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Green extraction techniques of bioactive components from cocoa shell

NIKA PAVLOVIĆ*[1], MARTINA JAKOVLJEVIĆ[2], MAJA MIŠKULIN[1], MAJA MOLNAR[2], ĐURĐICA AČKAR[2], STELA JOKIĆ[2]

[1]*Josip Juraj Strossmayer University of Osijek, Faculty of Medicine Osijek, Josipa Huttlera 4, 31000 Osijek, Croatia*
[2]*Josip Juraj Strossmayer University of Osijek, Faculty of Food Technology Osijek, Franje Kuhača 20, 31000 Osijek, Croatia*

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ABSTRACT

The aim of this study was to demonstrate that certain types of extraction techniques can give extracts with various bioactive components in different concentrations. Four green extraction techniques were used in this study: supercritical CO_2 extraction, ultrasound-assisted extraction, cold atmospheric plasma extraction and extraction using deep eutectic solvents. Those modern techniques gave better yields of bioactive components and showed better antioxidant activity of obtained extracts than classical Soxhlet extraction. The bioactive components in obtained extracts were quantified by High Performance Liquid Chromatography. Supercritical CO_2 extraction gave the highest yields for theobromine content, while ultrasound-assisted extraction with 50% aqueous ethanol solution gave the highest caffeine yields during 30 min of extraction, 35 Hz and 60 °C. The extraction with deep eutectic solvent mixture of choline chloride: oxalic acid and 50% pure H_2O at room temperature during 180 min of extraction time gave the highest total phenol content while the same mixture under the same conditions, but during longer extraction time (360 min), gave the highest antioxidant activity.
Cocoa shell (CS), due to its nutritional value and bioactive components, has a potential to become a desirable raw material in a large spectrum of functional and pharmaceutical products.

Introduction

Food industry waste is a growing problem, both economically and ecologically. By-products in the food industry may contain valuable bioactive components and their utilization as raw material in some other production could result in less waste. That is the reason for today's growing interest in utilizing food industry by-products for different purposes (Jokić et al., 2017). Given the serious environmental side effects over the years due to the use of classical techniques, technologists have begun to design less harmful technologies.
The development of green technologies contributed to cheap, fast procedures that are safe for the environment (Armenta, Garrigues and de la Guardia, 2015). In comparison to classical extraction such as a conventional method using organic solvents, green extraction technologies are more frequently used in food production and processing. One of these innovative technologies is definitely supercritical fluid extraction (SFE). The discovery of supercritical solvents has made a breakthrough and they gradually begin to replace toxic and ecologically unacceptable organic solvents (Jokić et al., 2011). It is a substance with both liquid and gas properties that occur above critical temperature and pressure of the component (Hitchen and Dean, 1993). Some advantages of supercritical solvent are better diffusion, lower viscosity and less surface tension with better penetration into material which contains the desired valuable components. This process provides better

