Optimization of the Extraction of Procyanidin B-2 Rich Extract from Unfermented Cocoa Using Response Surface Methodology and Interaction of Procyanidin B-2 Rich Cocoa Extract with Collagenase and Elastase as Biomarkers of Skin Aging

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OPTIMIZATION OF THE EXTRACTION OF PROCYANIDIN B-2 RICH EXTRACT FROM UNFERMENTED COCOA USING RESPONSE SURFACE METHODOLOGY AND INTERACTION OF PROCYANIDIN B-2 RICH COCOA EXTRACT WITH COLLAGENASE AND ELASTASE AS BIOMARKERS OF SKIN AGING

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Master of Science

in

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by

Marco Eduardo Toc Sagra
B.S., Zamorano University, 2013
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ABSTRACT

Skin ageing is a natural process accelerated by environmental exposure that generate reactive oxygen species and activates enzymes that degrade the integrity of the dermis. Cocoa powder (*Theobroma cacao*) is a good source of polyphenols, particularly procyanidins and flavan-3-ols, with potential enzyme inhibitory activities. In this study we determined whether cocoa extraction standardized to its procyanidin B-2 content has a potential application as anti-ageing bioactive compound. Oil-bath-assisted extraction (OBAE) and microwave-assisted extraction (MAE) were optimized for procyanidin B-2 rich extract using response surface methodology and water as solvent. Methanol (70% in water) extraction was the control to which other extraction methods were compared. The molecular masses distributions of the extracts were evaluated using MALDI-TOF-MS. The best extraction method was compared to methanol extraction through the extract inhibitory effect on collagenase and elastase. OBAE optimal conditions were 1:16 cocoa:water ratio, 48 min, and 115°C; while, MAE optimal conditions were 1:16 cocoa:water ratio, 13 min, and 115°C. The procyanidin B-2 content from OBAE (8.90 mg/g cocoa) was significantly (*P* < 0.05) greater than MAE (7.64 mg/g cocoa) or methanol (7.07 mg/g cocoa) extract. Methanol extract showed more compounds peak distribution than OBAE or MAE. Most of the compounds extracted were below 1000 Da regardless of the method of extraction. Methanol extraction was more effective in inhibiting collagenase or elastase activities than OBAE than epigallocatechin gallate, a well-known natural inhibitor of these two enzymes. Regardless of the method of extraction, this study has shown promising results for the use of cocoa extract as an active ingredient for the cosmetic industry.
CHAPTER 1: INTRODUCTION

Skin ageing is a complex process associated with genetic, hormonal and extrinsic factors. The extrinsic factors resulting from environmental exposure are relevant due to the generation of reactive oxygen species which activates matrix metalloproteinases (i.e. collagenases) and serine proteases (i.e. elastases) that degrade the extracellular matrix proteins in the dermis such as collagen and elastin (Scharffetter–Kochanek et al., 2000).

The demand for products designed to help in the prevention and treatment of skin ageing continues to grow (Manela-Azulay & Bagatin, 2009). Several studies have demonstrated that certain compounds present in plants have anti-ageing effect, as a consequence, plant extracts are used in cosmetic formulation (Aburjai & Natsheh, 2003). For instance, Lee et al. (1999) evaluated the inhibitory effects of 150 plant extracts on elastase activity. It was found that extracts from plants such as Areca catechu, Cinnamonum cassia and Myristica fragrans showed a high inhibition against elastase. This study concluded that the anti-elastase properties of these extracts might be useful for application in cosmetics. Similarly, a recent study carried out by do Nascimento Pedrosa et al. (2016) found that flavonoid extract from Libidibia ferrea has high potential as a cosmetic ingredient because it significantly inhibited the activity of elastase, hyaluronidase and collagenase, enzymes that cause skin degradation.
The method of extraction plays an important role in the isolation of the bioactive compounds. There are several methods that encompass the extraction of bioactive compounds such as microwave-assisted extraction and solid-solvent extraction. However, the best extraction conditions to isolate plant bioactive compounds are needed.

Cocoa powder contain theobromine, caffeine, and polyphenols, particularly procyanidins and flavan-3-ols (Kadow et al., 2013). Procyanidins act as potent antioxidants and can provide health benefits such as reducing the risk of cardiovascular disease, cancer and other diseases (Khan et al., 2014; Mellor et al., 2010; Lamuela-Raventós et al., 2005). However, the optimization of cocoa bioactive compounds extraction and their evaluation as anti-ageing has not been investigated before. Thus, in this study we determined whether cocoa extraction standardized to its procyanidin-B2 content can inhibit collagenase or elastase as a skin ageing biomarker. Specifically, the aims of the study were to:

1) Determine the best extraction conditions for procyanidin B-2 by oil-bath-assisted extraction, or microwave assisted extraction using response surface methodology and water as solvent. Comparison was made with methanol extraction.

2) Evaluate the effect of temperature and the molecular mass distribution of bioactive compounds extracted using MALDI-TOF-MS.

3) Evaluate the inhibitory effect of cocoa extracts on the activity of collagenase or elastase.
CHAPTER 2: LITERATURE REVIEW

2.1 Characterization of phenolic compounds: procyanidins

Phenolic compounds are considered the most important and diverse group of compounds in the plant kingdom. These molecules are synthesized during the normal growth of the plant, as well as in response of stress, UV radiation, among others (Naczk & Shahidi, 2004). The amount of phenolic compounds in plants is strongly dependent on the variety, climate, soil composition, geographic location and storage conditions, among other factors (Belitz et al., 2009).

A phenolic compound is one or more aromatic rings, in this case a benzene, which have one or more hydroxyl groups directly linked to it. Harbone and Simmonds (1964) developed a complete phenolic compound classification which is widely used nowadays. They classified these compounds into groups based on the number of carbons in the molecule as shown in Table 1.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Class</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₆</td>
<td>Simple phenolics</td>
</tr>
<tr>
<td>C₆–C₁</td>
<td>Phenolic acids and related compounds</td>
</tr>
<tr>
<td>C₆–C₂</td>
<td>Acetophenones and phenylacetic acids</td>
</tr>
<tr>
<td>C₆–C₃</td>
<td>Cinnamic acids, coumarins, isocoumarins, and chromones</td>
</tr>
<tr>
<td>C₁₅</td>
<td>Chalcones, aurones, dihydrochalcones</td>
</tr>
<tr>
<td>C₁₅</td>
<td>Flavans</td>
</tr>
<tr>
<td>C₁₅</td>
<td>Flavanones</td>
</tr>
<tr>
<td>C₁₅</td>
<td>Flavanonols</td>
</tr>
<tr>
<td>C₁₅</td>
<td>Anthocyanidins</td>
</tr>
<tr>
<td>C₁₅</td>
<td>Anthocyanins</td>
</tr>
<tr>
<td>C₃₀</td>
<td>Biflavonyls</td>
</tr>
<tr>
<td>C₆–C₁–C₆, C₆–C₂–C₆</td>
<td>Benzophenones, xanthones, stilbenes</td>
</tr>
<tr>
<td>C₆, C₁₀, C₁₄</td>
<td>Quinones</td>
</tr>
<tr>
<td>C₁₈</td>
<td>Betacyanins</td>
</tr>
<tr>
<td>Lignans, Neolignans</td>
<td>Dimers or oligomers</td>
</tr>
<tr>
<td>Lignin</td>
<td>Polymers</td>
</tr>
<tr>
<td>Tannins</td>
<td>Oligomers or polymers</td>
</tr>
<tr>
<td>Phlobaphenes</td>
<td>Polymers</td>
</tr>
</tbody>
</table>
Typically, when discussing about phenols in plant foods, flavonoids are the predominant class because they account for approximately 66% of the dietary phenols (Robbins, 2003). There are more than 6000 flavonoids and divided in 7 families which include flavones, flavonol, isoflavones, flavanones, flavanonol, flavanol and anthocyanidin (Harborne & Williams, 2000). Flavonoids have in common an A-, B-, and C-ring, and are depicted with the A-ring on the left-hand side (Figure 1).

Figure 1: Chemical structures of common flavonoids (de Pascual-Teresa, S., Moreno, D. A., & García-Viguera, C., 2010).

Amongst flavonoids, catechin and epicatechin (flavan-3-ols) are known collectively as flavanols and represent the largest class of monomeric units (de Pascual-Teresa, S., Moreno, D. A., & García-Viguera, C., 2010). However, flavanols are commonly found in their polymerized forms known as condensed tannins or proanthocyanidins (Vermerris & Nicholson, 2008).
According to Hemingway and Karchesy (1989), proanthocyanidins are divided into two categories based on their A ring classification: phloroglucinol and resorcinol (Figure 2). The proapigeninidins, propelargonidins, proluteolinidins, procyanidins, prodelphinidins, and protricetinidin all possess a phloroglucinol type A ring. Meanwhile, proguibourtinidin, proteracacidin, profisetinidin, promelacacidin and prorobinetinidin all possess a resorcinol type A ring. Proapigeninidins, lacking a hydroxyl group at C-3, and proluteolinidins are considered rare. Proteracacidin has not been found in plants while profisetinidin is generally restricted to leguminosae and anacardiaceae.

