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ULTRASOUND-ASSISTED EXTRACTION OF ACTIVE COMPOUNDS FROM COCOA BEAN SHELL

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Summary

In today's overcrowded world with declining food supplies and the constant struggle against waste accumulation, scientists are increasingly trying to discover new ways to solve these problems. Like many industries, the food industry generates, disposes and accumulates waste thus creating an environmental and economic problem. In this study the one of the green extraction technologies, ultrasound-assisted extraction (UAE), for isolation of bioactive compounds from cocoa bean shell (CBS), a by-product in the chocolate production, was applied. Different temperature (40, 60, 80 °C), extraction time (30, 60, 90 minutes), liquid/solid ratio (10, 30, 50 mL/g) and ultrasound power (30, 50, 70 %) were used to obtain the cocoa bean shell extracts. Six active compounds were detected in the extracts by high performance liquid chromatography with a diode array detector as follows: theobromine (2.077-5.916 mg/g), gallic acid (0.110-1.407 mg/g), caffeine (0.276-0.785 mg/g), catechin (0.033-0.457 mg/g), while the highest obtained concentrations for epicatechin and caffeic acid were 0.100 and 0.527 mg/g of CBS. The highest total phenolic content (TPC) and % scavenging activity measured were 132.897 mgGAE/$g_{extr.}$ and 86.377%. From all investigated parameters, liquid/solid ratio had the greatest influence on the concentrations of obtained compounds. Study proved how UAE is an efficient method for the extraction of bioactive compounds from food by-product - CBS. It should also be emphasized that such application could find the purpose at the industrial level for the discarded waste that still contains valuable compounds, while the enriched extracts could be further used as raw material in other processes.

Keywords: cocoa bean shell, by-products, bioactive compounds, ultrasound-assisted extraction

Introduction

By-products of the food industry that are discarded in the production process as inedible parts are considered as waste material. Such waste accumulates andbecoming an increasing economic and environmental problem today (Jokić et al., 2018; Pavlović et al., 2019; Viganó et al., 2015). Consequently, people are starting to think in the direction of utilizing this type of waste and its possible application. Various authors state that by-products still contain significant amounts of useful bioactive compounds which have been proven to possess the antioxidant, anti-inflammatory, as well as antiviral activities (Viganó et al., 2015). One such by-product is CBS in the production of chocolate and chocolate products (Hamzat and Adeola, 2011). Okiyama et al. (2017) and Panak Balentić et al. (2018) gave a detailed insight regarding new uses of CBS in the food industry, feedstuff for livestock, usage as biofuel as well as adsorbent or composite while Rojo-Poveda et al. (2020) focused on the latest advances of CBS applications for human health mostly from a nutritional and biofunctional point of view. Interesting chemical and nutritional composition of CBS opens up some new possibilities not only as food, feed or industrial usage but also had potential in medical applications (Rojo-Poveda, 2020). It has been scientifically proven that significant amounts of certain phenolic compounds, which are found in the cotyledons of cocoa beans, are lost during the process of fermentation, due to migration into the CBS (Hernández-Hernández et al., 2019, Utami, Armunanto & Rahardjo, 2016), as well as some methylxantines (Beckett, 2009). Green extraction techniques, as highly efficient processes, can provide higher yields from renewable sources without hazardous chemicals (Mustafa and Turner, 2012). By using alternative solvents (mainly water-based), with lower energy consumption and a reduced number of experiments in the extraction process, it is possible to obtain stable extracts without contaminants (Chemat et al., 2012). With longer extraction time, and usage of organic solvents, conventional extraction techniques are uneconomical (Ramos et al., 2002) and demanding at the same time. The reason is mostly the frequent need to purify such extracts, less solvent selectivity and small concentrations of desired thermolabile compounds (Mustafa and Turner, 2011). Ultrasound-assisted extraction (UAE) significantly reduces the time required to extract specific compounds, with higher yields achieved and better quality of the extract. It is considered to be a good option for the extraction of different organic compounds from various matrices because it provides more efficient contact between the solid matrix and the solvent due to increased pressure (better penetration and transport) and

