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ORIGINAL PAPER

Ultrasound-Assisted Method for Extraction of Theobromine and Caffeine from Cacao Seeds and Chocolate Products

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Abstract An ultrasound-assisted method for extraction of theobromine (TB) and caffeine (CF) from cacao seeds and chocolate products was developed. This extraction was carried out in 100 mL of heated water at 80 °C by using a 12.7-mm i.d. ultrasound probe and applying 240 W of power for 180 s. In order to quantify TB and CF, the aqueous extracts were treated with Carrez reagent to remove interferences. Then, 10 mL of the aqueous extract was adjusted at pH 12.5 and 10 mL of chloroform solvent was added. Ultrasound radiation was applied at 160 W of power for 30 s in order to transfer CF to the organic phase. The resulting emulsion was centrifuged at 6,000 rpm for 5 min to separate the phases. The photometric monitoring was at 276 nm for CF and at 273 nm for TB. This extraction method is simpler than the conventional liquid–liquid extraction which uses separatory funnels, and obtains similar results. By applying ultrasound in cacao seeds, the efficiency of extraction increased by 57.7 % for CF and 43.6 % for TB and by between 12 and 23 % for both analytes in chocolate products, in comparison with those results obtained by a conventional stirring extraction method.

Keywords Ultrasound-assisted extraction · Theobromine · Caffeine · Cacao seeds · Chocolate products

Introduction

The isolation of the target analytes from a solid matrix is one of the most critical analytical steps. Problems arise such as the possibility of analyte loss or contamination during sample preparation. Besides, it is a time-consuming process and involves high solvent consumption. The conventional Soxhlet extraction used to extract analytes from solid samples is a well-established model. However, despite being straightforward and cheap, it is slow and tedious.

In the last decade, there has been an increasing demand for new extraction techniques enabling automation, shorter extraction time, and reduction of organic solvents consumption (Sterbová et al. 2004; Valdez-Flores and Cañizares-Macías 2007). Advances in preparation of solid samples have brought about a great number of new techniques such as ultrasonic radiation, which shortens the steps of the analytical process (extraction, reaction, synthesis, and mixing) in comparison with other methods where this energy is not used (Cañizares-Macías et al. 2004; Valdez-Flores and Cañizares-Macías 2007; Chandrapala et al. 2013; Pingret et al. 2012).

Sound waves produce mechanical vibrations in a solid, liquid, or gas and are intrinsically different from electromagnetic waves. Thus, while the latter (radio waves, infrared rays, X-rays, gamma rays, visible or ultraviolet light rays) can pass through a vacuum, sound waves must travel in matter as they involve expansion and compression cycles travelling in an medium. In a liquid, the expansion cycles produce a negative pressure (Suslick 1990). Ultrasonic radiation is generated by immersing the reactor into an ultrasonic bath or directly introducing the source, i.e., a probe (Hong et al. 1999) or a transducer (Vinodgopal et al. 1998) into the reactor. An ultrasonic cleaning bath is the most widely used and cheapest source of ultrasound, but it does not produce better sonochemical effects than probe-type ultrasonic irradiation (Goel et al. 2004).

The most important effects of ultrasound on liquid–solid systems are mechanical and attributed to symmetric as well as asymmetric cavitations. In addition, shockwaves that have potential to create microscopic turbulence are produced within interfacial films surrounding nearby solid particles also referred to as micro-streaming (Thompson and Doraiswamy 1999; Hamdaoui et al. 2008). Asymmetric

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collapse leads to the micro-jet formation of solvent that collides with the solid surface with tremendous force, resulting in newly exposed and highly reactive surfaces, as well as corrosion and erosion. These phenomena increase the rate of mass transfer near the surface and enhance the reaction rate (Song et al. 2009). Ultrasound is also known to facilitate extraction processes (Luque de Castro and Priego-Capote 2006; Horžić et al. 2012). The effects of ultrasound on leaching are also essentially primarily related to cavitation. Thus, the implosion of bubbles formed during ultrasound application produces rapid adiabatic compression of gases and vapors within the bubbles or cavities, resulting in an increase of temperature and pressure. The increased temperature enhances the solubility of the analytes in the leachant and facilitates their diffusion from the sample matrix to the outer region; on the other hand, the increased pressure facilitates the penetration of the leachant into the sample matrix, and the analytes transfer from the matrix to liquid phase at the interface.

