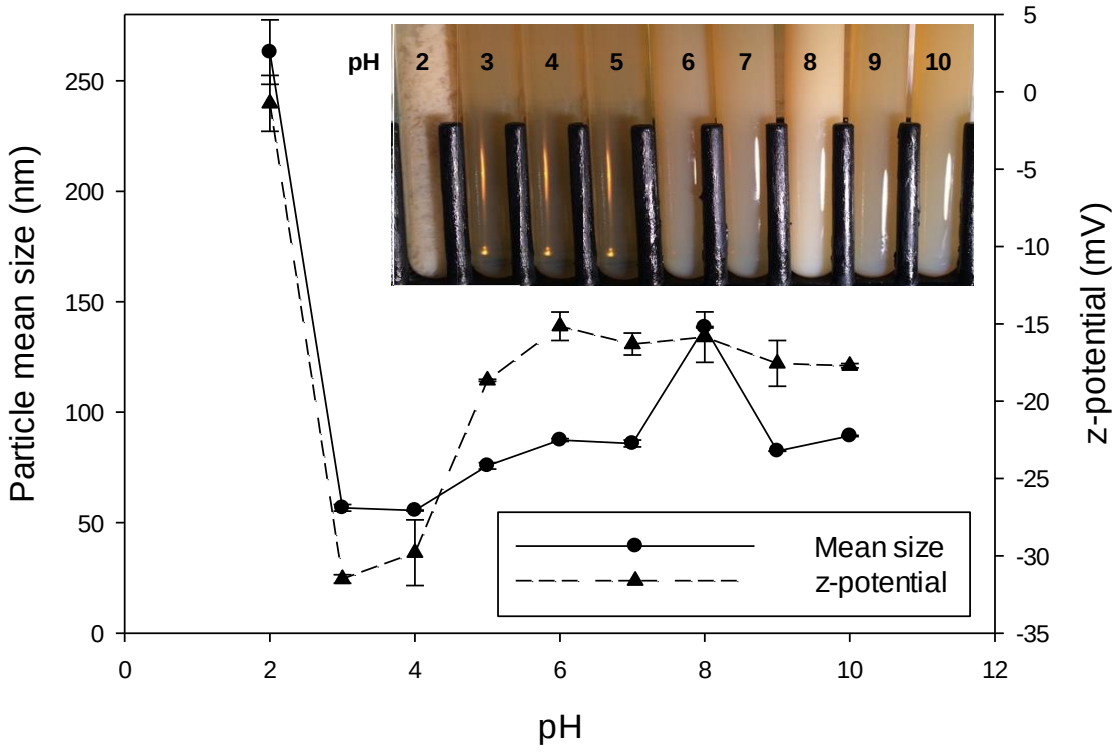

Figure S2. Influence of pH on the physical stability (mean particle size and ζ-potential) of soy liposomes.

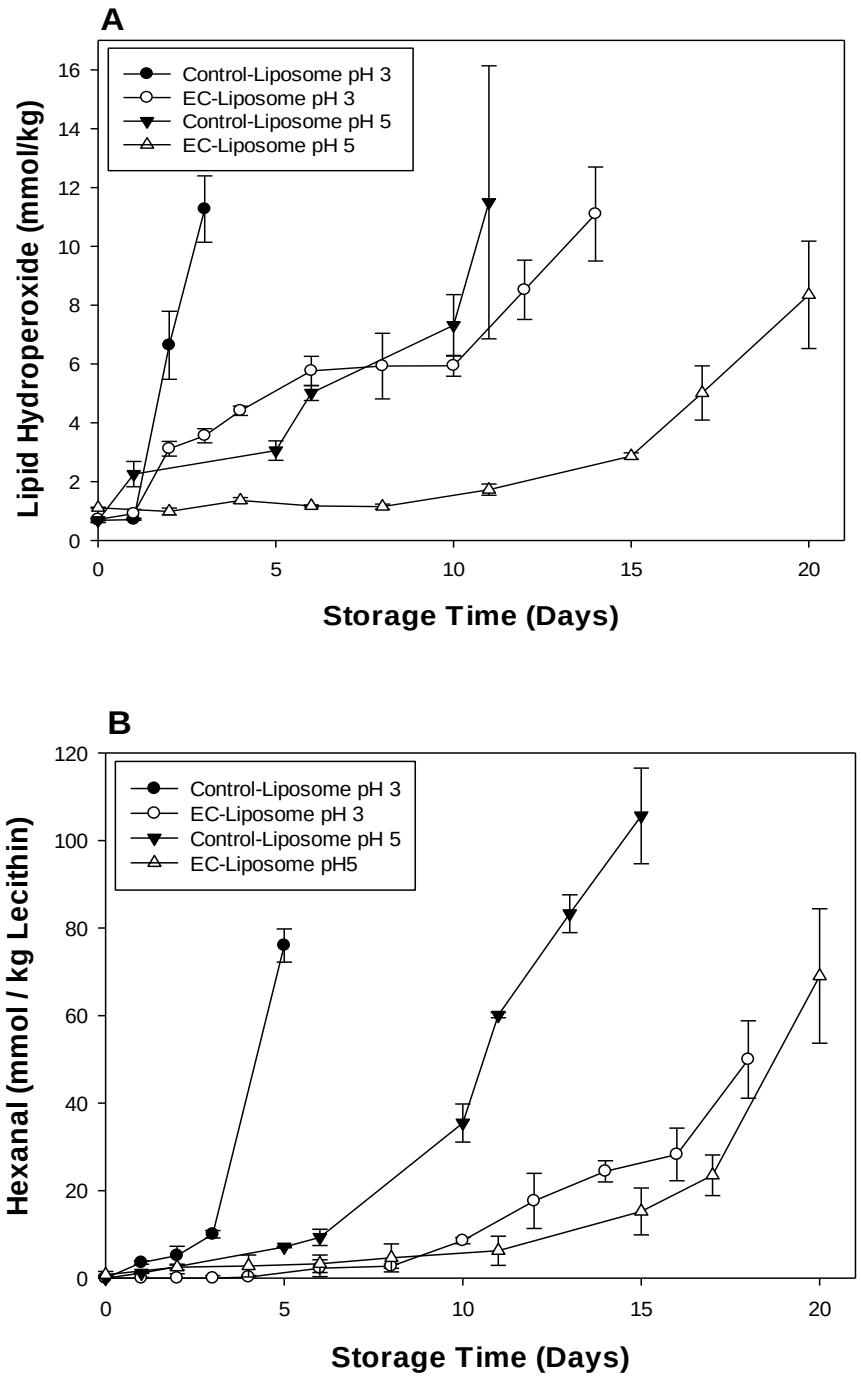

Figure S3. Oxidative stability of non-loaded and epicatechin-loaded (EC, 125 µM) liposomes determined by (A) lipid hydroperoxide formation and (B) hexanal formation.

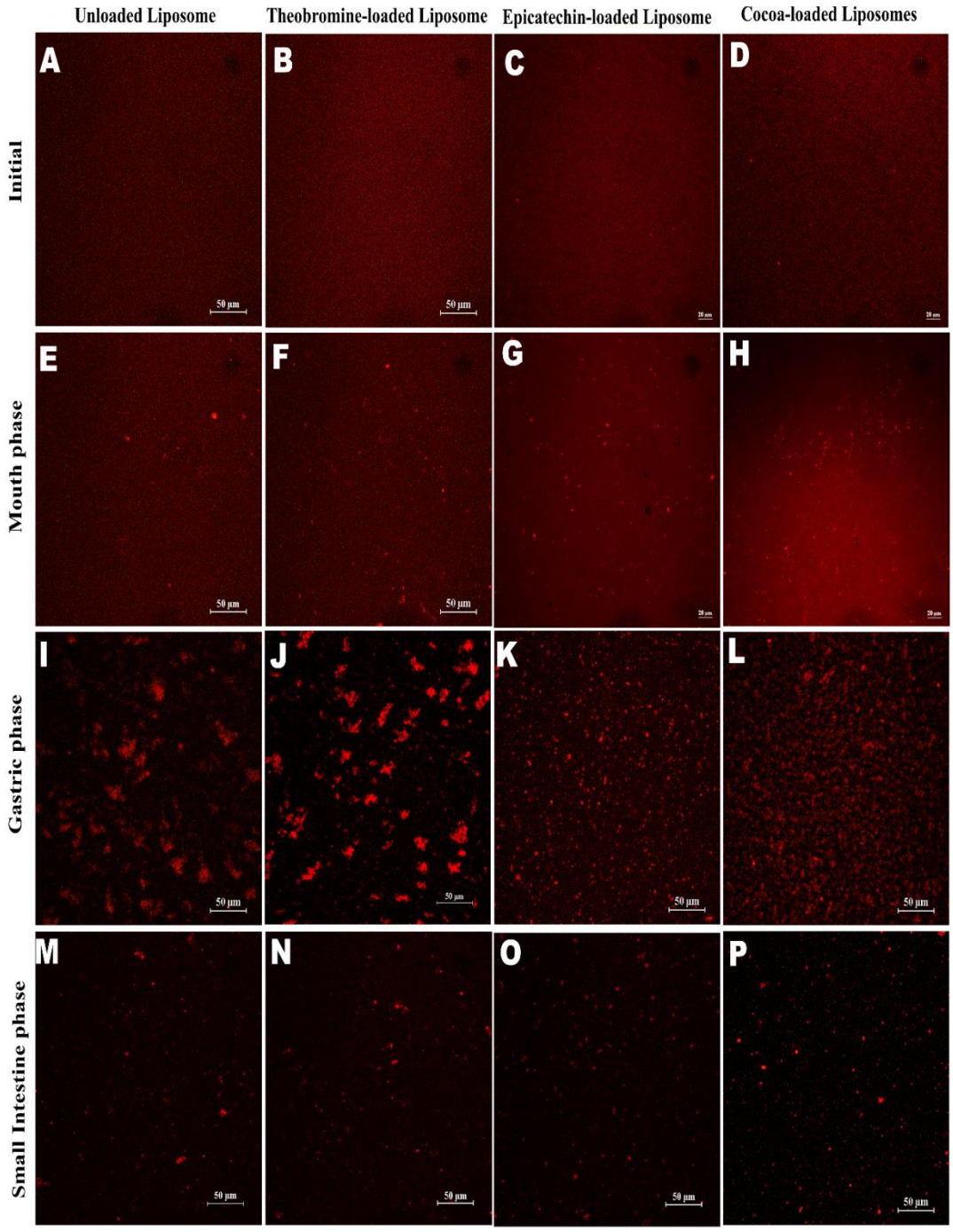

Figure S4. Confocal optical microscopy images of (A) non-loaded, (B) theobromine-, (C) epicatechin-, and (D) cocoa extract-loaded liposomes before and after simulated gastrointestinal digestion. Red color showed the location of the lipid phase.

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CHAPTER 7

INSIGHT OF STABILITY OF PROCYANIDINS IN FREE AND LIPOSOMAL FORM UNDER *IN VITRO* DIGESTION MODEL: STUDY OF BIOACCESSIBILITY, KINETIC RELEASE PROFILE, TRANSFORMATION AND ANTIOXIDANT ACTIVITY

ABSTRACT

Small unilamellar and multilamellar liposomes loaded with polymeric (epi)catechins up to pentamers together with a raw cocoa extract were produced. Bioaccessibility, kinetic release profile, and transformation under *in vitro* gastrointestinal conditions by UHPLC-DAD-QTOF-MS/MS were monitored. Results show that all the procyanidins underwent depolymerization and epimerization into small molecular oligomers and mainly to (epi)catechin subunits. Moreover, all the liposome formulations presented higher bioaccessibility and antioxidant activity than their respective counterparts in non-encapsulated form. Similar results were obtained with procyanidins from cocoa extract-loaded liposomes, where the time taken to attain a peak burst effect on liposomes after gastric phase was influenced by the polyphenol structure. Namely, the bioaccessibility of dimer, trimer, and tetramer fractions from cocoa-loaded liposomes were 4.5, 2.1, and 9.3-fold higher than those from the non-encapsulated cocoa extract. Indeed, the high release amount at the end of digestion together with the formation of low molecular weight compounds metabolites was responsible for their effectiveness to act as free radical scavenger, in particular, after the digestion process. Overall, the procyanidins release profile was dependent on their chemical structure and physicochemical interaction with the lipid carrier. These results

confirmed that liposomes are efficient carriers to stabilize and transport the procyanidins with the aim to enhance their bioaccessibility in a controlled release rate.

Keywords: Cocoa Procyanidins; *in vitro* digestion; liposomes; bioaccessibility

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8.1. INTRODUCTION

Procyanidins (PCs) are found in cocoa-derived products and mainly consist of polymeric flavan-3-ol subunits linked through $C_4 \rightarrow C_8$ and $C_4 \rightarrow C_6$ bonds, which are referred as B–type procyanidins (Serra et al., 2010). Occasionally, A–type ($C_2 \rightarrow O \rightarrow C_7$) polymers can also be found (Domínguez-Rodríguez, Marina, & Plaza, 2017). Flavan-3-ol monomers and procyanidins oligomers from dimers to tetradecamers constitute about 60% of total cocoa polyphenol content (Jalil & Ismail, 2008; Toro-Uribe, López-Giraldo, & Decker, 2018).

Consumption of procyanidins has been related to improved health status because of their antioxidant activity and chelating properties, as well as their role in the prevention of cardiovascular diseases or cancer, among other diseases (Aron & Kennedy, 2008; Jeong & Kong, 2004). The daily intake of cocoa is estimated between 53.6 and 450.7 mg per person for U.S. and Spanish population, respectively (Serrano, Puupponen-Pimiä, Dauer, Aura, & Saura-Calixto, 2009). In general, polyphenols are consumed through food matrix and therefore need to be released from ingested food as a result of the action of digestive enzymes and physical-mechanical actions of peristaltic movements (Epriliati & Ginjom, 2012). The amount that travels along the gastrointestinal tract and reaches the intestine is referred to as bioaccessible. The bioaccessible

fraction can exert its action through systemic circulation upon absorption or thanks to the action of the gut microbiota (Fogliano et al., 2011). After that, the absorbed compounds appear in plasma and urine primarily as glucuronidated, methylated and sulphated metabolites (Serra et al., 2010). The importance of transforming flavonoids into compounds with higher bioactive effect or smaller compounds has been emphasized by Abd El Mohsen et al., (2002) who found that glucuronide and 3-*O*-methylglucuronide metabolites from epicatechin can pass through the blood-brain barrier and act at a cerebral level.

The bioavailability of flavonoids is a complex issue due to their bio-chemical features that largely depend on its molecular weight and chemical structure (e.g., degree of polymerization, glycosylation, conjugation, esterification, and stereochemistry configuration) (Fogliano et al., 2011; Motilva et al., 2016; Serra et al., 2010; Zhu et al., 2002), as well as on the composition of the food matrix (e.g., fat, protein, carbohydrates content) (Ortega, Reguant, Romero, MaciÃ, & Motilva, 2009; Serra et al., 2010). Several reports agreed in catechin being absorbed in human and animals cell organs; for example, Zhu et al.(Zhu et al., 2002) reported that epicatechin and catechin in *in vitro* or *in vivo* digestion systems were highly stable in the gastric phase and were present as conjugated analytes in plasma (Motilva et al., 2016; Ortega et al., 2009; Serra et al., 2009). Moreover, Fraga, et al., (1987) found that (+)-catechin was 17, 50, 50, 67, 167, and 300-fold more efficient than caffeic acid, chlorogenic acid, apigenin, quercetin, kaempferol, and quercitrin, respectively, in an *in vivo* oxidative stress model. However, information regarding oligomeric and polymeric procyanidins is not completely clear. For instance, several studies indicated that absorption of PCs is limited by their degree of polymerization (DP) and only oligomers with DP ≤ 3 can be absorbed by the epithelial cell. In fact, PCs were 10- to 100-fold less absorbed than their monomeric constituents alone (Aron & Kennedy, 2008). This finding is

in agreement with the “rule of 5”, that is, poor absorption or permeation is more likely when the molecular weight is greater than 500 and more than 5 H-bond donors are present (Lipinski, Lombardo, Dominy, & Feeney, 2012). Other studies reported that procyanidins were not hydrolyzed into bioavailable monomers (Donovan et al., 2007), and that conjugated or methylated forms of procyanidins were not evident (Appeldoorn, Vincken, Aura, Hollman, & Gruppen, 2009). On the contrary, Spencer et al., (2000) investigated the *in vitro* pre-absorption of procyanidins from trimers to hexamers and found that there was a time-dependent decomposition (up to 80%) of all oligomers tested forming a mixture of monomeric compounds. More recently, Mendoza-Wilson et al., (2016) reported absorption of epicatechin (0.17%), dimers (0.13%), trimers (0.1%), tetramers (0.05%) and pentamers (0.04%) on IEC-18 cell line.

These contrasting results could be due to different reasons; for example, low stability of PCs under food processing and storage conditions (Fang & Bhandari, 2010), pH-dependence (Watson, Preedy, & Zibadi, 2012), low concentration on food matrix and marked loss during food processing, and interaction of polyphenols with other food constituents, which impact their absorption magnitude (Bouayed, Deußer, Hoffmann, & Bohn, 2012). Furthermore, the bioavailability of polyphenols in the cell culture medium is much worse than in organic solvents or water (L. Chen, Cao, & Xiao, 2018), which leads to poor lipid solubility and limited ability to modulate pro-oxidant and pro-inflammatory pathways, thus reducing their biological activity (L. Chen et al., 2018). A better understanding of digestive stability, bioaccessibility, antioxidant status, absorption, and tissue distribution of long-chain procyanidins is still essential to explain the large discrepancies about the bioavailability of oligomeric forms observed from previous studies. Indeed, works related with PCs should be treated carefully because their potential activity *in vivo* is conditioned by their poor bioavailability, but they can have an enhanced biological action thanks

to microbiota metabolization (Watson et al., 2012). Additional problems limiting these type of studies are the lack of standards, together with the extraction, isolation and analytical challenges for natural polymers.

In this sense, entrapping bioactive compounds into a delivery system could help to overcome the above-mentioned limitations and may provide food formulators and manufacturers with protective mechanisms that could maintain the active molecular form until the time of consumption and delivering to the target site. Several delivery systems, for example, nanoemulsions (Aditya et al., 2015), W/O/W emulsions (X. Chen et al., 2018), spray drying (Ferreira, Rocha, & Coelho, 2007), coated (Altin, Gültekin-Özgüven, & Ozcelik, 2018) and uncoated liposomes (Toro-Uribe et al., 2018), and electrospraying (Paximada, Echegoyen, Koutinas, Mandala, & Lagaron, 2017) have been assayed. Many works highlighted that substantially improved bioavailability of active ingredients could be obtained by reducing the drop size to 100 (or less) – 1000 nm ranges (Acosta, 2009). Since an effective delivery system depends on the characteristics of active ingredient, liposomes-based systems can be produced in a range of sizes (from nano to micrometer sizes) and are suitable to encapsulate and deliver both lipophilic and polar compounds.

In previous studies, we established and optimized the methodology to fractionate procyanidins from cocoa up to decamers with a high degree of purity and to encapsulate cocoa procyanidins into liposomes (Toro-Uribe et al., 2018), which led to high efficiency of encapsulation (> 90%), greater physiochemical stability and antioxidant activity by increasing the degree of polymerization. As a further step, the aim of this study was to evaluate the *in vitro* bioaccessibility, kinetic release profile, and transformation of both isolated procyanidins and crude cocoa extract encapsulated into liposomes and to compare the obtained results to their corresponding free non-encapsulated counterparts.