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increased temperature (improves solubility and diffusion) (Grumezescu and Holban, 2017; Chemat et al., 2011). Mechanical effect of cavitation leads to cell wall damage, which facilitates the penetration of solvent to cell content and mass transfer (Drmić and Režek Jambrak, 2010; Patist and Bates, 2008). In the field of Sustainable Chemistry, the advantages of UAE are the use of non-toxic solvents, reduced energy consumption, shorter time and lower temperature of the process what is suitable for the extraction of thermolabile compounds (Medina-Torres et al., 2017).

The main objective of this research was to investigate the impact of the UAE process on the extraction of selected bioactive compounds from CBS as well as to determine a % scavenging activity and TPC in obtained CBS extracts. Those highly enriched CBS extracts could possibly be further used as functional food or as raw material in different industries.

Material and methods

Chemicals

All standards and chemicals were purchased from commercial suppliers and were of analytical grade. Solvents were purchased from J.T. Baker (Phillipsburg, USA). All standards for HPLC analysis, including theobromine standard (purity ≥98%), theophylline (purity≥99%), gallic acid (purity≥99%), epicatechin (purity ≥98%), catechin (purity ≥99%), and caffeic acid (purity ≥99%), were purchased from Sigma Aldrich (Germany), while the caffeine standard (≥98%) was purchased from Dr. Ehrenstorfer (Augsburg, Germany). 2,2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma-Aldrich (St. Louis, MI, USA).

Material

CBS was obtained from Kandit d.o.o. Chocolate Factory, Osijek, Croatia, in the summer of 2017. The material was primarly separated from the cotyledon and roasted at 135 °C for 55 min. The geographical origin of CBS was West Africa blend.

UAE extraction of bioactive compounds from CBS

Prior to the extraction process, the samples of CBS were crushed in an IKA A11 Basic laboratory mill to increase the matrix area. After grinding and weighing 1 g of CBS, different concentrations of samples were prepared for extraction by addition of water as solvent. UAE of bioactive compounds from CBS was performed on an ELMA ultrasonic bath, Elmasonic P 120 H (Elma Schmidbauer GmbH, Germany). The extraction was performed at a frequency of 37 kHz in sweep mode for homogeneous distribution of ultrasonic waves within the water bath. Twenty-nine experiments, including five replicates, were performed according to the Response Surface Methodology (RSM) and the Box-Behnken design (BBD). The effect of temperature, extraction time, liquid/solid ratio and ultrasound power on the yield of bioactive compounds in the obtained CBS extracts were examined. The temperature was set from 40 °C to 80 °C, liquid/solid ratio at 10 to 50 mL/g, ultrasound power from 30 to 70 % and the extraction time from 30 to 90 minutes.

Determination of bioactive compounds of CBS by HPLC Analysis

Immediately after the extraction, the obtained extracts were diluted with water (1:10) and filtered through a 0.2 μm polytetrafluoroethylene (PTFE) syringe filter. Identification and quantification of the obtained bioactive compounds in extracts was performed on the High-Performance Liquid Chromatograph (HPLC) (Infinity 1260 Agilent Technologies, Santa Clara, USA), which contained an autosampler G7129A, quaternary pump G7111B 1260 and diode array detector (DAD) G7117C 1260 DAD HS. The column used for the measurement was Zorbax C18 150 mm x 4.6 mm x 5μm, which was thermostated on 30 °C. The wavelenght was set to 276 nm and the injection volume was 20 μL. Mobile phase was gradient, starting by 1% formic acid and acetonitrile (95:5) at the beginning, changing to (80:29) till 9 min, and returning to (95:5) till 13 min. The flow was set to 1 mL/min and the analysis was done in triplicate.