Cacao is one of the most consumed seeds as ingredient in beverages, pastries, or candies. Cacao products are rich in polyphenols, especially procyanidins, whose concentrations vary depending on their origin (Niemenak et al. 2006; Komes et al. 2012) and processing conditions (Summa et al. 2006). Procyanidins have been found to be health beneficial because of their antioxidant (Othman et al. 2007) and anti-inflammatory properties (Ramiro et al. 2005). Flavonoids from *Theobroma cacao* downregulate inflammatory mediators and anti-atherosclerotic activity (Vinson et al. 2006). In addition to procyanidins, cacao samples also contain alkaloids such as theobromine (TB) and caffeine (CF). These compounds affect the flavor of cacao products (Luna et al. 2002) and have received increasing attention due to their physiological effects (Buchelli et al. 2001). Their importance lies on their incidence over the central nervous system and on the fact that each person reacts differently according to the ingested amount (Yamada et al. 2009).

TB and CF in cacao are found in a quantity of 0.2 and 1.2 %, respectively, but these values depend on variety, harvest, curing process, etc. Therefore, the extraction procedure and quantification of these analytes are important in order to know about the origin and quality of cacao.

Due to the high solubility of TB and CF in water, the extraction of these compounds has been carried out by using this solvent, which allows simple extraction procedures. AOAC method describes the extraction of TB and CF from cacao seeds and cacao products using 95 mL of hot water (100 °C) and by stirring for 25 min (AOAC Official Method 2005). Brunetto et al. (2007) have carried out the extraction of both analytes using hot water at 80 °C and stirring for 5 min. Reflux with water for 60 min has also been used (Lo Coco et al. 2007), but the results have been similar to those obtained by stirring for 5 min (González-Nuñez and Cañizares-Macías 2011). On the other hand, during these extraction processes, interferents such as polyphenols, proteins, and other compounds with high molecular weight are also extracted and must therefore be removed from the extracts by addition of Carrez I and Carrez II reagents, as they are measured at the same TB and CF wavelengths (Shufen et al. 1990).

In this paper, an aqueous extraction method applying ultrasound to cacao seeds and products of chocolate to extract TB and CF has been developed. For the extraction, an ultrasound probe was used, and the results were compared with those obtained without using ultrasonic waves. The quantification was carried out by UV spectroscopy; therefore, it was necessary to separate CF from aqueous extract with chloroform. This process was carried out by applying ultrasound radiation with a probe while power and irradiation time were evaluated.

Material and Methods

Instrumentation

Ultrasonic irradiation was applied by means of a Branson 450 digital sonifier (20 kHz, 400 W, Danbury, CT, USA) equipped with a cylindrical titanium alloy microprobe (13 mm diameter). A UV–vis spectrophotometer Cary 3 from Varian (Sydney, Australia) was used to measure CF and TB at 276 and 273 nm, respectively. A magnetic stirring (Comarec, Mexico City) with controlled temperature, a centrifuge (HETTICH model EBA 20, Buckinghamshire, England), an oven (Rios Rocha, Mexico City), and a pH meter (Oakton, Vernon Hills, IL, USA) were also used in the treatment of the samples.

Statgraphics Plus 4.0 software (Statistical Graphics, Rockville, MD) was used for the designing of tests and data analysis.