![Figure 2: Types of proanthocyanidins (Hemingway and Karchesy, 1989).](image)
Procyanidins are considered the most abundant proanthocyanidins found in nature. The most common are the procyanidins type “B”. Procyanidins can be oligomers (dimers to tetramers) or polymers (pentamers or more units). Procyanidins can be formed by either catechin or epicatechin and are typically joined by a C4-C8 linkage 4→8 bond (epicatechin-(4β→8)-catechin) as shown in Figure 3. The flavan-3-ols and procyanidins are found abundantly in fruits such as apricots, sour cherries, grapes, blackberries and cocoa (Jaganath & Crozier, 2010; Lamuela-Raventós et al., 2005).

![Chemical structure of monomeric catechin (a), dimeric procyanidin B2 (b) and polymeric procyanidin (c)](image)

Figure 3: Chemical structures of monomeric catechin (a), dimeric procyanidin B2 (b) and polymeric procyanidin (c) (Terra, X. et al., 2007).

### 2.2 Cocoa composition

Cacao (*Theobroma cacao*) is an important source of income for producers and processor and it is considered a gourmet food for consumers. According to International Cacao Organization (2013), small cacao farms provide more than 90% of world cacao production which accounts for about 6 millions of cacao farmers. Cote d’Ivoire, Ghana, and Indonesia dominate the global cocoa bean production, covering around two-thirds of the world production (World
Commercially there are three varieties of cacao: Criollo, Forastero, and Trinitario (Motamayor et al., 2002). The most common product of cacao is the cocoa. Europe contains 39.1% of the cocoa processors (World cocoa Foundation, 2012.) indicating that cacao is generally cultivated in some countries but processed in others.

The cocoa composition depends on the cacao variety, soil, climate, crop management, and postharvest processing (de Brito et al., 2001). For that reason cocoa and its derived products (cocoa powder, cocoa liquor and chocolates) contain different amounts of theobroma and polyphenolic compounds such as simple phenols, benzoquinones, phenolic acids, acetophenones, phenylacetic acids, hydroxycinnamic acids, phenylpropenes, coumarin, chromones, naphtoquinones, xanthones, stilbenes, anthraquinones, flavonoids, lignans and lignins. Cocoa has more phenolic compounds and a higher antioxidant capacity than teas and red wine (Lee et al., 2003; Krähmer et al., 2015).

The polyphenolics compounds in cacao are stored in the polyphenolic cells, a type of parenchyma cells from cotyledons (Afoakwa et al., 2008). These compounds confer astringent and bitter sensations and contribute significantly to the green and fruity flavors of cocoa liquors (Norr-Soffalina et al., 2009). The main groups of polyphenols are catechins and epicatechins (37%), procyanidins (58%) and anthocyanins (4%). Thus, the polyphenols in cocoa beans contribute to about 12–18% of the dry weight of the whole bean (Hii et al., 2009). The dimers to hexamers predominate, but polymers with 18 monomeric units can be also found in cocoa products (Misnawi et al., 2003).
The most important proanthocyanidins are B1, B2, B3, B4, B5, C1, and D (Andújar et al., 2012). According to Kadow and others (2013), cocoa beans also contain methylxanthines such as theobromine and caffeine. Theobromine is the major alkaloid of cocoa and represents 2% to 3% of the dry weight; meanwhile, caffeine is found in small amount at 0.2%.

### 2.3 Effect of processing on cocoa chemical composition

Clotilde Hue et al. (2014) analyzed 700 cocoa samples from three different countries (Ecuador, Madagascar, and Dominican Republic) and developed a quantitative and efficient model for predicting flavan-3-ol content during cacao seeds postharvest processing. This study found that the total flavan-3-ol concentration fell from an average of 33.3 mg/g in unfermented samples to an average of 6.2 mg/g at the end of the fermentation which means a loss of 82% of total flavan-3-ol content just during the fermentation process. In addition to fermentation, other process such as drying, roasting, and alkalization of cocoa powder can result in a considerable loss of polyphenols compounds (Hurst et al. 2011; Miller et al. 2008; Misnawi, S., & Sulistyowati, M., 2008). Gu and collaborators (2006) reported that natural cocoa powder (32.19-48.70 mg/g) contains more procyanidins than alkalized cocoa powder (7.02-10.82 mg/g). This means that the alkalization process significantly reduces the content of procyanidins as well as theobromine and caffeine.

### 2.4 Cocoa and human health

According to the International Cocoa Organization, the worldwide consumption of cocoa was around 731,000 tons in 2011 and the consumption is continuously growing. European region accounted for 48% of the world consumption of cocoa followed by the Americas with 33% (International Cocoa Organization, 2013). The worldwide annual sales of cocoa contributes almost $83 billion to the global economy.
Cocoa has the highest flavanol content of all foods on a per-weight basis and is a significant contributor to the total dietary intake of flavonoids (Lee et al., 2003). As a result, there is increasing evidence suggesting that cocoa may act as a potent antioxidant and with enhanced health benefits.

Cocoa increases nitric oxide (NO) bioavailability (Heiss et al., 2005; Vlachopoulos et al., 2005) (Figure 4). A decreased NO bioavailability is a main contributor to endothelial dysfunction, which, in turn, has been suggested to be the earliest triggering event in atherogenesis (Smith et al., 2006a; Smith et al., 2006b). Additionally, an impaired endothelium-dependent vasorelaxation can increase the risk for developing atherosclerosis and its clinical sequelae, i.e., essential hypertension, hypercholesterolemia, type 2 diabetes, obesity, and ageing (Cooke, 2003). A study carried by Monagas et al. (2009), evaluated the effects of cocoa consumption on cellular and serum biomarkers related to atherosclerosis in high-risk patients. This study found that in monocytes expression, a biomarker for atherosclerosis, was significantly lower after cocoa powder intake than without cocoa intake. Thus, the findings of this study suggested that the intake of cocoa polyphenols may modulate inflammatory mediators in patients at high risk of cardiovascular disease which may contribute to the overall health benefits. Accordingly, several studies show evidence that cocoa consumption reduces the expression of cardiovascular disease inflammatory markers (Mao and colleagues, 2000 and 2002; Khan et al., 2012; Osakabe et al., 2001; Murphy et al., 2003; Ramiro et al., 2005).
Figure 4: Nitric oxide bioavailability protects the vascular endothelium and is increased by cocoa flavonoids (Visioli et al., 2009).

Cocoa consumption provides anti-inflammatory and other health benefits. A study carried by Martin et al. (2009) found that the ingestion of dark chocolate by human subjects with high anxiety profile, exhibited stress parameters comparable with the low-stress subjects. This study confirmed that chocolate alleviates stress. Several studies have reported that flavanol-rich cocoa decreases blood pressure and improves insulin sensitivity (Grassi et al., 2005; Taubert et al., 2003; Ruzaidi et al., 2005).

Ultimately, it has been shown that cocoa consumption is associated with endogenous skin health (Neukam et al., 2007; Heinrich et al., 2006). For instance, Heinrich and collaborators (2007) found that a flavanol-rich cocoa drink given for up to 12 weeks to volunteers, significantly decreased UV induced erythema from a solar simulator, increased the blood flow of cutaneous and subcutaneous tissues, and increased the skin density and hydration.
2.4.1 Skin composition

The skin is the largest organ of the body which counts for a total area of about 1.5 to 2.0 m² in adults (Balasubramani, Kumar & Babu, 2001). The skin is an integument composed of multilayered tissue and cell types arranged to form a complex multifunctional organ (Marieb & Hoehn, 2007). The anatomical regions of the skin can be divided into three interconnected regions: the epidermis, the dermis, and the hypodermis (Figure 5).

Figure 5: Anatomical regions of the skin (Marieb & Hoehn, 2007)
The epidermis is the outermost region of the skin. The thick of the epidermis is site-specific; however, it can range from 0.07 mm to 0.12 mm. Besides providing a barrier property, the epidermis is an avascular region which has a very sparse extracellular matrix (Narayan, 2009). The epidermis follows a continual regeneration in a process termed keratinization or cornification (Smack, Korge & James, 1994). Thus, this process arranges the deeper layers of the epidermis into an epidermal ridge that increases the contact area between the epidermis and dermis (Haake, Scott, & Holbrook, 2001).

Below the epidermis is the dermis. The dermis is the richest region with extracellular matrix (ECM) components. The ECM is basically a substance produced and secreted by cells into their surrounding medium. Collagen is the most abundant protein in the dermis, accounting for approximately 75% of the dry weight of skin (Haake, Scott, & Holbrook, 2001; Tzaphlidou, 2004). However, the four major components of ECM are: (1) structural proteins such as the collagens and elastin (although collagen can also have functional properties); (2) multidomain adhesive glycoproteins such as fibronectin, vitronectin, and laminin; (3) glycosaminoglycans such as hyaluronan and proteoglycans such as glypicans, and perlecan; and (4) matricellular proteins such as osteonectin, thrombospondin and osteopontin (Schultz & Wysocki, 2009). Thus, these components are responsible for imparting strength, elasticity, density and compliance to the skin.
Last but not least, is the hypodermis. The hypodermis is also referred to as the subcutaneous layer and is located directly below the dermis. The primary component of this region is adipose tissue (Haake, Scott, & Holbrook, 2001). There is a distinct border between the dermis and the hypodermis; however, the two regions are structurally and functionally interconnected through the rich vascular, neural, and lymphatic networks that supplies the regions (Braverman, 2000; Skobe & Detmar, 2000). The primary functions of the hypodermis include energy storage and release, insulation, mechanical protection, and the maintenance of body contours (Katz, et al., 1999).