*Corresponding author E-mail: nika.felicita@gmail.com

selectivity and the ability to control the solubility of supercritical fluid itself by changing the pressure and temperature, and thus easily removing the solvent from the extract. SFE belongs to "clean technology" due to the fact that environmentally damaging secondary products are not produced (Jokić et al., 2011). This more rapid extraction is more suitable extraction technique today due to better reproducibility compared to classic extraction techniques (Valadez-Carmona et al., 2018). The speed of the extraction depends on the viscosity and diffusivity of the mobile phase. Lower viscosity and high diffusivity mean faster extraction while changing the pressure and/or temperature, density of the supercritical fluid could be modified. Solvents used in SFE processes are generally known as safe (GRAS) what is also another advantage. Most frequently used supercritical fluid in 90% cases is carbon dioxide (CO_2) due to relatively low critical temperature (32 °C) and critical pressure (74 bar). This nonflammable, nontoxic, low cost, high purity solvent could be easily removed from the extract and is usually more suitable for lipophilic compounds while its disadvantage is the lack of polarity for extraction of polar analytes. SFE can also be used with solid or liquid samples. The main step of SFE is the separation and the extraction of compounds of interest. Bioactive compounds are recovered due to constant flow of supercritical fluid in a compressed form. Before entering the extraction cell, supercritical fluid is preheated during passing through the heat exchanger while extraction cells are regularly immersed in water baths for temperature stabilization. The mixture of solvent and extract is transferred to flash tanks from extraction cells, where pressure is quickly decreased, which causes minimization of fluid density and solvent power reduction (Grumezescu and Holban, 2017). Another alternative to classical extraction is ultrasound-assisted extraction (UAE) which uses sonification as a pre-treatment step. It is also environment-friendly, clean, flexible, versatile and easy to use extraction method which requires low investment costs compared to some other novel green extraction technologies. It is suitable for the extraction of different molecules such as polysaccharides, peptides, essential oils, dyes, pigments and other various bioactive components. The principle of UAE is acoustic cavitation (Tiwari, 2015). Under the influence of ultrasound, physical and chemical properties of the plant material change, what leads to releasing of extractable compounds. UAE uses small amounts of solvent together with shorter working time. This type of extraction is very suitable for the food industry due to its high reproducibility, solvent reduction, simple manipulation and higher purity of the final product than obtained by classical methods (Chemat, Zill-E-Huma and Khan, 2011). Cold atmospheric plasma extraction (CAPAE), as a non-thermal technology for food processing, gained importance over the past few years. This economical, versatile and environmental friendly extraction technique has proved to be effective in numerous food industry applications in removing toxins and decontamination of foods, enzyme inactivation and modifications in food packaging. As heat treatment could lead to some undesirable effects like colour and texture change with loss of nutritive components, the use of CAPAE could be of great importance. Plasma is composed from ions, free electrons, atoms and molecules in fundamental or excited states. It is a quasi-neutral ionized state of gas based on the thermal equilibrium. The important roles for the efficiency of CAPAE process in food processing are plasma source, electrode design, pressure, voltage, the time of the treatment, distance between electrodes and reactive gas (Pankaj, 2018). Deep eutectic solvents (DES) are a mixture of two or more compounds that have lower melting point together than individually. They include a quaternary ammonium salt as a hydrogen acceptor (HBA) and a hydrogen bond donor (HBD) (Bajkacz and Adamek, 2017). DES have an enormous advantage in possibility of preparing solvents in large number of combinations. This almost non-toxic extraction possesses great extraction properties like tunable viscosity, liquid state below 273 K, cheap and simple preparation (Owczarek et al., 2016). Many DES have been created to replace volatile, unsafe organic solvents and are used successfully in different fields as for extraction of bioactive components from various sources (Zainal-Abidin et al., 2017).

As a major by-product in the cocoa bean industry, cocoa shell (CS) has been disposed as a waste for years (Awarikabey, Amponsah and Woode, 2014; Bruna et al., 2009) and since it contains bioactive components in certain amounts, it is considered to be a good and inexpensive source as a raw material (Bruna et al., 2009). It could be applied in the food industry as a natural component of great diversity. The use of CS as a source of fibers, extracts rich in polyphenol components, applications as a natural colorant and flavouring agent was also reviewed in recent paper by Okiyama, et al. (2017). Methylxanthines like theobromine and caffeine were also found in CS and despite the fact that their amounts were lower than in cocoa beans, significant amounts of these components confirm the use of CS as a new source of those beneficial components (Barbosa-Pereira, Guglielmetti and Zeppa, 2018). It is known that theobromine (3,7-dimethylxantine) is a brain stimulant and diuretic with a potential in blood

pressure reduction with also some unclear synergistic interactions with polyphenols that should be investigated (Plaza et al., 2017). It is also demonstrated that theobromine induces a wide range of reproductive and developmental aberrations. In biological organisms there are numerous routes of theobromine degradation pathways that are species-dependent (Adamafio, 2013). The caffeine (1,3,7-trimethylxanthine) content is commonly around 10-15% of theobromine content in cocoa beans, while theophylline could be found in traces. The amount of every individual methylxanthine depends on the genotype of the cocoa tree. During the cocoa maturation, both methylxanthines increase their concentration, more in beans than in the shells. The increase of theobromine is also usually much higher than caffeine (Bentil, 2012). Caffeine is known to be a central nervous system stimulant even more than theobromine and also respiratory, diuretic and skeletal muscle stimulant. There are various concerns about methylxanthine toxicity which depends on specific compound, type of living organism and their sensitivity to methylxanthines what may be genetically originated (Monteiro et al., 2016).
The aim of this study was to investigate the effect of four different innovative green extraction techniques (SFE, UAE, CAPAE and DES) on the caffeine and theobromine content in CS, as well as an antioxidant activity (AA) and total phenol content (TPC) with a comparison to conventional Soxhlet extraction technique.