Reagents and Solutions

All used reagents were analytical grade. In order to remove polyphenols and proteins, the obtained aqueous extracts from cacao seeds and chocolate samples were treated with Carrez I solution [24 g of $Zn(CH_3COO)_2$ (Sigma, Toluca, Mexico) was weighed and dissolved into 100 mL of a 3 % CH_3COOH solution (Merck, Naucalpan, Mexico)] and $NaHCO_3$ powder (Baker-Mallinkroft, Mexico City). Fats were removed from the cacao seeds with petroleum ether (Baker-Mallinkroft, Mexico City). Chloroform solvent (Baker-Mallinkroft, Mexico City) was used to carry out the extraction of CF from the aqueous extracts. A TB (Fluka, Toluca, Mexico) aqueous stock solution of 50 $\mu g mL^{-1}$ was used to set the TB standard solutions. Chloroform as solvent

was used in order to prepare the CF stock solution (Sigma, Toluca, Mexico) of 50 $\mu g mL^{-1}$. For the extractions with chloroform, the pH of the aqueous extracts was adjusted at 12.5–12.7 with 1.0 $mol L^{-1}$ NaOH.

Samples

The used samples were: (a) cacao seeds from Comalcalco, Tabasco, Mexico, bought at the local market; (b) cacao peels obtained from the Comalcalco cacao seeds; and (c) cacao products such as chocolate bars, chocolate beverages, and commercial cocoa powder.

Samples Treatment

Solid samples of cacao were ground into soft powder in a ceramic mortar. Then, fats were removed with three portions of 5 mL of petroleum ether, shaken for 1 min, and allowed to stand for about 5 min each time. The petroleum ether extracts were combined and then filtered off, and the solvent excess was removed with N_2. All analyses were carried out using 1 g of defatted sample.

Liquid samples were analyzed without previous treatment using 10 mL of sample.

Procedures

Extraction of TB and CF from Cacao Seeds and Chocolate Products

Ultrasound-assisted extraction method (UAEM) was compared with a validated previously stirring method (SM) (González-Nuñez and Cañizares-Macías 2011).

Stirring Method One hundred milliliters of hot distilled water (80 °C) was added to 1 g of sample. The mixture was kept at 80 ° C and stirred for 5 min. Then, 5 mL of Carrez I was added, and it was stirred for 1 min. The mixture was left to settle until it reached room temperature; later, $NaHCO_3$ was added until precipitation stopped. The mixture was filtered off, and the remaining filtrate was transferred into a 100-mL volumetric flask and diluted to volume with distilled water. CF was separated from the aqueous extracts by carrying out multiples extractions with chloroform and using separatory funnels (see "Manual extraction" under "Extraction of CF from Aqueous Extracts" section; Shufen et al. 1990; González-Nuñez and Cañizares-Macías 2011).

Extraction of TB and CF from Cacao Seeds by the Proposed Ultrasound-Assisted Extraction Method One gram of solid sample was weighed and poured into a 250-mL beaker, and 100 mL of distilled water at 80 °C was added. The ultrasound probe was placed at 1 cm from the top surface of the liquid phase to allow a better interaction between the sample and the extractant. The ultrasound extraction was carried out at 240 W for 180 s. Next, 5 mL of Carrez I reagent was added, and it was stirred for 1 min. Then, $NaHCO_3$ was added until precipitation stopped. The mixture was filtered off, and the remaining filtrate was transferred into a 100-mL volumetric flask and diluted to volume with distilled water (solution A). CF was extracted from aqueous extracts with chloroform by applying ultrasound and using the probe (see "Proposed ultrasound extraction" under "Extraction of CF from Aqueous Extracts" section).

For liquid samples, 10 mL of beverage or syrup was diluted up to 100 mL with distilled water. They were directly treated with Carrez I reagent and $NaHCO_3$ and filtered off.

Validation of the Proposed UAEM for CF and TB A multifactorial design methodology based on a factorial design 2^3 type V resolution allowing four freedom degrees and involving eight randomized runs plus three centre points was built, in order to carry out a screening study of the main factors affecting the extraction of TB and CF of cacao seeds. Therefore, power, irradiation time, and extractant volume were evaluated. The ANOVA test was calculated by Statgraphics software. Top and bottom values used in the factorial design were established according to previous studies. The evaluated values for the factorial design were: extraction volume (V), 25 mL and 70 mL; irradiation time (IT), 30 and 180 s; and power (P), 40 and 120 W.