2.4.2 Skin ageing

Skin ageing is a complex process. The causes of skin ageing can be divided into two main types: intrinsic and extrinsic. Variations in individual genetic are thought to govern intrinsic ageing. By definition, the intrinsic ageing happens as time passes. Also, this type of ageing is inevitable and, thus, apparently is not subjected to manipulation through changes in human behavior (Baumann, 2007). Telomeres, the specialized structures found at the ends of eukaryotic chromosomes, play an essential role in the intrinsic ageing (Geserick & Blasco, 2006). In fact, the telomeric erosion (or shortening in length) through age causes the intrinsic ageing.

Conversely, extrinsic ageing is caused by factors occurring externally to the human body, such as smoking, excessive alcohol consumption, poor nutrition, and the exposure to the sun. Here, the rate of ageing is believed to be proportional to the severity of exposure to these factors. Of these external factors, sun exposure (photoaging) is considered to be by far the most significantly deleterious to the skin. Indeed, 80% of facial ageing is believed to be due to chronic sun exposure (Uitto, 1997; Jenkins, 2002).
Photoaging of the skin mainly affects collagen fibers, elastin fibers and glycoaminoglycans from the ECM resulting in visible changes in the skin such as wrinkles, pigmentation and changes in thickness (Jenkins, 2002). Moreover, there is evidence that suggests that extrinsic factors, especially UV exposure (UVB: 290-320 nm; UVA: 320-400 nm), could induce the production of reactive oxygen species (ROS) and its free radical activity is related to ageing (Masaki, Atsumi & Sakurai, 1995; Benedetto, 1998; Valencia & Kochevar, 2008). Thus, the ROS induce the expression of proteinases, such as matrix metalloproteinases and serine proteases, which are responsible for remodelling the extracellular matrix (Scharffetter-Kochanek et al., 1993; Wlaschek et al., 1994).

**2.4.3 Effects of plant extract on skin ageing**

As mention before, a key factor for extrinsic skin ageing is the activation of matrix metalloproteinases (MMPs) and serine proteases which degrade the ECM located mostly in the dermis. MMPs are part of a group of transmembrane zinc containing endopeptidases which include collagenases and gelatinases (Thring, Hili, & Naughton, 2009). The collagenases degrade collagen also aggrecan, fibronectin, and laminin. The collagenase reduce the tensile strength of the skin which is imparted by collagen.

Another proteolytic system involved in the degradation of ECM are the serine proteases, specially the elastase. Elastase degrades the elastin fibers, and plays a critical role in inflammatory processes (Wiedow et al., 1990). Elastin, due to its unique elastic recoil properties, is vital for providing elasticity to the skin (Thring, Hili, & Naughton, 2009). Thus, the elastase activity reduces the elasticity of the skin, allowing the skin return to its original structure (Tzaphlidou, 2004).
In terms of anti-ageing, finding inhibitors of collagenase and elastase enzymes can be useful to prevent the sagging skin, wrinkles and pigmentation. The use of plant extracts in cosmetic formulations have been increasing during the past years, mostly because of the poor image of animal-derived extracts and consumers’ awareness to use product with less chemicals additives (Aburjai & Natsheh, 2003).

As a result, there is an increased interest to prove the effectiveness of different plant extracts as anti-ageing. For instance, Vayalil and collaborators (2004) evaluated the effect of green tea polyphenols in mice during 8 weeks of UVB irradiation on the expression of MMPs. This study found that oral administration of green tea polyphenols inhibited the expression of matrix degrading MMP, such as MMP-2 (67%), MMP-3 (63%), MMP-7 (62%), and MMP-9 (60%) in hairless mouse skin, supporting the idea that a plant extract could be useful to attenuate solar UVB light-induced premature skin ageing. Sin & Kim (2005), suggested that certain flavonoids, particularly the flavonols, may prevent collagen breakdown by inhibiting collagenase in inflamed skin as well as photoaged skin. Likewise, Thring, Hili & Naughton (2009) evaluated the anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. Twelve of twenty plant extracts exhibited high or satisfactory anti-collagenase or anti-elastase activities and nine of the extracts had inhibitory activity against both enzymes. Therefore, it can be say that plant extracts can be a promising ingredient to prevent skin ageing without incurring to side effect that can undermine the body health.
2.5 Method for extracting bioactive compounds from plants

The method for extracting bioactive compounds from plant materials is a key step. Common factors affecting extraction processes include the matrix or plant material, type of solvent, temperature, pressure and time (Hernandez et al., 2009). Thus, a better understanding of the dynamics of these factors will help to find the optimal conditions for the extraction of the compound of interest (Aruoma O. I., 2013; Lordan, S., Ross, R. P., & Stanton, C., 2011; Asl, M. N., & Hosseinzadeh, H., 2008; Polya, G., 2003). Azmir et al. (2013) classified the extraction of plant bioactive compounds in two main groups including conventional extraction and non-conventional extraction methods.

2.5.1 Conventional extraction methods

Conventional methods use the extracting power of different solvents, the application of heat and/or shear force to extract the bioactive compounds from plants. The extraction efficiency of any conventional method mainly depends on the choice of solvents (Cowan, 1999). The polarity of the targeted compound is the most important factor for solvent choice. However, it is also necessary to consider the molecular affinity between the solvent and solute, environmental safety, human toxicity and financial feasibility. The methods that belong to this group are soxhlet extraction, maceration, and hydrodistillation.

Soxhlet extraction: Soxhlet extraction was first developed by German chemist Franz Ritter Von Soxhlet (1879). This method was designed mainly for the extraction of lipids from solid materials. However, nowadays it is widely used for the extraction of bioactive compounds and is considered a standard method for comparing new alternative extraction methods.
According to Royal Society of Chemistry, soxhlet extraction is the process of transferring the partially soluble components of a solid to the liquid phase using a Soxhlet extractor. The solid is placed in a filter paper thimble which is then placed into the main chamber of the Soxhlet extractor. The solvent (heated to reflux) travels into the main chamber and the partially soluble components are slowly transferred to the solvent.

**Maceration:** Maceration became popular because it is an inexpensive method to extract bioactive compounds from plants. This method is considered a traditional method because it has been used for long time at home and on small scale for the extraction of bioactive compounds from medicinal plants (Handa *et al.*, 2008). Maceration generally consists of three steps.

Firstly, the plant material is ground into small particles in order to increase the surface area for proper mixing with solvent. Secondly, the solvent is mixed with the sample and placed in a closed vessel. The solvent has to have the ability to solubilize the compound of interest. Thirdly, the solvent is evaporated and the compounds of interest are recovered. Maceration is a process of shear and diffusion between the sample and solvent. If the bioactive compounds are not completely extracted, the maceration process can be repeated several times to increase the yield.

**Hydrodistillation:** Hydrodistillation is also considered a traditional method for the extraction of bioactive compounds and essential oils from plants. This method does not involve the use of organic solvents and there are three types of hydrodistillation methods including water distillation, water and steam distillation and direct steam distillation (Vankar, 2004).
In this method, the plant material is mixed with water and heated to boiling point. Here, the hot water and steam act as the main influential factors to extract the bioactive compounds from plant tissue. Then, an indirect cooling by water condenses the vapor mixture of water and oil. Condensed mixture flows from the condenser to a separator, where oil and bioactive compounds separate automatically from the water (Silva et al., 2005). Basically, this method involves hydrodistillation, hydrolysis and decomposition by heat. The only problem of hydrodistillation is that high temperature can denature thermolabile compounds.

2.5.2 Non-conventional extraction methods

Non-conventional extraction methods were introduced as a result of the challenges facing conventional extraction methods such as the requirement for costly and high purity solvent, longer extraction time, low extraction selectivity, evaporation of huge amount of solvents, and thermal decomposition of thermolabile compounds (De Castro & García-Ayuso, 1998).

Some of these promising methods are considered “green techniques” due to the fact that these methods use less hazardous chemicals and have high efficiency on the use of energy (Azmir et al., 2013). Some of the methods that belong to this group are ultrasound assisted extraction, microwave-assisted extraction, and pulsed electric field assisted extraction.

**Ultrasound assisted extraction:** Ultrasound assisted extraction (UAE) of plant bioactive compounds disrupts cells due to the exposure to high sonication intensities. The UAE may be carried out only in a liquid to liquid containing solid material. Usually, waves of 20 kHz to 100 MHz pass through the medium and creates compression and expansion. This process produces a phenomenon called cavitation, which means production, growth and collapse of bubbles (Chemat, F. et al., 2017). UAE is widely used nowadays. Espada-Bellido et al. (2017) optimized the ultrasound-assisted extraction of anthocyanins and total phenolic compounds in mulberry...
(Morus nigra). This study found that the extraction temperature and solvent composition were the most influential parameters for anthocyanins and phenolic compounds. The optimized conditions for anthocyanins were 48 °C and 76% and for phenolic compounds the conditions were 64 °C and 61%. Other study carried out by Alzorqi et al. (2017) showed that the optimal extraction conditions for β-D-glucan polysaccharides from Ganoderma lucidum were found to be an ultrasonic power of 590 W, a time of 58 min and a temperature of 81 °C. Therefore, the temperature, pressure, frequency and time of sonication are key factors to consider when using UAE optimization.