Materials and methods

Material and chemicals

CS was collected from chocolate factory Kandit d.o.o., Osijek, Croatia in 2017. The country of origin of obtained cocoa was Gana and Ivory Coast. All solvents used for the extraction procedures were of analytical grade and purchased from J.T. Baker (PA, USA). CO_2 that was used for SFE extraction was 99.97% (w/w) pure, obtained from Messer, Osijek, Croatia. Theobromine standard for the chromatographic analysis was purchased from Sigma-Aldrich, Lot No: ASB-00020248-D01 while caffeine standard was purchased from Dr. Ehrenstorfer, Lot No: 124289. The Folin-Ciocalteu reagent as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and gallic acid were purchased from Sigma-Aldrich (USA). Before each extraction, CS was grounded using laboratory mill and sieved using vertical sieve shaker (Labortechnik GmbH, IImenau, Germany).

Extraction techniques

Soxhlet extraction

The initial oil content of CS was measured by automatic extraction systems Soxterm by Gerdhart using petroleum ether as an organic solvent. 5 g of ground CS was extracted with 120 mL of solvent. The temperature of water bath was 100 °C. The end of the extraction was checked by immersing the glass rod in the extractor and dripping on the filter paper. The extraction was over when there was no greasy stain left, the Soxhlet thimble was removed and the solvent was distilled. The measurement was done in duplicate.

Supercritical CO_2 extraction (SC-CO_2)

The 100 g of grounded CS was placed into extraction vessel. During the process, the CS extract was collected in previously weighted glass tubes. The extraction was performed at 300 bar for 30 minutes at 2 kg CO_2/h mass flow rate and at two different temperatures: 40 °C and 60 °C. The SFE system and scheme of the process was explained elsewhere (Jokić et al., 2014; Jokić et al., 2015). The measurement was done in duplicate.

Ultrasound assisted extraction (UAE)

The grounded sample of CS was weighed and placed in ultrasound water bath ELMA, Elmasonic P 120 H. All extractions were conducted in period of 30 min time at 35 kHz frequency. The four different solvents were used in UAE: pure H_2O, 50%, 75% and 96% aqueous ethanol solutions. The analysis was performed at room temperature (25 °C), 40 °C and 60 °C, respectively. The measurement was done in duplicate.

Cold atmospheric plasma extraction (CAPAE)

The grounded sample of CS was weighed and placed in glass container for the extraction in newly constructed instrument for CAPAE at The Faculty of Food Technology Osijek. The extraction was performed with pure H_2O as an extraction solvent, for 30 minutes on two different frequencies, 70 and 100 Hz. The measurement was done in duplicate.

Extraction with deep eutectic solvents (DES)

Grounded CS sample was weighed and placed together with a small stir bar in 2 mL tubes for the extraction with DES mixture which contained eutectic solvents choline chloride: oxalic acid and different amounts of H_2O (0%, 25% and 50%). The DES mixture was prepared in the following way: choline chloride and oxalic acid were

weighed and placed in a glass beaker. The mixture was stirred and heated on a low temperature until liquid (about one hour). After that, it was mixed with H_2O in previously mentioned amounts and placed in tubes with CS for extraction. The extraction was performed at room temperature and 1100 rpm in different periods of time (60, 180 and 360 minutes). The measurement was done in duplicate.

Determination of theobromine and caffeine content (HPLC)