8.2. MATERIALS AND METHODS

8.2.1. Samples and reagents. All chemicals used were of analytical grade with no further purification. L-α-Phosphatidylcholine from soybean (Type IV-S ≥ 30% PC), (+)- catechin hydrate, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), triton X-100, trolox ((±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), potassium persulfate, potassium chloride, potassium dihydrogen phosphate, sodium bicarbonate, magnesium chloride hexahydrate, ammonium carbonate, calcium chloride, sodium acetate trihydrate, hydrochloric acid, glacial acetic acid, ethanol, methanol, sodium hydroxide, porcine pepsin, porcine pancreatic lipase, and bile salts were obtained from Sigma Aldrich (Sigma–Aldrich, Steinheim, Germany). (–)-Epicatechin (purity ≥ 99%) were purchased from ChromaDex Inc. (Irvine, CA, USA). Procyanidin dimer B2 ((-)-epicatechin (4β-8)-(-)-epicatechin) and trimer C1 ((-)-epicatechin (4β-8)-(-)-epicatechin (4β-8)-(-)-epicatechin) were obtained from Cymit quimica (Barcelona, Spain). Milli-Q water (Millipore system, Billerica, MA, USA) and deionized water were used for the preparation of all solutions.

A polyphenols-enriched crude cocoa extract was recovered from unfermented cocoa beans with low polyphenol oxidase activity according to our previous published procedure (Toro-Uribe et al., 2018). Isolated procyanidin consisted of dimer, trimer, tetramer, and pentamer fractions obtained by a semi-preparative HPLC instrument employing a Develosil Diol column (250 × 10 mm, 5 μm, Nomura Chemical Co., Seto, Japan) connected to guard column Develosil Diol (10 × 8 mm, 5 μm, Nomura Chemical Co., Seto, Japan) as recently published.

Liposome preparation

Liposomes were prepared according to Toro-Uribe et al., (2018) Briefly, the bioactive compounds were dissolved in ethanol (2.5% v/v) and then added to sodium acetate-acetic acid buffer (0.1 M; pH 5.0 ± 0.1). After that, soybean-lecithin was added for a final concentration of 5.0 % (w/v). The sample was stirred at 25 ºC until complete dissolution, followed by homogenization with a high shear blender (T10 Ultra-Turrax, IKA, Staufen, Germany) at 20,000 rpm for 2 min. The multilamellar liposomes were produced by sonication (model 505, Fischer Scientific, USA) for 7 min in an ice bath (amplitude 75 %, 10 s on/off pulses). The final liposomal solution was filtered through 0.45 µm hydrophilic PTFE filter (Millipore, Milford, MA, USA). Liposome without active ingredient (control) was also prepared.

All the bioactive compounds (both encapsulated and non-encapsulated) were tested at a concentration of 1000 µM. For the cocoa extract, the concentrations of the catechins and procyanidins were calculated from the UHPLC-DAD profiles. For example, in the cocoa extract, the percentage of each polyphenol and procyanidin was determined, and then the molecular weight of each compound was used to calculate its molarity, and the sum of all the compounds was used as the total molarity of the total catechins and procyanidins.

8.2.2. Microstructure and morphology analysis. Transmission electron microscopy (TEM) was used to examine the morphology and to confirm the mean particle size of the samples. To do so, fresh liposomes (50 µl) were absorbed onto Formvar carbon-coated 200 mesh thick grid (Ted Pella Inc., USA) for fixation for 2 min. Then, the grid was stained with uranyl acetate aqueous solution (2 %) for 1 min and air-dried at room temperature, and the excess of liquid was removed with filter paper. Grid-sample was examined using a transmission electron microscope (JEOL JEM 1010, Tokyo, Japan) operating at an acceleration voltage of 100 keV, equipped with a Gatan ES1000W digital camera. The images were processed using Digital Micrograph software.

8.2.3. Encapsulation efficiency of liposomes. To measure the encapsulation efficiency (EE), the bioactive present in aqueous phase was separated from the liposome samples. The samples were transferred to Optiseal bell-top ultracentrifuge tubes (Beckman Coulter, USA) and ultracentrifuged (Beckman L-70, 70 Ti rotor, Beckman Instruments Inc, CA, USA) at 50,000 rpm, 4 ºC for 2 h according to the procedure of Toro-Uribe et al., (2018) Then, the supernatant (200 µL) was carefully collected and analyzed by UHPLC-DAD as described later. Therefore, the EE was expressed in weight percent (%) and calculated using the following equation:

$$EE\ (\%) = \frac{C_i - C_t}{C_i} * \ 100$$

where C_t is the sample concentration in the supernatant and C_i is the total concentration added to the system.

8.2.4. In vitro digestion stability of liposomes. Two groups of samples were subjected to a digestion study: fractions dissolved in an aqueous system and the same fractions loaded into liposomes. Empty liposomes and aqueous system were also used as control samples. The liquid formulations were passed through simulated gastric and small intestine phases according to the standardized static gastrointestinal method proposed by Minekus et al., (2014).

8.2.4.1. Gastric phase. Samples (10 mL) were mixed with 7.5 mL of 1.25-fold concentrated electrolyte solution. Then, 1.6 mL of ca. 2500 UmL-1 porcine pepsin stock solution and 5.0 µL of 0.3 M CaCl2 were added, followed by verification of pH 3 with 1 M HCl. Finally, the volume was made up to 20 mL with deionized water. The electrolyte solution (1.25-fold concentrated) consisted of 6.9 mM KCl, 0.9 mM KH2PO4, 25mM NaHCO3, 47.2 mM NaCl, 0.1 mM MgCl2(H2O)6, 0.5 mM (NH4)2CO3, and 15.6 HCl mM. Finally, the headspace of the sample was flushed with nitrogen and incubated in a rotary shaker (Infors HT Multitron Standard, Switzerland) at 100 rpm, for 2 h at 37 °C.

8.2.4.2. Small intestine phase After the gastric phase, the sample was exposed to the simulated small intestine digestion. The sample (15 mL) was mixed with 8.25 mL of intestinal electrolyte solution (1.25-fold concentrated). Then, 3.75 mL of 800 U/mL pancreatin solution, 1.87 mL fresh bile and 30 µL of 0.3 M $CaCl_2$ were added. Then, the pH was adjusted to 7 with 1 M NaOH, and the volume was made up to 30 mL with deionized water. The intestinal electrolyte solution consisted of 6.8 mM KCl, 0.8 mM KH_2PO_4, 85mM $NaHCO_3$, 38.4 mM NaCl, 0.33 mM $MgCl_2(H_2O)_6$, and 8.4 mM HCl. Finally, the headspace of the sample was flushed with nitrogen and incubated in a rotary shaker (Infors HT Multitron Standard, Switzerland) at 100 rpm, for 6 h at 37°C.

8.2.5. Chromatographic analysis. Chromatographic separation was performed in an UHPLC system Agilent (1290 infinity series II, Agilent Tech., Germany) consisting of a binary pump delivery system, a solvent degasser, a thermostated column compartment, and a diode-array detector (high UV sensitive 60 mm path-length flow cell). Separation was carried out on a C_{18} reverse phase Agilent Zorbax Eclipse Plus column (50 × 2.1 mm, 1.8 µm) connected to Agilent Zorbax SB-C_8 guard column (5 × 2.1 mm, 1.8 µm) at a column temperature of 55 ºC. The mobile

phase consisted of water (0.01% formic acid, solvent A) and acetonitrile (0.01% formic acid, solvent B) at a flow rate of 0.7 mL min^{-1}. The linear gradient was as follows: 0 min, 0% B; 3.9 min, 1.5% B; 4.0 min, 4% B; 11.0 min, 10 %B, 14.0 min, 35% B; 14.2 min, 100% B; 16.5 min, 100% B; 17.0 min, 0% B; 23 min, 0% B. Injection of blank samples between runs was also carried out. UV detection was set at 280 nm. System control and data analysis were processed using OpenLab CDS software (Agilent ChemStation). All the samples were injected in triplicate. The content of (+)-catechin, (–)-epicatechin, dimer B2, trimer C1 were calculated from commercial standards ($r^2 \geq 0.99$, standard curve); moreover, the calibration curve ($r^2 \geq 0.99$) for tetramers and pentamers were calculated from isolated procyanidin fractions with a purity higher than 90%.

Mass spectrometry characterization was performed on an Agilent UHPLC (1290 Infinity series I, Agilent Tech. Santa Clara, CA, USA) connected to a QTOF'MS detector (model 6540, Agilent). The separation of the samples was performed using the above-mentioned method. Data were collected in negative ESI mode and the QTOF-MS instrument was operated in full scan mode from *m/z* 25-3200 using the following settings: capillary voltage, 4000 V; acquisition rate, 2 spectra per second; nebulizer pressure, 40 psi; drying gas, 10 L/min; temperature, 350 ºC. During the analysis, two reference masses ($C_5H_4N_4$ and $C_{18}H_{19}O_6N_3P_3F_{24}$) were used. Thus, *m/z* 119.0363 and *m/z* 966.0007 for negative mode were employed. These masses were continuously infused into the system to allow constant mass correction. Data treatment was performed using Mass-Hunter Qualitative Analysis (Agilent, B.07.00). MS characterization features were analyzed using commercial standards reagents, extraction ion compound tool, and exact mass databases searched against the METLIN and HMBD databases.

8.2.6. Kinetic release profile. Analysis of remaining compounds during the simulated gastrointestinal conditions was performed. To do so, 200 µL aliquots were taken at every 30 min

during simulated gastric digestion, and every 1 h during duodenum simulated digestion. All the aliquots were immediately snap frozen in liquid nitrogen and subsequently stored at -86°C (ULT Ultralow freezer, Haier, Quingdao, China) until further analysis. For chromatography analysis, the samples were properly diluted and adjusted to pH ~5. After that, the samples were centrifuged at 13400 rpm, for 30 min at 4°C (Heraeus Fresco 21, Thermo Scientific, Hamburg, Germany). The supernatant was carefully collected (considered as the total amount of compound remaining) and analyzed by UHPLC-DAD as described above. The *in vitro* release profile was plotted as a function of time as follows:

$$Remaining\ (\%) = \frac{C_t}{C_i} * 100$$

where C_t is the sample concentration for each time point, and C_i is the initial concentration loaded.

8.2.7. Measurement of bioaccessibility of liposomes. At the end of *in vitro* digestion, 200 µL of the digested compounds were taken and used to measure their bioaccessibility. In the case of liposomes, to completely enhance the breakage of the lipid membrane, the sample was frozen in liquid N_2 and thawed 5 times. Then, the sample was diluted with a solution containing 1% Triton X-100, vortexed and centrifuged (Heraeus Fresco 21, Thermo Scientific, Hamburg, Germany) at 13400 rpm, 4°C, for 30 min. The supernatant was carefully collected and filtered through a 0.45 µm hydrophilic Durapore PVDF membrane (Millipore, USA). The collected sample was considered as the "micelle" fraction, in which the bioactive compounds were solubilized. The amount was quantified by UHPLC-DAD, and the bioaccessibility was calculated as follows:

$$Bioaccessibility\ (\%) = \frac{C_{Digesta}}{C_i} * 100$$

where $C_{Digesta}$ and C_i are the concentrations of tested samples in the mixed micelle phase and the initial concentration loaded, respectively.

8.2.8. Assessment of procyanidins transformation. Samples before and after the *in vitro* digestion process at specific time points were analyzed by UHPLC-QTOF-MS as describe above. For each sample, extracted ion chromatogram (EIC) with *m/z* 289.0727, 577.1358, 865.1993, 1153.2624, and 1442.3301 for monomers, dimers, trimers, tetramers, and pentamers, respectively, were studied. Data were expressed as percentage (%) and treated as follows:

$$Contribution_i\ (\%) = \frac{AUC_i - AUC_{Blank}}{AUC_T - AUC_{Blank}} * k$$

where AUC is the area under the curve of the samples and blank sample, *i* is the individual isomeric procyanidin selected by EIC, T is the total sum of the areas under the curve, and *k* is the sample dilution factor during the digestion process.

8.2.9. Free radical scavenging activity assays. DPPH and ABTS antioxidant activities of the samples before and after the gastric and duodenal phases of digestion were analyzed by measuring their abilities to scavenge the $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ free radicals. DPPH assay was measured according to the procedure described by Brand-Williams, Cuvelier, & Berset (1995). A daily-working DPPH solution (745 µL of 68.5 µM) was mixed with 100 µL of sample. After 1h of reaction, 300 µL of sample was taken and placed in 96 well microplates to measure their total absorbance. The decrease in absorbance was determined at 517 nm using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) controlled by Gen5 software (Gen5 v. 2.04 BioTek Inst. Inc). Methanol and $DPPH^{\bullet}$ solution without test samples were used as blank and control solutions, respectively. Results were expressed as % radical scavenging and calculated as follows:

$$DPPH\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} * 100$$

The ability to scavenge the $ABTS^{\bullet+}$ free-cation radicals was measured to Re et al., (1999) The ABTS (7 mM in 5 mM phosphate buffered saline pH 7.4) was mixed with 2.5 mM potassium persulfate, and this mixture was allowed to stand in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffered (pH 7.4) to an absorbance of 0.700 ± 0.002 at 734 nm, to form the test reagent. Test sample (10 µL) was mixed with 300 µL of test reagent and placed in 96 well microplates. The reaction mixture was incubated at room temperature for 45 min, and the absorbance at 734 nm using a microplate reader (Synergy HT, BioTek Instruments, Winooski, VT, USA) controlled by Gen5 software (Gen5 v. 2.04 BioTek Inst. Inc). Appropriate solvent blanks were run in each assay. A Trolox calibration curve (0.48 – 125 µM; r^2 = 0.99) was used as a reference standard, and the results were expressed as µmol Trolox equivalents per µmol of sample.

8.2.10. Statistical Analysis. All results were expressed as mean ± standard deviation from at least three independent experiments. Statistical analysis was done using GraphPad Prism V. 6.0 (GraphPad Soft. Inc., La Jolla, California, USA). The significance of variability between results was determined by one-way ANOVA, and individual differences between groups were tested by Tukey´s post-hoc test at 5% level of significance using IBM SPSS Statistics version 25 (IBM Corporation, New York).

8.3. RESULTS

8.3.1. Liposome physicochemical properties. As mentioned, this work aims to study an improvement on the bioaccessibility of procyanidins under simulated gastrointestinal conditions. To do so, isolated procyanidins from dimers to pentamers, together with a

procyanidin-rich crude cocoa extract were incorporated into a liposome carrier. In a recently published work, the physicochemical characteristics of the procyanidins-loaded liposomes (Toro-Uribe et al., 2018) analyzed by dynamic light scattering showed that dimer- to pentamer-loaded liposomes had particle size distribution ranging from 60-84 nm and ζ-potential between -21 to -23 mV; moreover, control- and cocoa extract-loaded liposomes particle size distribution and ζ-potential were 75.2 ± 1.5 and 87.3 ± 0.8 nm, and -18.7 ± 0.1 and 22.4 ± 2.1 mV, respectively.

Based on literature, it is expected that samples loaded into liposomes show better performance than those in non-encapsulated form. Indeed, particle sizes below 500 nm increased the uptake and absorption of the encapsulated target (Acosta, 2009). To confirm the particle sizes obtained from dynamic light scattering, transmission electron microscopy was also used. As can been seen in Figure 1, all the samples displayed particle sizes lower than 150 nm, together with the formation of clusters through the contact of interlayer lipid bilayers. Differences among the encapsulated bioactive agents were also observed. For instance, control liposomes (Fig. 1A) are formed by small unilamellar vesicles with highly organized structures while procyanidins and cocoa extract did not. As can be seen, the nature of procyanidins (Fig. 1 B-E) had a significant impact on both particle size distribution and capture volume of the aqueous core of the vesicle. In general, cocoa extract-loaded liposomes displayed the most diverse structure (Fig. 1F), which is formed by small multilamellar vesicles linked through bilayer heterojunctions. Cross-section particle sizes ranged from 30 to 115 nm, membrane thickness around 40 Å and fully hydrated bilayers over 60 Å, which is in line with Polozova et al., (2005) These findings suggest that the high diversity of cocoa polyphenols as well as other components found in the crude extract influence the interaction with

the lipid membrane and establish chemical and physical interactions in different magnitude, thus, playing an important role in the permeability, diffusion, and release rate of the encapsulated agent.

In agreement with these results, the encapsulation efficiency (EE) of the compounds increased with the degree of polymerization (Table 1). While monomers have an EE around 45% (Toro-Uribe et al., 2018), dimers, trimers, tetramers, and pentamers presented higher EE being equal to 93.5, 90.3, 88.2 and 92.0 %, respectively. This trend was also similar for the procyanidins from cocoa extract-loaded liposomes. That is, the longer the procyanidin polymer, the higher the partitioning into the liposomes due to their association with the lipid bilayer, that is affected by their stereochemical structures, bonding capacities, and polarities (Huang & Frankel, 1997; Kajiya, Kumazawa, & Nakayama, 2001; Toro-Uribe et al., 2018).

8.3.2. Identification and characterization of cocoa procyanidins. Prior to the digestion assay, the characterization of both isolated procyanidins and procyanidins from cocoa extract was carried out by high-resolution mass spectrometry using UHPLC-QTOF in negative ionization mode as well as UHPLC-DAD as shown in Figure 2. As expected, major alkaloids in cocoa extract were theobromine followed by caffeine. Among the flavan-3-ol diastereoisomers, (+)-catechin and (-)-epicatechin were the most abundant, showing both catechins the same diagnostic ions (Table 2). As previously documented in the literature, the MS/MS fragmentation of these monomers followed retro Diels-Alder (RDA), and Heterocyclic Ring Fission (HRF) mechanisms (Li & Deinzer, 2007; Said et al., 2017). For example, (epi)catechins showed MS/MS ions at *m/z* 271.0624, 245.0830, 167.0343, 165.0201, 151.0405, 137.0245, 125.0247, and 121.0294. Therefore, the product ions identified at *m/z* 271 (-18 Da) and 245 (-42 Da, HC≡C−OH) were consistent with the expected fragmentation patterns (Table 2, Fig. S1A). The HRF mechanism was confirmed after the elimination of the A ring, that is, *m/z* 165 $[M-H-125]^-$. The RDA mechanism results after the

dissociation of C ring producing the product ion *m/z* 137 and 151. In addition, benzofuran ring fragmentation could be present due to the product ions at *m/z* 167→ 121 $[M-H-122]^-$(Fig. S1A).