**Microwave-assisted extraction:** The principle of heating using microwave energy is based on the direct effect of microwaves on molecules by ionic and dipole rotation. Microwaves are electromagnetic fields in the frequency range from 300 MHz to 300 GHz. Electromagnetic energy is converted to heat following ionic conduction and dipole rotation mechanisms (Jain et al., 2009). During ionic conduction mechanism heat is generated because of the resistance of medium to flow ion. On the other hand, ions keep their direction along field signs which change frequently. This frequent change of directions results in collision between molecules and consequently generates heat (Azmir et al., 2013).

One of the main advantages using microwave-assisted extraction (MAE) is the reduction of extraction time. This can mainly be attributed to the difference in heating performance employed by the microwave technique and conventional heating. In conventional heating a finite period of time is needed to heat the vessel before the heat is transferred to the solution, while microwaves heat the solution directly. This keeps the temperature gradient to a minimum and accelerates the speed of heating (Eskilsson & Björklund, 2000).
MAE is also recognized as a green technology because it reduces the use of organic solvent (Alupului et al., 2012). The drawbacks of MAE is that the extraction solvent must be able to absorb microwaves otherwise the extraction will not carry out. Also, if a large volume is treated, it will take long time for the vessel to cool.

The most commonly studied parameters are solvent composition, solvent volume, extraction temperature, extraction time and matrix characteristics including water content. Li et al. (2011) found that the optimal extraction conditions for grape seed polyphenols were ethanol concentration (47.2%), liquid:solid ratio (45.3:1) and time (4.6 min). Furthermore, Pinela et al. (2016) determined that the best condition which provided tomato extracts with high potential as nutraceuticals or as active ingredients in the design of functional foods were time = 20 min, temperature = 180 °C, ethanol concentration = 0%, and solid/liquid ratio = 45 g/L.

By considering practical aspects, MAE is a strong competitor to other recent sample preparation methods. Also, MAE could be considered as a moderate expensive method due to the equipment used; however, improvement of the technology and the lowering of prices will make this method more accessible to industries.

**Pulsed electric field assisted extraction:** Similar to ultrasound assisted extraction, the pulsed electric field (PEF)-assisted extraction destroys cell membrane structure to increase the extraction of bioactive compounds. However, in PEF an electrical treatment of short time (from several nanoseconds to several milliseconds) with pulse electric field strength from 100 to 300 V/cm to 20–80 kV/cm is applied to the sample (Fincan & Dejmek, 2002, and Vorobiev & Lebovka, 2008).
At high electric fields (> 20 kV/cm), it can be considered an alternative method to traditional thermal processing methods which inactivate spoilage and pathogenic microorganisms and quality related enzymes, with the advantage of retaining or minimally modifying sensorial, nutritional and health-promoting attributes of liquid food products (Sánchez-Vega, Elez-Martínez, & Martín-Bellos, 2014).

There are many successful examples of PEF applications on plant materials. The effectiveness of PEF treatment strictly depends on the process parameters, including field strength, specific energy input, pulse number, treatment temperature and properties of the materials to be treated (Heinz et al., 2003).

Segovia et al. (2015) evaluated the effect of PEF (0–5 kV/cm) on the aqueous extraction of polyphenols and antioxidant compounds from borage (Borago officinalis L.) leaves. It was found that the PEF treatments increased the total polyphenol content between 1.3 and 6.6 times, compared to the control. Lopez et al. (2008) evaluated the effects of PEF at 5 and 10 kV/cm on the total polyphenols in grape skin. The results showed that the total polyphenols with respect to the control, increased when the electric field strength raised from 5 to 10 kV/cm.
CHAPTER 3: MATERIALS AND METHODS

3.1 Unfermented cocoa

Unfermented cocoa (*Theobroma cacao*) powder was donated by Mars Chocolate North America, Inc. (Hackettstown, NJ). The cocoa powder was not treated with alkali, known as Dutch process.

3.2 Experimental design

Oil-bath-assisted extraction (OBAE) and microwave-assisted extraction (MAE) were optimized using response surface methodology (RSM). The OBAE unit (Figure 6) was built in Biological and Agricultural Engineering Department. The OBAE unit consisted of three 316 stainless steel seal vessel coupled with a thermocouple type K. The vessels were submerged in mineral oil, heated using a hot plate, and the temperature was controlled with PicoLog® software. Each vessel had a capacity of 200 mL but it was filled with 150 mL of cocoa:water solution. For MAE was used an Ethos Plus® microwave unit (Milestone microwave laboratory system, Shelton, CT). The vessels were of Teflon. Each vessel had a capacity of 250 mL but it was filled with 200 mL of cocoa:water solution.

Figure 6: Extractor unit used in oil-bath-assisted extraction.
A rotatable ($\alpha = 1.682$) central composite design (CCD) was used to determine the optimal conditions of OBAE or MAE for procyanidin B-2 extraction. CCD is a highly efficient, robust design, and provides reliable information on the effect of the variables with reduced number of total experimental runs. The three independent factors studied in OBAE or MAE were cocoa:water ratio ($X_1$), extraction time ($X_2$) and extraction temperature ($X_3$), which were coded at three levels as shown in Table 2.

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>Coded levels</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Oil-bath-assisted extraction (OBAE)</strong></td>
<td>-1 0 1</td>
</tr>
<tr>
<td>$X_1$: Ratio (cocoa : water)*</td>
<td>9 12.5 16</td>
</tr>
<tr>
<td>$X_2$: Time (min)</td>
<td>15 31.5 48</td>
</tr>
<tr>
<td>$X_3$: Temperature (°C)</td>
<td>48 81.5 115</td>
</tr>
<tr>
<td><strong>Microwave-assisted extraction (MAE)</strong></td>
<td>-1 0 1</td>
</tr>
<tr>
<td>$X_1$: Ratio (cocoa : water)*</td>
<td>9 12.5 16</td>
</tr>
<tr>
<td>$X_2$: Time (min)</td>
<td>6 9.5 13</td>
</tr>
<tr>
<td>$X_3$: Temperature (°C)</td>
<td>48 81.5 115</td>
</tr>
</tbody>
</table>

*Ratio cocoa : water means one unit of cocoa powder per 9, 12.5 or 16 units of water.

The CCD consisted of 8 factorial points, 6 axial points and 6 central points, which totaled 20 experimental runs for each method of extraction, OBAE or MAE. The extractions were carried in triplicate. No pressure was applied during the extractions and pH $6.5 \pm 0.1$ was constant. A second-order polynomial model shown in Eq. 1 (Khuri & Mukhopadhyay, 2010) was fitted to OBAE or MAE.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i x_i + \sum_{i=1}^{3} \beta_{ii} x_i^2 + \sum_{i<j=1}^{3} \beta_{ij} x_i x_j \quad (Eq. 1)$$
where $Y$ is the predicted response (concentration of procyanidin B-2 in mg/g cocoa), $\beta_0$ is the regression coefficient for the intercept (a constant), $\beta_i$ is the coefficient for the linear effect, $\beta_{ij}$ is the coefficient for the quadratic effect, $\beta_{ij}$ is the coefficient for the interaction effect of variables $i$ and $j; X_i$ and $X_j$ are independent variables. The JMP® Pro software Version 12.1.0 (SAS Institute Inc., Cary, NC, USA) was used for the statistical analysis and generation of the response surface plots.

In order to obtain the cocoa extract, the mixture of cocoa and water, prepared by OBAE or MAE, was centrifuged at 4327 x g for 30 min and 25 °C. The supernatant was filtered using a syringe and disposable filter device (25 mm GD/x, PTFE filter media, 0.45 µm). The cocoa extract was placed into an amber vial and was ready for analysis. Cocoa extraction at room temperature using 70% v/v methanol, a ratio of 1:16 (cocoa powder:methanol) and three time-extraction, was the control method of extraction.

### 3.3 UHPLC analysis of cocoa extracts

Ultra High Performance Liquid Chromatography (UHPLC) was performed with a Thermo Scientific Ultimate 3000 focused+ (Waltham, MA). Analysis of procyanidin B-2, theobromine, caffeine or monomeric ((+)-Catechin and (-)-Epicatechin) was done by using a reverse phase column Acquity HSS T3 C18 (50 mm _ 2.1 i.d., 1.8 µm particle size) (Waters, Milford, MA, USA). The cocoa extracts were separated using a binary phase where eluent A was water/acetic acid (99.5/0.5, v/v) and eluent B was acetonitrile. Injections of 2.5 µL were used and the oven was held at a temperature of 30 °C. The flow gradient was shown in Table 3.
Table 3: Flow gradient and eluents distribution over time in the UHPLC analysis of cocoa extracts.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Flow (mL/min)</th>
<th>%B</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.300</td>
<td>10</td>
</tr>
<tr>
<td>1.000</td>
<td>0.300</td>
<td>13</td>
</tr>
<tr>
<td>3.750</td>
<td>0.100</td>
<td>15</td>
</tr>
<tr>
<td>3.875</td>
<td>0.100</td>
<td>100</td>
</tr>
<tr>
<td>6.250</td>
<td>0.100</td>
<td>100</td>
</tr>
<tr>
<td>6.375</td>
<td>0.300</td>
<td>10</td>
</tr>
<tr>
<td>10.000</td>
<td>0.300</td>
<td>10</td>
</tr>
</tbody>
</table>

Procyanidin B-2 standard was purchased from ChromaDex (Irvine, CA) and the standards of theobromine, caffeine, (+)-Catechin and (-)-Epicatechin from Sigma-Aldrich (St. Louis, MO). The standards and cocoa extracts were analyzed at 273 nm wavelength. Chromeleon TM 7.2 Chromatography Data System (CDS) SOFTWARE (Thermo Scientific, Waltham, MA) was used to identify and quantify the peaks of interest by its specific retention time. Standard curves were developed to determine the concentration of the compounds in the cocoa extract. A linear response was obtained for procyanidin B-2 in the concentration range of 25-1000 μg/mL. For theobromine, caffeine, (+)-Catechin or (-)-Epicatechin the linear response was obtained in the concentration range of 50-1000 μg/mL.

