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Ionic Liquid-Based Ultrasonic-Assisted Extraction of Alkaloids from Cacao (*Theobroma cacao*)

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Recent advances in the solid-liquid extraction have brought a large number of new techniques as ultrasonic assisted extraction (UAE). Different solvents such as alcohols, acetonitrile and chloroform are reported as extractors of added-value compounds. Ionic liquids (ILs) have been used the last few years as a non-conventional solvent for the extraction of alkaloids from natural sources. This work aims to extract efficiently alkaloids such as Caffeine (CF) and Theobromine (TB) from cacao, using protic ionic liquids (PILs) based on ultrasonic assisted extraction (PIL-UAE). In order to optimize the extraction, a 2^3 factorial design was applied using three parameters (extraction time, ratio of mass/volume, solvent concentration). The extraction method was applied to cacao seeds, using 2-hydroxy ethylammonium acetate (2HEAA) as PIL. The results indicated that protic ionic liquids showed remarkable effects on the extraction yields of alkaloids. The main variable that influences the extraction process of the alkaloids is the solid / liquid ratio; however, the ultrasonic power has no effect.

1. Introduction

Alkaloids are organic compounds found in natural products such as cocoa (*Theobroma cacao*), which can produce physiological effects on the human body. Among the alkaloids that cacao possesses, theobromine, caffeine and theophylline are important active ingredients (Yamada et al., 2009) that can be used as raw materials for a large number of industrialized products used by the pharmaceutical, cosmetic and food industry. The structure of alkaloids is depicted in the Figure 1.

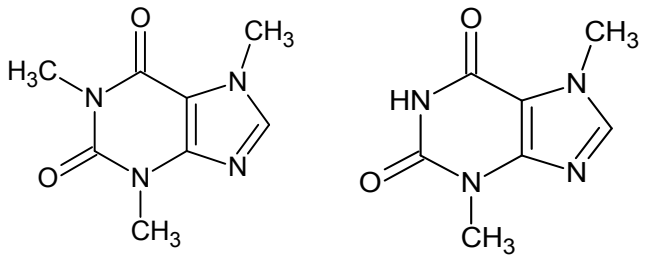

Figure 1: Molecular structure of different alkaloid (caffeine and theobromine).

Nevertheless, for a biomolecule to be used industrially, firstly it is necessary to extract it from several matrices (Azmir et al., 2013). For this process, it is necessary to develop techniques for identification, quantification, isolation, extraction and purification that are economically viable (Huie, 2002), and additionally, compatible with the principles of green chemistry, especially in the use of safe chemical compounds and solvents with

high energy-efficient. In this sense, ionic liquid can be an excellent alternative to volatility organic solvents, in extraction protocols more environmentally friendly because has low vapor pressures, thermal and chemical stability, non-flammable and non-corrosive, beside to act as a catalyst properties (Bogdanov et al., 2015).
Ionic liquids (ILs) are molten salts with a melting point below 100 ^{o}C, which contain at least one organic cation and organic or inorganic anion (Losetty et al., 2017). A large combination of cations (cationic family and alkyl chain) and anions allows building about one million different ionic liquids, conferring the denomination of "design solvents" (Baker et al., 2005). Owing to their excellent physiochemical properties, ILs has been investigated as potential candidates for the extraction of value-added compounds from natural sources (Passos et al., 2014; Bogdanov and Svinyarov, 2013). IL can be divided into two broad categories: aprotic (AIL) and protic ionic liquids (PIL). The subset PILs are synthetized by means of proton transfer from a Brønsted acid with a Brønsted base, generally have higher conductivity and fluidity, as well as lower melting points than the AIL; they are also cheaper and their synthesis does not involve the formation of by-products (Ghandi, 2014, Álvarez et al., 2010). These new solvents have accelerated research in different process of chemistry and chemical engineering. The unique properties allow the application of IL or IL-based materials in extraction of bioactive compounds from plants. They are also used in liquid-liquid extraction (LLE), ultrasonic-assisted extraction (UAE), microwave-assisted extraction (MAE) high performance liquid chromatography (HPLC) and solid-phase extraction (SPE) among others (Tang et al., 2012).
Recent advances in the preparation of solid samples have brought a large number of new techniques, such as the ultrasonic assisted extraction method (UAE), which reduces the process steps (extraction, reaction, synthesis, and mixing) in comparison with other methods in which this energy is not used (Peralta-Jiménez; Cañizares-Macías, 2013, Chemat et al., 2011). This technique is one of the most widely explored in laboratories and industrial scale, due to its high efficiency in extracting components, low solvent consumption, simple operation and low pollution to the environment (González-Centeno et al., 2015; Tao and Sun, 2015, Tao et al., 2014). UAE is a system that uses acoustic energy and solvent to promote the extraction process of the target compounds of several plant structures (Minjares-Fuentes et al., 2014).
This work is addressed in the extraction of alkaloids such as caffeine and theobromine from cocoa, using protic ionic liquids in ultrasonic assisted extraction protocols (PIL-UAE). For this, a planning of experiments 23 with three replicates at the central point was applied.