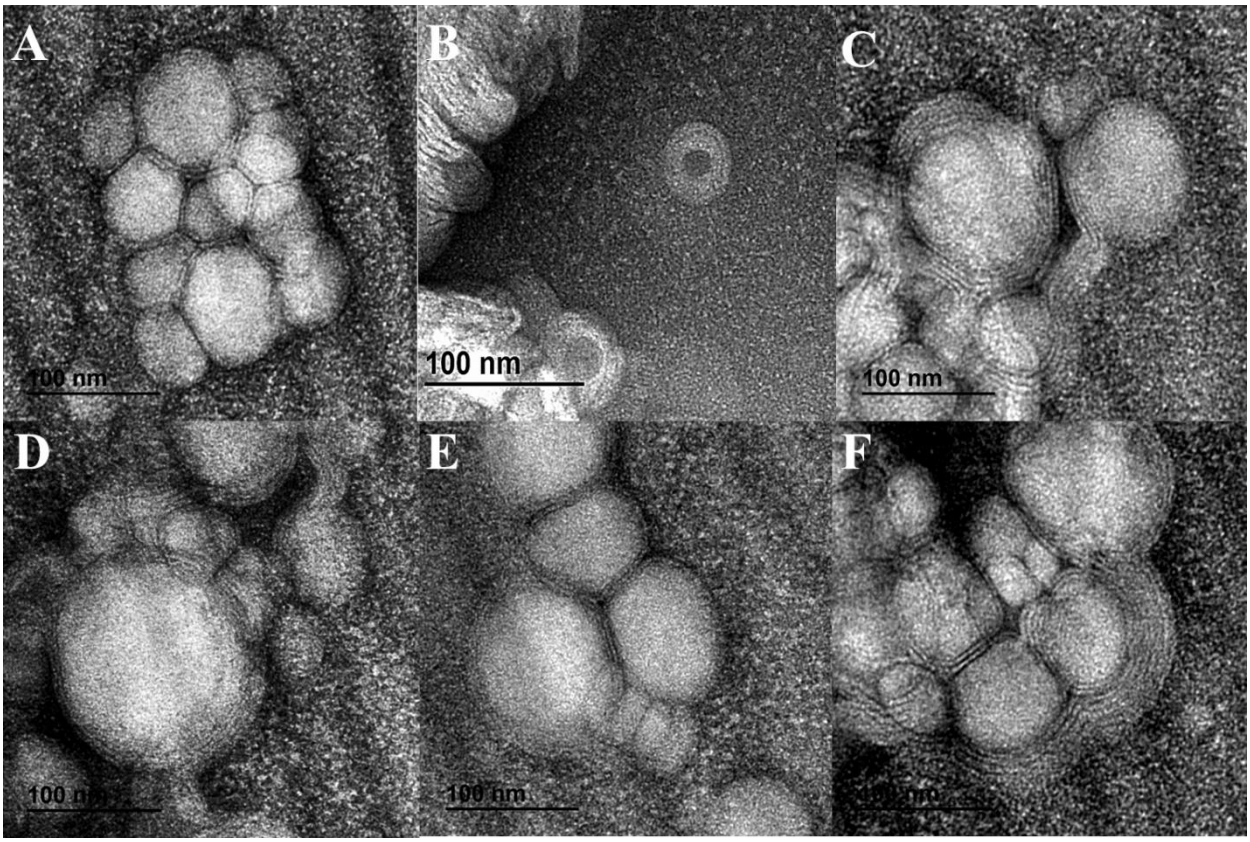


Figure 1. TEM images of (A) empty liposome and (B) dimer-, (C) trimer-, (D) tetramer-, (E) pentamer- and (F) cocoa extract-loaded liposomes.

Table 1. Encapsulation efficiency, remaining concentration and bioaccessibility for free and encapsulated samples before simulated digestion and during simulated *in vitro* gastric and intestinal phases.

Compounds		**Bioaccessibility (%)**	**Total depolymerization into Monomers (µM)**

	Encapsulation Efficiency (%)				
			Initial (t=0h)	Gastric (t=2h)	Duodenum (t=8h)
Samples in free system					
Dimer	-	8.3±4.7[a]	< LOD	8.8±0.2[a,A]	2.6±0.1[a,B]
Trimer	-	2.1±0.1[b]	< LOD	51.4±2.1[b,A]	17.0±2.6[b,B]
Tetramer	-	2.1±0.0[b]	< LOD	26.8±1.1[c,A]	3.7±0.6[a,B]
Pentamer	-	3.9±0.9[a,b]	< LOD	5.3±0.1[d,A]	2.2±0.3[a,B]
Procyanidins-loaded Liposomes					
Dimer	93.5±8.8[a]	11.7±1.8[a]	< LOD	14.4±0.7[a]	-
Trimer	90.3±1.6[a]	5.0±0.5[b]	< LOD	21.7±6.0[b,A]	7.8±0.3[a,B]
Tetramer	88.2±6.0[a]	5.6±0.1b[b]	< LOD	6.1±0.4[c,A]	8.9±2.8[a,A]
Pentamer	92.0±5.5[a]	8.8±0.3[c]	< LOD	12.3±0.2[d]	-
Free cocoa extract			590.8±5.5[A]	506.3±72.9[A]	72.8±22.7[B]
Dimer	-	1.3±0.1[a]			
Trimer	-	4.1±0.1[b]			
Tetramer	-	0.7±0.1[a]			
Pentamer	-	9.3±0.1[c]			
Cocoa-loaded liposomes			276.5±78.1[A]	41.6±4.0[B]	164.1±11.8
Dimer	95.1±0.9[a]	5.9±0.4[a]			
Trimer	93.4±0.5[b]	8.4±0.5[b]			
Tetramer	91.3±0.5[c]	6.5±0.7[a]			

Pentamer	95.6±0.8[a]	8.8±0.6[b]	

Samples denoted with different lower-case letters (a, b, c) were significantly different ($p < 0.05$) between same digestion phases for the different bioactive compounds. Samples with different capital letters (A, B, C) indicate significant differences ($p<0.05$) between different digestion phases for the same bioactive compounds. LOD, Limit of detection.

Table 2. Identification of observed molecular ions and MS/MS fragmentation pattern of procyanidins present in the studied crude cocoa extract and contents of each compound (peak area) before simulated gastrointestinal digestion and at the end of each *in vitro* digestion phase for free and encapsulated into liposomes samples.

Peak No	Ret. Time (min)	Procyanidin	$[M-H]^-$ (measured) (*m/z*)	Error (ppm)	MS/MS product ions (*m/z*)	Cocoa Extract Initial (0 h)		Cocoa Extract Gastric (2h)		Cocoa Extract Duodenal (8 h)	
						Free	Lipo	Free	Lipo	Free	Lipo
1	1.772	Trimer 1	865.1968	-0.64	739.1659, 576.1148, 289.0717	0.25	0.07	0.59	0.00	0.00	0.28
2	2.439	Dimer 1	577.1349	0.44	482.0666, 433.1368, 125.0241	0.06	0.03	0.11	0.00	0.00	0.55
3	3.295	Dimer 2	577.1370	-0.77	292.0480, 248.0564, 204.0678	0.12	0.07	0.23	0.00	0.00	0.46

4	4.042	Dimer 3	577.1379	-0.77	289.0703, 125.0229	245.0809,	0.08	0.03	0.17	0.00	0.00	0.08
5	4.042	(+)-Catechin	289.0738	-2.20	245.0820, 125.0238	203.0713,	7.32	6.43	13.73	0.00	3.15	73.45
6	4.215	Dimer 4	577.1365	-0.77	407.0765, 245.0397	289.0708,	0.33	0.09	0.49	0.00	0.00	2.01
7	4.566	Dimer 5	577.1515	0.09	577.1372, 289.0706	407.0760,	0.62	0.20	0.92	0.00	0.00	1.37
8	4.847	Dimer 6	577.1356	-0.77	425.0822, 289.0666	407.0698,	0.46	0.14	0.80	0.00	0.00	1.76
9	5.237	Trimer 2	865.1989	-0.64	739.1762, 425.0890	575.1205,	0.33	0.09	0.44	0.00	0.00	1.00
10	5.274	Dimer 7	577.1374	-2.16	425.0880, 125.0233	287.0537,	0.76	0.28	0.81	0.00	0.00	1.70
11	5.469	Trimer 3	865.2014	-0.64	739.1661, 426.0921	575.1192,	0.98	0.12	0.69	0.00	0.00	0.86
12	5.577	Dimer 8	577.1375	-2.16	425.0884, 137.0254	287.0566,	0.27	0.06	0.52	0.00	0.00	0.17
13	5.860	Dimer 9	577.1349	-2.16	425.0874, 289.0711	407.0758,	9.42	14.13	47.21	0.62	3.12	54.64
14	6.096	Trimer 4	865.1992	-0.64	713.1514, 425.0857	577.1351,	2.45	0.81	4.95	0.00	0.00	2.31
15	6.161	(-)-Epicatechin	289.0742	-3.24	245.0830, 125.0246	203.0720,	32.78	60.90	116.3	1.74	43.4	483.9
16	6.574	Dimer 10	577.1391	-2.16	289.0749, 165.0216	269.0492,	3.05	1.13	5.22	0.45	0.00	18.29

17	6.893	Tetramer 1	1153.2653	0.55	1001.2265, 575.1241	863.1825,	0.31	0.04	0.36	0.00	0.00	0.36
18	7.314	Tetramer 2	1153.2635	0.55	983.2044, 575.1183	865.1945,	1.14	0.16	1.41	0.00	0.00	1.16
19	7.774	Trimer 5	865.1976	-0.64	695.1397, 425.0909	577.1332,	1.04	0.22	1.88	0.45	0.00	5.49
20	8.020	Trimer 6	865.2012	-0.64	713.1515, 425.0892	575.1210,	14.38	6.64	42.10	4.30	0.25	75.20
21	8.501	Dimer 11	577.1352	-2.16	407.0758, 245.0885	289.0715,	0.37	0.07	0.63	0.00	0.00	1.44
22	8.457	Pentamer 1	1441.3194	0.71	720.6587, 289.0723	575.1215,	0.05	0.00	0.16	0.00	0.00	0.00
23	8.869	Dimer 12	576.1296	-2.16	451.1039, 289.0724	407.0782,	0.34	0.08	0.57	0.00	0.00	1.33
24	8.871	Tetramer 3	1153.2662	0.55	865.2012, 287.0574	575.1217,	7.12	1.24	13.48	1.73	0.00	13.12
25	9.087	Pentamer 2	1441.3289	0.71	984.2082, 577.1312	865.1901,	0.17	0.00	0.17	0.00	0.00	0.00
26	9.218	Trimer 7	865.2003	-0.64	713.1550, 425.0893	575.1212,	1.96	0.47	3.78	0.00	0.00	3.84
27	9.221	Dimer 13	577.1364	-2.16	432.0949, 245.0837	289.0711,	0.29	0.09	0.77	0.00	0.00	1.76
28	9.499	Pentamer 3	1442.3282	0.71	1153.2544, 575.1244	863.1833,	0.24	0.00	0.43	0.00	0.00	0.00
29	9.806	Pentamer 4	1442.3237	0.71	1153.2555, 575.1234	865.1917,	1.21	0.04	1.51	0.00	0.00	0.00
30	9.957	Pentamer 5	1442.3244	0.71	1153.2555, 575.1234	865.1917,	0.33	0.18	0.51	0.19	0.00	2.27
31	10.034	Tetramer 4	1153.2630	0.55	984.2067, 577.1336	863.1792,	0.76	0.06	0.92	0.00	0.00	1.68

32	10.490	Pentamer 6	1441.3235	0.71	720.1571, 425.0866	577.1345,	0.09	0.00	0.16	0.00	0.00	0.00
33	10.877	Dimer 14	576.1273	-2.16	407.0768, 151.0383	289.0705,	0.28	0.10	0.47	0.00	0.00	3.54
34	11.437	Dimer 15	577.1359	-2.16	407.0771, 245.0446	289.0720,	6.75	4.18	15.47	0.00	0.00	58.71
35	11.942	Dimer 16	577.1346	-2.51	439.1029, 287.0591	329.0610,	0.81	0.38	1.89	0.00	0.00	10.88
36	12.026	Trimer 8	865.2008	-0.64	713.1515, 425.0882	577.1357,	2.30	0.98	4.29	0.00	0.00	1.90
37	12.349	Pentamer 7	1441.3213	0.71	720.6587, 425.0857	635.6309,	0.14	0.00	0.10	0.00	0.00	0.00
38	12.403	Dimer 17	577.1356	-2.16	407.0777, 243.0286	289.0720,	0.36	0.36	0.67	0.00	0.00	8.10
39	12.458	Tetramer 5	1153.2615	0.55	865.2003, 413.0883	575.1198,	0.30	0.14	0.36	0.00	0.00	2.91
Total Monomers (%)							40	67	46	18	93	67
Total Dimers (%)							24	21	27	11	6	20
Total Trimers (%)							24	9	21	50	1	11
Total Tetramers (%)							10	2	6	18	0	2
Total Pentamer (%)							2	0	1	2	0	0

where catechins, dimers, trimers, tetramers, and pentamers had the following chemical formula: $C_{15}H_{14}O_6$, $C_{30}H_{26}O_{12}$, $C_{45}H_{38}O_{18}$, $C_{60}H_{50}O_{24}$, and $C_{75}H_{62}O_{30}$, respectively. CE, collision energy.

Regarding oligomeric flavan-3-ols, as can be observed in Figure 2 and Table 2, the great diversity of procyanidin isomers was confirmed and the presence of polymers up to dodecamers could be determined. Larger polymers were observed but could not be fragmented due to their

lower concentration and co-elution as an unresolved hump at the end of the analysis. Major procyanidin oligomers peaks corresponded to dimer B2 (peak 13, *m/z* 577.1348), trimer C1 (peak 20 *m/z* 865.2012), tetramer (peak 24 *m/z* 1153.2662), and pentamer (peak 30 *m/z* 1442.3244) (Table 2). Hexamer at *m/z* 1730.3923 and doubly-charged molecular ions ($[M\text{-}2H]^{2-}$) at *m/z* 1008.7250 (11.213 min), 1152.7555 (11.734 min), 1296.7858 (12.073 min), and 1440.8102 (12.349 min), corresponding to heptamer, octamer, nonamer, and decamer structures, respectively, were also identified. Moreover, $[M\text{-}3H]^{3-}$ triply-charged ions detected at *m/z* 1056.8999 and 1152.2523 were assigned to undecamer (13.351 min) and dodecamer (13.015 min), respectively.

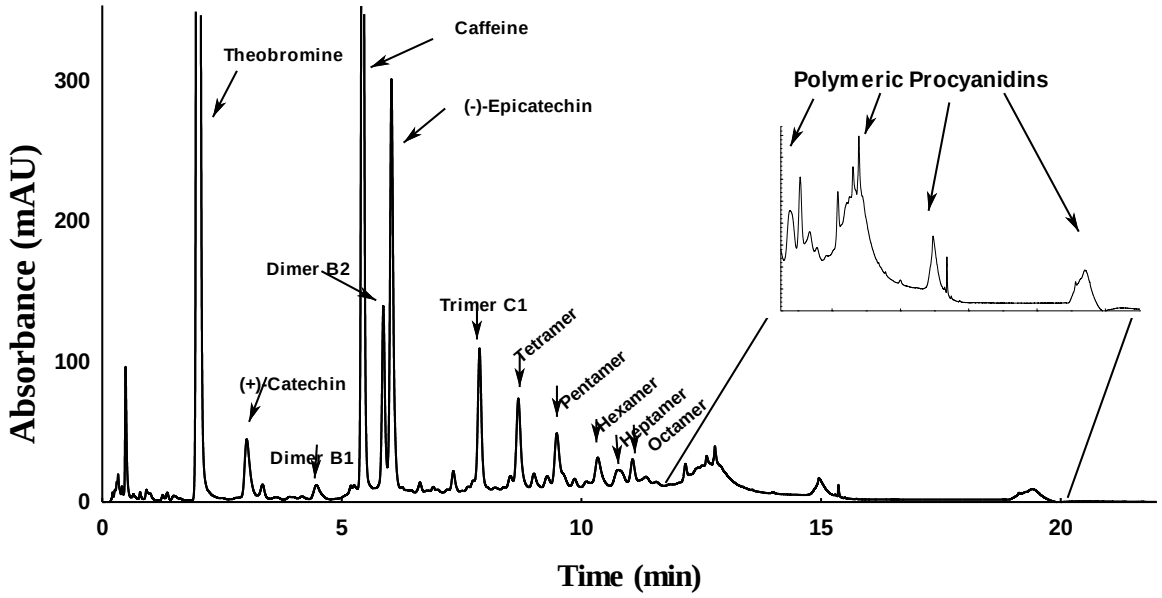
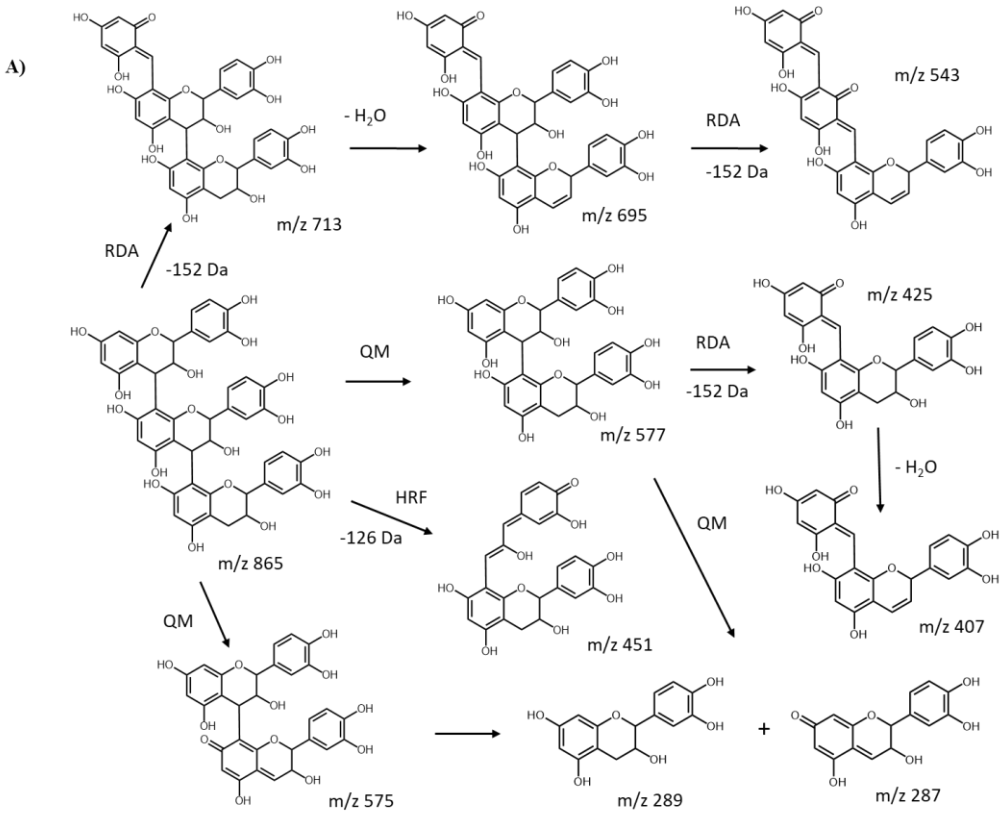


Figure 2. Chromatogram (280 nm) of the crude cocoa extract obtained by reversed phase UHPLC-DAD.

Seventeen, eight, five and seven peaks were detected with the same molecular ion for dimers, trimers, tetramers and pentamers in the extracted ion chromatogram (Table 2). These compounds were tentatively assigned as B-type procyanidins with (epi)catechin as a monomeric unit (either top or terminal unit). These findings reinforced the high diversity of chemical structure of cocoa

procyanidins because of the possible combinations between the interflavan linkages (C_4–C_8 or C_4–C_6) together with the stereoisomers and rotational isomers configuration. As reported in literature, HRF and RDA fragmentation mechanisms provide useful structural information about the degree of hydroxylation on the B-ring and binding site between the two monomeric units (Fabre, Rustan, De Hoffmann, & Quetin-Leclercq, 2001; Gu et al., 2003), whereas the quinone methide (QM) mechanism defines the cleavage of the interflavan bond (top and based unit) (Gu et al., 2003).