3.4 MALDI-TOF mass spectrometric analysis of cocoa extracts

A saturated solution of 2,5-Dihydroxybenzoic acid (DHB; Sigma-Aldrich, St. Louis, MO, USA) dissolved in a 0.1% trifluoroacetic acid (TFA) in water was used as the matrix. A 0.5 μL solution of cocoa extract was first deposited on the MALDI target plate followed by deposition of 0.5 μL of matrix on the top of the sample. The matrix and sample were mixed and allowed to dry at room temperature. MALDI-TOF MS measurements were performed on an Ultraflextreme (Bruker Daltonics, Billerica, MA, USA).
Mass spectra were recorded in positive ion reflectron mode with an accelerating voltage of 25 kV and analyzed in the mass range of 150–4500 Da. The spectra were acquired after calibration of the instrument with a peptide standard (Peptide Calibration Standard II, Bruker Daltonics, MA, USA). A minimum of 500 laser shots per sample was used to generate each mass spectrum.

### 3.5 Collagenase inhibition assay

The assay was adapted from Pastorino et al., (2017) using a 96-well plate. This assay was performed using a 50 mM Tricine (pH 7.5 with 400 mM NaCl and 10 mM CaCl$_2$) buffer. A stock solution of *Clostridium histolyticum* collagenase (ChC - EC.3.4.23.3, Sigma-Aldrich, St. Louis, MO, USA) at 0.72 U/mL was used. *Clostridium histolyticum* collagenase was dissolved in cold (2-8 °C) ultrapure water immediately before use. N-[3-(2-furyl) acryloyl- Leu-Gly-Pro-Ala (FALGPA, Sigma-Aldrich, USA) was used as substrate. FALGPA was dissolved in 50 mM Tricine buffer to give a stock solution of 4.19 mM. This stock solution was also prepared immediately before use.

Briefly, 55 μL of the treatment (Table 4) was incubated with 50 μL of *Clostridium histolyticum* collagenase and 77 μL of tricine buffer for 20 min at 37 °C. Subsequently, 43 μL of FALGPA was added to initiate the reaction. The final reaction mixture per well was 225 μL where the concentration of *Clostridium histolyticum* collagenase and FALGPA were 0.16 U/mL and 0.8 mM, respectively. The ChC inhibitory activities of the individual treatment was measured by continuously monitoring the decrease in absorbance of FALGPA at 335 nm for 20 min after starting the reaction.
An AF2200 plate reader (Eppendorf, Hauppauge, NY, USA) was used to measure the absorbance. Initial velocities were calculated from the slope of the absorbance change during the first 10 min of hydrolysis at 37 °C. Each treatment was performed in triplicate and the percentage of inhibition activity was calculated according to Eq. (2). IC₅₀ values were determined from dose–effect curves.

\[
\text{Inhibition} \, (\%) = \frac{\text{Initial velocity}_{\text{control}} - \text{Initial velocity}_{\text{treatment}}}{\text{Initial velocity}_{\text{control}}} \times 100
\]  

(Eq. 2)

### 3.6 Elastase inhibition assay

The assay was adapted from Pastorino et al., (2017) using a 96-well plate. This assay was performed using a 200 mM Tris HCl (pH 8.0 at 25°C) buffer. A stock solution of porcine pancreatic elastase (PPE - EC3.4.21.36, Sigma- Aldrich, St. Louis, MO, USA) at 0.09 U/mL was used. Porcine pancreatic elastase was dissolved in cold (2-8 °C) buffer immediately before use. N-succinyl-Ala–Ala-Ala-p-nitroanilide (AAAPVN, Sigma-Aldrich, St. Louis, MO, USA) was used as substrate. AAAPVN was dissolved in 200 mM Tris HCl buffer to make a stock solution of 1.83 mM. This solution was also prepared immediately before use.

Briefly, 55 μL of the treatment (Table 4) was incubated with 50 μL of porcine pancreatic elastase and 77 μL of Tris HCl buffer for 20 min at 25 °C. Subsequently, 43 μL of AAAPVN was added to initiate the reaction. The final reaction mixture per well was 225 μL where the concentrations of porcine pancreatic elastase and AAAPVN were 0.02 U/mL and 0.35 mM, respectively. The PPE inhibitory activities of the individual treatment was measured by continuously monitoring the decrease in absorbance of AAAPVN at 410 nm for 20 min after starting the reaction.
An AF2200 plate reader (Eppendorf, Hauppauge, NY, USA) was used to measure the absorbance. Initial velocities were calculated from the slope of the absorbance change during the first 10 min of hydrolysis at 25 °C. Each treatment was performed in triplicate and the percentage of inhibition activity was calculated according to Eq. (2). IC₅₀ values were determined from dose–effect curves.

Table 4: Description of the treatments evaluated for the enzyme inhibition assays.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Description</th>
<th>Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cocoa extract 1</td>
<td>The best optimum extract between OBAE and MAE</td>
<td>0.10, 4.11, 16.46, 65.82 and 131.64 μg procyanidin B-2/mL</td>
</tr>
<tr>
<td>Cocoa extract 2</td>
<td>Methanol extract (methanol was removed then was dissolved in water)</td>
<td>0.12, 4.72, 18.89, 75.58 and 119.97 μg procyanidin B-2/mL</td>
</tr>
<tr>
<td>Standard 1</td>
<td>Procyanidin B-2</td>
<td>0.17, 1.71, 17.11, 68.44 and 244.44 μg/mL</td>
</tr>
<tr>
<td>Standard 2</td>
<td>Theobromine</td>
<td>0.17, 1.71, 17.11, 68.44 and 244.44 μg/mL</td>
</tr>
<tr>
<td>Standard 3</td>
<td>Caffeine</td>
<td>0.17, 1.71, 17.11, 68.44 and 244.44 μg/mL</td>
</tr>
<tr>
<td>Positive</td>
<td>Epigallocatechin gallate</td>
<td>0.17, 1.71, 17.11, 68.44 and 244.44 μg/mL</td>
</tr>
<tr>
<td>Negative</td>
<td>Enzyme and substrate; No extract or standards, instead it was buffer.</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td>Substrate only; No extract or standards and no enzyme, instead it was buffer.</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4: RESULTS AND DISCUSSION

4.1 Optimization of oil-bath-assisted extraction (OBAE) or microwave-assisted extraction (MAE) conditions for procyanidin B-2 rich extract

4.1.1 UHPLC analysis

The UHPLC analysis was used to quantify the procyanidin B-2 content in the cocoa extracts from the experimental runs according to the central composite design. The UHPLC method was similar to that used by Ortega and collaborators (2008). However, the gradient elution time took only 2 min, instead of 20 min, to analyze the cocoa extracts. According to Wren (2005), many factors come into play when there is a need to obtain high separation efficiency such as column characteristics, gradient time, flow rate, and analyte characteristics. Thus, the conditions used in one UHPLC instrument not necessarily will work in other UHPLC instrument. In this case, this method not only was fast, but also efficient in reducing the use of solvents (reduced cost) for the analysis.

Cocoa is a complex matrix containing theobromine and flavanols. For this study, we only analyzed for theobromine, caffeine, catechin, procyanidin B-2 and epicatechin. Consequently, standard curves were built for each biocompound and were determined its concentration in cocoa extracts. It were achieved high coefficients of determination or $R^2$ (Figure 7), which means a high confidence to determine the concentration of the biocompounds as a function to the peak area (mAU*min) integrated in the chromatogram.
Figure 7: Standard curves of (a) theobromine, (b) caffeine, (c) catechin, (d) procyanidin B-2 and (e) epicatechin.
In all the experimental runs from OBAE or MAE, the compounds of interest followed the same elution times as shown in Figure 8. Theobromine was the compound that always presented the highest distribution in the cocoa extract for both OBAE and MAE; meanwhile, caffeine, catechin, procyanidin B-2 and epicatechin slightly varied (data not shown). This pattern is consistent with the results obtained by Cooper et al. (2007) and Ortega et al. (2010).

Figure 8: UHPLC chromatogram from cocoa extracts measured at 273 nm. UHPLC analysis was performed with Chromeleon TM 7.2 Chromatography Data System (CDS) SOFTWARE (Thermo Scientific, Waltham, MA).

4.1.2 Response surface methodology for OBAE

Experimental data response and predicted values for procyanidin B-2 rich extract (mg/g cocoa) using OBAE are presented in Table 5. The analysis of variance (ANOVA) for procyanidin B-2 extraction yield showed that experimental data had a correlation coefficient ($R^2$) of 0.9401, which suggested that a high degree of correlation was achieved given that the $R^2$ value was higher than 0.8 (Mirhosseini et al., 2009). That means, the calculated model successfully fitted 94.01% of the procyanidin B-2 rich extracts in the linear regression model.
A high R² along with a no significant lack of fit (P > 0.05), indicated that the response variable significantly (P < 0.05) fitted the second order model and was adequate to represent the relationship between the response and the independent variables.

