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COMPARATIVE STUDY OF ULTRASOUND AND MACERATION TECHNIQUES FOR THE EXTRACTION OF POLYPHENOLS FROM COCOA BEANS (*Theobroma cacao* L.)

ESTUDIO COMPARATIVO ENTRE LAS TÉCNICAS DE ULTRASONIDO Y MACERACIÓN PARA LA EXTRACCIÓN DE POLIFENOLES DEL GRANO DE CACAO (*Theobroma cacao* L.)

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Abstract

Polyphenols have gained significant interest in recent years due to their antioxidant capacity and their important role in disease prevention. In this work, the phenolic content and antioxidant properties of cocoa extracts prepared by maceration and ultrasound-assisted extraction were studied. The polyphenol content was determined using the Folin-Ciocalteu reagent and the antioxidant activity was estimated by DPPH (2,2-diphenyl-2-picrylhydrazyl) and FRAP (Ferric reducing/antioxidant power) assays. The identification and quantification of (+)-catechin and (−)-epicatechin were performed using high-performance liquid chromatography (HPLC). The results showed that by using ultrasonic radiation, it was possible to obtain higher polyphenol contents from both the husk and cotyledon of cocoa. Furthermore, extracts obtained by sonication showed the highest antioxidant activity, thus proving that this activity depends directly on the total phenolic content. The contents of (+)-catechin and (−)-epicatechin were higher in the cotyledon extracts compared to those of the husk.

Keywords: cocoa, polyphenols, extraction, ultrasound, antioxidant activity.

Resumen

Los polifenoles han ganado un interés significativo en años recientes debido a su capacidad antioxidante y a su papel importante en la prevención de enfermedades. En este trabajo, el contenido fenólico y las propiedades antioxidantes de extractos de cacao preparados por maceración y extracción asistida por ultrasonido, fueron estudiados. El contenido de polifenoles fue determinado empleando el reactivo de Folin-Ciocalteu y la actividad antioxidante fue estimada por las técnicas de DPPH (2,2-diphenyl-2-picrylhydrazyl) y FRAP (Ferric reducing/antioxidant power). La identificación y cuantificación de (+)-catequina y (−)-epicatequina fueron realizadas a través de la cromatografía de líquidos de alta resolución (HPLC). Los resultados mostraron que mediante el uso de radiación ultrasónica, fue posible extraer un mayor contenido de polifenoles tanto para cascarilla como para cotiledón de cacao. Además, los extractos obtenidos por sonicación presentaron la mayor actividad antioxidante, demostrando así que esta actividad depende directamente del contenido fenólico total. Los contenidos de (+)-catequina y (−)-epicatequina fueron mayores en los extractos obtenidos del cotiledón con respecto a aquellos obtenidos de la cascarilla.

Palabras clave: cacao, polifenoles, extracción, ultrasonido, actividad antioxidante.

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1 Introduction

Oxidation reactions occur naturally in the human body and are formed as byproducts of respiration and oxidative metabolism in all cells of aerobic organisms. Although oxygen is necessary for aerobic cells to generate energy, it has the disadvantage of producing small amounts of reactive oxygen species (ROS) that can cause damage to macromolecules. This is related to a number of chronic degenerative diseases such as cancer, rheumatoid arthritis, Alzheimer's, atherosclerosis, emphysema, cirrhosis and diabetes among others, which share common pathogenic ROS as a key factor (Mora-Huerta *et al*., 2010; Yoshihara *et al*., 2010). Because the body is vulnerable to these radicals, antioxidants are needed to decrease the concentration of these reactive species to prevent the negative effects mentioned above. Antioxidants in the body are primarily derived from diet and can promote good health. Thus, due to their significant role in disease prevention, there has been an increasing interest in recent years in the study of certain fruits, vegetables and grains with high antioxidant contents to boost their consumption (Wootton-Beard and Ryan, 2011).