Identification and quantification of CS bioactive components was done according to Pura Naik (2001) with slight modifications. The sample extracts were filtered through 0.2 μm PTFE filter and placed in apparatus for the chromatographic analysis. The measurement was done on reversed-phase High Performance Liquid Chromatography (HPLC) Infinity 1290 Agilent Technologies (USA) instrument containing an autosampler G4226A, quaternary pump G4204A and diode array detector (DAD) G4212A. Injection volume of the sample was 20 μL while wavelength was set to 273 nm. The separation was carried out at room temperature using an Agilent Eclipse C18 column, 4.6 x 250mm packed with 5μm diameter particles. The calculations concerning the quantitative analysis were performed with the external standardization by measuring the peak areas and were integrated automatically by the computer using the Agilent HPLC Data Analysis software program based on calibrations of five standards. RP-HPLC analysis was performed by isocratic elution with a flow rate of 1.0 mL min^{-1} and the mobile phase consisted of water: acetonitrile (80:20 v/v). The run time was set to 10 min. The limit of detection (LOD) for theobromine was measured to be 0.42 mg/kg and the limit of quantification (LOQ) 1.28 mg/kg, while for caffeine LOD was 1.24 mg/kg and LOQ 3.75 mg/kg, respectively. Correlation factors for both parameters were 0.9999. The analysis was conducted in three replications.

Determination of total phenol content (TPC)

TPC was measured by the Folin-Ciocalteu spectrophotometric method and expressed in milligrams of gallic acid per gram of extract. The analysis was done on Selecta UV-2005 spectrophotometer, Serial No. 0462575 while wavelength was set to 765 nm. 20 μL of diluted sample was mixed with 1580 μL H_2O, 100 μl Folin-Ciocalteu reagent and 300 μLNa_2CO_3 in micro centrifuge tubes and placed in thermostat in 40 °C. The blank was prepared in the same way only with pure H_2O instead of the sample.

Determination of % DPPH scavenging activity

The 500 μL of the 1 mg/mL diluted sample was mixed with 4500 μL methanol and aliquots were prepared with fresh DPPH solution (0.2 mM). After 30 min, the absorbance was determined at 517 nm. The determination was performed in triplicate and expressed as % scavenging activity (% DPPH).

Data analysis

All measurements were performed in triplicate and the results were analyzed by Kruskal Wallis Test and the statistical significance of each factor is represented with *p*-value.

Results and discussion

The results obtained by classic Soxhlet extraction method for the theobromine and caffeine concentrations are given in Table 1. Theobromine concentration was 0.61 mg/kg while the caffeine concentration was 1.46 mg/kg. Mazzutti et al. (2017) highlighted the well-known theory how high temperature during Soxhlet extraction reduces surface tension and viscosity of the solvent, allowing active substances to access inside of the solid material, thus increasing solute solubilization. Also, this conventional extraction recovers mainly lipids while methylxantines are more soluble in aqueous fractions. This is the reason for the low yield of these components obtained by this extraction method.
Results in our study related to CS extracts obtained by different green extraction techniques (SFE, UAE, CAPAE and DES) are given in Tables 2-5.

Table 1. Theobromine and caffeine concentrations obtained by conventional extraction methods

Extraction type	Theobromine (mg/kg)	Caffeine (mg/kg)
Soxhlet	0.61	1.46

Influence of extraction technique on theobromine and caffeine content in CS

In SFE, the effect of temperature (40 and 60 °C) on the extraction yield of theobromine and caffeine, as well as on the TPC and on AA of CS extracts was examined. The pressure (300 bar), CO_2 flow rate (2 kg/h) as well as extraction time (30 min) were kept as constants. The results represented in Table 2 show how the obtained theobromine concentration was 5870.96±0.18 mg/kg at 40 °C and 6345.37±0.25 mg/kg at 60 °C. Caffeine content, on the other hand, was also high, 537.42±0.27 mg/kg at 40 °C and 649.65±0.02 mg/kg at 60 °C. This increase in yields for both components at higher temperatures could be explained due the fact that with temperature increase at constant density of the solvent (CO_2) the solubility of these components increases because of increased vapour pressure of those substances. Due to some small differences in structure and consequently their molecular interactions, SFE was more suitable for caffeine than for theobromine what is evident through the increase in their concentrations (Johannsen and Brunner, 1994).