Table 5: Observed responses and predicted values for procyanidin B-2 using OBAE.

<table>
<thead>
<tr>
<th>Run</th>
<th>X₁: Ratio (Cocoa:Water)</th>
<th>X₂: Time (min)</th>
<th>X₃: Temperature (°C)</th>
<th>Response P. B-2 (mg/g cocoa)</th>
<th>Predicted P. B-2 (mg/g cocoa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>12.50</td>
<td>31.50</td>
<td>81.50</td>
<td>6.74 ± 1.83</td>
<td>6.48</td>
</tr>
<tr>
<td>2</td>
<td>12.50</td>
<td>31.50</td>
<td>25.16</td>
<td>4.80 ± 0.09</td>
<td>4.67</td>
</tr>
<tr>
<td>3</td>
<td>12.50</td>
<td>31.50</td>
<td>81.50</td>
<td>6.68 ± 0.96</td>
<td>6.48</td>
</tr>
<tr>
<td>4</td>
<td>9.00</td>
<td>15.00</td>
<td>115.00</td>
<td>7.63 ± 0.23</td>
<td>7.32</td>
</tr>
<tr>
<td>5</td>
<td>12.50</td>
<td>31.50</td>
<td>81.50</td>
<td>7.06 ± 0.18</td>
<td>6.48</td>
</tr>
<tr>
<td>6</td>
<td>16.00</td>
<td>48.00</td>
<td>48.00</td>
<td>5.90 ± 0.90</td>
<td>5.98</td>
</tr>
<tr>
<td>7</td>
<td>9.00</td>
<td>48.00</td>
<td>115.00</td>
<td>7.30 ± 0.23</td>
<td>7.11</td>
</tr>
<tr>
<td>8</td>
<td>9.00</td>
<td>15.00</td>
<td>48.00</td>
<td>5.15 ± 0.07</td>
<td>4.90</td>
</tr>
<tr>
<td>9</td>
<td>16.00</td>
<td>15.00</td>
<td>48.00</td>
<td>5.15 ± 0.26</td>
<td>5.13</td>
</tr>
<tr>
<td>10</td>
<td>12.50</td>
<td>3.75</td>
<td>81.50</td>
<td>5.45 ± 1.52</td>
<td>5.99</td>
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<tr>
<td>11</td>
<td>9.00</td>
<td>48.00</td>
<td>48.00</td>
<td>4.66 ± 1.11</td>
<td>4.86</td>
</tr>
<tr>
<td>12</td>
<td>12.50</td>
<td>31.50</td>
<td>81.50</td>
<td>6.09 ± 0.23</td>
<td>6.48</td>
</tr>
<tr>
<td>13</td>
<td>12.50</td>
<td>31.50</td>
<td>137.84</td>
<td>8.01 ± 1.90</td>
<td>9.08</td>
</tr>
<tr>
<td>14</td>
<td>16.00</td>
<td>48.00</td>
<td>115.00</td>
<td>8.76 ± 0.18</td>
<td>8.80</td>
</tr>
<tr>
<td>15</td>
<td>6.61</td>
<td>31.50</td>
<td>81.50</td>
<td>5.40 ± 0.13</td>
<td>5.63</td>
</tr>
<tr>
<td>16</td>
<td>12.50</td>
<td>59.25</td>
<td>81.50</td>
<td>6.73 ± 0.49</td>
<td>6.54</td>
</tr>
<tr>
<td>17</td>
<td>18.39</td>
<td>31.50</td>
<td>81.50</td>
<td>7.17 ± 0.27</td>
<td>7.23</td>
</tr>
<tr>
<td>18</td>
<td>12.50</td>
<td>31.50</td>
<td>81.50</td>
<td>6.19 ± 0.26</td>
<td>6.48</td>
</tr>
<tr>
<td>19</td>
<td>12.50</td>
<td>31.50</td>
<td>81.50</td>
<td>6.33 ± 0.29</td>
<td>6.48</td>
</tr>
<tr>
<td>20</td>
<td>16.00</td>
<td>15.00</td>
<td>115.00</td>
<td>8.92 ± 0.48</td>
<td>8.11</td>
</tr>
</tbody>
</table>
The extraction yield of procyanidin B-2 was most significantly affected by temperature 
\( X_3 \) (P = 0.00000) and ratio cocoa:water \( X_1 \) (P = 0.00000), followed by time \( X_2 \) (P = 
0.00259). Some quadratic and interaction parameters were significant (P < 0.05). Neglecting the 
non-significant parameters, the final predictive equation obtained was given below:

\[
Y = 6.4843 + 0.4775 \left( \frac{(X_1 - 12.5)}{3.5} \right) + 0.1616 \left( \frac{(X_2 - 31.5)}{16.5} \right) + 1.3092 \left( \frac{(X_3 - 81.5)}{33.5} \right) \\
+ 0.2229 \left( \frac{(X_1 - 12.5)}{3.5} \right) \left( \frac{(X_2 - 31.5)}{16.5} \right) + 0.1413 \left( \frac{(X_1 - 12.5)}{3.5} \right) \left( \frac{(X_3 - 81.5)}{33.5} \right) \\
+ 0.1382 \left( \frac{(X_3 - 81.5)}{33.5} \right)^2 \quad (Eq. 3)
\]

The effects of the independent variables and their mutual interaction on the extraction 
yield of procyanidin B-2 can also be seen on three dimensional surface plot and contour plot as 
shown in Figure 9. These graphs were generated by plotting the predicted values in y-axis 
against two independent variables in the x-axis and z-axis, respectively. Figure 9a shows the 
interaction between temperature and extraction ratio on the yield of procyanidin B-2. While 
Figure 9b shows the interaction between temperature and extraction time on the yield of 
procyanidin B-2. As can been seen in Fig. 9, an increase in temperature (from 25 to 137 °C), 
ratio cocoa:water (from 1:6 to 1:18) and time (from 3 to 60 min), significantly enhanced the 
extraction yield of procyanidin B-2. This trend is in accordance with the extractions carried out 
by Yang et al. (2009) and Aliakbarian et al. (2012).
4.1.3 Response surface methodology for MAE

The experimental data response and predicted values for procyanidin B-2 rich extract (mg/g cocoa) using MAE are presented in Table 6. According to the ANOVA, MAE’s extractions also had a high $R^2$ (0.9513) and no significant lack of fit ($P > 0.05$); thus, it also indicate that the yield of procyanidin B-2 followed a second order model.
Here, the extraction yield for procyanidin B-2 was also mostly affected by temperature ($X_3$) ($P = 0.00000$), ratio cocoa:water ($X_1$) ($P = 0.00000$) and, followed by time ($X_2$) ($P = 0.00124$). Also, some quadratic and interaction parameters were significant ($P < 0.05$). Thus, the final predictive equation obtained was given below:

$$Y = 5.1064 + 0.8410 \left( \frac{(X_1 - 12.5)}{3.5} \right) + 0.1604 \left( \frac{(X_2 - 9.5)}{3.5} \right) + 1.1287 \left( \frac{(X_3 - 81.5)}{33.5} \right)
+ 0.1563 \left( \frac{(X_2 - 9.5)}{3.5} \right) \left( \frac{(X_3 - 81.5)}{33.5} \right) + 0.31 \left( \frac{(X_3 - 81.5)}{33.5} \right)^2$$

\textit{(Eq.4)}

---

Table 6: Observed responses and predicted values for procyanidin B-2 using MAE.