With the aim of evaluating possible changes in the studied analytes by the ultrasonic radiation, two mixtures of known concentration of CF and TB (2 and 10 $mg L^{-1}$) under the same extraction conditions of the samples were analyzed.

In order to study the intra-laboratory reproducibility and repeatability, two independent extractions were also carried out for 7 days. The results were analyzed using an ANOVA test. A study of recoveries adding 2.5 mg of each analyte to 1 g of sample was also carried out.

Measurement of TB and CF from Aqueous Extracts

Extraction of CF from Aqueous Extracts With the purpose of simplifying the CF extraction from aqueous extracts using chloroform solvent as extractant, a novel ultrasound method using a probe was proposed. The method was compared with the conventional liquid–liquid extraction method, which uses separatory funnels and chloroform solvent.

Manual extraction: First of all, 50 mL of the diluted aqueous extracts (see "Stirring method" section) was transferred into a 100-mL separatory funnel, and about 5.5 mL of 1 mol L^{-1} NaOH was added so as to regulate

the pH between 12.5 and 12.7. Then, these extracts were treated with four portions of 5 mL of chloroform, shaken for 1 min, and left to stand for about 5 min each time. After that, the chloroform extracts were combined in a 25-mL volumetric flask and diluted to volume with chloroform. Next, 1 mL of this solution was transferred into another 25 mL volumetric flask and diluted to volume with chloroform so that CF would be measured at 276 nm. The aqueous phase was next transferred into a 100-mL volumetric flask and diluted to volume with distilled water and filtered off. Ten milliliters was transferred into another 100-mL volumetric flask, and 50 mL of distilled water and 0.55 mL 10 % HCl were added. Then, the solution was diluted with distilled water to volume. Finally, 1 mL was transferred into a 25-mL volumetric flask and diluted to volume with distilled water in order to measure TB at 273 (González-Nuñez and Cañizares-Macías 2011).

Proposed ultrasound extraction:

The conventional liquid–liquid extraction is laborious and requires a great amount of organic solvent. With the aim of both simplifying the liquid–liquid extraction and of decreasing the amount of organic solvent, an ultrasound-assisted extraction method was developed. To begin with, 10 mL of diluted aqueous extract (see "Extraction of TB and CF from Cacao Seeds by the Proposed Ultrasound-Assisted Extraction Method" section) was transferred into a 50-mL beaker, and about 5.5 mL of 1 molL^{-1} NaOH was added to regulate the pH between 12.5 and 12.7. Then, 10 mL of chloroform was added, and the ultrasound probe was introduced into the glass at 0.5 cm from the top surface of the liquid. Next, a power of 160 W for 30 s was applied. After that, the mixture was separated and centrifuged at 6,000 rpm for 5 min, and 1 mL of chloroform phase was transferred into a 10-mL volumetric flask and diluted to volume with chloroform in order to measure CF. Finally, in order to analyze TB, 1 mL of the aqueous solution was transferred into a 50-mL volumetric flask and diluted to volume with distilled water.

Standard solutions with different concentrations of CF and TB treated under the same extraction conditions of the aqueous extracts were analyzed in order to validate the method, and recoveries were calculated.

Results and Discussions

Calibration Graphics

Two calibration curves were constructed in order to quantify TB and CF in cacao seeds and chocolate products. CF standards were prepared by using chloroform solvent, whereas for the TB ones, aqueous solutions were used. The obtained linear equation for TB was: Abs=0.06 (±0.0003)[TB]+0.018(±0.0035) with a linear range between 1 and 20 μgmL^{-1}, a detection limit of 0.08 μgmL^{-1}, and a regression coefficient of 0.9999. For CF, the equation was: Abs=0.05(±0.001)[CF]−0.009(±0.011) with a regression coefficient of 0.9999, a detection limit of 0.30 μgmL^{-1}, and linear range from 3 to 20 μgmL^{-1}.