In the UAE, the effect of temperature (room temp., 40 and 60 °C) and type of extraction solvent on the extraction yield of theobromine and caffeine, as well as on the TPC and AA of CS extracts were examined and represented in Table 3. The frequency and extraction time were kept as constants, at 35 kHz and 30 min. The UAE showed to be most suitable extraction method for caffeine extraction compared to other extraction methods. Both components gave very good yields in pure H_2O, 50%-aqueous ethanol and 70% ethanol aqueous solutions, while in 96% aqueous solution the yields were not satisfactory. 50%-aqueous ethanol solution gave the highest yields for caffeine (911.70±2.70 mg/kg at room temperature, 913.10±0.45 mg/kg at 40 °C and 994.70±0.73 mg/kg at 60 °C, respectively) while 70% aqueous- ethanol solution also gave good caffeine yields but slightly lower concentrations (822.08±0.40 mg/kg at room temperature, 965.9±0.19 mg/kg at 40 °C and 884.60±0.54 mg/kg at 60 °C, respectively). Those results show that again more caffeine was obtained than with other green extraction methods in this study. With pure H_2O as a solvent for UAE, caffeine content in CS was found to be 506.99±0.40 mg/kg at room temperature, 550.13±0.82 mg/kg at 40 °C and 749.60±0.21 mg/kg at 60 °C, respectively, what could be considered also as a very good yield. Neither theobromine nor caffeine gave good results with 96% ethanol as an extraction solvent (caffeine was found to be 37.6±4.35 mg/kg at room temperature, 71.75±2.82 mg/kg for the extraction at 40 °C and a little bit higher value on the temperature at 60 °C, 189.30±3.43 mg/kg, respectively, while theobromine was not even found).

Table 2. Theobromine and caffeine concentrations obtained from CS by SFE during constant pressure (300 bar), extraction time (30 minutes) and flow (2 kg CO_2/h)

Extraction parameters	**Theobromine (mg/kg)**	**Caffeine (mg/kg)**	**Total phenols** (mg GAE/g extract)	**% DPPH** scavenging activity
40 °C	5 870.96±0.18	537.42±0.27	10.85±1.18	15.90±1.66
60 °C	6 345.37±0.25	649.65±0.02	12.36±2.04	15.07±0.36

Table 3. Theobromine and caffeine concentrations obtained from CS by different UAE conditions during constant frequency (35 kHz) and time (30 minutes)

Extraction solvent	**Extraction parameters**	**Theobromine (mg/kg)**	**Caffeine (mg/kg)**	**Total phenols** (mg GAE/g extract)	**% DPPH** scavenging activity
H_2O	Room temp.	4 454.29±0.49	506.99±0.40	24.95±0.44	39.98±0.64
	40 °C	4 526.57±7.80	550.13±0.82	17.26±2.47	41.13±1.63
	60 °C	4 955.88±1.12	749.60±0.21	15.97±1.18	47.06±0.80
50%-aqueous ethanol	Room temp.	2 056.90±1.23	911.70±2.70	14.18±2.47	62.41±0.68
	40 °C	2 136.60±1.22	913.10±0.45	14.28±0.44	72.24±0.46
	60 °C	2 501.10±1.92	994.70±0.73	15.46±0.77	91.87±0.34
70%-aqueous ethanol	Room temp.	392.97±0.98	822.08±0.40	11.36±2.35	47.31±0.44
	40 °C	432.70±1.26	965.90±0.19	14.95±1.94	69.67±0.63
	60 °C	462.84±5.52	884.60±0.54	15.72±2.47	70.28±1.64
96%-aqueous ethanol	Room temp.	-	37.60±4.35	10.08±0.77	5.59±1.28
	40 °C	-	71.75±2.82	12.36±0.78	6.36±1.97
	60 °C	-	189.30±3.43	14.69±1.54	7.04±0.24