Cocoa is recognized as a major dietary source of antioxidants because of its high phenolic compound (procyanidins and flavonols mainly) content (Tomás-Barberán *et al*., 2007). It has even been observed that cocoa-based products contain a higher antioxidant capacity and greater amounts of flavonoids per serving than tea or red wine (Jonfia-Essien *et al*., 2008). It is possible to distinguish three main groups of polyphenols in cocoa: catechins or flavan-3-ols (37%), anthocyanins (4%) and proanthocyanidins (58%) (Belščak *et al*., 2009). The main catechin is (−)-epicatechin, which constitutes approximately 35% of the total polyphenol content of cocoa. In addition to the cotyledon, the husk (a byproduct of the chocolate industry) also contains a significant amount of phenolic compounds (Lecumberri *et al*., 2006).

The extraction of phenolic compounds from plant materials is influenced by the compounds' chemical nature, extraction method, sample size, time and storage conditions as well as the presence of interfering substances such as proteins and carbohydrates (Koffi *et al*., 2010; García-Márquez *et al*., 2012). It has been reported that the use of aqueous solutions of methanol, ethanol and acetone dramatically improves the extraction of polyphenols compared to a single-compound solvent system (Yilmaz and Toledo, 2006). The most reported extraction methods are maceration with solvents, hot-water extraction, alkaline extraction, resin-based extraction, enzyme-assisted extraction, extractions based on gamma and electron-beam irradiation and extraction using supercritical fluids. However, some of these methods can cause a loss of bioactive compounds due to the use of high temperatures and long extraction times; or, in the case of irradiation, it can represent a health risk if the proper care is not taken (Liu *et al*., 2005). These shortcomings have led to the use of new sustainable innovative green techniques that increase extraction efficiency, reduce time and energy-consuming procedures and contribute to environmental preservation by reducing the use of water and solvents, fossil energy and generation of hazardous substances, such as microwave and ultrasound-assisted extraction (Chemat *et al*., 2011). The use of ultrasonic radiation (20-100 kHz) to extract natural compounds provides high reproducibility, easy handling, low solvent and energy consumption, low-temperature processing and a lower loss of bioactive compounds (Pan *et al*., 2011). Compared with other novel extraction techniques such as microwave-assisted extraction, the ultrasound apparatus is cheaper and its operation is easier. Furthermore, the ultrasound-assisted extraction, like Soxhlet extraction, can be used with any solvent for extracting a wide variety of natural compounds (Wang and Weller, 2006). Ultrasound can facilitate swelling and hydration of vegetal tissue, allowing high diffusion rates across the cell wall and enhancing the mass transfer. On the other hand, cavitation produced by ultrasonic waves can also disrupt the cell wall, facilitating the release of contents (Vinatoru, 2001). Khan *et al*., (2010) reported higher extraction yields of polyphenols from orange peel using an ultrasonic processor operated at a frequency of 25 kHz. According to Jacques *et al*. (2007), sonication is a simpler, faster and more effective technique than maceration to extract organic compounds from *Ilex paraguariensis* leaves. Currently, there are no reports in the literature on the extraction of polyphenols from cocoa beans using ultrasonic radiation. Therefore, the objective of this study was to evaluate the effect of ultrasonic treatment on the total phenolic content and antioxidant activity of extracts from cocoa husk and cotyledon. In addition, a comparison was made with respect to the traditional method.

2 Methodology

2.1 Materials and reagents

The cocoa beans were purchased at a local market in Mexico City. The reagents 2,2-diphenyl-1-picrylhydrazyl (DPPH), Folin-Ciocalteu reagent, gallic acid, Tris-HCl buffer, 2,4,6-Tris (2-pyridyl)-s-triazine (TPTZ) and ferric chloride hexahydrate were purchased from Sigma-Aldrich (USA). Acetic acid, ascorbic acid and sodium acetate were obtained from JT Baker (Mexico). Standards and solvents for HPLC analysis were provided by Sigma-Aldrich.