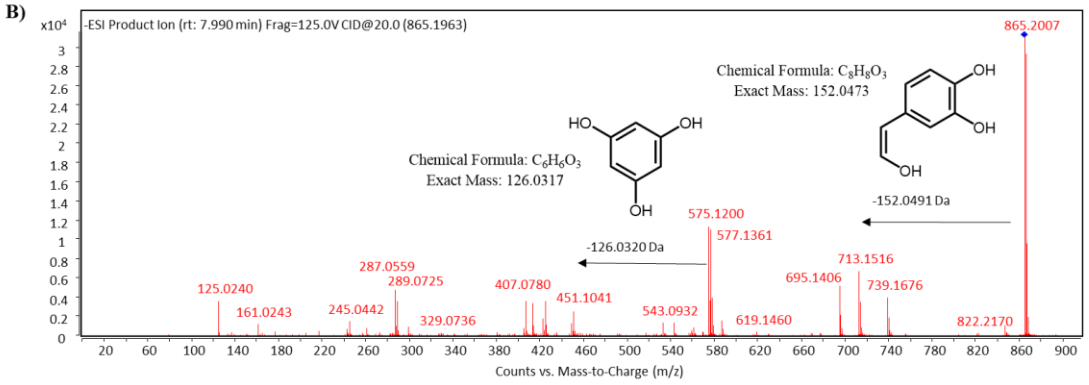


Figure 3. (A) Schematic MS/MS fragmentation pattern for a type-B procyanidin trimer (peak 20) under negative ionization mode. (B) MS/MS product ion spectrum corresponding to the proposed fragmentation pathway.

Based on these mechanisms, the MS/MS fragmentation patterns of the detected procyanidins was assigned to the different oligomers. For instance, Figure 3 shows the proposed fragmentation pathway that support the assignation of the product ion spectrum to a trimeric B-type procyanidin (formed by three flavan-3-ol units). The MS/MS product ions at *m/z* 575 and 577 are derived from the QM elimination of the monomer on the base and top unit of the trimer, respectively. Secondary ion at *m/z* 713 is the result of the elimination of B-ring from the flavan-3-ol trough RDA reaction. Subsequent fragmentation of *m/z* 577 ion produce the fragment at *m/z* 425 by RDA mechanism or *m/z* 451 after HRF fission (phloroglucinol molecule loss). The fragment ion at *m/z* 695 results from the neutral loss (-152 Da) related to RDA reaction and subsequent loss of a water molecule most likely from the C-ring (free 3-OH). Ions at *m/z* 289 and 287 ions correspond to the top/base unit after QM reaction.

The same behavior was observed for dimers, tetramers and pentamers, observing fragments related to the loss of a flavan-3-ol unit on the extension or terminal site through QM reaction followed by another loss of a monomeric unit (Fig. S1). Likewise, fragments derived from HRF fission were also detected. On the basis of these observations, dimers, tetramers and pentamers were formed by (epi)catechin-(epi)catechin units linked through $C_4 \rightarrow C_8$ bonds (B-type configuration).

These observations highlight the importance of high-resolution MS as useful tool for the characterization of cocoa proanthocyanidins as well as further studies involving their complete characterization, isolation, and metabolomic fate.

8.3.3. In vitro digestion of procyanidins with and without being encapsulated into liposomes. All the samples were evaluated for their bioaccessibility and release profile under simulated gastric and small intestine digestion phases. Considering that the isolated procyanidins fractions consisted of several stereoisomers, the transformation of the main compound in each group, that is, dimer B2 (peak 13), trimer C1 (peak 20), tetramer (peak 24), and pentamer (peak 29), was followed (Figures 4 and 5). As can be seen in Figure 4, the different formulations had significantly different release profile proving the effect of liposome carrier. Free non-encapsulated procyanidins (DP 3 to 5) underwent extensive transformation (e.g., epimerization, oxidation, degradation) during the transit time whereas the dimer was the most stable oligomer at acidic pH. Thereby, 88.5 ± 4.6, 46.8 ± 1.6, 66.1 ± 1.4, and 40.3 ± 0.6 % of the initial concentration for free dimer, trimer, tetramer, and pentamer, respectively, remained at the end of the gastric phase (0 to 2h). However, the stability of these compounds was greatly compromised under alkaline conditions (2 to 8h). In fact, the degradation of flavan-3-ols under digestive conditions appears to be most directly correlated to pH rather than to digestive enzymes activity (Bouayed et al., 2012). During the exposure to duodenal phase, these compounds were reduced up to 98% of the initial concentration. These findings are in line with previous works showing that several phenolic compounds (e.g., monomeric flavonoids, quercetin, and procyanidins) rarely exceeded 1 µM in plasma (Bouayed et al., 2012; Mendoza-Wilson et al., 2016; Motilva et al., 2016). This is because the proanthocyanidins *per se* had very limited bioavailability as only trace amounts of dimers could be accumulated in plasma due to lower apical and basolateral permeation values of procyanidins (DP 2 to 6), which confirmed their low absorption (Serra et al., 2009; Zumdick, Deters, & Hensel, 2012).

According to UHPLC-DAD-QTOF-MS analyses, the degradation mechanism of procyanidins consisted of depolymerization into small molecular weight compounds, which was in agreement with Ortega et al., (2009) Thus, degradation of oligomers into monomers was evidenced. For instance, free pentamer exposed to gastric phase led to depolymerization of 7.6, 1.9, and 4.1%, whereas for pentamer-loaded liposomes was 0.3, 0.3, and 2.6 % for tetramer, trimer, and dimer respectively. The depolymerization reaction into flavan-3-ols units was observed for all the tested samples. Moreover, at the end of the gastric phase the concentration of catechin plus epicatechin was 8.8, 51.4, 26.8, and 5.3 µM, whereas at the end of duodenal phase were 3.4-, 3.0-, 7.2-, and 2.4- fold lower for free dimer, trimer, tetramer, and pentamer, respectively (Table 1). These results reinforced the pH-dependence and high dissociation of procyanidins by hydrolysis processes, in particular for samples in non-encapsulated form.

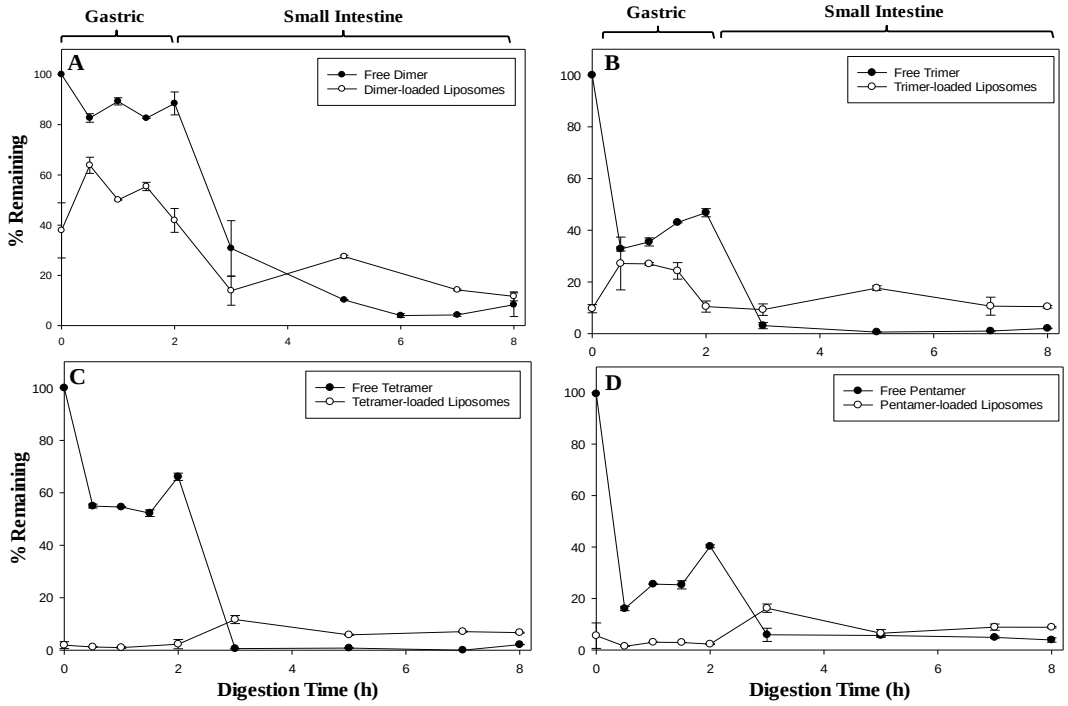


Figure 4. Concentration remaining (%) during simulated *in vitro* digestion of (A) dimer, (B) trimer, (C) tetramer, and (D) pentamer samples non-encapsulated (free) and encapsulated into liposomes.

A peak of concentration for free oligomers during the acidic environment (around 2 h) was also found (Figure 4). These phenomena could be a consequence of epimerization of isomers to the most stable procyanidins, for example, dimer B1 to B4 into dimer B2. It can be concluded that procyanidins with different degree of polymerization and isomeric configuration possessed different activities during the digestion process. Regarding procyanidins-loaded liposomes, their analysis is quite difficult, and the data should be treated carefully due to many processes occurring simultaneously during digestion; for instance, release and transformation of the bioactive agent from the carrier to the medium, oxidation and degradation of the bioactive agent, interaction of the lipid carrier and the bioactive agent with the digestive proteins, and lipolysis of the lipid membrane. Thereby, in this work, the amount of the target compound released into the medium is considered as the fraction bioaccessible through the process.

As can be seen in Figure 4, different release profiles were also observed. Low concentration of procyanidins-loaded liposomes before digestion was available, which is in agreement with their efficiency of encapsulation (> 88%) that affects the concentration of procyanidin as free molecule in the system. During the gastric phase, a higher amount of dimer (41.9%)- and trimer (10.5%)-loaded liposomes was released (Fig. 4A-B) while the opposite was observed for tetramer (2.3%)- and pentamer (2.3%)-loaded liposomes (Fig. 4C-D). These findings suggest that release is polymer size-dependent; moreover, high stability at acidic pH for the higher oligomers-loaded liposomes could be due to the greater partition coefficient and long-chain size of these compounds (Toro-Uribe et al., 2018). Therefore, they may be distributed into the aqueous core of liposomes and

establish interaction forces with the lipid bilayer surface (Toro-Uribe et al., 2018). Liposome boost for all liposome formulation was enhanced during the duodenal phase (between 3 – 5h). This demonstrates that liposomes have a controlled release rate and were efficient to transport polymeric catechins. In order to demonstrate the previous assumption, the bioaccessibility of each sample was assayed. Hence, the bioaccessibility was 1.4, 2.4, 2.7, 2.3-fold higher for dimer-, trimer-, tetramer-, pentamer-loaded liposomes than for their non-encapsulated counterparts. Like free procyanidins' behavior, the isomeric forms were epimerized to most stable structure (data not shown), but degradation rate of procyanidin into small molecular polymers was lower for liposome formulations than those in free form. These results confirmed that lipid bilayer was useful to protect the target structure during the digestion process. Thus, encapsulation into liposomes could be an effective means to enhance the biological activity in a specific site of action in the body.

***8.3.4. In vitro simulated* digestion of procyanidins from cocoa extract with and without being encapsulated into liposomes.** In order to investigate the differences observed among all the isolated procyanidins, we also studied the release profile at different time points, bioaccessibility and depolymerization trends for individual procyanidins contained in the crude cocoa extract both encapsulated into liposomes and dissolved in an aqueous system. In this case, again, the effect of the formulation type on the transit time was significant ($p<0.05$) when comparing the release profile of the procyanidins from the free cocoa extract and those in the liposome system (Figure 5). For instance, at gastric phase, losses up to 62% were noted for free cocoa extract while the same compounds displayed low degradation (<4.2%) in the encapsulated form. This phenomenon is related to the hydration of lipid film (lipid swelling), leading to higher entrapment efficiency and further protection by the lipid bilayer.

Furthermore, it can be hypothesized that under the gastric environment the liposome membrane rigidity was not completely perturbated and, thus, liposomes were able to hold the encapsulated bioactive and release them in the latter digestion stage. Comparison of Figure 4 and 5 suggested that isolated procyanidins-loaded liposomes and procyanidins from cocoa extract-loaded liposomes showed a maximum peak concentration at different time points. This may imply that the presence of other non-polyphenolic compounds in the latter case accelerated the diffusion of procyanidins from cocoa extract to the external medium.

Once again, samples exposed to acidic pH-triggered depolymerization of oligomers mainly for those in non-encapsulated form. Further insight of depolymerization process is shown in Fig. S2 and S3, where the isomeric forms yielded to the formation of most stable procyanidins. Moreover, a high relative abundance for these compounds at the end of digestion process (Fig. S2 and S3, duodenal phase) could be also seen. Analysis by UHPLC-QTOF-MS (Table 2) confirmed that the sum of monomers (61%) and dimers (60 %) formed during the gastric phase was significantly higher ($p<0.05$) than those in liposome system. On the contrary, trimers, tetramers and pentamers from cocoa extract-loaded liposomes were 58, 67, and 50% higher than those in free cocoa extract. These results highlighted that liposomes protect the target bioactive in its original nature. These findings were also confirmed by their quantification using HPLC-DAD, in which the concentration of catechins (506.3 µM *vs* 41.6 µM) at acidic pH was significantly ($p<0.05$) greater in free cocoa extract than in its encapsulated form (Table 1). Nevertheless, this trend was the opposite for duodenal phase were the sum of catechins were 55.6 % higher as well as the bioaccessibility of oligomers in the cocoa-loaded liposomes (Table 1).

A concentration boost effect was again observed during the small intestine phase (around 3 h), which agreed with the controlled release profile for liposome formulations found by Pachis et al.,

(2017). As shown in Figure 4 and 5, the time taken to attain the peak burst effect after gastric phase was influenced by the polyphenol structure, because of high diversity, and interindividual variability observed among of procyanidins samples that impacted their encapsulation efficiency, diffusion rate kinetic and final bioaccessibility.

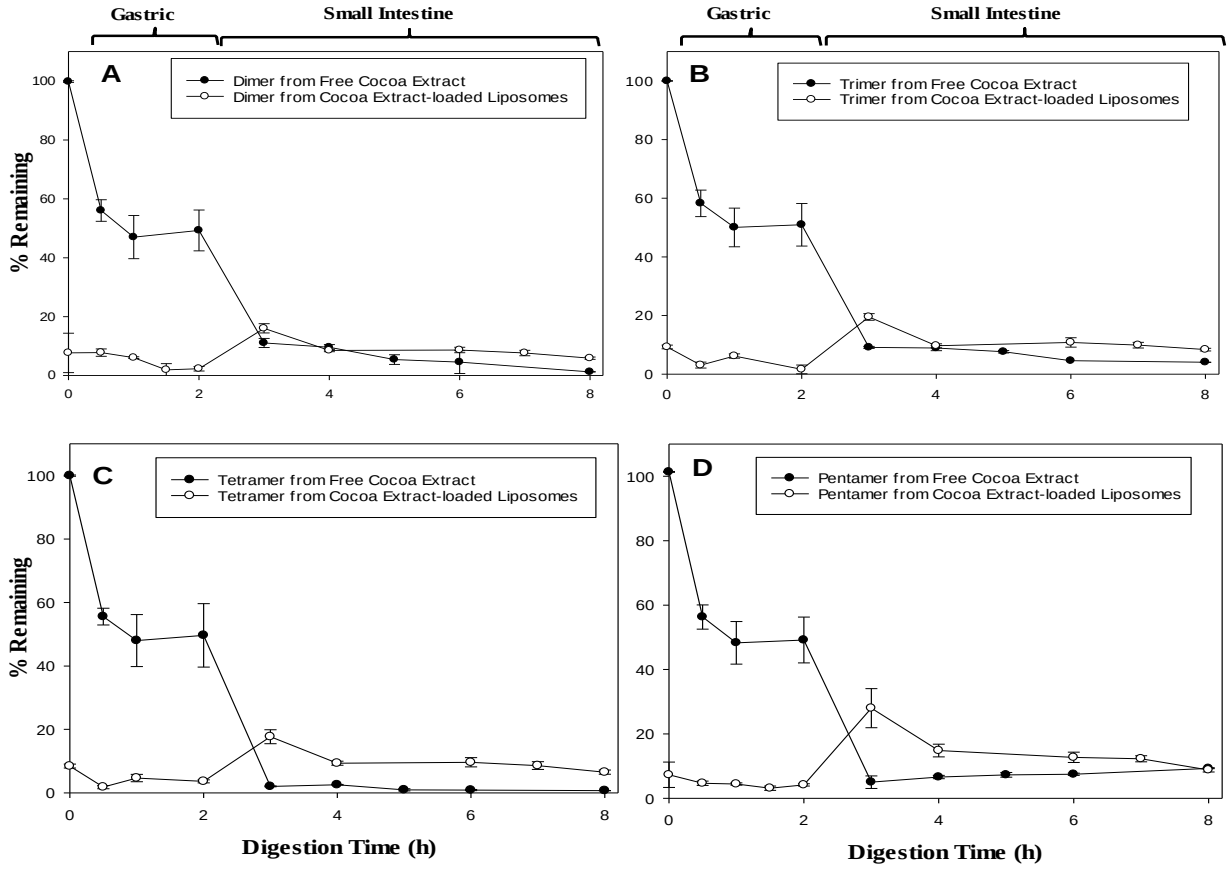


Figure 5. Concentration remaining (%) during simulated *in vitro* digestion of (A) dimer, (B) trimer, (C) tetramer, and (D) pentamer, from free cocoa extract and cocoa extract-loaded liposomes.

As expected, at this stage, oligomers in the free cocoa extract underwent intense transformation or degradation. For instance, degradation of dimers and trimers from free cocoa extract were 70 and 90% higher than those in the encapsulated forms (Table 2). Concerning the bioaccessibility, procyanidins from cocoa extract-loaded liposomes showed higher bioaccessibility among the

cocoa extract formulations except for pentamer which was slightly higher (1.05-fold) in the free cocoa extract. The higher bioaccessibilities of liposomes would be a result of several factors: i) liposome membrane by itself acts as a physical barrier; ii) a higher presence of phospholipids led to more significant amount of micelles that solubilize the phenolic compounds; iii) after the swelling effect, the liposomes were subjected to change of osmolarity and pH, which cause their rupture and the release of the encapsulated bioactives into the supernatant, and; iv) the breakdown of liposomes due to bile salts acting as biological surfactants.

In agreement with our results, the effectiveness of liposomes has been previously reported in the literature. For example, Mancini et al., (2018) demonstrated that phospholipid nanoformulations were interesting candidates to deliver antioxidants and monoamine oxidase A inhibitors into the brain. Moreover, Ortega et al., (2009) compared the digestion of two formulations of cocoa with different fat content. This study proved that greater fat content on the sample favored the formation of fat-forming micellar structures and the incorporation of the cocoa phenols into the lipid phase, achieving a protective effect during duodenal digestion.

Based on the above results, it was possible to prove that liposomes were efficient carriers to stabilize and transport cocoa procyanidins in order to enhance their bioaccessibility in a controlled release rate. The release profile was dependent on their chemical structure (e.g., the degree of polymerization and stereochemical configuration) and physicochemical interaction with the lipid carrier. Furthermore, the difference among the liposome samples suggests that different polymer size and lamellarity opens up the possibility to fine-tune the effectiveness of this system to control drug release kinetics of hydrophilic compounds.