Optimization of the Extraction of TB and CF from Cacao Seeds by the Proposed Ultrasound Method (UAEM)

The cacao seeds used for the analysis were high quality with regard to moisture (4.5±0.15 %), pH (5.1±0.25), and fats (24.6±3.2 %), in accordance with the specifications of the Mexican norm (NMX-FF-103-2003) (Mexican Norm NMX-FF-103-SCFI-2003 2003). Fats were removed from all the samples as previous studies demonstrated best results for the extraction of TB and CF from cacao seeds when fats are not present (González-Nuñez and Cañizares-Macías 2011). The factorial design to evaluate power, irradiation time, as well as extractant volume was constructed in accordance with previous studies, according to which, volumes lower than 25 mL prevent a good mixing between the extractant and the sample. Therefore, 70 mL as maximum volume was selected to minimize the extractant amount and to allow a good mixing. Powers lower than 40 W caused very low extraction of caffeine but, as it considerably increased from this value up, it was selected as minimum value. The maximum power value 120 W was selected to evaluate the effect of power without damaging the probe. The irradiation time values were selected with the aim of decreasing the extraction time in comparison with the

Table 1 Matrix employed for studying the simultaneous effect of three variables, power (*P*), irradiation time (IT), and volume of extractant (*V*), on extraction of theobromine (TB) and caffeine from cacao seeds (CF) using an ultrasound extraction-assisted method (UEAM)

Test	*V* (mL)	*P* (W)	IT (s)	CF[a] ($mg\,g^{-1}$)	TB[a] ($mg\,g^{-1}$)
1	−1 (25)	+1 (120)	+1 (180)	0.62±0.05	10.99±0.12
2	0 (47.5)	0 (80)	0 (105)	0.75±0.02	13.61±0.09
3	+1 (70)	−1 (40)	+1 (180)	0.64±0.01	13.84±0.15
4	+1 (70)	+1 (120)	−1 (30)	1.03±0.05	16.59±0.09
5	0 (47.5)	0 (80)	0 (105)	0.75±0.04	15.63±0.13
6	−1 (25)	−1 (40)	−1 (30)	0.25±0.02	6.86±0.18
7	−1 (25)	−1 (40)	+1 (180)	0.25±0.03	11.94±0.12
8	−1 (25)	+1 (120)	−1 (30)	0.59±0.04	13.65±0.15
9	+1 (70)	+1 (120)	+1 (180)	0.92±0.03	17.46±0.20
10	+1 (70)	−1 (40)	−1 (30)	0.94±0.02	13.81±0.08
11	0 (47.5)	0 (80)	0 (105)	0.71±0.04	14.46±0.22

Results, coded and real values (in parenthesis) of the variables are indicated

[a] Mean±standard deviation
(n=3)

batch method, but at the same time allowing enough contact time between sample and solvent. Table 1 shows the experimental design used with three central points. The ANOVA results are shown in Tables 2 and 3. The ANOVA table partitions the variability in TB and CF into separate pieces for each one of the effects. This then proves the statistical significance of each effect by comparing the mean square against an estimate of the experimental error. The study was carried out at 95 % confidence level so *P* values are less than 0.05, indicating that they are significantly different from zero. The extraction volume was statistically significant for CF and TB, but only power was significant for CF. Nevertheless, at higher power, the TB extraction increased, and besides, when the power was low and the irradiation time was long, the extraction of TB and CF increased. Consequently, a second factorial design (2^2) was carried out by increasing the power and maintaining the same values above described for the irradiation time and by using 70 mL of extractant. In Table 4, the matrix employed is shown. After performing the experiments and measuring the response of the ANOVA, tests showed that both factors were not statistically significant. However, with higher irradiation and power, and after longer time, the extracted CF amount increased. Therefore, in order to improve the extraction, power was increased at 240 W (maximum power recommended for the used probe), and the irradiation time was tested at 180 and at 300 s. With this power, the extraction of TB and CF of cacao seeds increased 5 and 2 %, respectively. The increase in irradiation time did not improve the extraction of the analytes. A final test was carried out, in which the volume of extractant at 100 mL was increased. This volume was selected for two reasons: (1) because, in line with the first experimental design, the volume of extractant was an important factor of the extraction for both analytes; and (2) since this volume had been proved already in the batch method as optimum. The extraction of both analytes was higher, between 2 and 3.2 % for TB and CF, respectively.