2. Material and Methods

Dried cocoa seeds samples were kindly donated by farmers in the municipality of Ihéus-Bahia, Brazil. The samples were ground in a mill (Marconi mod. MA 340/A), sieved (45 mesh), packed in polypropylene bags, and finally stored for the next tasks.
Based on our previous experience in the field of valuable compounds isolation, we selected the PIL 2-hydroxy ethylammonium acetate (2HEAA) as extractant. The ionic liquid was synthetized by an equimolar neutralization reaction between ethanolamine (Sigma, mass fraction purity 0.96) and acetic acid (Dinâmica, mass fraction purity 0.997). The amine solution in water amine (1 mL of water per every 1 mL of amine) was placed in a flask made entirely of glass and connected with an addition funnel containing the acetic acid solution in water (3 mL of water per every 1 mL of acid). The purity of the reagents was considered when calculating the quantities to be combined. These chemical reactions are highly exothermic; thereupon; the flask was mounted in an ice bath to avoid the temperature increase. The acetic acid was added dropwise to the flask under stirring with a magnetic stir bar. The mixture were per-evaporated (Tecnal mod. TE-211) at 40 ^{o}C and under vacuum (5.0 KPa) for 6 hours. The water residue was then removed in high vacuum (20 Pa) at 50 ^{o}C for overnight (Edwards RV5). The water content was determined by coulometric Karl-Fisher titration using a Mettler-Toledo 870 KT Titrino Plus. The purity of protic ionic liquid was analysed in 1H NMR spectra were recorded on a Bruker AC-200 NMR (operating at 200 MHz).
The milled samples was added to an aqueous solution of 2HEAA, and then the extraction of alkaloids were carried out on an ultrasound (QSonica Q500 USA) for 3 minutes with ultrasonic pulses every 10 seconds under different conditions of ultrasonic power (W), solid/liquid ratio (g/g) and PIL concentration (M). After the extraction, the samples were centrifuged at 4500 rpm for 10 minutes to remove the finely suspend particles.
The supernatants were filtered through Millipore filter with 45 µm pore diameter. Subsequently, the filtrate was diluted (1:10) in mobile phase (water and acetonitrile - 80:20) and 20 µL (at a rate flow rate of 0.8 mL.min^{-1}, isocratic mode) of the solution were injected into the chromatographic system (Varian ProStar 210) with UV-VIS detector, at 272 nm, C18 column type for the procedure for the identification and quantification of alkaloids. In order to optimize the alkaloids extraction was applied the response methodology (factorial 2^3 factorial with three central points) according to Rodrigues and Iemma (2015). The variables to optimize were theobromine and theophylline concentration (mg.L^{-1}), and ultrasonic power (W), solid/liquid ratio (g/g) and PIL concentration (M) were chosen as the control factor (Table 1).

Table 1: Extraction conditions according to response surface methodology

Entry	X1, Power (W)	X2, Solid/liquid (g/g)	X3, Concentration (M)
1	-1 (100)	-1 (1:6)	-1 (1.5)
2	+1 (300)	-1 (1:6)	-1 (1.5)
3	-1 (100)	+1 (1:14)	-1 (1.5)
4	+1 (300)	+1 (1:14)	-1 (1.5)
5	-1 (100)	-1 (1:6)	+1 (4.5)
6	+1 (300)	-1 (1:6)	+1 (4.5)
7	-1 (100)	+1 (1:14)	+1 (4.5)
8	+1 (300)	+1 (1:14)	+1 (4.5)
9	0 (200)	0 (1:10)	0 (3.0)
10	0 (200)	0 (1:10)	0 (3.0)
11	0 (200)	0 (1:10)	0 (3.0)

The analysis of variance (ANOVA) was applied for the determination of significant variables. ANOVA consists of classified and cross-classified statistical results and was tested by the means of a specified classification difference, which was carried out by Fisher's statistical test (F-test). The F-value is defined as the ratio of the mean square of regression (MRR) and the error (MRe) (F=MRR/MRe), representing the significance of each controlled variable on the tested model. The regression equations were also submitted to the F-test to determine the coefficient of R^2. The mathematical adjustment of the obtained model was measured using the coefficient of determination. The response surface methodology (RSM) was also utilized to predict the optimization of total anthocyanins extraction. The STATISTICA® 7.0 software was used for the RSM.