2.2 Preparation of extracts

Cocoa beans were husked manually and fractions (husk and cotyledon) ground separately in a disc mill (148-2, The Bauer Bros Co., USA). In the case of cotyledon, fat was removed from the material by soaking in hexane for 24 h at room temperature. The extraction of phenolic compounds from both fractions was performed by maceration and ultrasound application. Maceration: the plant material was subjected to a first extraction using a water-methanol solution (1:1 ratio) for 2 h at room temperature and under constant stirring. Then, the mixture was centrifuged (3,000 rpm, 15 min) and filtered. The residue was recovered for a second extraction (2 h) with an acetone-water solution (70:30 ratio), repeating the centrifugation and combining the supernatants with those obtained previously. Ultrasound: the process was similar to that described above except that instead of soaking for 2 h, the mixture of plant-solvent material was subjected to two 30-min periods of ultrasonic radiation (25 kHz) in an ultrasonic bath (TI-H-5, Elma, Germany) using the same solvent systems. The extraction conditions used in both methods were established after preliminary tests. In all the extractions the ratio sample-solvent was 1:20. Finally, the cocoa extracts were concentrated in a rotary evaporator (40-60 rpm, 50°C) (RE-500, Yamato, Japan) and dried under vacuum for 24 h at 30°C.

2.3 Determination of total phenol content

The total phenolic content was calculated from the reduction capacity of Folin-Ciocolteau using gallic acid as a standard (Waterhouse 2002). A sample volume of 100 μL was added to 7 mL distilled water, followed by the addition of 500 μL of Folin-Ciocalteu reagent (2N). The final solution was allowed to stand for 3 min at room temperature. Thereafter, 1.5 mL of a solution of sodium carbonate was added (20% w/v). After 90 min of rest in the dark, the absorbance was determined at a wavelength of 760 nm (Cary 50, Varian, USA). The results were expressed as milligram equivalents of gallic acid g^{-1} of dry matter.

2.4 DPPH radical scavenging capacity

The antiradical capacity was determined by the DPPH assay following the methodology proposed by Othman *et al*. (2007), with some modifications. A 2-mL aliquot of extract was mixed with 500 μL of 0.1M Tris-HCl Buffer by vortex mixing for 5 s. To this solution, 2 mL of a 200-μM solution of DPPH were added. After 30 min, the absorbance was determined at 517 nm. The percentage of DPPH reduction was calculated using the Eq. (1).

$$Inhibition\ (\%) = \left(1 - \frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) \times 100 \quad (1)$$

The EC_{50} value was determined from the data in the graph of the DPPH reduction effect against the extract concentration. The EC_{50} was determined as the necessary amount of the extract studied to reduce the concentration of DPPH by 50%, using ascorbic acid as a control.

2.5 Ferric reducing/antioxidant power (FRAP) assay

The antioxidant capacity was determined using the FRAP test (Thaipong *et al*., 2006). This method determines the antioxidant capacity of polyphenols to reduce TPTZ-Fe^{3+} complex. The FRAP reagent was prepared by mixing 25 mL of a 0.3 M acetate buffer (pH 3.6), 2.5 mL of TPTZ solution (0.01M) and 2.5 mL of a solution of $FeCl_3 \cdot 6H_2O$ (0.02M) at 37°C. A 150-μL extract sample was mixed with 2850 μL of FRAP solution and allowed to stand for 30 min in the dark. The absorbance was read at a wavelength of 593 nm. The results were reported in μM ascorbic acid equivalents.

2.6 HPLC analysis of cocoa extracts

The HPLC-analyses were carried out in an Agilent 1200 chromatograph (Agilent Technologies, Germany) equipped with a quaternary pump and a multiple wavelength detector coupled to an HP

Chem Station (rev. B.04.01) data-processing station. Separations were conducted on a Zorbax Eclipse XDB-C18 with dimensions 4.6x150 mm and 5 μm particle size. The mobile phase consisted of water/formic acid (99.9/0.1) as eluent A and methanol/acetonitrile (50/50) as eluent B. The system was run with a gradient program: 10-60%B in 15 min, followed by isocratic elution with 60% B for 5 min. Column temperature was set at 30°C, flow rate was 400 μl min^{-1} and the injection volume was 5 μL. Samples were previously dissolved in a mixture of water/methanol (70%) and filtered through a 0.45 μm membrane filter. The peaks of (+)-catechin and (−)-epicatechin were identified by comparing the retention times of samples with those of standards. Chromatograms were recorded at 280 nm. Standard calibration curves were also prepared and used for quantitative analysis.