As a result, the selected optimum conditions of extraction of TB and CF by the proposed ultrasound extraction method (UAEM) were: *P*=240 W, IT=180 s, and *V*=100 mL.

Finally, another test adding Carrez I reagent before and after the extraction using the optimum ultrasound extraction conditions was conducted. The extraction showed better results when the reagent was added afterwards as, when added before, a precipitate was formed and stirring was no efficient.

The precision of the proposed method in terms of within-laboratory reproducibility and repeatability was assessed by using a single experimental setup with duplicates. The repeatability and reproducibility values, expressed as relative standard deviation, showed high precision: 1.11 and 2.90 % for TB, and 4.03 and 4.87 % for CF, respectively.

Table 2 Analysis of variance (ANOVA) for theobromine (TB) at 95 % of confidence level from the results of the factorial design of Table 1

Source	Sum of squares	DF	Mean square	*P* value
A: *V* (mL)	41.6785	1	41.6785	0.0311
B: *P* (W)	18.7272	1	18.7272	0.0942
C: IT (s)	1.3778	1	1.3778	0.5852
AB	0.0392	1	0.0392	0.9252
AC	0.2888	1	0.2888	0.7995
BC	5.95125	1	5.95125	0.2854
Total error	15.6846	4	3.92115	
Total (corr.)	83.7473	10		

V volume, *P* power, *IT* irradiation time

Table 3 Analysis of variance (ANOVA) for caffeine (CF) at 95 % of confidence level from the results of the factorial design of Table 1

Source	Sum of squares	DF	Mean square	*P* value
A: *V* (mL)	0.41405	1	0.41405	0.0007
B: *P* (W)	0.1458	1	0.1458	0.0051
C: IT (s)	0.01805	1	0.01805	0.1217
AB	0.01445	1	0.01445	0.1546
AC	0.0242	1	0.0242	0.0859
BC	0.00605	1	0.00605	0.3201
Total error	0.0188182	4	0.00470455	
Total (corr.)	0.641418	10		

V volume, *P* power, *IT* irradiation time

The recoveries obtained with standards in order to evaluate possible changes in the studied analytes under the same extraction conditions of the samples were: between 99.8 and 102.25 % for TB, and between 98.5 and 101.15 % for CF. These results show that the obtained values are within the precision of the method.

Comparative Study Between the UAEM and the SM

Cacao seeds were also analyzed by the SM, and the results were compared with those provided by the UAEM. *F* and *t* tests were conducted using the values of Table 5. The *F* test showed that there was no difference in variances, so the *t* test was calculated through an equal variance. The proposed null hypothesis was: media values of TB and CF in the samples are the same for both methods. The results showed that the values of the media were statistically different as the UAEM proposed method extracted a higher amount of the studied analytes than the SM [the calculated *t* value was higher than the critical value (t4=2.78)]. With the UAEM, the extraction efficiency improved 57 % for CF and 40 % for TB, and also, the extraction time decreased 60 %. The values were calculated using the average of all measurements.

Table 4 Matrix employed for studying the simultaneous effect of the power (*P*) and irradiation time (IT) on extraction of theobromine (TB) and caffeine (CF) from cacao seeds (CF) using ultrasound extraction-assisted method (UEAM)

Test	*P* (W)	IT (s)	CF[a] ($mg\,g^{-1}$)	TB[a] ($mg\,g^{-1}$)
1	0 (180)	0 (105)	1.06±0.02	18.15±0.14
2	−1 (160)	−1 (30)	0.64±0.01	17.69±0.09
3	0 (180)	0 (105)	1.11±0.01	17.23±0.12
4	+1 (200)	+1 (180)	2.02±0.02	20.59±0.24
5	0 (180)	0 (105)	1.22±0.02	17.49±0.13
6	−1 (160)	+1 (180)	0.69±0.03	19.66±0.16
7	+1 (200)	−1 (30)	1.13±0.02	19.04±0.20