Determination of % scavenging activity of obtained extracts

The antiradical activity of the obtained extracts was determined using the prior described DPPH procedure (Jokić et al., 2016) on a UV/Vis spectrophotometer (Thermo Spectronic, Cambridge, Great Britain). Measurement solutions were prepared by adding 1.2 mL of CBS (10 mg/mL) and 0.5 mL 0.2 mM freshly prepared DPPH solution in methanol. The samples were left to incubate for 30 minutes in the dark space at ambient temperature, after which the apsorbance was measured at 517 nm. All measurements were done in triplicate.

Determination of total phenol content of obtained extracts

The total phenol content (TPC) of the extracts was determined according to modified spectrophotometric method using Folin-Ciocalteu reagent, with gallic acid as a calibration standard (Jakobek et al., 2007) on a Selecta

UV/Vis spectrophotometer (UV-2005, Spain). Measurement solutions were prepared by adding 20 µl of sample, 1580 µl distilled water, 100 µl Folin-Ciocalteu reagent and 300 µl Na_2CO_3 solution (200 g/L). Prepared samples were placed in a thermostat at 40 °C for 30 minutes after which the measurement was performed. The results were calculated according to the calibration curves derived from the three analyzes and expressed as mg gallic acid (GAE) equivalent per g of extract. The measurement was performed in triplicate at the wavelength of 765 nm.

Statistical Analysis

A BBD with four numerical factors at three levels was used for statistical analysis of the obtained parameters. The design consisted of 29 experiments with five replications at the center point. For the statistical analysis of obtained experimental data the commercial Design-Expert® software, v.9 (Stat Ease Inc. Minneapolis, MN, USA) was used as well as the analysis of variance (ANOVA) to estimate the quality of the obtained models. The test of the statistically significant difference was based on the total error criteria with the level of confidence of 95.0%. The same software was used to generate the response plots in order to better understand the correlation of independent and response variables.

Results and discussion

The results obtained by UAE extraction method for isolation of selected bioactive compounds from CBS are given in Table 1. In our previous study (Pavlović et al., 2019) we investigated the extraction of bioactive compounds from CBS, specifically methylxantines, (theobromine and caffeine) by selected green extraction techniques (supercritical CO_2 extraction, UAE, cold atmospheric plasma extraction, and deep eutectic solvent extraction) in comparison to conventional Soxhlet extraction. It was proved the significantly better efficiency of innovative green extraction techniques compared to conventional Soxhlet extraction in the isolation of targeted compounds as well as the better antioxidant activity of obtained extracts. Opposite to the new technologies, the conventional method did not show practicable due to long duration of the process and the application of high temperatures. In addition to this preliminary research as well as to the available literature, eleven (11) compounds were analyzed in this study that were assumed to be pottentially present in CBS (theobromine, caffeine, theophylline, gallic acid, epicatechin, epigalocatechin, catechin, chlorogenic acid, caffeic acid, vanillin, and 5-Hydroxymethylfurfural (5-HMF).

Table 1. Bioactive compounds, TPC and % scavenging activity of CBS in obtained UAE extracts