8.3.5. Antioxidant activity. $DPPH^{\bullet}$ radical scavenging and $ABTS^{\cdot+}$ free-cation radicals scavenging assays were used for the assessment of the antioxidant activity of the different studied

samples during *in vitro* simulated gastric and duodenal digestion. In general, both encapsulated and non-encapsulated cocoa extracts presented the highest antioxidant activity among the studied systems, which could be a result of the presence of procyanidins together with other polyphenols (Table 3). DPPH radical scavenging activity was significantly reduced during the transit time, in particular for all the free formulations. In general, the antioxidant activity of free procyanidins and those from the free cocoa extract was reduced more than 30% during the gastric time and remained quite similar during the intestinal phase (except for free tetramer). Correlating this phenomenon with Figure 4, data suggested that the hydrolysis of higher oligomers into their terminal subunits during exposition of acidic pH still contribute to good radical scavenging activity. Although small concentration (low bioaccessibility) was measured for all the compounds at the end of digestion stage (Table 1), the high antioxidant magnitude could be a consequence of the formation of autooxidation products, which is in agreement with Donovan et al., (2007).

Once again, DPPH assay reinforced the good performance of liposomes. During duodenal phase, all samples loaded into liposomes were significantly more antioxidant than their respective non-encapsulated ones. Indeed, dimer-, trimer-, tetramer-, pentamer-, and cocoa extract-loaded samples were 2.0, 1.7, 1.2, 1.9, >1.3-fold higher than those compounds in free system. Moreover, it is well-known that at alkaline pH the radical scavenging capacity of the catechins increases because donation becomes much easier following an electron donation mechanism (Muzolf, Szymusiak, Gliszczynska-Swiglo, Rietjens, & Tyrakowska, 2008). However, as the only differences between both formulations is the lipid membrane, we could hypothesize that the high scavenging activity for the procyanidins-loaded liposomes was not only by the protection of lipid bilayer (high bioaccessibility) but also for the high amount of solubilized micelles that influence the rate of the $DPPH^{\bullet}$ free radical scavenging. In line with this results, Noipa et al., (2011) showed

that the magnitude and reaction rate between $DPPH^{\bullet}$ and antioxidant was greater for micelle solutions compared to methanolic solutions. The same authors explained that the abstraction of phenol H-atom by $DPPH^{\bullet}$ occurred inside the micelle which was more lipophilic than outside; in consequence, rates of scavenging of $DPPH^{\bullet}$ by the antioxidant are high in the micelle system.

The same behavior was observed in the case of $ABTS^{\cdot+}$ free-cation radicals scavenging. At physiological pH, dimer-, trimer-, tetramer-, and pentamer-loaded liposomes were 1.2, 3.1, 1.3, and 1.7-fold higher than their respective free compounds.

This trend was also similar for cocoa extract-loaded liposomes, being 1.5-fold higher than free cocoa extract. Although it has been well reported in the literature that ABTS assays is pH dependent, our results also suggested that the formation of unknown products during the duodenal phase could contribute to a high free scavenge activity. This is in line with Z. Wu et al., (2015) who found higher $ABTS^{\cdot+}$ cation radical scavenging activity for four types of tea as a result of small molecular phenolic compounds and breakdown after digestion process.

Regarding samples loaded into liposomes, we speculated that the high content of solubilized micelles could form a mixture of charged micelles together with different interaction forces (e.g., electrostatic, hydrophilic and hydrophobic). In this sense, newly formed compounds during digestion, either due to pH or enzymes activity, could be associated with the micelle increasing the antioxidant activity. Indeed, Y. Wu & Wang (2017) reported that conjugated micelles provide a more appropriate microenvironment to donate H atoms to reduce $ABTS^{\cdot+}$ into non-radical form easily. Finally, it is worth to mention that the high antioxidant effectiveness (both DPPH and ABTS assays) was also consistent with Figures 4 and 5 and high bioaccessibility results (Table 1) for the encapsulated samples.

Table 3. ABTS (µmol Trolox/ µmol sample) and DPPH (scavenging activity, %) antioxidant activities of the studied compounds before simulated gastrointestinal digestion and at the end of each *in vitro* digestion phase.

ABTS Assay	Free Compounds			Compounds-loaded Liposomes		
Compound	Initial	Gastric	Duodenum	Initial	Gastric	Duodenum
Dimer	0.91 ± 0.00^{a}	1.23 ± 0.05^{a}	11.66 ± 0.15^{a}	1.05 ± 0.01^{a}	4.80 ± 0.00^{a}	14.27 ± 0.28^{a}
Trimer	0.44 ± 0.01^{b}	2.34 ± 0.05^{b}	4.10 ± 0.05^{b}	0.70 ± 0.01^{b}	4.74 ± 0.02^{a}	12.69 ± 0.50^{a}
Tetramer	2.12 ± 0.02^{c}	3.54 ± 0.04^{c}	4.18 ± 0.06^{b}	2.64 ± 0.02^{c}	4.87 ± 0.09^{a}	5.26 ± 0.26^{b}
Pentamer	2.51 ± 0.00^{d}	1.52 ± 0.03^{a}	4.15 ± 0.03^{b}	3.03 ± 0.00^{d}	1.57 ± 0.06^{b}	7.15 ± 0.10^{c}
Cocoa extract	0.62 ± 0.03^{e}	3.70 ± 0.05^{c}	8.85 ± 0.07^{c}	1.66 ± 0.03^{e}	4.97 ± 0.02^{a}	13.41 ± 0.40^{a}
DPPH Assay						
Dimer	>LOQ	50.89 ± 0.10^{a}	44.83 ± 0.18^{a,b}	>LOQ	49.71 ± 1.43^{a,b}	91.31 ± 4.87^{a}
Trimer	>LOQ	57.54 ± 1.40^{a}	48.01 ± 1.07^{b}	>LOQ	51.13 ± 0.54^{b}	83.04 ± 0.55^{b}
Tetramer	>LOQ	51.66 ± 0.37^{a}	71.78 ± 0.57^{c}	>LOQ	43.48 ± 0.97^{c}	85.60 ± 0.80^{a,b}
Pentamer	>LOQ	55.89 ± 1.94^{a}	43.83 ± 1.58^{a}	>LOQ	46.13 ± 1.08^{a,c}	82.72 ± 3.57^{b}
Cocoa extract	>LOQ	80.25 ± 10.63^{b}	81.54 ± 1.76^{d}	>LOQ	93.08 ± 3.04^{d}	>LOQ

Means within a column (comparison between same digestion phases for the different bioactive compounds) with different letter are significantly different ($p < 0.05$).

8.4. CONCLUSIONS

Stability and *in vitro* bioaccessibility of procyanidins up to pentamers and cocoa extract encapsulated into liposomes and compared with their respective counterparts in aqueous system were studied. It is emphasized that liposomes were efficient to hold the encapsulated bioactive compounds to enhance their bioaccessibility and played an important role on the release profile, burst effect, and bioaccessibility of procyanidins at the end of digestion. Moreover, cocoa extract matrix influenced in different levels the release rate and the bioaccessibility of the individual studied procyanidins. It was proven that the release rate of oligomeric procyanidins depends on the chemical structure and physicochemical interaction with the lipid carrier. Based on the strong scavenging free radical activity at the end of *in vitro* digestion, it is suggested that polyphenols do

not act alone because they could interact with the micelle system, thus increasing their solubility together with the formation of unknown compounds. As a result of depolymerization of procyanidins, the formation of small molecules may lead to a higher bioavailability and intestinal uptake.

8.5. ACKNOWLEDGEMENTS

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8.6. SUPPLEMENTARY INFORMATION

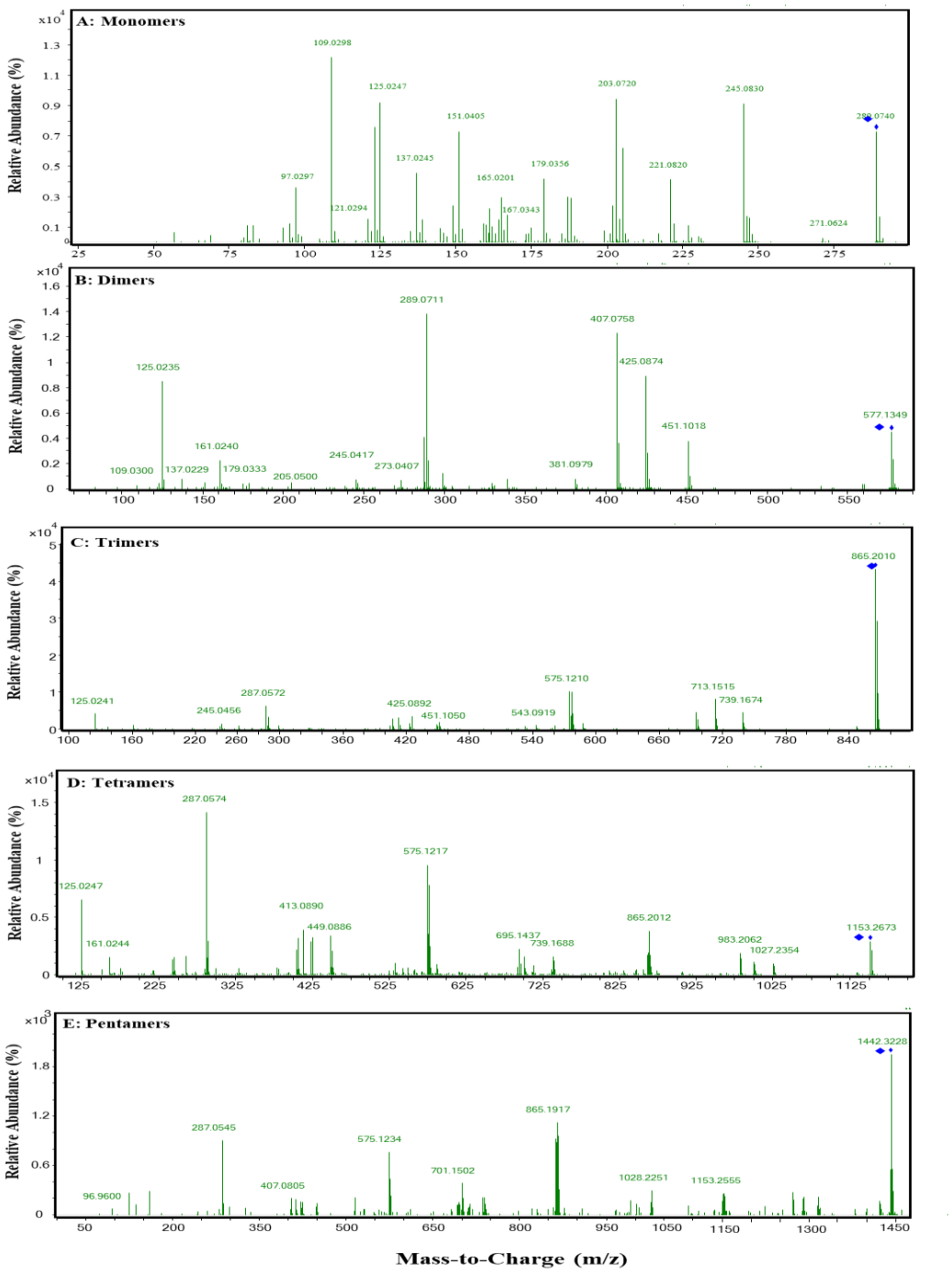

Fig. S1. Schematic MS/MS fragmentation pattern for A) monomers, B) dimers, C) trimers, D) tetramers, and E) pentamers from cocoa extract under negative ionization mode.

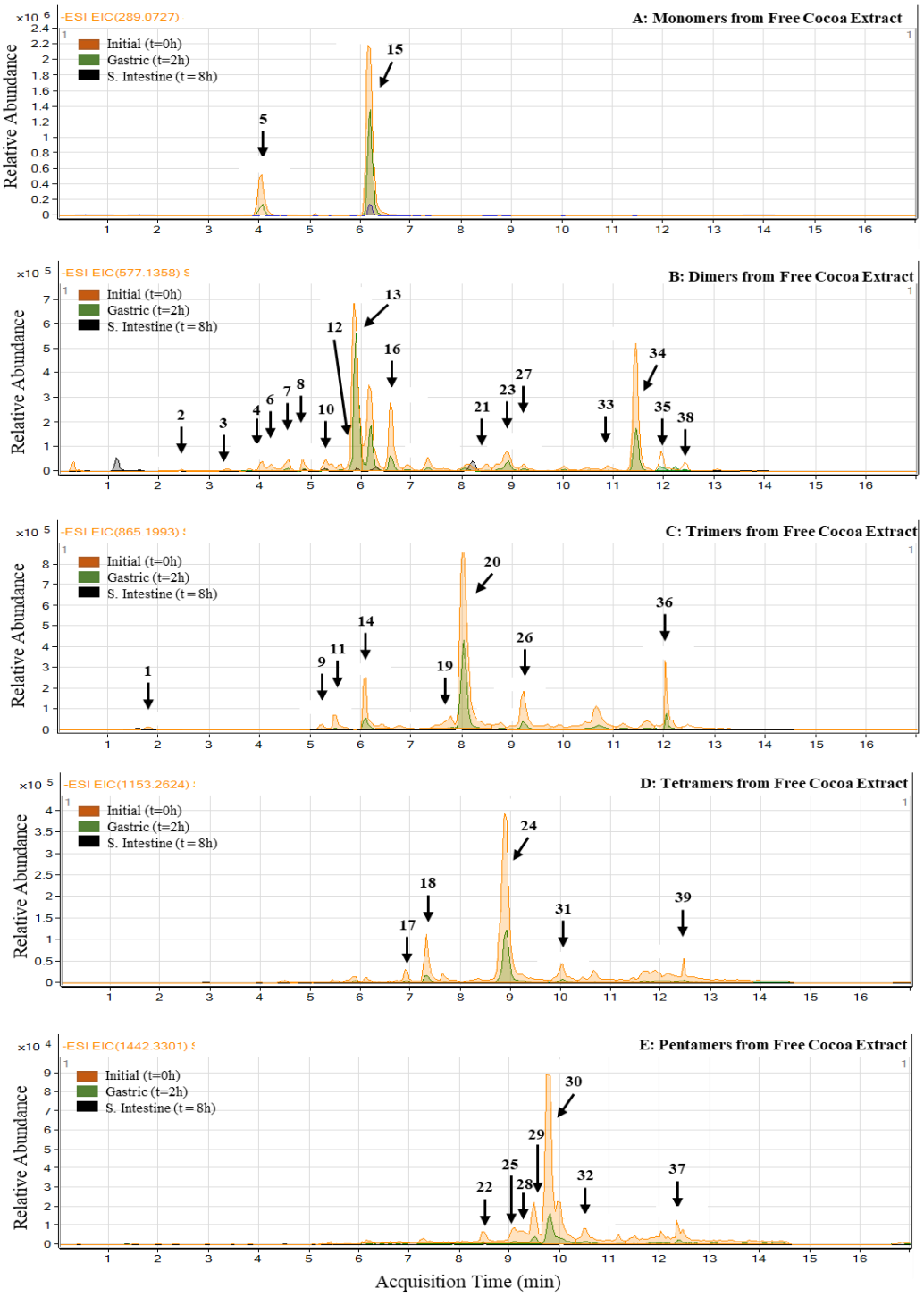

Fig. S2. UHPLC-QTOF-MS characterization and depolymerization of procyanidins during simulated *in vitro* gastrointestinal model for (A) monomers, (B) dimers, (C) trimers, (D) tetramers, and (E) pentamers contained in the non-encapsulated cocoa extract.

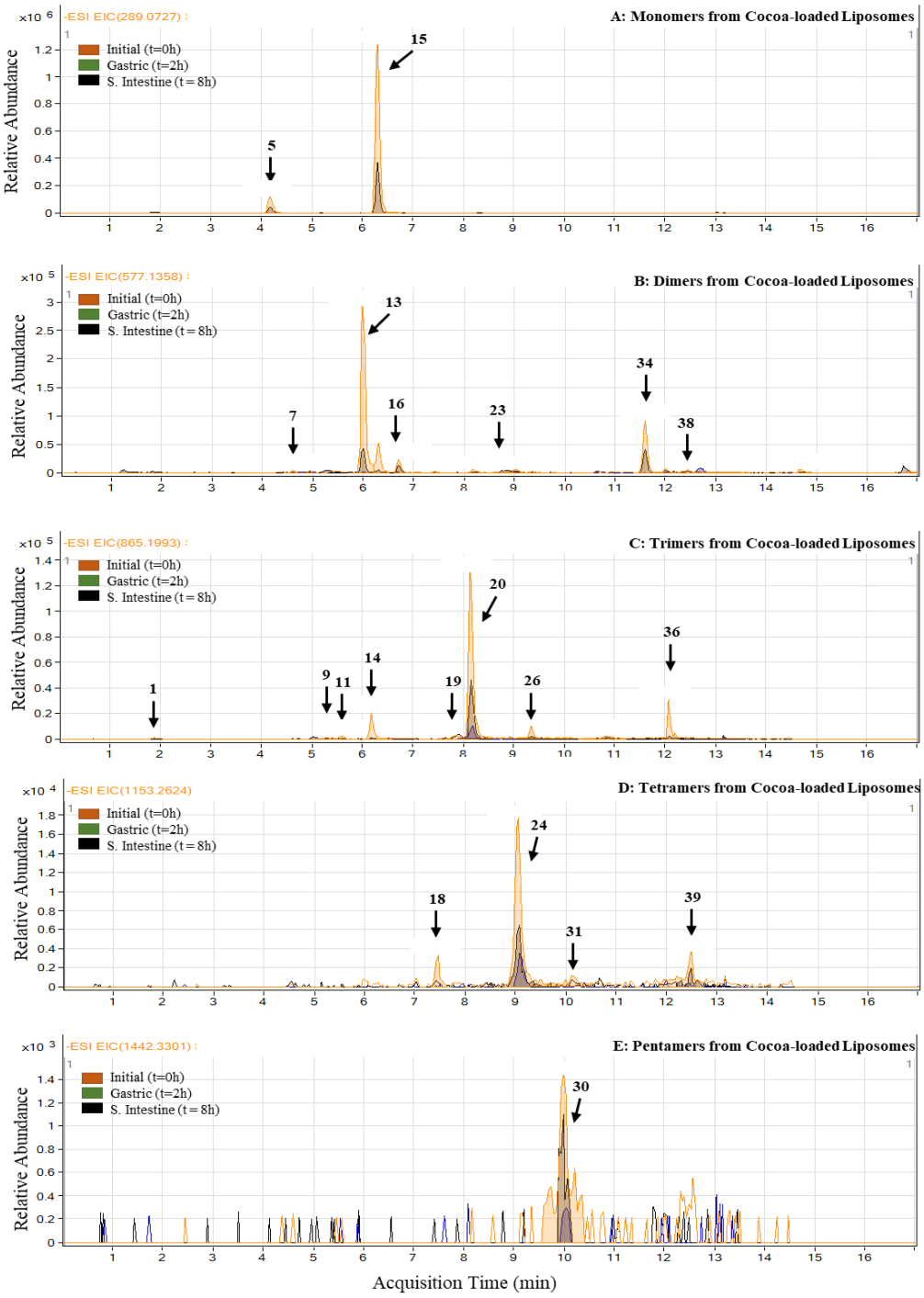

Fig. S3. UHPLC-QTOF-MS characterization and depolymerization of procyanidins during simulated *in vitro* gastrointestinal model for (A) monomers, (B) dimers, (C) trimers, (D) tetramers, and (E) pentamers contained in the cocoa extract-loaded liposomes.

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COMPREHENSIVE DISCUSSION

This Ph.D. aimed to enhance *in vitro* bioaccessibility of procyanidins through liposome-based delivery system preferably using food-grade solvents. Considering the hydrophilic character of procyanidins, physicochemical instability and rather low bioavailability, this task has been a big challenge. Further insight into each one of the stages of this research is stated in **Chapters 1** to **7**.