Results, coded and real values (in parenthesis) of the variables are indicated. Volume of extractant was adjusted at 70 mL

[a] Mean±standard deviation (n=3)

Table 5 Determination of caffeine (CF) and theobromine (TB) in cacao seeds using the ultrasound-assisted extraction method (UAEM) and the stirring method (SM)

Sample	UAEM[a]		SM[a]	
	CF ($mg\,g^{-1}$)	TB ($mg\,g^{-1}$)	CF ($mg\,g^{-1}$)	TB ($mg\,g^{-1}$)
Cacao seeds	2.16±0.02	25.91±0.14	1.37±0.02	18.04±0.18
	1.91±0.02	25.19±0.10	1.16±0.015	18.54±0.14

Optimal conditions by UAEM: 240 W, 180 s, and 100 mL of water at 80 °C

[a] Mean±standard deviation (n=3)

Evaluation of the Extraction of CF from the Aqueous Extracts of Cacao Seeds Using an Ultrasound Probe

At the same time that the optimization of the TB and CF extraction from cacao seeds was carried out, a study aiming to separate CF and TB with chloroform from the aqueous extracts using the ultrasound probe was also performed. For the optimization, 10 mL of aqueous extract was used; this value was selected as the minimum volume for a good sonication with the 12.7-mm I.D. probe. In order to extract CF from the aqueous phase to the chloroformic phase and keep TB in the aqueous phase, the pH of the aqueous solutions was adjusted at 12.5–12.7 (Shufen et al. 1990). Four combinations of the three key parameters for the extraction of CF such as power, irradiation time, and volume of chloroform were evaluated. The values were selected with the aim of decreasing the extraction time in comparison with separatory funnel extraction. The ultrasound probe was placed at 0.5 cm from the top surface of the liquid. In all the cases, an emulsion was formed within a few seconds after the ultrasound application. As the emulsion existed since the beginning of the extraction, emulsions were disrupted by centrifugation at 6,000 rpm for 5 min after the ultrasonic radiation. The results of the tests are found in Table 6 and showed no statistical difference for any parameter. However, at 160 W, 25 mL, and 40 s, the values were a little higher. With the aim to minimize the method even more, lower volumes of chloroform and shorter irradiation times were tested. A volume of chloroform of 10 mL and an irradiation time of 30 s at 160 W of power were enough to extract CF with efficiency, which was proved by a recovery study using seven aqueous mixtures containing 5 $\mu g\,mL^{-1}$ of CF and 10 $\mu g\,mL^{-1}$ of TB. The solutions were treated under the same conditions used by the proposed method. The results showed recoveries between 99.30 and 102.23 %

Table 6 Evaluation of extraction of caffeine (CF) from aqueous extracts of cacao seeds by applying ultrasonic radiation by means of a probe of 12.7 mm i.d.

Volume of choloroform (mL)	Power (W)	Irradiation time (s)	CF[a] ($mg\,g^{-1}$)	TB[a] ($mg\,g^{-1}$)
25	120	50	2.18±0.07	19.77±0.12
25	160	40	2.21±0.11	19.89±0.24
50	120	50	2.19±0.08	19.85±0.12
50	160	40	2.21±0.05	19.82±0.16

TB theobromine

[a] Mean ± standard deviation (n=3)

for both analytes. The method allowed a decrease in both analysis time and volume of chloroform, simplifying the extraction in addition. The extraction by ultrasound was compared with the manual liquid–liquid extraction using separatory funnels. With the proposed method, the volume of sample decreased from 50 to 10 mL, the chloroform volume decreased from 20 to 10 mL, and the extraction time from 20 min to 30 s in comparison with the manual extraction method.