RUN	T (°C)	t (min)	Liquid/solid ratio (mL/g)	Power (%)	Gallic acid (mg/g)	Theobromine (mg/g)	Caffeine (mg/g)	Caffeic acid (mg/g)	Catehin (mg/g)	Epicatechin (mg/g)	TPC (mgGAE/g extract)	% scavenging activity
1.	40	60	30	30	0.504	2.077	0.276	0.000	0.165	traces	54.692	36.420
2.	60	90	30	70	0.517	2.696	0.410	0.255	0.266	0.077	87.000	65.108
3.	40	60	10	50	0.904	3.767	0.486	0.000	0.065	0.024	21.103	14.624
4.	40	90	30	50	0.622	2.493	0.360	0.000	0.317	0.055	82.385	56.907
5.	60	60	30	50	0.368	4.122	0.295	0.274	0.409	0.082	92.385	67.703
6.	40	60	30	70	0.642	3.120	0.444	0.398	0.342	0.020	96.487	64.783
7.	60	90	10	50	0.968	3.872	0.497	0.000	0.080	0.005	23.667	16.923
8.	80	60	30	70	0.738	4.098	0.580	0.447	0.457	0.029	132.897	74.943
9.	60	60	30	50	0.807	4.545	0.618	0.527	0.333	0.060	108.282	85.106
10.	60	30	30	70	0.749	3.424	0.486	0.443	0.321	0.078	109.821	73.244
11.	40	60	50	50	1.197	5.916	0.570	0.000	0.134	0.042	91.872	74.812
12.	80	90	30	50	0.727	3.725	0.537	0.431	0.354	0.100	123.410	86.377
13.	60	60	30	50	0.570	3.610	0.508	0.434	0.309	0.085	114.436	70.598
14.	60	60	50	30	1.077	5.195	0.726	0.000	0.208	0.054	118.538	82.326
15.	60	60	30	50	0.769	4.134	0.589	0.000	0.336	0.067	91.615	73.795
16.	60	60	50	70	1.407	5.431	0.785	0.000	0.341	traces	108.282	77.594
17.	60	30	10	50	0.866	3.642	0.433	0.000	0.033	traces	21.872	13.683
18.	40	30	30	50	0.605	2.255	0.315	0.000	0.185	0.023	78.538	59.304
19.	60	30	30	30	0.584	3.325	0.486	0.392	0.245	0.000	105.718	68.167
20.	60	60	10	70	0.110	2.916	0.698	0.000	0.111	0.012	34.436	27.471
21.	60	90	30	30	0.469	3.419	0.472	0.381	0.446	traces	93.410	81.569
22.	80	30	30	50	0.520	2.402	0.358	0.000	0.289	traces	91.872	82.941
23.	60	90	50	50	1.249	5.600	0.587	0.000	0.175	0.023	124.179	14.034
24.	60	60	30	50	0.785	3.456	0.537	0.000	0.340	traces	101.103	81.815
25.	80	60	50	50	1.080	4.128	0.678	0.000	0.247	0.070	123.923	85.485
26.	80	60	30	30	0.586	2.568	0.400	0.000	0.243	0.036	105.974	74.944
27.	60	60	10	30	0.854	3.193	0.439	0.000	0.044	0.006	20.590	14.130
28.	60	30	50	50	1.118	5.297	0.646	0.000	0.170	0.023	106.487	76.097
29.	80	60	10	50	0.687	3.234	0.421	0.000	0.051	0.006	22.128	17.334

T: temperature; t: time; % scavenging activity.

Processes like roasting could eventually lead to thermal degradation and consequently to formation of 5-HMF which could have adverse effect on human health (Kowalski et al., 2013). According to the obtained results shown in Table 1, the most abundant compound in CBS extracts obtained by UAE was theobromine, followed by gallic acid, caffeine, catechin, epicatechin and finally caffeic acid in traces. Theobromine, as the most abundant methylxanthine, was extracted in concentrations from 2.077 mg/g to 5.916 mg/g, while caffeine was extracted in concentrations from 0.276 mg/g to 0.785 mg/g. The highest concentration of theobromine (5.916 mg/g) was extracted under conditions with 50% ultrasonic power, 50 mL/g liquid/solid ratio, temperature 40 °C in the extraction time of 60 minutes. The highest concentration of caffeine (0.785 mg/g) was extracted under conditions of 70% ultrasonic power, 50 mL/g liquid/solid ratio, at a temperature of 60 °C in the extraction time of 60 minutes. The most abundant phenolic compounds extracted by this technique were gallic acid in concentrations from 0.110 mg/g to 1.407 mg/g and catechin from 0.033 mg/g to 0.457 mg/g. The highest concentrations of caffeic acid and epicatechin were 0.527 mg/g and 0.100 mg/g depending on the given extraction parameters. The highest concentration of gallic acid (1.407 mg/g) was extracted at 70% ultrasonic power, 50 mL/g liquid/solid ratio, at 60 °C in the extraction time of 60 minutes. The highest concentration of catechin (0.457 mg/g) was extracted at 70% ultrasonic power, 30 mL/g liquid/solid ratio, at 80 °C in the extraction time of 60 minutes. The extract with the highest catechin concentration was also the extract with the highest TPC (132.897 mgGAE/$g_{extr.}$) while the extract with the highest epicatechin concentration was also the extract with highest % scavenging acitvity (86.377%). The highest concentration of caffeic acid (0.527 mg/g) was extracted at 50% ultrasonic power, 30 mL/g liquid/solid ratio, at 60 °C in the extraction time of 60 minutes. The highest concentration of epicatechin (0.100 mg/g) was extracted at 50% ultrasonic power, 30 mL/g liquid/solid ratio, at 80 °C in the extraction time of 90 minutes. Other phenolic compounds that were found in low concentrations in the CBS extracts were below the limit of detection and could not be quantified while theophylline and 5-HMF were not even detected.