3. Results and discussion

A surface response analysis by a 2^3 factorial was carried to evaluate the optimal extraction conditions (Figure 2 for theobromine and Figure 3 for caffeine). This tool allow to test simultaneously several operational conditions with reduced number of experiments and to find interactions between process variable (Rodrigues and Ienna, 2005). The variables were selected based on the literature (Peralta-Jiménez and Cañizares-Macías, 2013; Cláudio et al., 2013).

The surface responses with their respective contour curve revealed the relationship between dependent and independent variable. The concentration of theobromine ranged between 95.0 $mg.L^{-1}$ (power – 300 W, solid/liquid ratio – 1:14 and PIL concentration – 1.5 M) and 282.2 $mg.L^{-1}$ (power – 300 W, solid/liquid ratio – 1:6 and PIL concentration – 4.5 M), while the caffeine concentration ranged between 5.4 $mg.L^{-1}$ (power – 100 W, solid/liquid ratio – 1:14 and PIL concentration – 4.5 M) and 53.4 $mg.L^{-1}$ (power – 100 W, solid/liquid ratio – 1:6 and PIL concentration – 4.5 M). The average of theobromine and caffeine in the central points (power – 200 W, solid/liquid ratio – 1:10 and PIL concentration – 3.0 M) were 137.9 ± 1.5 $mg.L^{-1}$ and 18.3 ± 0.2 $mg.L^{-1}$, respectively. Carrillo et al. (2014) observed a content of theobromine (201.8 – 271.7 $mg.L^{-1}$) and caffeine (20.9 – 53.7 $mg.L^{-1}$) from different samples of Colombian cocoa, demonstrating that protic ionic liquid like 2HEAA has a high capacity to extract these alkaloids.

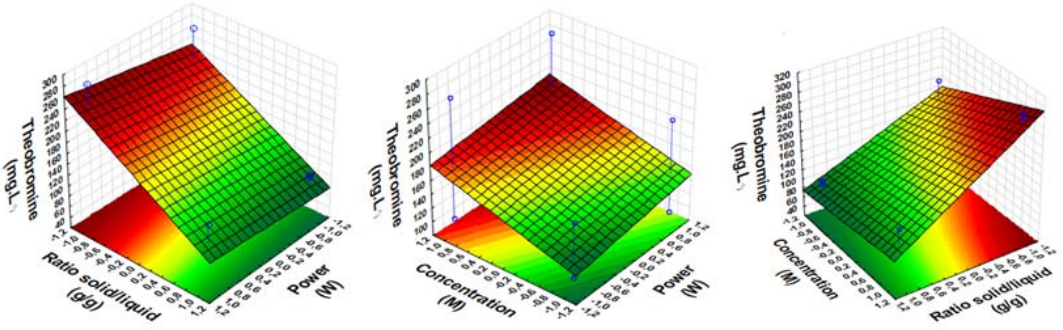

Figure 2: Response and contour surface for theobromine extraction in ultrasound-assisted extraction using the ionic liquid 2HEAA at 3 min with ultrasonic pulse every 10 seconds.

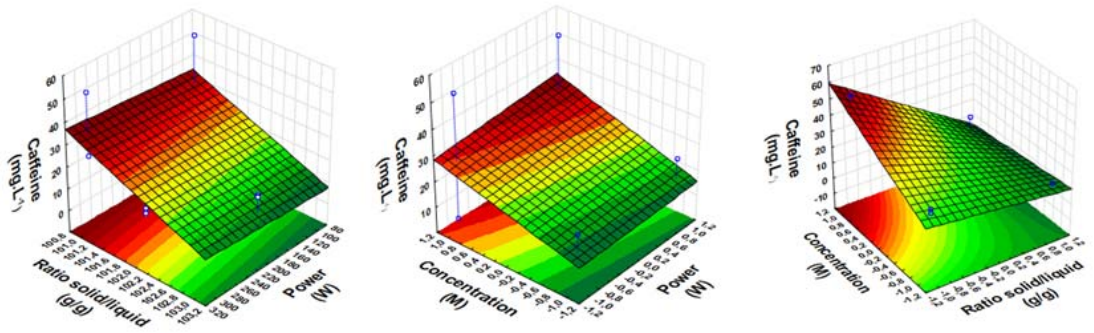

Figure 3: Response and contour surface for caffeine extraction in ultrasound-assisted extraction using the ionic liquid 2HEAA at 3 min with ultrasonic pulse every 10 seconds.