By observing the theobromine yield on the other hand with UAE, best yields were achieved at 60 °C and with pure H_2O as a solvent (4955.88±1.12 mg/kg, while with lowering the temperature the yield decreased (4826.57±7.80 mg/kg at 40 °C and 4454.29±0.49 mg/kg at room temperature). Those results point to findings how temperature as a variable parameter in both extraction methods, UAE and SFE, influenced significantly the yields of both components. Also, these results were expected given the fact that UAE depends on the applied frequency, ultrasound intensity, time of the extraction as well as solvent polarity (Drmić and Režek Jambrak, 2010). The effect of frequency (70 and 100 Hz) on the extraction yield of theobromine and caffeine, as well as on the TPC and on AA of CS extracts obtained by CAPAE was examined. Pure H_2O was used as the extraction solvent during the 30 min of the extraction along with the room temperature. The results represented in Table 4 for CAPAE extraction of those bioactive components from CS demonstrate the fact that with the decrease of frequency for 30 Hz (from 100 Hz to 70 Hz) the yield for both components increase. This can be explained with the fact that lower frequencies cause micro ruptures of cell walls in the material due to cavitation phenomena, allowing better penetration of the solvent what consequently gives higher mass yields (Drmić and Režek Jambrak, 2010). In comparison to other extraction yields, CAPAE was the second best green extraction method used in this study for extraction of theobromine from CS (5612.00±0.22 mg/kg during 30 minutes, at frequency of 70 Hz and pure H_2O as an extraction solvent, respectively). The effect of different amounts of pure H_2O and choline chloride: oxalic acid mixture as a DES solvent (0, 25, 50% H_2O) together with different extraction time (60, 180 and 360 min), on the extraction yield of theobromine, caffeine, TPC and AA from CS extracts was examined. The results represented in Table 5 show how 50% eutectic mixture, during 180 minutes gave the best theobromine yield (4045.15±0.13 mg/kg) while longer extraction (360 min) gave slightly less theobromine yield (4042.65±0.06 mg/kg), but the highest caffeine yield (579.20±1.20 mg/kg). Lower extraction yields for both components could be due to higher viscosity of the DES mixture and consequently lower mass transport. That also confirms the yield increase for both components due to the increase of H_2O content in the mixture (Alañón et al., 2018). According to Adamafio (2013) theobromine content varies in CS from 5000 – 21000 mg/kg depending on the plant geographical and varietal differences. It has been documented how cocoa from Africa contains the highest theobromine content. Beckett et al. (2011) mentions that mean theobromine concentration in CS could range from 0.2-1.3% with mean value of 0.87%, while mean caffeine concentration was 0.13%, with wide range from 0.04 – 0.3%. Hartati (2010) established how CS is a potential source of protein and theobromine for utilization in animal feed due to hydrotropic extraction method. Author stated how CS contains 5500 mg/kg theobromine. Hernández-Hernández et al. (2018) indicated the fact that the concentration of theobromine in unfermented CS was 3900±0.15 mg/kg while in fermented CS it was much higher 12000±0.10 mg/kg, emphasizing that the most of theobromine is contained in cotyledon while in CS only about 17.75% (unfermented sample).

Table 4. Theobromine and caffeine concentrations obtained from CS by different CAPAE conditions during constant time (30 minutes) and H_2O as an extraction solvent

Frequency (Hz)	**Theobromine (mg/kg)**	**Caffeine (mg/kg)**	**Total phenols** (mg GAE/g extract)	**% DPPH** scavenging activity
100	4 443.95±0.55	536.48±0.11	16.23±2.66	32.21±1.21
70	5 612.00±0.22	591.10±0.85	17.00±0.76	32.05±1.53

Table 5. Theobromine and caffeine concentrations obtained by different DES* conditions

Extraction parameters		**Theobromine (mg/kg)**	**Caffeine (mg/kg)**	**Total phenols**	**% DPPH** scavenging activity
solvent	**Time (min)**				
0% H_2O	60	260.65±0.22	17.65±2.07	10.21±1.75	82.84±1.96
	180	345.55±0.00	23.35±0.55	12.90±1.23	83.25±1.13
	360	532.10±0.14	24.20±0.25	10.59±1.61	85.01±1.06
25% H_2O	60	3699.85±0.34	506.78±0.50	29.82±1.68	84.75±1.67
	180	3513.30±0.06	502.15±0.20	31.38±1.84	88.08±1.53
	360	3 173.70±0.23	511.55±0.94	30.04±2.35	89.99±1.45
50% H_2O	60	3643.85±0.43	535.75±0.37	32.39±2.05	82.34±1.46
	180	4045.15±0.13	565.70±0.17	37.90±1.96	89.35±1.40
	360	4042.65±0.06	579.20±1.20	34.83±1.62	92.27±0.76

*choline chloride:oxalic acid and H_2O mixture

During the fermentation most of theobromine, however, passes to CS (55% of total value). Arlorio et al. (2005) determined 12900±1.8 mg/kg of theobromine content in mixed CS from different geographic regions. He also mentions the fact that phenolic compounds are stored in cotyledons of the cocoa seeds and that during the fermentation process the loss of these compounds occurs due to diffusion process, creating the CS rich in these components. Barbosa-Pereira, Guglielmetti and Zeppa (2018) also highlighted that the composition of bioactive compounds from different CS extracts together with their antioxidant properties depends on the origin, variety and industrial processing of the raw material. They obtained theobromine concentrations from CS with pulsed electric field assisted extraction (PEF) in range of 4640±0.12 mg/kg and 10920±0.33 mg/kg respectively, depending on the origin of the cocoa. Caffeine in the CS was determined to be in the range between 1590 and 4210 mg/kg also depending on these factors.