If we look at other similar studies, Esclapez et al. (2011) mentioned that temperature, as one of the important UAE parameter, affects the yield of each compound individually, due to improved mass transfer. McDonell and Tiwari (2017) noted that increased temperature reduces solvent viscosity due to the increased kinetic energy of molecules in the solution. Although some studies have shown that fewer phenolic compounds can be extracted at higher temperatures due to their possible thermal degradation or polymerization, this is not the case in this study. Such processes generally occur at much higher temperatures than in the above experiment (110 °C) (De la Calle and Costas -Rodriguez, 2017). McDonnel and Tiwari (2017) gave an insight into the effectiveness of UAE in the extraction of polyphenols, antioxidants and other bioactive compounds from different matrices (pomegranate peel, grapefruit seeds, olive leaves, etc.). Yusof et al. (2019) applied different concentrations of ethanol (70-90 v/v%), temperature (45-65 °C) and different extraction time (30-60 min) for the extraction of flavonoids from Malasian CBS by UAE. Except epicatechin, authors detected the certain flavonoid oligomers (procyanidin B2, procyanidin C1, procyanidin B4, procyanidin A2, procyanidin trimer, procyanidin tetramer, procyanidin pentamer) in the CBS by the ultra-high-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-QTOF-MS). The calculated optimal extraction conditions for these compounds were 80% aqueous ethanol solution, temperature of 55 °C and the extraction time of 45 minutes. Quiroz-Reyes et al. (2013) demonstrated better UAE efficiency unlike maceration due to the higher antioxidant activity in UAE extracts as well as a good correlation between antioxidant activity and TPC. They also pointed out the higher content of catechins and epicatechins in extracts of cocoa bean cotyledons compared to CBS extracts. They stated how UAE is an excellent extraction method for isolating antioxidants given the shorter extraction time, higher reproducibility as well as small lost of bioactive compounds. Oliveira et al. (2018) extracted methylxanthines (caffeine and theobromine) from cocoa beans by proton ionic liquid 2HEAA (2-hydroxy ethylammonium acetate) and ultrasound. They emphasized excellent efficiency of proton ionic liquids in the extraction of these compounds and highlighted the solid/liquid ratio as the main influential variable in the extraction, while the power of ultrasound had no significant effect, which is also the case in this study. Jiménez and Cañizares-Macías (2013) proved that the UAE is more efficient in isolating caffeine (by 57.7%) and theobromine (by 43.6%) from cocoa beans in comparison to conventional extraction by mixing. As a reason for more efficient extraction, they pointed out the importance of ultrasonic waves that do not cause modification of the extract. Dent et al. (2015) also