<table>
<thead>
<tr>
<th>Run</th>
<th>$X_1$: Ratio (Cocoa:Water)</th>
<th>$X_2$: Time (min)</th>
<th>$X_3$: Temperature (°C)</th>
<th>Response P. B-2 (mg/g cocoa)</th>
<th>Predicted P. B-2 (mg/g cocoa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18.39</td>
<td>9.50</td>
<td>81.50</td>
<td>6.25 ± 0.30</td>
<td>6.29</td>
</tr>
<tr>
<td>2</td>
<td>12.50</td>
<td>9.50</td>
<td>81.50</td>
<td>5.32 ± 0.25</td>
<td>5.11</td>
</tr>
<tr>
<td>3</td>
<td>12.50</td>
<td>9.50</td>
<td>81.50</td>
<td>5.38 ± 0.20</td>
<td>5.11</td>
</tr>
<tr>
<td>4</td>
<td>16.00</td>
<td>13.00</td>
<td>48.00</td>
<td>5.37 ± 0.33</td>
<td>4.99</td>
</tr>
<tr>
<td>5</td>
<td>12.50</td>
<td>9.50</td>
<td>81.50</td>
<td>5.32 ± 0.25</td>
<td>5.11</td>
</tr>
<tr>
<td>6</td>
<td>12.50</td>
<td>9.50</td>
<td>81.50</td>
<td>5.19 ± 0.11</td>
<td>5.11</td>
</tr>
<tr>
<td>7</td>
<td>9.00</td>
<td>6.00</td>
<td>48.00</td>
<td>3.75 ± 0.08</td>
<td>3.49</td>
</tr>
<tr>
<td>8</td>
<td>16.00</td>
<td>6.00</td>
<td>48.00</td>
<td>4.76 ± 0.40</td>
<td>4.83</td>
</tr>
<tr>
<td>9</td>
<td>12.50</td>
<td>9.50</td>
<td>25.16</td>
<td>3.69 ± 0.06</td>
<td>4.07</td>
</tr>
<tr>
<td>10</td>
<td>6.61</td>
<td>9.50</td>
<td>81.50</td>
<td>3.23 ± 0.06</td>
<td>3.46</td>
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<tr>
<td>11</td>
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<td>9.50</td>
<td>137.84</td>
<td>8.00 ± 0.17</td>
<td>7.87</td>
</tr>
<tr>
<td>12</td>
<td>16.00</td>
<td>13.00</td>
<td>115.00</td>
<td>7.66 ± 0.24</td>
<td>7.75</td>
</tr>
<tr>
<td>13</td>
<td>9.00</td>
<td>13.00</td>
<td>48.00</td>
<td>3.59 ± 0.61</td>
<td>3.35</td>
</tr>
<tr>
<td>14</td>
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<td>81.50</td>
<td>4.90 ± 0.16</td>
<td>5.27</td>
</tr>
<tr>
<td>15</td>
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<td>9.50</td>
<td>81.50</td>
<td>4.74 ± 0.07</td>
<td>5.11</td>
</tr>
<tr>
<td>16</td>
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<td>6.00</td>
<td>115.00</td>
<td>5.08 ± 0.22</td>
<td>5.24</td>
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<td>4.86 ± 0.44</td>
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<tr>
<td>18</td>
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<td>81.50</td>
<td>4.74 ± 0.11</td>
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<td>19</td>
<td>16.00</td>
<td>6.00</td>
<td>115.00</td>
<td>6.94 ± 0.21</td>
<td>6.97</td>
</tr>
<tr>
<td>20</td>
<td>9.00</td>
<td>13.00</td>
<td>115.00</td>
<td>5.99 ± 0.15</td>
<td>5.73</td>
</tr>
</tbody>
</table>
Additional to the equation obtained, the effects of the independent variables and their mutual interaction on the extraction yield of procyanidin B-2 are shown in Figure 10. In this case, an increase in temperature (from 25 to 137 °C), and cocoa:water ratio (from 1:6 to 1:18), showed an abrupt increase in the extraction yield of procyanidin B-2. However, an increase in time (from 3 to 15 min) showed a slight increase in the yield of procyanidin B-2. This difference could be partly caused by the ability of the microwave energy to rapidly heat materials (Eskilsson & Björklund, 2000) and the lower range of extraction time compared with OBAE. This results also followed similar curve as the MAE extractions carried out by Hayat et al. (2009) and Krishnaswamy et al. (2013).

Figure 10: Response surface plots showing the effects of extraction temperature vs ratio (a) and extraction temperature vs extraction time (b) on the extraction yield of procyanidin B-2 by MAE.
4.1.4 Comparison between methanol extraction and the optimized conditions for OBAE and MAE

No optimization of procyanidin B-2 extraction from cocoa powder has been done before. According to the results of the response surface methodology, it can be concluded that the optimized conditions for both, OBAE and MAE, were at 1:16 cocoa:water ratio and 115 °C (Figure 11); however, MAE offered the shorter extraction time (13 min) compared to OBAE (48 min). The shorten extraction time could be associated to the mode of heat transfer. Conventional extraction such as OBAE, is a method based on heat transfer via conduction and convection which makes it a time-consuming process. In contrast, MAE is a fast extraction process because no medium is required for the radiative heat transfer since the energy transfer is through molecular interaction by electromagnetic waves. Thus, microwave energy is delivered rapidly and efficiently to the extraction solvent and plant materials (Criado et al., 2004; Zhou & Liu, 2006). MAE is relatively cost-effective when compared to other methods of extraction; however, the equipment and their operation could be very expensive (Zhang, Yang & Wang, 2011).

![Figure 11: Maximized desirability predictor plot for oil-bath-assisted extraction (a) or microwave-assisted extraction (b).](image)

(a)  
![Graph](image)  
(b)  
![Graph](image)
To validate the adequacy of the model equation (Eq. 3 and Eq. 4), a verification experiment was carried out under the optimal conditions by OBAE or MAE showed in Figure 11. Under the optimal conditions, the model predicted an extraction yield of 8.79 mg of procyanidin B-2/g cocoa by OBAE and 7.74 mg procyanidin B-2/g cocoa by MAE. Similarly to the predicted values, the verification experiments yielded 8.90 mg of procyanidin B-2/g cocoa extracted by OBAE and 7.64 mg/g cocoa extracted by MAE (Figure 12). The good correlation between the predicted and the verified results, undoubtedly confirmed that the model was adequate to predict the yield of procyanidin B-2 in the optimized conditions. On the other hand, the model predicted a 40% less yield of procyanidin B-2/g cocoa when extracted at 25 °C than when extracted at 115 °C.

In this study, comparison of procyanidin B-2 extracted by 70% methanol for 1 h, was used as the control to which other extraction methods were compared. Methanol at 70% solution in water is commonly used as the conventional extraction method (Astill, et al., 2001; Hayat et al., 2009; Singh et al., 2011). In fact, it was reported that methanol is a better extraction solvent for phenolic compounds than acetone or ethanol because methanol polarity increases the phenolic compounds solubility (Pellati et al., 2004; Jaiswal et al., 2012; Alothman, Bhat & Karim, 2009; Naczk & Shahidi, 2006). Methanol extraction had a yield of 7.07 mg of procyanidin B-2 rich extract /g cocoa; however, it was not significantly (P < 0.05) higher than the yield obtained by the optimum conditions of OBAE or MAE.
The concentration and the profile of compounds present in cocoa extracts obtained by methanol, OBAE or MAE are shown in Figure 12. At first sight, it can be observed that theobromine was the compound with the highest abundance and caffeine had the lowest abundance. The distribution of theobromine, caffeine, catechin, procyanidin B-2 and epicatechin are the same as reported by Ortega and collaborators (2010).

Extraction efficiency is commonly a function of process conditions (e.g., temperature, extraction time, solvent-to-solid ratio, etc.). Liyana-Pathirana and Shahidi (2005) suggested that increasing temperature may favor extraction by enhancing the solubility of phenolic compounds. It can also favor extraction by increasing the diffusion coefficient, and decreasing the viscosity coefficient of the solute-solvent (Cacace & Mazza, 2003; Bimakr et al., 2011). This might be the main reason why procyanidin B-2 yield changed significantly when temperature was increased from 25 to 138 °C. Therefore, it can be demonstrated that using water in OBAE or MAE, more procyanidin B-2 rich mixture can be extracted compared to methanol extraction. Consequently, OBAE or MAE could be good alternatives to recover almost all flavanols and alkaloids present in cocoa powder.
Figure 12: Theobromine, caffeine, catechin, procyanidin B-2 rich and epicatechin content on the cocoa extract obtained by methanol, and under OBAE or MAE best conditions.
4.2 MALDI-TOF mass spectrometric analysis of the best extraction condition by OBAE or MAE

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) is an analytical method specially used for the characterization of the molecular mass distribution in complex mixtures (Hanton, 2001). The principle behind MALDI-TOF-MS is to irradiate a sample–matrix crystals with a laser beam of high irradiance power (10⁶Wcm⁻²) and short pulse width (few nanoseconds) to simultaneously desorb and ionize the sample and matrix molecules into gas. Immediately, the packs of ions are accelerated by a fixed electrical potential into the analyzer (1–2 m flight path), they hit the detector and a spectrum of molecular masses profile is created (Monagas et al., 2010). Thus, MALDI-TOF-MS produces only a singly charged molecular ion for each parent molecule and allows detection of high masses with precision (Krueger, Vestling, & Reed, 2004).

MALDI-TOF-MS has been extensively used to identify or characterize the structural diversity of polyphenolic compounds in many foods and beverages. For instance, Shoji and collaborators (2006) characterized the procyanidins from apple juice according to the degree of polymerization. Meanwhile, Yang & Chien (2000) determined the molecular mass distribution of flavanols in grape seeds. However, the molecular masses distribution of bioactive compounds in cocoa extracts obtained by methanol, oil-bath and microwave-assisted extraction, have not been compared before.
Figure 13: MALDI-TOF-MS positive reflectron mode mass spectra of methanol extract (a), and the best condition extracts by OBAE (b) or MAE (c).
The mass spectra for methanol extracts obtained at 25 °C, and the best condition for OBAE and MAE with water at 115 °C are shown in Figure 13. All spectra shows a mass distribution ranging between 150 up to 1400 Da, however, most molecules are located below 1000 Da.

All the compounds of interest including theobromine (180.16 Da), caffeine (194.19 Da), catechin (290.27 Da), epicatechin (290.27 Da), and procyanidin B-2 (579.52 Da) were identified in all spectra; however, the peaks’ signal intensity were low. Ion suppression could be the cause of the low signal intensity, especially with a complex sample such as cocoa extract. In a mixture, some analytes have higher affinities and successfully compete for the available protons for charge than others (Duncan, Roder & Hunsucker, 2008). Thus, the ion suppression make difficult to quantify the molecules of interests. For this study, only a qualitative analysis of the mass distribution was performed.