By observing the concentrations obtained from all four green extraction techniques, it can be concluded that the best recovery for theobromine was obtained by SFE extraction (including both extraction conditions). In comparison to conventional Soxhlet extraction, all green techniques showed drastically higher yields for both components along with TPC and AA.

Table 6. Differences in theobromine concentrations in CS extracts due to the type of the extraction

Type of extraction	Number of samples	Median	Interquartile range (25%-75%)	p^*
Soxhlet	10	0.609	0.585-0.634	
UAE	24	194.980	0-3443.680	
CAPAE	4	5034.150	4437.768-5612.000	<0.001
SFE	4	6100.840	5869.350-6354.305	
DES	18	3224.450	400.075-3816.350	

*Kruskal Wallis Test

Table 7. Differences in caffeine concentrations in CS extracts due to the type of the extraction

Type of extraction	Number of samples	Median	Interquartile range (25%-75%)	p^*
Soxhlet	10	1.463	1.413-1.513	
UAE	24	785.475	271.053-912.750	
CAPAE	4	563.910	536.360-591.100	<0.001
SFE	4	597.135	533.323-650.140	
DES	18	488.700	24.900-549.400	

*Kruskal Wallis Test

Table 8. Differences in TPC content in CS extracts due to the type of extraction

Type of extraction	Number of samples	Median	Interquartile range (25%-75%)	p^*
Soxhlet	10	0.000	0	
UAE	24	14.820	12.815-15.908	
CAPAE	4	16.615	16.230-17.000	<0.001
SFE	4	11.605	10.850-12.360	
DES	18	30.040	12.323-33.000	

*Kruskal Wallis Test

Table 9. Differences in AA of CS extracts due to the type of extraction

Type of extraction	Number of samples	Median	Interquartile range (25%-75%)	p^*
Soxhlet	10	0.000	0	
UAE	24	47.185	15.275-70.128	
CAPAE	4	32.130	32.050-32.210	<0.001
SFE	4	15.485	15.070-15.900	
DES	18	88.080	84.375-89.510	

*Kruskal Wallis Test

Influence of extraction technique on antioxidant activity and total phenols in CS

Extracts obtained by DES with 50% H_2O and eutectic mixture in 360 min extraction time showed the highest AA (92.27±0.76%), along with the highest TPC but during 180 min time (37.90±1.96 mg GAE/g extract) respectively (Table 5). In all DES extracts there was a slight increase of AA with the increase of extraction time. Therefore, it can be concluded that extraction time significantly influenced on AA, regardless of the composition of the selected DES. In comparison with other extraction methods, this extraction gave the highest TPC for all three selected extractions (60,180 and 360 min). UAE (Table 3) showed the second highest AA for extracts obtained with 50% ethanol aqueous solution at 60 °C (91.87±0.34%), what is almost the same value as the first one obtained by DES. By observing the AA of UAE extracts there was also an increase along with the temperature increase. The highest TPC gained by UAE was obtained by pure H_2O as an extraction solvent, at room temperature (24.95±0.44 mg GAE/g extract, respectively). UAE with 96% ethanol also gave the lowest TPC value at room temperature (10.08±0.77 mg GAE/g extract, respectively) what is also the lowest TPC value obtained by green extraction techniques in this study. The lowest AA was obtained with UAE and 96% ethanol as the extraction solvent (5.59±1.28%). CAPAE (Table 4) showed that with frequency increase there was a slight increase in AA (32.05±1.53% for 70 Hz and 32.21±1.21% for 100 Hz, respectively) and TPC decrease (17.00±0.76 mg GAE/g extract for 70 Hz and 16.23±2.66 mg GAE/g extract for 100 Hz, respectively). SFE extracts (Table 2) showed relatively small AA, almost identical but with a slight decrease, although temperature rise occurred (15.90±1.66% for 40 °C and 15.07±0.36% for 60 °C, respectively).