compared some conventional methods for extraction of phenolic compounds from sage (*Salvia officinalis* L.) using two different UAE techniques (ultrasonic device with direct mixing and direct sonication with a probe). Studies have shown that UAE with ultrasonic device with direct mixing achieved the highest yield of total and individual polyphenols while direct sonication was also more efficient than conventional extractions. Bamba et al. (2018) were the first who to investigate the influence of UAE conditions on the yield of phenolic compounds from blueberries (*Vaccinium angustifolium*) as well as the antioxidant activity of the obtained extracts. They proved that the efficiency of this extraction largely depends on the ethanol content in the aqueous extract, the solid/liquid ratio, temperature, time and pH. UAE with a 50% ethanol gave higher yields of flavonoids and anthocyanins as well as higher TPC, while a decrease in the solid/liquid content increased the polyphenol content as well as the antioxidant activity of the extracts. With the use of 50% ethanol and higher temperatures, the total flavonoids and antioxidant activity increased while the TPC decreased. The weakly basic pH had a positive effect on the antioxidant activity and TPC in contrast to the acidic pH, while the anthocyanin content reduced. Thus, prolonged extraction time in water increased the anthocyanin content. The authors also stated the possibility of different phenolic fractions with selected UAE conditions. Papoutsis et al. (2018) optimized the UAE for the isolation of rutin from lemon by-products (*Citrus limon* L.) (residual endocarp, seeds, and exocarp). The results of total TPC, total flavonoids and antioxidant activity were compared with hot water-assisted extraction and conventional extraction with organic solvents. Hot water extraction has been shown to be the most effective in isolating the highest flavonoid content and the highest antioxidant activity while UAE and conventional extraction showed similar results. However, they recommended use of UAE due to the shorter duration of extraction.

Response Surface Analysis and Process Optimization

To explore the influence of process parameters on the extraction yield, TPC and % scavenging acitvity of selected compounds in CBS extracts, a response surface analysis was performed. The results showed that six responses (theobromine, caffeine, gallic acid, catechin, TPC and % scavenging activity) (Table 1) were detected in all 29 experiments and they were included in further statistical analysis. The selected responses were evaluated by analysis of variance (ANOVA) and the results are summarized in Tables 2-7. For methylxanthines (theobromine and caffeine, respectively), a statistically significant effect was shown in linear term of liquid/solid ratio (p = 0.0003 and 0.0068), as well as in its quadratic term (p = 0.0041 and 0.0079). The quadratic term of temperature also showed a significant influence on these two compounds (p = 0.0203 and 0.0436). The model F-value of 3.96 for theobromine and 2.94 for caffeine imply that model is significant. There is only a 0.73% chance for theobromine and 2.63% chance for caffeine that an F-value this large could occur due to noise (Tables 2 and 3). For gallic acid, statistically significant effect was shown in linear term of liquid/solid ratio (p = 0.0008), as well as in its quadratic term (p = 0.0005). The interaction between the liquid/solid ratio and the ultrasound power (X_3X_4), also showed a statistically significant effect (p = 0.0119). The model F-value of 3.70 for gallic acid implies that model is significant. There is only a 1.00% chance that an F-value this large could occur due to noise (Table 4). Three process parameters for catechin showed statistically significant effect in linear term, temperature (p = 0.0486), liquid/solid ratio (p = 0.0005) and ultrasound power (p = 0.0291) while the quadratic term of the liquid/solid ratio also showed statistically significant effect (p <0.0001). The interaction between the extraction time and the ultrasound power (X_2X_4) also showed statistically significant effect (p = 0.0437). The model F-value of 8.03 for catechin implies that model is significant. There is only a 0.02% chance that an F-value this large could occur due to noise (Table 5). TPC (Table 6) as well as in the antioxidant activity (Table 7), except for the linear term (p <0.0001 for both responses), the quadratic term of the liquid/solid ratio showed a statistically significant effect (p <0.0001 and 0.0002). Temperature also showed statistically significant effect for both responses (p = 0.0014 and 0.0404). The interaction between the extraction time and the liquid/solid ratio (X_2X_3) showed statistically significant effect only for the antioxidant activity (p = 0.0436). The model F-value of 14.83 for TPC and 5.44 for % scavenging activity imply that models are significant. There is only a 0.01% chance for TPC and 0.16% chance for % scavenging activity that an F-value this large could occur due to noise.