Cocoa has been identified as a polyphenol-containing food since the 1960s (Robinson, Ranalli, & Phillips, 1961) and is recently ranked as the fourth richest dietary source of polyphenols, just behind cloves, peppermint and star anise (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010). The main polyphenols in cocoa are (epi)catechins, proanthocyanidins, flavanol glycosides, and anthocyanins (Cienfuegos-Jovellanos et al., 2009; Kelm, Johnson, Robbins, Hammerstone, & Schmitz, 2006; Tomas-Barberán et al., 2007). Procyanidins are, by far, the major flavonoids in cocoa. These compounds are exclusively composed of (epi)catechin units and are characterized for possessing a high degree of polymerization (Kelm et al., 2006). The benefits of consumption of procyanidins are due to the positive health effects they conferred, as it is well-explained in the literature (Aron & Kennedy, 2008; Jeong & Kong, 2004; Yu & Ahmedna, 2013). Thereby, there is increasing interest in the incorporation of natural antioxidants to enriched food.

The pre-treatment process is the first step to extract bioactive compounds from plant materials. In our specific case, unfermented cocoa beans contain a significant amount of polyphenol oxidase enzyme (52 – 75 U_{PPO} mg^{-1}) (Lee, Lee, & Karim, 1991; Misnawi, Selamat, Bakar, & Saari, 2002), which could affect the polyphenols' extraction yield. Polyphenol oxidase (PPO) is located in the chloroplasts, therefore, its action takes places during cell-damage treatment where the oxygen is available (Araji et al., 2014; Cheema & Sommerhalter, 2015). After the physical damage, PPO

accelerates the oxidation and degradation of polyphenols and their derivatives (Li, Chen, Zhang, & Fu, 2017). Up to date, several inactivation methodologies have been used, for example, thermal treatment (El-Shimi, 1993; López-Malo, Palou, Barbosa-Cánovas, Welti-Chanes, & Swanson, 1998; Schweiggert, Schieber, & Carle, 2005), UV-radiation (Lante, Tinello, & Nicoletto, 2016), and high hydrostatic pressure treatment (López-Malo et al., 1998; Rastogi, Eshtiaghi, & Knorr, 2009). However, these works are focused on either the characterization/isolation of PPO enzyme or enzyme inhibition strategies without evaluating the effect of the process on the recovery of bioactive compounds.

Thereby, thermal treatment to inhibit the action of PPO was implemented. To do so, the conditions to enhance high PPO inactivation while maintaining a high level of polyphenols were determined. Thus, type and concentration of inhibitor (L-cysteine: ascorbic acid), temperature, and time were studied. The relationship of these factors was evaluated through 2^3 surface design + central points + start points. To determine the impact of enzyme inactivation treatment on the total recovery of polyphenols, not only the specific PPO activity was measured but also the content of total polyphenol non-inactivated beans was determined. These two parameters were used as response variables. The results showed a non-linear relationship between the response variables. So, models such as quadratic (r^2 = 0.61), exponential (r^2 = 0.56), and logarithmic (r^2 = 0.59) did not explains properly the experimental data. For this reason, a new equation (Eq. 1) represented by Γ was established.

Γ correlates high polyphenol content with reduced PPO activity –expressed in percentage– in an inverse relationship, as follows:

$$\Gamma_i = \frac{\text{Total Polyphenol}_i\ (\%)}{100 - \text{PPO Inhibition}_i\ (\%)} \quad (Eq.\ 1)$$

Based on the new response variable, Γ, typical exponential profile was observed, in other words, Γ increased by increasing the enzyme inhibition and polyphenol content until saturation value was reached. Analysis of variance showed a good coefficient of multiple determination of r^2= 0.891. The good performance of the model was confirmed by the root mean square error (RMSE), which was lower than 0.4. In general, all factors studied presented significant ($p<0.05$) impact on Γ, in particular, the length of treatment. Synergic effect of ascorbic acid and L-cysteine as an efficient solution to prevent enzymatic browning reactions was also confirmed, which was in agreement with previous works (Dudley & Hotchkiss, 1989; Siddiq & Dolan, 2017). Thereby, the maximum PPO reduction was 93.3 ± 2.1% for a total polyphenol content equal to 94.9 ± 4.09 mg gallic acid equivalent per g_{cocoa}, that is, 55.6 % higher than non-treated samples.

The affinity of PPO for catechins and catechol was also evaluated. Results showed that all the substrates were oxidized displaying Michaelis-Menten kinetics, being most suitable for catechol > epicatechin > catechin. To prove the efficacy of the inactivation process, cocoa beans with reduced PPO activity were obtained. Thus, it was confirmed that the affinity and catalytic power for catechol was reduced by 99.7 %. In light of these results, we hypothesized that the heat treatment together with inhibitor solution not only allowed to break down the enzyme-substrate complex but also led to the softening of the cell for increasing the polyphenols' extraction yield. To demonstrate the previous statement, scanning microscopy images were taken. As can be seen in **Figure 1**, the non-treated beans' cell wall had well oval-shape, solid and denser appearance, and the cellular content was embedded in it (**Fig. 1A**). When the sample was subjected to inactivation process, changes in the cell wall, such as larger, smooth, and fibrous appearance, together with more interspace and holes in the microstructure were observed (**Fig. 1B**). These observations confirmed

that the heat treatment and chemical inhibitors caused not only a cell denaturation but also changes in cell morphology for improving the pathway of polyphenols to the extraction solvent.

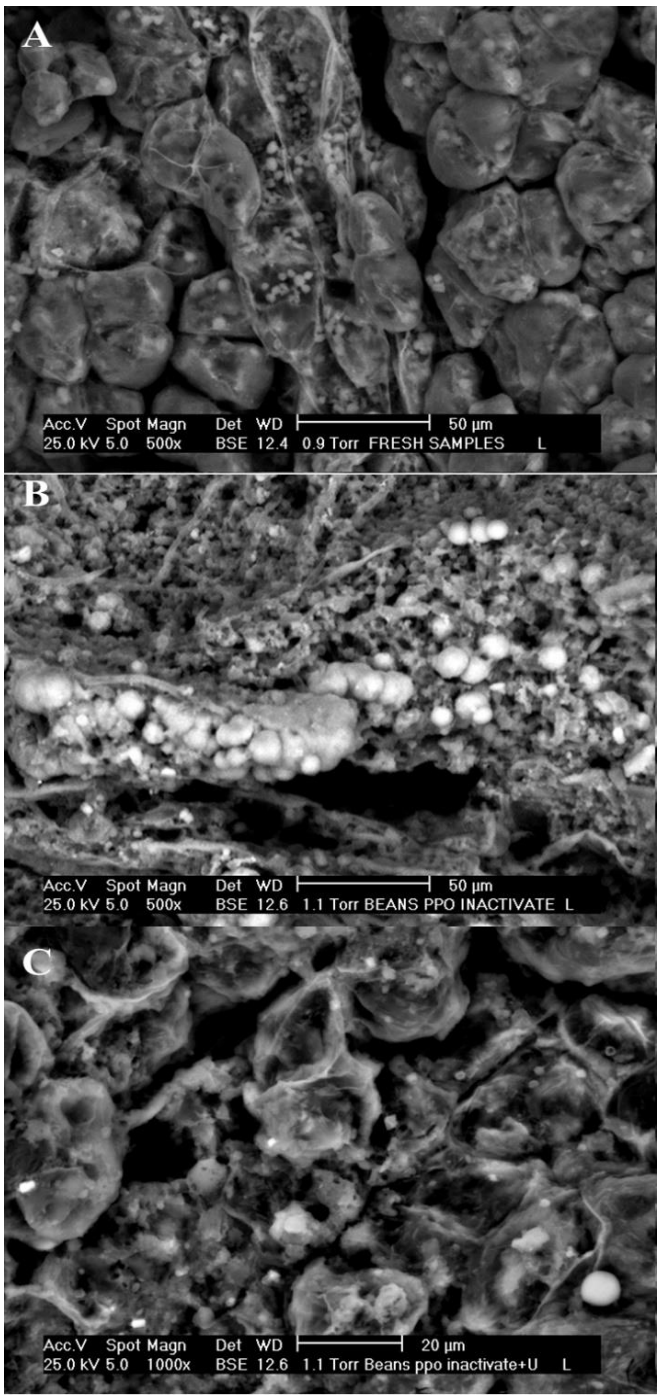

Figure 1. Microscopy images for the microstructure of a) non-treated cocoa beans, b) beans after PPO inhibition and c) beans after PPO inhibition and ultrasound treatment.

After enzyme inhibition treatment, the most favorable conditions for the recovery of polar polyphenols from cocoa beans were established. Solvent extractions are the most commonly used procedure to prepare extracts from vegetable matrices. It is well known that the solid-liquid extraction yield depends on many factors such as type and concentration of solvents, sample-to-solvent ratio, extraction time, temperature, pH of the system as well as on the chemical composition and physical characteristics of the samples (e.g., particle size).

In the specific case of proanthocyanidins, the literature review shows some works focusing on the optimization process. For instance, Shi et al., (2003) optimized the effect of single factors on the extraction of polyphenols from grape by aqueous ethanol. In line to this work, Bai et al., (2010) through a Box-Behnken design optimized the recovery of bioactive compounds from apples using microwave technology. Process variables such as pressure, CO_2 flow rate, and co-solvent for the extraction of polyphenols from grape seed were also optimized using supercritical fluid extraction (Da Porto & Natolino, 2017). Although these works can give us an idea about the behavior of procyanidins during the extraction process, in the particular case of cocoa, the nature and configuration of procyanidins are quite different between apple and grape seed sources. Indeed, cocoa extract contained low amounts of flavan-3-ols linked to galloyl or glucose moieties and its polymers are mainly formed by (epi)catechin subunits. In this regard, Sarmento et al., (2008) established the conditions to concentrate polyphenols from cocoa seed using supercritical CO_2 connected to a polymeric membrane module. This work assayed several combinations of pressure and concentration of ethanol as well as different types of membranes. However, complete information about the chemical characterization and amount of lipid and polar polyphenols recovered is not fully detailed. Process optimization from cocoa beans is also reported, but these studies are focusing on processing conditions for chocolate manufacturing (Gültekin-Özgüven,

Berktaş, & Özçelik, 2016; Saltini, Akkerman, & Frosch, 2013; Żyżelewicz et al., 2016) or the impact on the degradation of polyphenol during fermentation, drying or alkalization process (Camu et al., 2008; Kyi et al., 2005).

It is also well known that the fat content in cocoa beans is higher than 50 wt% (Servent et al., 2018), which is suggested to act as a barrier for the isolation of polyphenols. Therefore, it is a prerequisite to employ a fat removal treatment before the extraction process. Although several methodologies can be used for this purpose (e.g., pressing, soxhlet, ultrasonic, and supercritical fluids), most of them employ toxic solvents or constitute an additional step that impacts the cost of the process. Furthermore, there is a need to establish an easier procedure and to maximize the solid-liquid extraction conditions for obtaining high recovery of polyphenols, in particular, flavonoids compounds.

Before polyphenol extraction, it is very common to reduce the particle size of samples by milling followed by reduction of water content by either oven-drying or preferentially freeze-drying. Freeze-drying allows to preserve higher levels of phenolic compounds, but it is the most expensive process to dehydrate a product (Ratti, 2001). In order to establish a cheap, robust and food-grade polyphenols' recovery process, the extraction stage in this Ph.D. dissertation was divided into (a) evaluation of drying process *vs* freeze-drying, (b) a new strategy to avoid the degreasing process, and (c) selection of the best extraction conditions to enhance recovery of polyphenols.

Beans with reduced enzyme activity were oven-dried at both 50 and 70 ºC and freeze-dried until a moisture content of < 4%. After that, total flavonoid (**TF**) content was measured. Differences among the samples were observed, where drying at 50 ºC for ca. 20 h had the lowest TF content while drying at 70 ºC (around 3h) and freeze-drying methods displayed similar

flavonoid yield ($p<0.05$). Our results suggest that flavonoids were more sensitive to drying time than temperature. Furthermore, this phenomenon could be as a result of epimerization-dependence at high temperatures for flavan-3-ols compounds as shown by Kothe, Zimmermann, & Galensa (2013).

As a strategy to avoid the lipid removal process, the samples were milled under frozen conditions and three different particle sizes were selected: (S_1) >0.42 mm, (S_2) 0.42-0.18 mm, and (S_3) <0.18 mm. The samples sonicated at specific time points (0 – 60 min) showed that the lower particle size (<0.18 mm) for 30 min had no significant difference ($p > 0.05$) against the control (defatted sample). Results confirmed that ultrasonic wave was able to increase the TF content, whereby there is an inverse relationship between the particle size and the extraction yield of flavonoids. Indeed, it has been published that the cavitation forces improve the polarity of the system; moreover, the bubbles in the liquid/solid extraction can explosively collapse and generate localized pressure causing plant tissue rupture, thus increase the mass transfer rate (Oroian & Escriche, 2015; Y. Sun, Liu, Chen, Ye, & Yu, 2011).

Beans with reduced enzyme activity, oven-dried at 70 °C for 3h, milling under cold conditions until reaching a particle size lower than 0.18 mm, and ultrasonicated for 30 min were used for the next step: the recovery of polyphenols. Solid-liquid extraction was optimized through 2^4 + central point + start point factorial design. The independent factors were temperature (30 to 70 °C), solute/solvent ratio (w/v) (1/120 to 1/24), the concentration of aqueous ethanol (0 to 100 v/v %) and acidification with citric acid at pH ranges from 2 to 6. As dependent factors total polyphenol, total flavonoid and total flavan-3-ol content were studied.

In general, the recovery of bioactive compounds from cocoa beans mainly depended on the linear effect of temperature and pH, linear and quadratic effect of ethanol/water ratio, and

solute/solvent ratio; besides interaction of these factors. The quadratic model showed a good coefficient of multiple determination of r^2 equal to 0.96, 0.95, and 0.91 for total polyphenol, flavonoid, and flavan-3-ols content, respectively. Experimental conditions providing the maximum response were 50% ethanol, 1/120 w/v solute/solvent ratio, pH 6 at 70 ºC for an extraction yield of 16.8 %, which was 12.8% higher than the non-treated cocoa sample. The resulting cocoa polyphenolic extract consisted of 122.34 ± 2.35 mg of gallic acid equivalent per g_{cocoa} for total polyphenol and 88.87 ± 0.78 and 62.57 ± 3.37 mg of epicatechin equivalent per g_{cocoa} for total flavonoid and flavan-3-ol, respectively.

Results can be summarized as follows:

- The temperature decreases the viscosity of the solvent, increases the solubility, diffusion rate and mass transfer of polyphenols
- Fick´s second law of diffusion predicts the influence of solute/solvent, that is, a final equilibrium (**Figure 2**) between the concentration of solute in the solid matrix and in the bulk solution after a specific time.
- Maximum extraction yield of polyphenols was enhanced using 50% ethanol due to different polarities of polyphenols affecting the kinetic and thermodynamics of the process
- The pH impacts the independent factors at different levels, thus influencing the stability of polyphenols and controlling the diffusion process.
- At optimal conditions, the recovery of flavonoids was 37% higher than the non-treated samples.

Another factor that also impacts the extraction yield, as well as energy requirements and cost of the process, is the extraction time. Therefore, a kinetic study and an adjustment to several kinetic

models was performed. Peleg model was the most suitable model ($r \geq 0.98$ and $RMSE \leq 0.71$) that properly describes the sorption/desorption process. As shown in **Figure 2**, the extraction curve shape had a faster extraction rate followed by a slower extraction rate and asymptotically approaching to equilibrium concentration. Equilibrium times for total polyphenol, flavonoid and flavan-3-ol were reached at 45, 39, and 34 min, respectively.

To confirm the high flavonoids' and polyphenols' extraction yield avoiding the degreasing process, SEM pictures were also taken. As can be seen in **Figure 1C**, it is confirmed that ultrasonic treatment led to morphological changes in the cell and greater intercellular spaces compared to the non-treated samples (**Fig. 1A**) and beans after PPO inhibition (**Fig. 1B**). Therefore, smaller fragments were dispersed within the cell, and the microstructure was more porous. As a consequence of these changes, higher penetration of solvent into the sample matrix increased the contact surface between solid and liquid phase and diffusion of solute into the solvent, in other words, faster diffusion rate of polyphenols from the solid phase to the solution was attained. Furthermore, a long-term study showed that cocoa beans treated at optimal conditions and stored at 4 ºC maintain their total phenol content (ca. 115 ± 5.4 mg GAE/g) and reduced PPO activity (ca. 89 ± 3.8 %) with no significant change over 2 years ($p<0.05$). Finally, food-grade, fast, and suitable method for recovery of polyphenols from cocoa beans avoiding the defatting process was fulfilled.

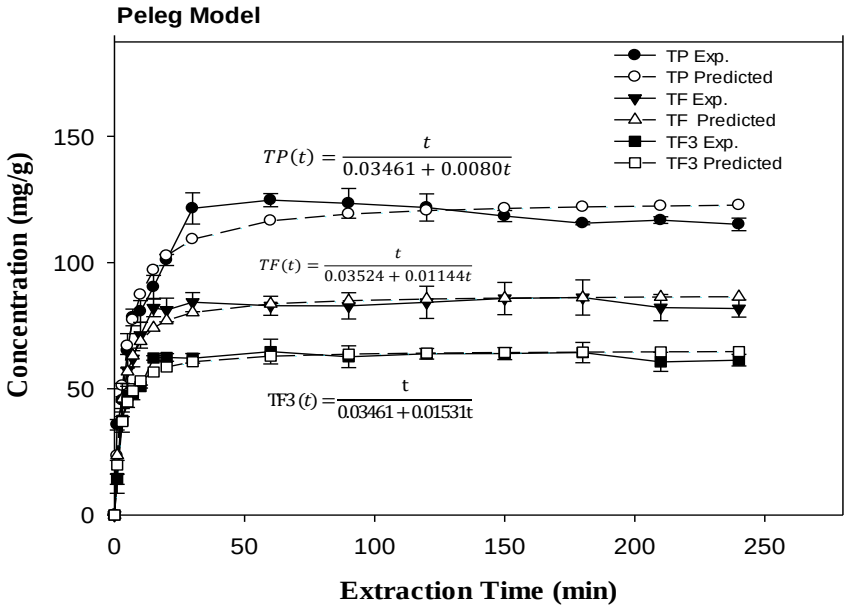

Figure 2. Experimental and calculated extraction curves for a total polyphenol (TP), total flavonoids (TF), and flavan-3-ols content (TF3) based on Peleg kinetic model.

Regarding characterization of polyphenolic extracts, several methods can be assayed such as spectrophotometric (Singleton, Orthofer, & Lamuela-Raventós, 1998; B. Sun, Ricardo-da-Silva, & Spranger, 1998; Zhishen, Mengcheng, & Jianming, 1999), NMR (Cuyckens & Claeys, 2004; Hansen et al., 1999), and HPLC by either normal phase (NP) (Gu, House, Wu, Ou, & Prior, 2006; Miller et al., 2006) or reversed phase (RP) (Tomas-Barberán et al., 2007; Toro-Uribe, Montero, López-Giraldo, Ibáñez, & Herrero, 2018) connected to tandem mass spectrometry detectors (Esatbeyoglu, Wray, & Winterhalter, 2015; Pedan, Fischer, & Rohn, 2016; Rodríguez-Carrasco, Gaspari, Graziani, Sandini, & Ritieni, 2018; Żyżelewicz et al., 2018). Recently, Robbins et al., (2009) developed an outstanding work for the separation of procyanidins according to their degree of polymerization employing a diol-column. Following this procedure, our cocoa extract was characterized, consisting of polymers with a degree of polymerization up to 14 (**Figure 3A**);

however, insight of isomeric forms cannot be assessed using normal phase. In this sense, a limited number of works reported the separation of proanthocyanidins and their isomeric forms by comprehensive liquid chromatography. For instance, LC×LC separation of cocoa procyanidins by stop-flow and offline modes has been published (Kalili & de Villiers, 2009; Kalili & De Villiers, 2013). In those works, the analysis consisted of two main steps: cocoa extract is fractionated in the first dimension and collected, and the collected fractions are analyzed individually into the second dimension. However, on-line comprehensive two-dimensional liquid chromatography approaches for procyanidins in a single run have not been reported yet.