2.7 Statistical analysis

All measurements were performed in triplicate and the results analyzed by ANOVA. Differences between means were detected by Duncan's multiple range test. Significant differences were considered at a level of $P < 0.05$. Linear regression tests were performed to determine correlations between data.

3 Results and discussion

3.1 Total phenol content

Figure 1 shows the total phenolic content of cocoa extracts obtained by ultrasound and maceration techniques. In the extraction of polyphenols from the husk, the sonication allowed the extraction of higher phenolic contents (25.34±1.82 mg g^{-1}) compared to the content extracted by maceration (17.85±1.33 mg g^{-1}). However, there were no significant differences ($P > 0.05$) between both methods. In the case of the cotyledon, the phenolic content results were significantly different ($P < 0.05$) between the two extraction methods. The polyphenol content varied from 91.06±0.86 to 135.92±3.77 mg g^{-1} for conventional and ultrasound methods respectively. According to Pan *et al*. (2011), the mechanical effects of sonication allow for a greater penetration of solvent into the cells, enhancing mass transfer. In the process of extraction, ultrasonic radiation can also break cell walls, facilitating the release of the compounds.

The results obtained by the traditional method are consistent with those reported in the literature

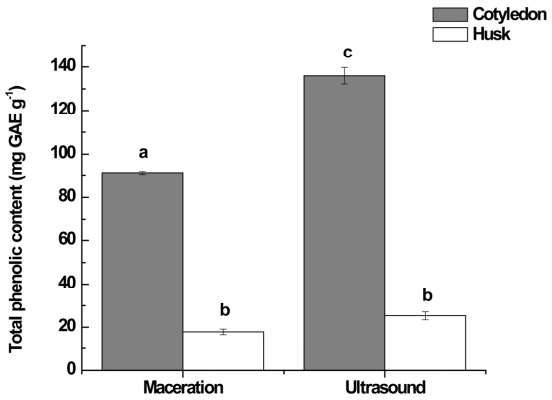

Fig. 1. Total phenolic content of cocoa extracts. Values are expressed as mean±sd ($n = 3$). Means with different letters were significantly different ($P < 0.05$).

for cocoa husk and cotyledon (Tomás-Barberán *et al*., 2007; Lecumberri *et al*., 2006). However, it is important to emphasize that the use of ultrasonic radiation greatly improved the extraction of polyphenols, especially in the case of cocoa cotyledon. The big difference in the phenolic content values obtained for both fractions of cocoa can be attributed mainly to the fact that the amount of polyphenols is not identical in the different parts of the cocoa bean. However, the moisture and particle size of the samples are two features that have an important influence on the efficiency of extraction (Wang and Weller 2006). The reduction in the particle size of the plant material will increase the number of cells directly exposed to extraction by solvent and ultrasonic cavitation (Vilkhu *et al*., 2008). In this case, the cotyledon samples presented higher moisture content and lower values of particle size than husk samples, which would explain the greater efficiency in polyphenol release.