Bruna et al. (2009) investigated AA of CS of different origin. TPC, ranged from 2.56 to 4.06 mg GAE/g dw, showed a significant difference between the CS of different origin and stated the fact that genetic diversity as well as few environmental factors (light intensity, humidity, temperature, the use of fertilizers, wounding and infections) could be the reason to those findings. They noted that high TPC in CS could be attributed to an optimal fermentation time. Also, they highlighted strong correlation between AA and polyphenol content which could be due to the antioxidant mechanisms of polyphenolic compounds towards free radicals. Considering that fact, they used solvent extraction (0.1% HCl:methanol (15:85 v/v). Barbosa-Pereira, Guglielmetti and Zeppa (2018) obtained higher values due to different extraction method (PEF) on CS of different origin. TPC varied from 17.88±0.87 mg GAE/g dw to 55.16±2.24 mg GAE/g dw. In comparison of AA from cocoa pods and CS extracted with 80% aqueous ethanol in water bath shaker for 30 min and 35 °C, Karim et al. (2014) showed that cocoa pod contains larger amount of TPC as well as flavonoid content (FC). The values for CS were similar to this study (16.30±1.04 mg GAE/g extract to 41.35±2.23 mg GAE/g extract). He also pointed that AA of the extracts was obtained from several bioactive compounds that act synergistically. Awarikabey, Amponsah and Woode (2014), by observing the AA of CS, also confirmed other researchers when it comes to degrading effect of plant metabolites at high temperatures. Their nib and shell extraction was based on water extraction for 30 min. Lower values were gained for different mixes of shells and nibs (4.55-5.53%) almost in the range of UAE extracts with 96% ethanol in this study (the lowest values here). Utami, Armunanto and Rahardjo (2016), on the other hand, observed TPC and AA of CS from different fermentation processes depending on various duration of the process. After separation from the bean, CS was extracted with acetone:water (70:30 v/v). Partially fermented cocoa beans (during 24 h) gave the highest TPC for CS (39.41±0.29 mg/GAE dw) and AA showing the great correlation for TPC and AA due to the highest scavenging activity of 88.67±1.12% and the lowest of 44.60±2.48% for CS in 120 h fermented cocoa beans. Due to these results they concluded that fermentation step surely affects TPC as well as AA. México Quiroz-Reyes et al. (2013) showed similar TPC of CS extracts obtained by ultrasound and maceration techniques (25.34±1.82 mg/g and 17.85±1.33 mg/g) with no significant differences ($p < 0.05$) between both methods. Valadez-Carmona et al. (2018) investigated SFE of CS at the optimal conditions (60 °C, 299 bar and 13.7% ethanol as a co-solvent). TPC was measured to be 12.97 mg GAE/g extract, similar to this study. They concluded that SFE is a potential and efficient technique for valorization of such materials as CS while avoiding toxic solvents in the meantime. Valadez-Carmona et al. (2017) also mentioned that CS is rich in phenolic compounds and investigated the effect of microwaves on TPC and AA. Cacao pod husk contained 323.7±26.5 mg GAE/ 100g dw and that is evidently much higher value than in CS.

The results showed that there were statistically significant differences among all examined measurements and among all extraction techniques ($p < 0.001$) on theobromine and caffeine content as well as on TPC and AA. Statistical data obtained by Kruskal Wallis Test are given in Tables 6-9.

Conclusion

Results of this study showed that the selected different green innovative extraction techniques have different influence on yield of theobromine and caffeine from CS as well as on AA and TPC of obtained extracts. The results indicate that the extraction mostly depends on solvent polarity, as well as on applied extraction parameters. Thus, the highest yield of theobromine was obtained by SFE technique, while UAE technique provided the highest caffeine yield. CS extracts obtained by DES technique showed the highest AA and TPC.

CS extracts, rich in bioactive components, could also be used as potential raw materials in further production (food, pharmaceutical and cosmetic). Also, by extraction of some components that may be toxic in higher concentrations (like theobromine in this case), it could be possible to obtain plant material with reduced level of theobromine and to use it potentially in animal feed without limitation.

The extraction of bioactive components from CS in order to obtain high-quality extracts could reduce the waste accumulation that has become a serious environmental and economic problem in the world.

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