The significance of the variable and the possible interaction between them was verified by the application of the variance analysis ANOVA (Table 2 and 3) and Pareto chart (Figure 3). The surface responses with their respective contour curve revealed the relationship between dependent and independent variable. The concentration of theobromine ranged between 95.0 $mg.L^{-1}$ (power – 300 W, solid/liquid ratio – 1:14 and PIL concentration – 1.5 M) and 282.2 $mg.L^{-1}$ (power – 300 W, solid/liquid ratio – 1:6 and PIL concentration – 4.5 M), while the caffeine concentration ranged between 5.4 $mg.L^{-1}$ (power – 100 W, solid/liquid ratio – 1:14 and PIL concentration – 4.5 M) and 53.4 $mg.L^{-1}$ (power – 100 W, solid/liquid ratio – 1:6 and PIL concentration – 4.5 M). The average of theobromine and caffeine in the central points (power – 200 W, solid/liquid ratio – 1:10 and PIL concentration – 3.0 M) were 137.9 ± 1.5 $mg.L^{-1}$ and 18.3 ± 0.2 $mg.L^{-1}$, respectively. Carrillo et al. (2014) observed a content of theobromine (201.8 – 271.7 $mg.L^{-1}$) and caffeine (20.9 – 53.7 $mg.L^{-1}$) from different samples of Colombian cocoa, demonstrating that protic ionic liquid like 2HEAA has a high capacity to extract these alkaloids.

Table 2: Variance analysis for Theobromine

Variable	SS	DF	MS	F-value	p-value
1- Power (W)	1046.53	1	1046.53	1.06	0.3620
2- Ratio S/L (g/g)	34282.71	1	34282.71	34.63	0.0042
3- PIL Concentration (M)	5045.10	1	545.1	5.10	0.0870
1 by 2	76.26	1	76.26	0.08	0.7951
1 by 3	0.03	1	0.03	0.00	0.9958
2 by 3	1262.53	1	1262.53	1.27	0.3219
Error	3959.81	4	989.95		
Total SS	45672.98	10			

SS = sum of square; DF = degree of freedom; MS = mean of square. R^2 = 0.9133; Adj. = 0.7832; MS residual = 989.9536

Table 3: Variance analysis for Caffeine

Variable	SS	DF	MS	F-value	p-value
1- Power (W)	18.00	1	18.00	0.74	0.4385
2- Ratio S/L (g/g)	1490.58	1	1490.58	61.18	0.0014
3- PIL Concentration (M)	389.21	1	389.21	15.97	0.0162
1 by 2	6.13	1	6.13	0.25	0.6424
1 by 3	3.92	1	3.92	0.16	0.7088
2 by 3	505.62	1	505.62	20.75	0.0104
Error	97.46	4	24.37		
Total SS	2510.91	10			

SS = sum of square; DF = degree of freedom; MS = mean of square. R^2 = 0.96119; Adj. = 0.90296; MS residual = 24.36477

The variance analysis (ANOVA) was applied to test the significand and adequacy of model. The significant process variables are those with the calculated Fischer test higher than the table Fischer test ($F_{cal} > F_{tab}$). The

experimental design of this work present F_{tab} of 3.44. Therefore, for the theobromine extraction (Table 1 and Figure 4a) the significant variable was the solid liquid ration with a negative effect (effect estimative = -5.88). While for the caffeine extraction (Table 2 and Figure 4b) the significant variables were solid/liquid ration with a negative effect (effect estimative = -7.82), PIL concentration with a positive effect (effect estimative = 4.00) and the interaction between these variable with a negative effect (effect estimative = -4.56). The negative effect indicates that increasing the variable value harms the extraction.
Taking into account a 95% confidence level ($p < 0.05$), the linear model is a good representation of the experimental data of theobromine and caffeine extractions. In these cases, high correlation coefficients (R^2), 0.9131 (theobromine) and 0.9612 (caffeine) were observed.
The model is also revealed that the best conditions are the lowest concentration of PIL, resulting in savings in the use of the extractor. The mass/volume ratio indicates that a higher biomass results in a greater quantitative of the alkaloids.