3.2 Antiradical capacity (DPPH assay)

Figure 2 shows the scavenging activity of extracts from the husk obtained using the proposed methods. It can be seen that the activity increased rapidly in the concentration range of 0.04-0.1 mg mL^{-1}, remaining constant at higher concentrations. The compounds extracted by ultrasound application showed greater inhibition activity toward the DPPH radical compared to that toward the extracts obtained by the conventional method. As for the compounds extracted from the cotyledon (Fig. 3.), the antiradical activity was greater in the range of 0.01-0.04 mg

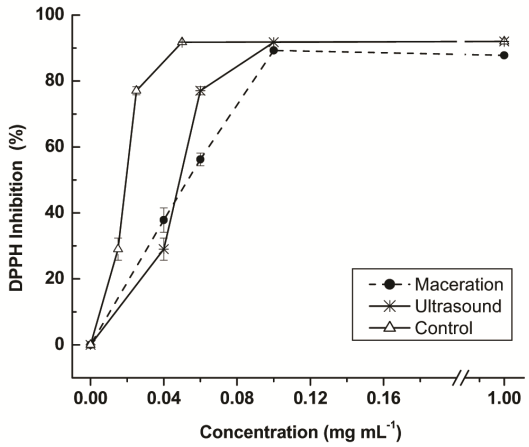

Fig. 2. Scavenging effect of cocoa husk extracts on DPPH radicals. Values are expressed as mean±sd (n = 3). Ascorbic acid was used as the control.

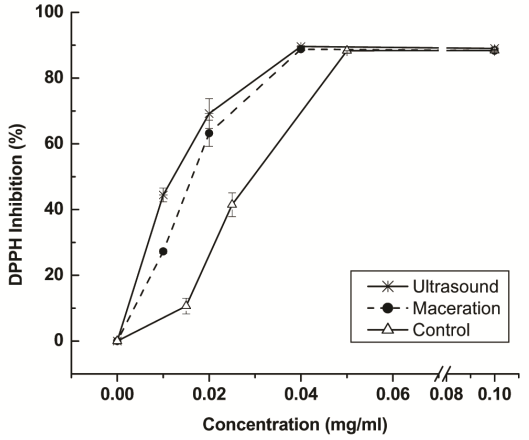

Fig. 3. Scavenging effect of cocoa cotyledon extracts on DPPH radicals. Values are expressed as mean±sd (n = 3). Ascorbic acid was used as the control.

mL^{-1}. The extracts obtained by sonication had higher antiradical activity than those obtained by maceration. It is noteworthy that the DPPH radical scavenging activity of the phenolic extracts obtained from the cotyledon was significantly higher ($P < 0.05$) than the activity exhibited by extracts obtained from the husk.

EC_{50} is defined as the amount of antioxidant necessary to decrease the initial concentration of DPPH radical by 50%. The lower the EC_{50} value is the greater the activity of extracts as DPPH radical scavengers (Wootton-Beard and Ryan, 2011). The EC_{50} value was determined by plotting the inhibition percentage of DPPH radical against the concentration of the extract. Table 1 shows the EC_{50} values for the different extracts. In the case of the husk, there were no significant differences between the EC_{50} values. However, the control had an EC_{50} value significantly lower ($P < 0.05$) than the values of the husk fraction extracts. On the other hand, the extracts of cotyledon had EC_{50} values lower than those obtained for the control. In this fraction, the extract obtained via ultrasound showed the lowest mean EC_{50} value, which was significantly different ($P < 0.05$) from that obtained by the maceration method. The analysis of variance revealed statistical differences between the EC_{50} values of the husk and cotyledon extracts. The results show good correlation ($R^2 = 0.72$) between the phenolic content and the antiradical activity of cocoa extracts. The EC_{50} results determined in this research are considerably lower than those reported by Othman *et al*. (2007), who published EC_{50} values in the range of 1.2-1.5 mg mL^{-1} for ethanol extracts of cocoa. These differences can be attributed to the variety of cocoa species used, production area and even the methodology used by the authors.

Table 1. Scavenging activity (EC50) of cocoa extracts on DPPH radicals.