Analysis of Samples

The proposed method was applied to chocolate products, and the results were compared with those obtained by the stirring method. The results of the analyzed samples and the obtained recoveries by the proposed ultrasound method are shown in Table 7. The values obtained for each sample were higher by the UAEM than by the SM itself: for chocolate products between 12 and 19 %, and for cacao seeds there was an increase of 40 % for TB and of 57 % for CF, with excellent recoveries. The amounts of TB and CF in the chocolate beverage and syrup were very low, showing that they were elaborated with an insignificant amount of cacao.

Conclusions

The proposed UAEM was more efficient than the stirring method commonly used for the extraction of these analytes. Moreover, the proposed method was applied in different samples of chocolate with very good results, demonstrating that the ultrasound waves improve the extraction and cause no modification in the extracts. Besides, the results showed a high amount of CF and TB in cacao peel and, therefore, it could be used for the elaboration of chocolate bars and chocolate derivatives instead of cacao seeds or cacao powder. Consequently, it is of paramount importance to establish precise and trustworthy methods such as the proposed ultrasound one for the extraction and measurement of CF and TB in cacao products.

On the other hand, the applications of ultrasound to extract CF from the aqueous extracts from chocolate

Table 7 Concentration obtained of theobromine (TB) and caffeine (CF) in chocolate products and cacao seeds by the proposed ultrasound-assisted extraction method (UAEM) and by the stirring method (SM)

Sample	Method					
	UAEM[a]			SM[a]		
	CF ($mg\,g^{-1}$)	Recovery (%)	TB ($mg\,g^{-1}$)	Recovery (%)	CF ($mg\,g^{-1}$)	TB ($mg\,g^{-1}$)
Cacao seeds	2.16±0.02	100.62±0.5	25.91±0.22	101.12±0.13	1.37±0.02	18.04±0.174
Commercial cocoa powder Hershey's	1.18±0.08	100.43±0.3	18.63±0.12	99.62±0.23	1.01±0.04	15.52±0.12
Chocolate bar 70 % Lindt Excellence	1.48±0.11	100.16±0.1	16.75±0.24	104.00±0.21	1.20±0.16	14.88±0.21
Chocolate bar 85 % Lindt Excellence	1.84±0.05	100.41±0.6	20.01±0.20	100.79±0.12	1.52±0.12	16.75±0.25
Chocolate bar 90 % Lindt Excellence	1.95±0.07	108.9±0.90	23.12±0.18	101.18±0.52	1.56±0.09	20.25±0.26
Chocolate bar 42 % Hershey's	0.95±0.03	101.14±1.0	10.75±0.14	104.60±0.32	0.80±0.08	9.50±0.30
Chocolate bar Turin Exoticas	1.04±0.12	100.09±0.20	9.38±0.21	99.09±0.60	0.85±0.16	8.29±0.15
Milky chocolate bar World table	0.49±0.05	107.49±0.30	3.80±0.24	103.74±0.21	0.40±0.04	3.40±0.19
Sugar free chocolate bar Chocozero	0.84±0.09	100.28±0.53	9.08±0.16	107.69±0.20	0.70±0.11	8.01±0.23
Chocolate bar[b] Nestle, Abuelita	1.33±0.10	98.65±0.62	16.22±0.12	100.07±0.18	1.09±0.09	14.40±0.12
Chocolate beverage Hershey's	0.15±0.08	101.20±0.21	0.34±0.15	100.93±0.21	0.13±0.02	0.28±0.03
Chocolate flavor syrup Great Value	0.12±0.08	102.74±0.10	0.24±0.14	100.92±0.25	0.09±0.02	0.21±0.05
Milk chocolate bar Nestle, Carlos V	0.55±0.05	101.23±0.44	2.36±0.15	102.25±0.14	0.47±0.06	2.10±0.25
Cacao peel	1.31±0.03	99.50±0.51	15.10±0.13	100.96±0.11	1.01±0.02	13.22±0.19

[a] Mean±standard deviation (n=3)

[b] Cacao paste (70 %), sugar, and almonds

products and cacao seeds allowed a very high precision. Also, the liquid–liquid extraction was simplified.

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