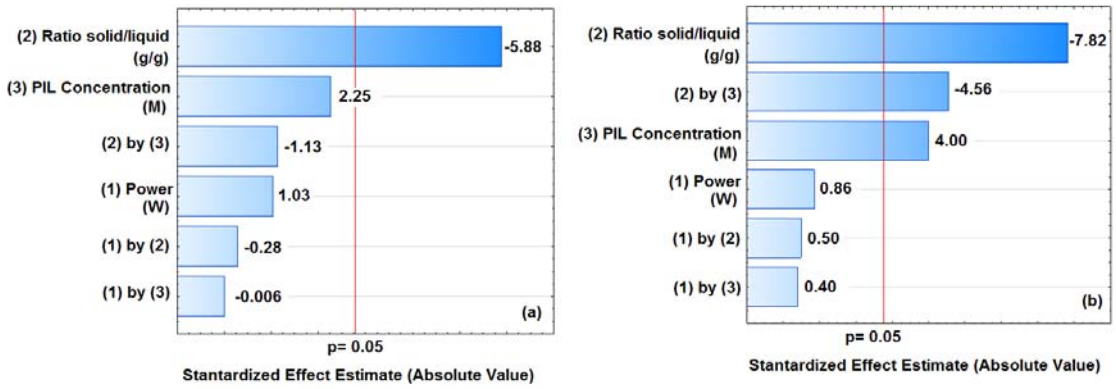

Figure 4: Pareto's chart obtained for (a) theobromine and (b) caffeine.

The potency variation does not interfere with the amount of alkaloid extraction (Figure 5), which means the use of a less expensive and energy-free technique, possibly in the optimum region. Any variation in potency results in quantitative similar alkaloids. In this sense, three extractions were carried out to investigate the effect of ultrasound power on alkaloids extraction. It was observed that the extractions no assisted by ultrasound provided the best extraction of the alkaloids, while the application of the ultrasound in two different values did not alter the extraction. This result corroborates the comparison between conventional extraction protocols and ultrasound assisted extraction using ultrasound bath and ultrasound probe performed by Horzic group (Horzic et al 2012). In fact, conventional process using water and aqueous ethanol solution (75 %) provides better extraction of theobromine and caffeine from yellow tea when compared to the ultrasound process in the bath. However, extraction protocols using ultrasound probe are more favourable in amplitudes 50 and 75%.

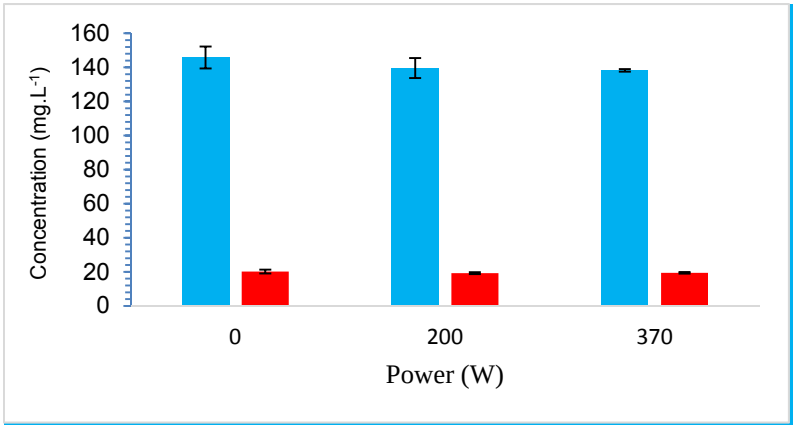

Figure 5: Effect of ultrasound power in alkaloids extraction using 1:10 solid/liquid ratio, 3M concentration of protic ionic liquid. ■ *Theobromine and* ■ *caffeine.*

4. Conclusions

The results indicated that protic ionic liquid could be used as an alternative to non-conventional solvents for the extraction of alkaloids such as theobromine and caffeine. The optimum conditions for extraction are the alkaloids are power – 300 W, solid/liquid ratio – 1:6 and PIL concentration – 4.5 M for theobromine (282.2 $mg.L^{-1}$) and power – 100 W, solid/liquid ratio – 1:6 and PIL concentration – 4.5 M for caffeine (53.4 $mg.L^{-1}$). The solid/liquid ratio influences the extraction process of the alkaloids; however, the ultrasonic power does not change this extraction.