Separation by focusing-modulated on-line comprehensive two-dimensional liquid chromatography coupled to tandem mass spectrometry was established. As shown in **Fig. 3B**, it is reinforced that LC×LC is a potent analytical tool since it combines two different separation dimensions for the separation of analytes. As the procyanidins are hydrophilic compounds, HILIC mode was selected to carry out the separation in the ^{1}D at low flow rate (20 $\mu L min^{-1}$). Subsequently, the sample was on-line separated by a short C_{18} partially-porous column in the ^{2}D at high flow rate (3.5 $mL min^{-1}$). Moreover, the use of trapping columns instead of loops in the interface was evaluated. Data showed significant focusing effect by trapping columns. As it is explained in **Chapter 3**, when trapping columns are used, peaks are slightly more separated as well as more intense, probably due to the minimization of the solvent strength mismatch produced and improvement of ^{2}D peak shapes.

Additionally, focusing configuration using active modulation, which consists of the use of additional make-up flow composed by a weak solvent for the ^{2}D was studied. The latter setup

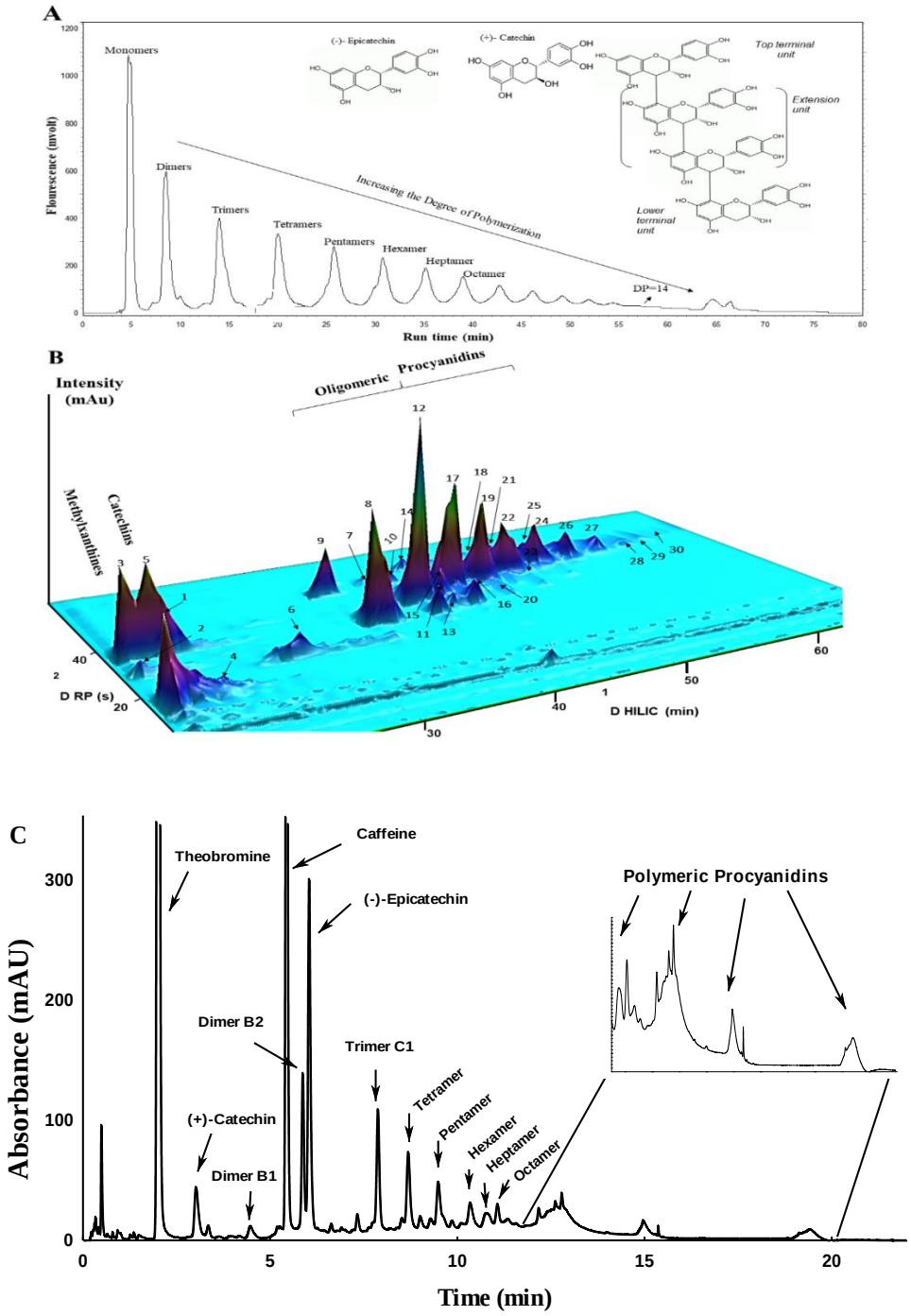

Figure 3. Separation of cocoa extract by (a) normal phase, (b) LCxLC, and (c) reverse phase. Number peaks are: 1, caffeine; 2, theophylline; 3, theobromine; 4, catechin; 5, epicatechin; 6, unknown compound; 7-9, dimers; 10-13, trimers; 14-17, tetramers; 18-20, pentamers; 21-23,

hexamers; 24-25, heptamers, 26, octamers; 27, nonamer, 28, decamer; 29, undecamer; and 30 dodecamer.

allowed complete elimination of solvent strength mismatch between ^{1}D and ^{2}D solvents at make-up flow rate of 100 $\mu L min^{-1}$.

According to the LC×LC chromatogram, theobromine and caffeine were the most abundant alkaloids while (-)-epicatechin followed by (+)-catechin were the significant flavan-3-ols. With regard to oligomeric procyanidins, these compounds are more difficult to assign due to the lack of commercial standards. In this regard, their tentative identification was based on mass spectra. Thus, 24 oligomeric procyanidins with a degree of polymerization up to 12 were effectively separated and tentatively identified (**Figure 3B**). In summary, the on-line method developed provides advantages such as full automation, faster analyses and less risk of analyte loss.

Analysis by reversed phases was also carried out. Two columns, C_{18}-phenyl (2.5 µm p.s.) and C_{18} (1.8 µm p.s.) were studied. Both columns allowed an excellent separation and resolution of alkaloids, flavan-3-ols, and oligomeric procyanidins up to octamers (**Figure 3C**). Moreover, the characterization was assessed by both ion trap and QTOF mass spectrometer detectors. As evidenced by the findings summarized in **Table 1**, the concentration of procyanidins obtained by diol-column was quite higher than those by C_{18} column. These differences could be as a consequence of all the isomeric procyanidins co-eluted in a single peak, thus increasing their intensity and concentration when diol-column is used. Overall, theobromine and (-)-epicatechin constitute 13.1 and 14.5% of major alkaloids and flavan-3-ols in the cocoa extract, respectively. In this regard, trimer was the most abundant procyanidin, being equal to 22.1% of whole cocoa extract, followed by tetramer (16.7%), pentamer (13.2%), dimer (9.1%), and hexamer (6.3%). Higher oligomers were detected but not quantified due to the lack of standards.

Table 1. Characterization of alkaloids, catechins, and procyanidins from cocoa beans obtained by using reverse and normal phases as well as mass pattern by UHPLC-QTOF-MS.

Compounds	Diol-Column	C_{18}-phenyl column	C_{18} column	UHPLC-QTOF-MS	
	Concentration (ppm)			MS (*m/z*)	MS/MS Fragments (*m/z*)
Alkaloids $[M+H]^+$					
Theobromine	5.6±0.1	7.8 ± 0.0	8.1±0.8	181.0724	158.0260, 122.9975, 104.0602
Caffeine	3.2±0.1	2.7 ± 0.0	3.1±0.4	195.0881	138.0663, 110.0668, 77.0365
Flavan-3-ols ($[M-H]^-$					
(+)-Catechin	0.7±0.0	0.6 ± 0.0	1.4±0.2	289.0738	245.0820, 203.0713, 1125.0238
(-)-Epicatechin	8.1±0.4	7.3 ± 0.1	8.6±0.6	289.0742	245.0830, 203.0720, 125.0246
Oligomeric proanthocyanidins $[M-H]^-$					
Dimer B1	*	*	0.3±0.2	577.1375	407.0771, 289.0731, 125.0248
Dimer B2	4.7±0.5	4.1 ± 0.0	5.9±0.2	577.1358	407.0781, 289.0727, 125.0246
Trimer C1	13.5±4.4	12.0 ± 0.3	11.1±0.9	865.1993	713.1509, 575.1200, 287.0564
Tetramer	9.7±3.0	9.3 ± 0.4	8.6±0.5	1153.2624	865.1971, 575.1197, 287,0564
Pentamer	8.1±3.1	6.8 ± 0.5	7.0±1.4	1442.3301	1153.2597, 863.1834, 287.0551
Hexamer	5.4±0.4	1.8 ± 0.0	*	1730.3923	1151.2449, 863.1826, 287.0723

Doubly-charged proanthocyanidins $[M-2H]^{2-}$					
Heptamer	*	*	*	1008.7250	863.1819, 577.1364, 289.0723
Octamer	*	*	*	1152.7555	865.2007, 575.1195, 287.0562
Nonamer	*	*	*	1296.7858	1152.2475, 863.1834, 287.0568
Decamer	*	*	*	1440.8102	1151.2432, 863.1846, 287.0578
Triply-charged proanthocyanidins $[M-3H]^{3-}$					
Undecamer	*	*	*	1056.8999	1008.2214, 865.1855, 575.1255
Dodecamer	*	*	*	1152.2624	865.1974, 575.1209, 287.0581

Data expressed as means of triplicate experiments. Concentration expressed as mg polyphenol per g cocoa beans (dry weight basis), *detected but not quantified. Fluorescence and UV detectors set at 230/321 nm emission/excitation wavelength and 280 nm, respectively.

In view of increasing interest of high polymeric procyanidins, the cocoa extract was fractionated by employing three different techniques: Sephadex LH-20 gel permeation, solid-phase separation by coupling Diol-C_{18} cartridges, and semi-preparative HPLC through both C_{18} and diol stationary phases. Sephadex LH-20 separation was based on Kennedy & Taylor (2003) whereas the solid-phase separation and semi-preparative LC was developed and optimized in the present Thesis. In general, classical techniques allowed good separation of alkaloids and procyanidins up to trimers but were inefficient for higher oligomeric forms. Regarding semi-preparative separations by HPLC, results are, by far, better than classical approaches. Interestingly, the solvents used in C_{18} separation consisted of water-acetic acid and ethanol. Therefore, a food-grade and cheap strategy to isolate procyanidins with high purity was developed. However, fractions enriched with polymer sizes higher than five (pentamer) had a purity lower than 80%.

Moreover, diol stationary phase employing acetonitrile-acetic acid and methanol-water-acetic acid as solvents was investigated. As explained in **Chapter 4**, eleven fractions up to decamers were collected and separated by increasing degree of polymerization. These fractions were formed exclusively by isomeric forms with impurities lower than 5%. Overall, B-type interflavan linkage was more frequent than A-type linkages and consistent with quinone methide (QM), retro-Diels-Alder (RDA), and heterocyclic ring fissions (HRF) fragmentation pathway mechanisms. In some fractions, lower concentrations of A-type procyanidins were found, e.g., trimer and tetramer with *m/z* 863.1868 (-2Da) and 1149.3679 (- 4Da), respectively. Interestingly, for the first time, the presence of *m/z* 739.1888, 1025.2326, and 1313.2987 were detected, which can be attributed to A-type procyanidins (one or two type) linked to glucose moiety (e.g., β-galactopyranose, α-arabinopyranose and glucopyranosyl); the presence of novel configuration of dimer (*m/z* 575.1195 and 561.1358), trimer (*m/z* 849.2045), tetramer (*m/z* 1313.2987), pentamer (*m/z* 1425.3268), and hexamer (*m/z* 864.1901, $[M-2H]^{-2}$) was determined as well. These observations highlight the importance of high-resolution MS as a useful tool for the characterization of cocoa proanthocyanidins as well as to be employed in further studies involving their complete characterization, isolation, and metabolomic fate.

Once the isolated procyanidins were obtained, the following step was the encapsulation process. In order to do this, liposome delivery system was selected mainly because of its amphiphilic nature, lower particle size and feasibility to be produced using GRAS components. Nevertheless, not only high encapsulation and stability should be considered during the incorporation of bioactive agents into lipid carriers but also chemical oxidation because the carrier is susceptible to lipid oxidation.

Since 1997, Huang & Frankel (1997) evaluated the oxidative effect of green tea catechins on soybean lecithin liposomes. They found that the antioxidant activity to prevent the formation of

primary or secondary lipid oxidation products depend on the dose tested and catechin configuration. This is because of the greater affinity of the polar tea catechin gallates for the polar surface of the lecithin bilayers, thus affording better protection against oxidation. In line with this work, grape seed extract was incorporated into chitosan-coated liposomes. This work reported that grape extract effectively inhibits the formation of free radicals, thus prolonging the oxidative stability of phospholipids. These examples highlight that the effectiveness of antioxidants is strongly related to the ability of the antioxidants to partition at the location where lipid oxidation is most prevalent (Choe & Min, 2009; Decker, Warner, Richards, & Shahidi, 2005).

To the best of our knowledge, there are not studies focusing on procyanidins with different degree of polymerization and their impact on physical properties, partitioning, and antioxidant activity within the liposomes. As already mentioned, cocoa procyanidins are unique in that they are highly formed by (epi)catechin units and can form oligomers and polymers of these units. These chemical characteristics could provide unique partitioning properties into liposomes.

Effective design of liposome system was studied as stated in **Chapter 5** and **6**. As well-explained in **Chapter 6**, two homogenization methods were examined. Overall, sonication had better performance than microfluidization. In addition, combination of several parameters (e.g., concentration of lecithin, pH strength, sample concentration, time and intensity of sonication) were independently assayed. Optimal formulation by sonication consisted of pulse at 75% intensity for 7 min (10 s on /off pulses) with soy lecithin (5 wt.%, pH 5.0 ± 0.1, 0.1 M). Besides, sonication requires less amount of sample (no loss during processing) and lower processing time, therefore, lower energy and production costs. Under these conditions, samples were highly stable compared to empty liposomes. For instance, epicatechin- and cocoa extract-loaded liposomes showed a lag phase of lipid oxidation higher than 200 days at 4 ºC and 70 days at 55 ºC.

Theobromine, caffeine, (-)-epicatechin, (+)-catechin, procyanidins up to hexamers, and cocoa extract-loaded liposomes were fabricated. In general, liposome samples were characterized to have a particle size lower than 130 nm, encapsulation efficiency lower than 0.1% for alkaloids, 37 – 55 % for monomers and 72 – 98 % for oligomers. In this order, ζ-potential ranged from -32 to -41 mV and -18 to -23 mV, for pH 3 and 5, respectively. To confirm the small particle size, transmission electron microscopy was assayed. As shown in **Figure 4**, all the samples displayed particle sizes smaller than 150 nm, together with the formation of clusters through the contact of interlayer lipid bilayers. An influence of encapsulated compounds in the structure of liposomes was also observed. For instance, control liposome (**Fig. 4A**) is formed by small unilamellar vesicles with highly organized structure while procyanidins and cocoa extract did not. As can be seen in **Figure 4 B-G**, the nature of procyanidins had a significant impact on both particle size distribution and capture volume of the aqueous core of the vesicle. In general, cocoa extract-loaded liposomes exhibited the most diverse structure (**Fig. 4H**), which is formed by small multilamellar vesicles linked through bilayer heterojunctions. Therefore, cross-section particle sizes ranged from 30 to 115 nm, membrane thickness was around 40 Å and fully hydrated bilayers were over 60 Å.

Finally, results showed that cocoa polyphenols and all procyanidins inhibited lipid hydroperoxide and hexanal formation; in fact, the antioxidant activity increased with increasing polymer chain size. The higher antioxidant activity of the isolated procyanidins was likely due to their increased metal chelating capacity and radical scavenging activity as determined by FRAP and DPPH assays, respectively; and the greater partitioning of oligomers into the lipids and encapsulation efficiency. The crude extract had the greatest antioxidant activity which could be due to the presence of other antioxidants or combinations of different polyphenols that could inhibit lipid oxidation synergistically.

The final stage of this Ph.D. focused on studying the bioaccessibility of encapsulated procyanidins up to pentamer and cocoa polyphenols *vs* to those samples attainable in aqueous solutions. Parameters such as particle size, ζ-potential, microscopy pictures, release kinetic profile, bioaccessibility, and antioxidant magnitude were assessed. In summary, the oral phase did not affect the sample stability significantly due to the short residence time (2 min). However, during the gastric (2 h) and duodenal (6 h) phases significant changes ($p<0.05$) were observed. For instance, during the gastric phase particle size increased while ζ-potential reduced. These phenomena were confirmed due to acidic environment that impacts the surface membrane ionization state and charge distribution of phosphatidylcholine heads; moreover, the swelling effect of liposomes at gastric phase led to the formation of a complex macromolecular structure with changes in the surface properties (**Chapter 6**). The main losses in all the formulations were observed during the intestinal phase because of the deprotonation of flavonoids at alkaline pH, which increased the radical scavenging capacity of samples. Indeed, strong antioxidant activity by ABTS and DPPH assays was found for all the samples, mainly for liposome formulations at the end of digestion.