OBAE’s (Fig. 13b) and MAE’s (Fig. 13c) spectra have a similar molecular masses distribution. In contrast, the spectra of methanol extract (Fig. 13a) seems to exhibit higher presence of peaks compared to OBAE’s and MAE’s spectra, especially in the regions between 600 Da to 700 Da and between 850 Da to 1000 Da. The reason could be that methanol as an extraction solvent is more efficient in extracting bioactives compounds than water. In fact, methanol not only possesses the swelling or break up power but also has the advantage to being miscible with a wide range of monomers and polymers compared to water (Metivier, Francis & Clydesdale, 1980; Dilli & Garnett, 1967).
On the other side, the extraction temperature seems to influence the mass distribution for both OBAE and MAE spectra. OBAE’s and MAE’s extract were obtained at 115 °C, meanwhile, methanol extract was obtained at 25 °C. OBAE and MAE spectra showed more peaks below 350 Da comparing to that from methanol spectra. The thermal decomposition which result in the formation of fragments or monomeric units splitted off from polymeric chains (Patras et al., 2010) could explain this difference. Wang and Xu (2007) evaluated the thermal degradation of blackberry anthocyanins in 8.90°Brix juice at 60, 70, 80, or 90 °C. The results indicated that the blackberry anthocyanins degraded more quickly with increasing temperature and the thermal degradation of blackberry anthocyanins followed a first-order reaction kinetics.

Other reason of different peaks distribution between methanol extract and water extract is the effectiveness of methanol in extracting a wider range of polyphenols than water. Ferran Sánchez-Rabaneda and collaborators (2003) extracted the cocoa bioactives compounds with methanol and characterized them using mass spectrometry. Besides flavanols, theobromine and caffeine, this study also identified anthocyanins (cyanidin glycosides) and flavonol glycosides such as quercetin-3-O-arabinose, isoquercitrin, quercetin-3-O-glucuronide and quercetin. Some compounds were naringenin (m/z 271), apigenin (m/z 269), luteolin-8-C-glucoside (m/z 427), apigenin-8-C-glucoside (m/z 431), quercetin-3-O-arabinoside (m/z 433), and quercetin-3-O-galactoside (m/z 463).

Cocoa extracted with methanol showed slight differences on the masses distribution spectra when compared to OBAE’s or MAE’s spectra. Methanol extract had more peaks than water extracts. However, further studies on the stabilization of cocoa extract are needed in order to evaluate the thermal degradation kinetics and the characterization of the unknown biocompounds.
4.3 Collagenase and elastase inhibitory activity of cocoa extracts

Skin is an organ directly exposed to the environment, as a consequence, the ageing processes resulting from environmental exposure is of considerable relevance, particularly due to the generation of reactive oxygen species (ROS) which activates enzymes such as collagenase and elastase. These enzymes degrade the extracellular matrix proteins in the dermis (collagen and elastin), affecting the skin’s three-dimensional integrity. (Scharffetter-Kochanek et al., 2000; Brenneisen et al., 1998).

To investigate the inhibitory effect of the cocoa extracts on collagenase or elastase, cocoa extracts were incubated with Clostridium histolyticum collagenase (ChC) or porcine pancreas elastase (PPE) and their respectively substrates FALGPA (N-[3-(2-furyl) acriloyl- Leu-Gly-Pro-Ala) and AAAPVN (N-succinyl-Ala–Ala-Ala-p-nitroanilide). The inhibitory potential of the cocoa extracts was examined by increasing dilutions, in order to establish dose-effect relationships, and to calculate the half maximal inhibitory concentrations (IC$_{50}$).

The effect of different cocoa extract concentrations and individual compounds on the enzyme activity of ChC or PPE is shown in Figure 14 (a and b), respectively. Methanol extract was dried, redisolved in water and analyzed for collagenase or elastase inhibitory activity. Also, we only evaluated procyanidin B-2 rich extract from optimal OBAE since it had significantly (P < 0.05) higher concentration of procyanidn B-2 than MAE (see Fig. 12). Epigallocatechin gallate (EGCG), a well-known natural inhibitor of collagenase and elastase, was used as the positive control (Madhan et al., 2007; Demeule et al., 2000).
Figure 14: Dose-effect inhibition of *Clostridium histolyticum* collagenase (a) and porcine pancreas elastase (b) activity by methanol extract, cocoa extract from OBAE best condition or individual phenolic compounds.

Cocoa extracts inhibited both enzymes in a dose-dependent manner. However, methanol extract showed a higher enzyme inhibitory activity than OBAE than EGCG, as shown by a low IC$_{50}$ value.

IC$_{50}$ value for methanol extract that inhibited ChC was 9.50 μg procyanidin B-2/mL. The concentrations of theobromine, caffeine, catechin, and epicatechin present in the IC$_{50}$ value were 25.84, 4.00, 10.04 and 12.18 μg/mL, respectively. EGCG had an IC$_{50}$ value of 18.4 μg/mL. Meanwhile, the IC$_{50}$ value for OBAE extract was 42 μg procyanidin B-2/mL. Here, the concentrations of theobromine, caffeine, catechin, and epicatechin were 95.11, 12.35, 43.75 and 45.29 μg/mL, respectively.
The only treatment that reached an IC\textsubscript{50} in the inhibition of PPE was methanol extract with a value of 76 μg procyanidin B-2/mL. The concentrations of theobromine, caffeine, catechin, and epicatechin were 206.76, 32.01, 80.31 and 97.43 μg/mL, respectively. EGCG and extract from OBAE barely reached a maximum PPE inhibition of 13% and 32%, respectively.

The comparison of these findings with those reported for other plant extracts is not straightforward due to the use of different extraction methods, different plant extracts and the varying assay conditions such as incubation times and types of substrates (Wittenauer \textit{et al.}, 2015). Also, the solvent may influence the extract composition. Hubert and collaborators (2016) investigated the ability of different trees’ bark extracts to reduce collagenase and elastase activity. Solid/liquid extractions of the barks were performed using n-heptane or methanol. They found that methanol extraction exhibited higher elastase and collagenase inhibitory activity than n-heptane extracts, indicating that bark extract obtained using non-polar solvent were less active. Therefore, solvent polarity may influence the compounds to be extracted and this could explain the fact that methanol extract of cocoa had the highest inhibitory activity against both enzymes.

Other possible reasons that methanol extract had higher enzyme inhibitory activity than OBAE’s extract could be due to the influence of extraction temperature. As mentioned before, methanol extract was prepared at room temperature (25 °C), meanwhile, OBAE’s extract was obtained at 115 °C. Xiao, Han & Shi (2008) investigated the extraction of flavonoids from \textit{Radix astragali} using microwave-assisted extraction. It was found that extractions performed at temperatures higher than 110 °C significantly degraded the flavonoids. Also, temperatures below 110 °C was chosen in order to avoid scorching of the plant material used.
The degradation of flavonoids was also observed in a kinetic study of catechin performed by Li, Taylor, Ferruzzi, & Mauer (2012). In this study they investigated the degradation behavior of catechin in green tea caused by pH, concentration and temperature. They mentioned that catechin degradation mechanism might be complex suggesting that multiple reactions, such as oxidation, isomerization, and cleavage reactions are involved. This study found that insoluble caffeine-catechin interaction complexes increased while temperature increased. Thus, catechin was considered lost due to the physical instability.

Besides the factors aforementioned, the reason why cocoa extracts exhibited higher enzyme inhibition compared to individual phenolic compounds, could be due to a synergistic effect. A study performed by Angelis and collaborators (2016), investigated the effective recovery of methanol-soluble metabolites of common spruce bark for the development of new dermo-cosmetic agents. They found that pure taxifolin, (+)-catechin and taxifolin 30-O-glucopyranoside did not have elastase inhibitory activity. However, once these naturally occurring compounds present in the spruce bark were together, they exhibited a significant reduction of elastase activity. This suggested that there could be a synergistic effect among these compounds and minor compounds contained in the spruce bark extraction. Regardless of the method of extraction, it can be demonstrated that cocoa extract could be a good source for potential applications as cosmetic ingredients because it showed promising results in the inhibition on the activity of collagenase and elastase, enzymes associated with skin ageing.
CHAPTER 5: SUMMARY AND CONCLUSION

The optimized conditions for both, OBAE and MAE, were at 1:16 cocoa:water ratio and 115°C; however, MAE offered the shorter extraction time (13 min) compared to OBAE (48 min). The procyanidin B-2 content of 8.90 mg/g cocoa for OBAE was significantly (P < 0.05) greater than 7.64 mg/g cocoa for MAE and 7.07 mg/g cocoa for methanol (70%) extraction under the same conditions.

Methanol extract exhibited different masses distribution compared to OBAE or MAE extract, especially in the regions between 600 Da and 700 Da and between 850 Da and 1000 Da. However, most of the compounds extracted had molecular masses below 1000 Da regardless of the method of extraction. The different distribution of masses could be explained by the presence of minor compounds such as anthocyanins (cyanidin glycosides) and flavonol glycosides (quercetin-3-O-arabinose, isoquercitrin, quercetin-3-O-glucuronide and quercetin) also present in cocoa powder or the fragmentation of polymeric procyanidins by high temperatures.

Methanol extract showed the highest inhibitory activity of collagenase or elastase, which was attributed to the synergistic effect of its phenolic components such as theobromine, caffeine, catechin, epicatechin, procyanidin B-2 and the presence of other minor compounds. In view of the importance of collagen and elastin in preventing the skin ageing processes, cocoa extract shown promising results for their use as an active ingredient for the cosmetic industry. However, further investigations are needed to support the potential enzyme inhibitory activity of cocoa extract.
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