Extraction method	EC_{50} (DPPH) mg mL^{-1}	
	Husk	Cotyledon
Maceration	0.0533 ± 0.0022^a	0.0164 ± 0.0012^c
Ultrasound	0.0486 ± 0.0018^a	0.0124 ± 0.0017^d
Control	0.0243 ± 0.0009^b	0.0243 ± 0.0009^b

Means with different letters were significantly different ($P < 0.05$).
Ascorbic acid was used as a control

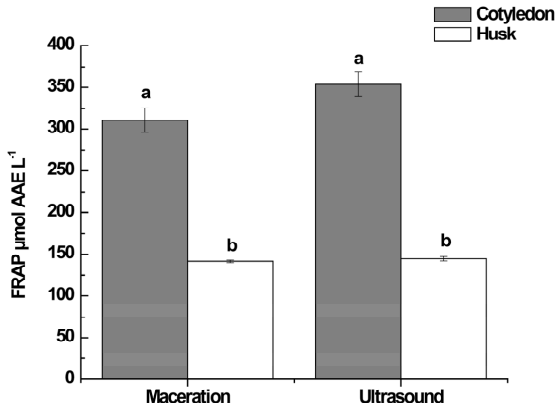

Fig. 4. Antioxidant capacity of cocoa extracts measured by FRAP assay. Concentration of sample was 0.10 mg mL^{-1}. Values are expressed as mean±sd (n = 3). Means with different letters were significantly different ($P < 0.05$).