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Reference

Álvarez, V.H., Mattedi S., Martin-Pastor M., Aznar, M., Iglesias. M., 2010, Synthesis and thermophysical properties of two new protic long-chain ionic liquids with the oleate anion, Fluid Phase Equilib. 299, 42-50.

Azmir J., Zaidul I.S.M., Rahman M.M., Sharif K.M., Mohamed A., Sahena F., Jahurul M.H.A., Ghafoor K., Norulaini N.A.N., Omar, A.K.M., 2013, Techniques for extraction of bioactive compounds from plant materials: A review. J. Food Eng. 117, 426-436.

Baker G.A., Baker S.N., Pandey S., Bright F.V., 2005, An analytical view of ionic liquids, Analyst, 130, 800-808.

Bogdanov M.G., Svinyarov I., 2013, Ionic liquid-supported solid-liquid extraction of bioactive alkaloids. II. Kinetics, modeling and mechanism of glaucine extraction from *Glaucium flavum* Cr. (Papaveraceae), Sep. Purif. Technol. 103, 279-288.

Bogdanov M.G., Keremedchieva R., Svinyarov I., 2015, Ionic liquid-supported solid-liquid extraction of bioactive alkaloids. III. Ionic liquid regeneration and glaucine recovery from ionic liquid-aqueous crude extract of *Glaucium flavum* Cr. (Papaveraceae), Sep. Purif. Technol. 155, 13-19.

Carillo L.C., Londoño-Londoño J. Gil A., 2014, Comparison of polyphenol, methylxanthines and antioxidant activity in Theobroma cacao beans from different cocoa-growing areas in Colombia, Food Res. Int. 60, 273-280.

Chemat F., Huma Z., Khan M.K., 2011, Applications of ultrasound in food technology: Processing, preservation and extraction, Ultrason. Sonochem. 18, 813-835.

Ghandi K., 2014. A Review of ionic liquids, their Limits and applications, Green Sustain. Chem. 4, 44-53.

González-Centen M.R., Comas-Serra F., Femenia A., Rosselló C., Simal S., 2015, Effect of power ultrasound application on aqueous extraction of phenolic compounds and antioxidant capacity from grape pomace (*Vitis vinifera* L.): Experimental kinetics and modelling, Ultrason. Sonochem. 22, 506-514.

Horzic D., Jambrak A.R., Belscak-Cvitanovic A., Komes D., Lelas V., 2012, Comparison of conventional and ultrasound assisted extraction techniques of yellow tea and bioactive composition of obtained extracts, Food Bioprocess Tech. 5, 2858-2870.

Huie C.W., 2002, A review of modern sample-preparation techniques for the extraction and analysis of medicinal plants, Anal. Bioana. Chem. 373, 23-30.

Losetty V., Matheswaran P., Wilfred C.D., 2017, Synthesis, thermophysical properties and COSMO-RS study of DBU based protic ionic liquids, The Journal of Chemical Thermodynamic, 105, 151-158.

Minjares-Fuentes R., Femenia A., Garau M.C., Meza-Velázquez J.A., Simal S., Rosselló C., 2014, Ultrasound-assisted extraction of pectins from grape pomace using citric acid: A response surface methodology approach, Carbohy. Polym. 106, 179-189.

Passos H., Freire M.G., Coutinho J.A.P., 2014, Ionic liquid solutions as extractive solvents for value-added compounds from biomass, Green Chem. 16, 4786-4815.

Peralta-Jiménez L., Cañizares-Macías M.P., 2013, Ultrasound-assisted method for extraction of theobromine and caffeine from cacao seeds and chocolate products, Food and Bioprocess Tech. 6, 3522-3529.

Rodrigues M.I., Iemma A.F. Eds., 2015, Experimental Design and Process Optimization. CRS Press, Londom, USA.

Tang B., Bi W., Tian M., Row K.H., 2012, Application of ionic liquid for extraction and separation of bioactive compounds from plants, Journal of Chromatogr. B, 904, 1-21.

Tao Y., Sun D.W., 2015, Enhancement of food processes by ultrasound: A review, Criti. Rev. Food Sci. Nutr. 55, 570-594.

Tao Y., Wu D., Zhang Q.A., Sun D.W., 2014, Ultrasound-assisted extraction of phenolics from wine lees: Modeling, optimization and stability of extracts during storage, Ultrason. Sonochem. 21, 706-715.

Yamada T., Yamada Y., Okano Y., Terashima T., Yokogoshi, H., 2009, Anxiolytic effects of short- and long-term administration of cacao mass on rat elevated T-maze test, J. Nutr. Biochem. 20, 948-955.