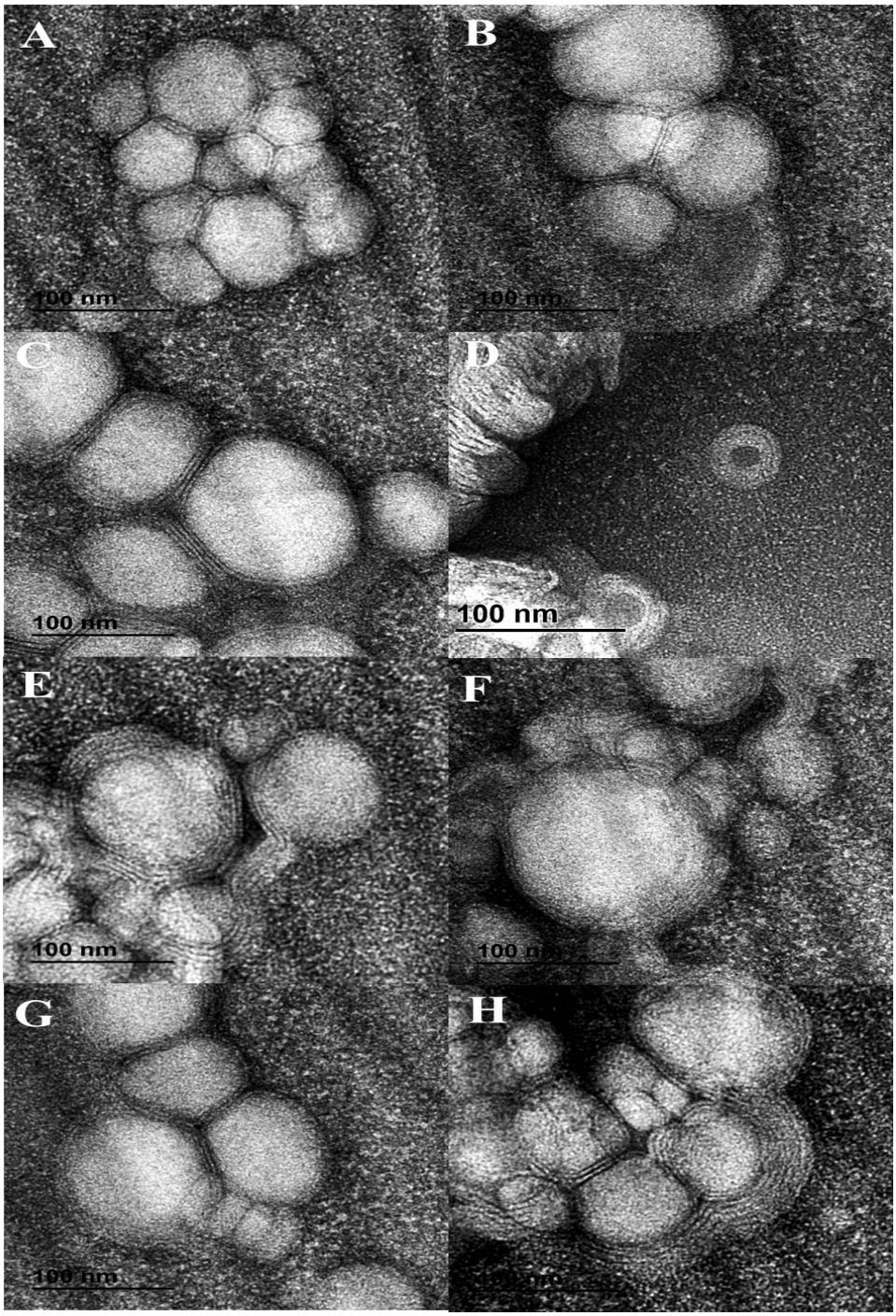

Figure 4. TEM picture of (A) empty liposome and (B) theobromine, (C) epicatechin, (D) dimer, (E) trimer, (F) tetramer, (G) pentamer, (H)cocoa extract-loaded liposomes.

Regarding kinetic release profile, all the samples in aqueous solution decreased their concentration along the transit time. On the contrary, a concentration burst effect during the small intestine phase for flavonoids-loaded liposomes was observed, it meant, prolonged and sustained drug-release profile. These facts were in line with our TEM observations, that is, changes on pH, osmolarity and interaction of digestive compounds (e.g., bile salts and pepsin) caused a perturbation of membrane (e.g., fully fused and breakage of lipid bilayers), thus mediating the release of bioactive agents into the target site.

Additionally, the analysis of samples by UHPLC-QTOF demonstrated:

- Epimerization of (+)-catechin → (-)-epicatechin, and (-)-epicatechin → (+)-catechin.
- Isomeric forms epimerized to the most stable structure.
- The degradation rate of procyanidin into small molecular polymer was lower for liposome formulations than those in free form.
- The time taken to attain peak burst effect on liposomes after gastric phase was influenced by the polyphenol structure. This is a result of high diversity, and interindividual variability observed among of procyanidins samples, thus impacting their diffusion rate kinetic and final bioaccessibility.
- The sum of oligomeric procyanidins was higher in liposome formulations than in aqueous system while the opposite during the gastric phase was observed.
- Degradation mechanism of procyanidins consisted of depolymerization process. Therefore, samples exposed to acidic pH-triggered depolymerization of oligomers into small molecular weight compounds, mainly for those in non-encapsulated form.

In summary, theobromine and caffeine were highly stable during the digestion, in particular for the formulations in an aqueous system. On the contrary, the procyanidins- and cocoa extract-loaded liposomes were more efficient than those in an aqueous system. For example, at gastric phase, losses up to 62% for procyanidins from cocoa extract in non-encapsulated form were seen while cocoa extract-loaded liposomes displayed low degradation (<4.2%). Bioaccessibility of catechin-, epicatechin-, dimer-, trimer-, tetramer-, pentamer-loaded liposomes were 2.0, 2.2, 1.4, 2.4, 2.7, and 2.3-fold higher than those compounds in non-encapsulated form. Similar trend was found for cocoa extract-loaded liposomes, where catechin, epicatechin, dimer, trimer, and tetramer were 2.3, 2.2, 4.5, 2.0, and 9.3-fold higher than same compounds from free cocoa extract. Furthermore, higher antioxidant status of liposome is due to the higher presence of phospholipids, which led to a more significant content of micelles that solubilize the phenolic compounds. That means that polyphenols do not act alone; therefore, they could be interacting with the micelle system to increase their solubility together with the formation of unknown compounds with high antioxidant activity as result of pH rather than enzymatic action during the gastrointestinal tract.

In terms of going beyond the highlights, this Ph.D. study confirmed the potential to advantages of encapsulating both oligomeric procyanidins and polyphenol extracts into liposome carrier. The liposome system was able to a) protect the target bioactive in its original nature and b) transport it to the intestinal phase with higher bioaccessibility than those compounds in non-encapsulated form, and c) may lead to a higher bioavailability and intestinal uptake.

GENERAL CONCLUSIONS

Based on the findings stated in **Chapters 1** to **4**, the conclusions are divided into three stages as follows:

a) Extraction, characterization, and isolation of polyphenols from cocoa beans:

- Two processes consisting on enzyme inhibition and recovery of polyphenols from cocoa beans were optimized. Both procedures were characterized by being suitable for food-grade applications. Operating conditions to avoid degreasing and lyophilization were established, thus leading to a more cost-effective option. The optimized factors allowed to reduce by 93.3% PPO activity and increase on 27.0 % polyphenol yield; therefore, total content of polyphenols, flavonoids, and flavan-3-ols on cocoa beans were 122.3 ± 2.4 $mgGAEg^{-1}$, 88.9 ± 0.8 $mgECEg^{-1}$and 62.6 ± 3.4 $mgECEg^{-1}$, respectively.
- The characterization of cocoa beans was carried out by both reverse and normal phase LC coupled to tandem mass spectrometry detectors. Furthermore, a focusing-modulated on-line comprehensive LC×LC-DAD-ESI-MS method for the characterization of procyanidins was developed. These techniques confirmed that cocoa extract is predominantly composed by theobromine, (–)-epicatechin and trimer as more abundant alkaloid, flavan-3-ol, and procyanidin, respectively. The major procyanidins were consistent with B-type configuration and in less extent A-type.
- Conventional separation techniques such as Sephadex LH-20 gel permeation and solid-phase separation were compared with semi-preparative liquid chromatography with

both C_{18}- and diol-columns. Analysis by UHPLC-DAD-QTOF-MS confirmed that semi-preparative separation was, by far, better than classical separations. Moreover, diol stationary phase was the most efficient fractionation method, isolating the procyanidins by increasing degree of polymerization.

b) Encapsulation and characterization of procyanidins- and cocoa extract-loaded liposomes:

- Liposome formulation based on food grade ingredients was fabricated by sonication method. Soy lecithin liposomes were formed by small multilamellar vesicles with particle sizes ranging from 30 – 150 nm and were highly efficient carrier system for catechins, procyanidins, and cocoa extract.
- Sonicated liposomes showed good physical and oxidative stability over time, mainly for oligomeric procyanidins and cocoa extract. It was confirmed that the polymer size impact on partitioning distribution, encapsulation efficiency, particle size and ζ-potential, thus influencing their antioxidant performance.
- FRAP, DPPH, and ORAC assays showed that by increasing the polymer size, also increased the antioxidant activity, which agreed with the ability of oligomeric procyanidins to control lipid oxidation on liposome system.

***In vitro* gastrointestinal digestion of unloaded and loaded-liposome formulations:**

- Samples-loaded liposomes played an important role on the release profile, concentration burst effect, and bioaccessibility of procyanidins at the end of digestion. Catechins, isolated procyanidins up to pentamers, and cocoa extract encapsulated into liposomes showed *in vitro* sustained release profile and high bioaccessibility compared to

those in non-encapsulated form. The opposite was observed for theobromine and caffeine which were more stable to digestion in non-encapsulated form.

- To understand the transformation of samples during digestion, analysis by UHPLC-DAD-QTOF-MS was carried out. It was demonstrated that the mechanism of polyphenols consisted of depolymerization into small molecular weight procyanidins, as well as, epimerization into the most stable structures. Moreover, TEM pictures confirmed that the gastric and duodenal environment impact on structure, arrangement, and stability of liposomes.
- Based on the strong scavenging free radical activity at the end of *in vitro* digestion, it is suggested that polyphenols do not act alone because they could interact with the micelle system, thus increasing their solubility together with the formation of unknown compounds.

This Ph.D. highlights the potential of food-grade liposome system to efficiently encapsulate and delivery bioactive compounds; in particular, to increase the solubility and *in vitro* bioaccessibility of catechins, procyanidins, and cocoa extract.

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LIST OF WORKS INCLUDED IN THE DOCTORAL DISSERTATION

I. VISITING RESEARCH FELLOW

1. University of Massachusetts

   Adviser: Eric A. Decker

   Group: Chenoweth Lab., Food Science Department

   Place: U.S.

   Year: 2015 – 2017

2. Universidad Autónoma de Madrid

   Adviser: Elena Ibañez,

   Co-adviser: Miguel Herrero

   Group: Foodomics Lab, Instituto de Investigación en Ciencias de la Alimentación.

   Place: Madrid

   Year: 2017 - 2018

II. PUBLISHED WORKS

A. Research Publications

1. Relationship between the physiochemical properties of cocoa procyanidins and their ability to inhibit lipid oxidation in liposomes.

   Authors: Said Toro-Uribe, Luis J. López Giraldo, Eric A. Decker

   Journal: Journal of Agricultural and Food Chemistry

   Year: 2018

2. Characterization of secondary metabolites from green cocoa beans using focusing-modulated comprehensive two-dimensional liquid chromatography coupled to tandem mass spectrometry.

   Authors: Said Toro-Uribe, Lidia Montero, Luis López-Giraldo, Elena Ibáñez, Miguel Herrero.

   Journal: Analytica Chimica Acta

   Year: 2018

3. Design, fabrication, characterization and *in vitro* digestion of alkaloid-, catechin-, and cocoa extract-loaded liposomes.

   Authors: Said Toro-Uribe, Elena Ibáñez, Eric A. Decker, David Julian McClements, Ruojie Zhang, Luis Javier López-Giraldo, Miguel Herrero.

   Journal: Journal of Agricultural and Food Chemistry

   Year: 2018

B. Book Chapters:

Encyclopedia of Analytical Science. Omics Technology: Foodomics. Third Edition.

Authors: Gerardo Álvarez-Rivera, Said Toro-Uribe, Elena Ibañez, Alejando Cifuentes, Mónica Bueno.

Publishers: Elsevier

Year: 2018

C. Other Publication(s):

Cocoa Husk as Source of Natural Phenolic Antioxidants: Comparison of Polyphenols and Antioxidant Activity in Theobroma Cacao Beans and Husk.

Authors: Said Toro-Uribe, Mauren R. Estupiñan, Luis Javier López-Giraldo

Journal: Facultad Nacional de Agronomía

Year: 2014

III. ARTICLES UNDER REVIEW

Research Publications

1. Preparative separation of procyanidins: comparative study of different fractionation techniques.

   Authors: Said Toro-Uribe, Miguel Herrero, Eric A. Decker, Luis López-Giraldo, Elena Ibáñez.

   Journal: Journal of Chromatography A

   Year: 2018

2. Insight of polyphenol oxidase enzyme inhibition and total polyphenol recovery from cocoa beans.

   Authors: Said Toro-Uribe, María X. Quintanilla-Carvajal, Jhair Godoy-Chivatá, Elena Ibañez, Janeth A. Perea Villamil, Arley Villamizar Jaimes, María de Jesús Perea-Flores, Luis J. López-Giraldo.

   Journal: Food Research International

   Year: 2018

3. Food-safe process for high recovery of flavonoids from cocoa beans: antioxidant and HPLC- DAD-ESI-MS/MS analysis.

   Authors: Said Toro-Uribe, Jhair Godoy-Chivatá, Luis Javier López-Giraldo, Elena Ibañez, Eric A. Decker.

   Journal: Food Research International

   Year: 2018

4. Insight of stability of procyanidins in free and liposomal form under *in vitro* digestion model: study of bioaccessibility, kinetic release profile, transformation and antioxidant activity.

   Authors: Said Toro-Uribe, Luis Javier López-Giraldo, Gerardo Álvarez-Rivera, Elena Ibáñez, Miguel Herrero.

   Journal: Journal of Agricultural and Food Chemistry

   Year: 2018

IV. FORTHCOMING PUBLICATIONS

1. Characterization and antioxidant activity of cocoa procyanidins in oil-in-water emulsions.

   Authors: Said Toro-Uribe, Eric A. Decker

2. Identification of new A-type proanthocyanidins from cocoa beans by UHPLC-DAD-QTOF-MS.

   Authors: Said Toro-Uribe, Gerardo Álvarez, Aidé Perea Villamil, Luis J. López Giraldo, Elena Ibañez, Miguel Herrero.

3. Elucidation of new structures derived from *in vitro* digestion of cocoa procyanidins by UHPLC-QTOF-MS.

   Authors: Said Toro-Uribe, Gerardo Álvarez, Luis J. López Giraldo, Elena Ibañez, Miguel Herrero.

4. Effect of milk and chocolate beverage matrix on the bioaccessibility of cocoa procyanidins with and without liposome delivery system under *in vitro* digestion model.

   Authors: Said Toro-Uribe, David J. McClements, Luis J. López Giraldo, Elena Ibañez, Miguel Herrero.

5. Impact of Microencapsulation on lipid digestion and control bioaccessibility for alpha and beta carotenes: comparison between emulsions and powders with High Oleic Palm Oil

   Authors: María Ximena Quintanilla-Carvajal, C. Sandoval, Said Toro-Uribe, Ruojie Zhang, David J. McClements.

   Year: 2018

V. AWARDS

1. Eloy Valenzuela

   Authors: Said Toro-Uribe, Jhair E. Godoy, Arley Villamizar, Aidé Perea, Elena Ibañez, Luis J. López Giraldo.

   University: Universidad Industrial de Santander

   Year: 2018

1. Administrative Department of Science, Technology and Innovation (COLCIENCIAS)

   To: Said Toro-Uribe

   Grant: 567, National Scholarship

   Year: 2013 – 2017

2. Scholarship: Workshop Cocoa and Chocolate Processing

   To: Said Toro-Uribe

   Grant: VLIR-UOS Program

   University: Ghent University (Belgium)

   Year: 2015

VI. UNDERGRADUATE RESEARCH ADVISING

1. Análisis preliminar de la simulación y factibilidad económica del proceso de recuperación de polifenoles a partir de cascara de cacao.

   Author: Silvia F. Barragan Mantilla, Sergio A. Villamizar Delgado

   Adviser: Luis J. López-Giraldo, Said Toro-Uribe

   University: Universidad Industrial de Santander, Chem. Eng. Department

   Year: 2014

2. Obtención de antioxidantes a partir de grano de cacao: condiciones favorables de inactivación de la enzima polifenol oxidasa, extracción de polifenoles y ajuste de parámetros cinéticos.

   Author: Jhair Enrique Godoy Chivata

   Year: 2015

   Adviser: Luis J. López-Giraldo, Said Toro-Uribe

   University: Universidad Industrial de Santander, Chem. Eng. Department

   Award: Outstanding Undergraduate Thesis (Acuerdo No. 249. de 2016)

VII. CONFERENCE PRESENTATIONS (Ph.D. Related)

A) Oral Presentations

1. Isolation and Characterization of A- and B-type of Cocoa Proanthocyanidins by UHPLC-QTOF-MS/MS (forthcoming)

   Conference: XVIII Reunión de la Sociedad Española de Cromatografía y Técnicas Afines

   Authors: Said Toro-Uribe, Gerardo Álvarez, Aide Perea V., Luis J. López Giraldo, Elena Ibañez, Miguel Herrero.

   Place: Spain

   Year: 2018

2. Study of Cocoa Procyanidins: Extraction, Characterization, and Purification.

   Conference: 15th Instrumental Analysis Conference – Secyta

   Authors: Said Toro-Uribe, Lidia Montero, Luis J. López Giraldo, Elena Ibañez, Miguel Herrero.

   Place: Spain

   Year: 2017

3. Cocoa Diversity and Quality in Southern Mexico

   Conference: CacaoNet/INGENIC. Frontiers of Cacao Science and Technology for Cacao Quality, Productivity and Sustainability.

   Authors: Hugo F. Chavez Ayala, David Condori, Julio Geronimo, Said Toro-Uribe

   Place: PenState University, College of Agricultural Science – U.S

   Year: 2016

4. Process for Obtaining Polyphenols-Rich Extract from Mango By-products.

   Conference: 9th World Congress on Polyphenols Applications

   Authors: Said Toro-Uribe, Ana M. Rosso, Luis J. López Giraldo

   Place: Malta

   Year: 2015

5. Evaluation of Five Varieties of Cocoa Husk as a Source of Polyphenols: Effect of Solid-Liquid Extraction.

   Authors: Said Toro-Uribe, Mauren Estupiñan, Luis J. López Giraldo.

   Conference: 9th World Congress on Polyphenols Applications.

   Place: Malta

   Year: 2015

6. Cocoa Husk as a Source of Natural Antioxidants: Simulation, Economic Analysis, and Analytical Hierarchy Process.

   Conference: 9th Conference on Sustainable Development of Energy Water and Environment System

   Authors: Said Toro-Uribe, Ana M. Rosso, Luis J. López Giraldo

   Place: Istanbul

   Year: 2014

B) Poster Presentations

1. Antioxidant Effect of Cocoa Procyanidins-loaded Liposomes.

   Conference: A Foods for Health Conference

   Authors: Said Toro-Uribe, Eric A. Decker

   Place: Thailand

Year: 2018

2. Antioxidant Effect of Flavan-3-ols in Liposomes.

   Conference: Euro Fed Lipid

   Authors: Said Toro-Uribe, Luis J. López Giraldo, Eric A. Decker

   Place: Portugal

   Year: 2016

3. Extracción, Caracterización y Evaluación Antioxidante de Extractos Polifenólicos Obtenidos de la Cacota del Cacao: Simulación Preliminar del Proceso de Extracción.

   Conference: II Congreso Colombiano de Estudiantes de Ingeniería de Alimentos

   Authors: Said Toro-Uribe, Sergio Villamizar, Silvia Barragan, Luis J. López Giraldo.

   Place: Colombia

   Year: 2015

4. Cocoa Husk as a Source of Natural Antioxidants: Comparison of Polyphenol and Antioxidant Activity in *Theobroma Cacao* Beans and Husk.

   Conference: II Congreso Internacional de Investigación e Innovación en Ingenería, Ciencia y Tecnología de Alimentos

   Authors: Said Toro-Uribe, Mauren Estupiñan, Luis J. López Giraldo.

   Place: Colombia

   Year: 2014

5. Obtención de Productos de Valor Agregado a Partir de Cáscara de Cacao.

   Conference: Seminario Internacional de Cacao y Chocolate

   Authors: Said Toro-Uribe, Mauren Estupiñan, Luis J. López Giraldo.

Place: Colombia

Year: 2013