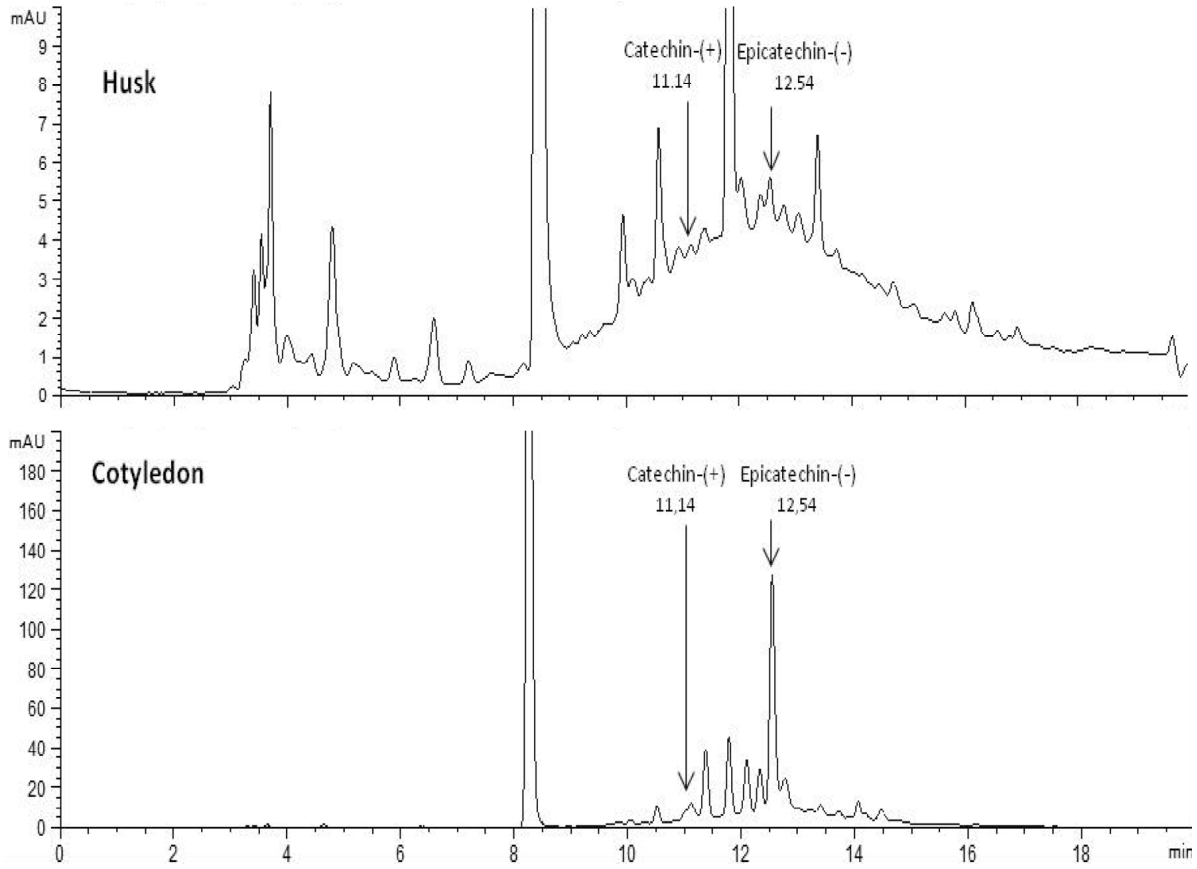

Fig. 5. Typical chromatograms of cocoa extracts obtained by ultrasound-assisted extraction.

3.3 Antioxidant activity (FRAP assay)

FRAP determination is based on the reduction of Fe^{3+}-TPTZ complex to Fe^{2+}-TPTZ complex. The reducing properties are generally associated with the presence of reducing agents, which have been shown to exert antioxidant action by breaking the free radical chain by donating a hydrogen atom. The FRAP results (Fig. 4.) indicate that the cotyledon extract obtained by ultrasound application had the highest antioxidant activity, reaching a value of 350±15 μME ascorbic acid L^{-1}. However, no significant differences between the methods used were detected. On the other hand, the FRAP values for the husk were located in the range of 141-148 μME of ascorbic acid L^{-1}. These values are significantly lower ($P < 0.05$) than that reported for cotyledon. The FRAP results correlate well with the total phenolic content ($R^2 = 0.78$) and DPPH ($R^2 = 0.98$). Othman *et al*. (2007) also reported a positive correlation between antioxidant activity (FRAP) and the polyphenol content of cocoa extracts.

Table 2. Concentration of (+)-catechin and (−)-epicatechin in cocoa fractions.

Treatment/cocoa fraction	Content (μg mg^{-1} dry extract) (+)-Catechin	(−)-Epicatechin
Maceration		
Cotyledon	4.62 ± 0.047^a	132.88 ± 0.245^a
Husk	0.28 ± 0.046^b	2.64 ± 0.018^b
Ultrasound		
Cotyledon	4.26 ± 0.025^a	144 ± 4.850^c
Husk	0.32 ± 0.083^b	2.77 ± 0.340^b

Means with different letters within the same column are significantly different ($p < 0.05$).

3.4 Identification and quantification (HPLC)

Using HPLC analysis it was possible to identify the presence of (+)-catechin and (−)-epicatechin in the cotyledon and husk samples of cocoa. Typical chromatograms of cocoa extracts obtained by sonication are shown in Fig. 5. The (+)-catechin and (−)-epicatechin contents were significantly higher ($P < 0.05$) in cotyledon than in husk for both extraction procedures (Table 2). Only in the case of (−)-epicatechin, the ANOVA showed significant differences between ultrasound and maceration methods. These results are compatible with total polyphenol quantification using the Folin-Ciocalteu method, and explain why the cotyledon presents greater efficiency in tests to determine free-radical scavenging activity and antioxidant capacity. According to Ortega *et al*. (2008), polyphenols belonging to the catechin group are mainly responsible for the antioxidant properties of cocoa.

Conclusion

The use of ultrasonic radiation facilitated the extraction of polyphenols from cocoa beans, increasing the content by 50% compared to the traditional method. In addition, the antioxidant activity measured by DPPH and FRAP methods was greater in the compounds obtained by sonication for husk and cotyledon fractions. The total phenolic content extracted from the cotyledon was significantly greater than that extracted from the husk fraction. The results demonstrate a positive correlation between the total phenolic content and antioxidant activity of cocoa extracts. Cocoa cotyledon presented significantly greater quantities of (+)-catechin and (−)-epicatechin as compared with the husk. Finally, we can conclude that the use of ultrasonic radiation is an excellent method for the extraction of natural antioxidants since it provides a short extraction time, it offers high reproducibility and low loss of bioactive compounds.

Acknowledgments

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