Table 2. Analysis of variance (ANOVA) for the response surface quadratic model for theobromine

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	23.72	14	1.69	3.96	0.0073*
X_1-Temperature	0.0232	1	0.0232	0.0543	0.8191
X_2-Time	0.1774	1	0.1774	0.4150	0.5298
X_3-Liquid/solid ratio	9.98	1	9.98	23.35	0.0003*
X_4-Power	0.3033	1	0.3033	0.7095	0.4138
X_1X_2	0.2946	1	0.2946	0.6892	0.4204
X_1X_3	0.3939	1	0.3939	0.9214	0.3534
X_1X_4	0.0591	1	0.0591	0.1383	0.7155
X_2X_3	0.0013	1	0.0013	0.0031	0.9563
X_2X_4	0.1690	1	0.1690	0.3953	0.5397
X_3X_4	0.0658	1	0.0658	0.1539	0.7008
X_1^2	2.93	1	2.93	6.85	0.0203*
X_2^2	0.9173	1	0.9173	2.15	0.1651
X_3^2	5.01	1	5.01	11.73	0.0041*
X_4^2	1.38	1	1.38	3.24	0.0935
Residual	5.98	14	0.4275		
Lack of Fit	5.21	10	0.5211	2.69	0.1760
Pure Error	0.7737	4	0.1934		
Cor Total	29.71	28			
R^2	0.7985				

Table 3. Analysis of variance (ANOVA) for the response surface quadratic model for caffeine

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	0.3512	14	0.0251	2.94	0.0263*
X_1-Temperature	0.0228	1	0.0228	2.67	0.1245
X_2-Time	0.0016	1	0.0016	0.1912	0.6686
X_3-Liquid/solid ratio	0.0859	1	0.0859	10.07	0.0068*
X_4-Power	0.0304	1	0.0304	3.56	0.0801
X_1X_2	0.0044	1	0.0044	0.5209	0.4823
X_1X_3	0.0075	1	0.0075	0.8850	0.3628
X_1X_4	0.0000	1	0.0000	0.0041	0.9496
X_2X_3	0.0038	1	0.0038	0.4422	0.5169
X_2X_4	0.0010	1	0.0010	0.1120	0.7429
X_3X_4	0.0099	1	0.0099	1.17	0.2985
X_1^2	0.0420	1	0.0420	4.92	0.0436*
X_2^2	0.0235	1	0.0235	2.76	0.1189
X_3^2	0.0818	1	0.0818	9.59	0.0079*
X_4^2	0.0018	1	0.0018	0.2136	0.6511
Residual	0.1194	14	0.0085		
Lack of Fit	0.0543	10	0.0054	0.3336	0.9275
Pure Error	0.0651	4	0.0163		
Cor Total	0.4706	28			
R^2	0.7463				

Table 4. Analysis of variance (ANOVA) for the response surface quadratic model for gallic acid

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	1.79	14	0.1277	3.70	0.0100*
X_1-Temperature	0.0015	1	0.0015	0.0444	0.8362
X_2-Time	0.0010	1	0.0010	0.0286	0.8681
X_3-Liquid/solid ratio	0.6523	1	0.6523	18.10	0.0008*
X_4-Power	0.0007	1	0.0007	0.0193	0.8915
X_1X_2	0.0091	1	0.0091	0.2625	0.6164
X_1X_3	0.0025	1	0.0025	0.0714	0.7932
X_1X_4	0.0000	1	0.0000	0.0014	0.9703

X_2X_3	0.0002	1	0.0002	0.0060	0.9392
X_2X_4	0.0035	1	0.0035	0.1001	0.7564
X_3X_4	0.2884	1	0.2884	8.35	0.0119*
X_1^2	0.0008	1	0.0008	0.0223	0.8835
X_2^2	0.0009	1	0.0009	0.0262	0.8738
X_3^2	0.6944	1	0.6944	20.10	0.0005*
X_4^2	0.0447	1	0.0447	1.29	0.2744
Residual	0.4837	14	0.0346		
Lack of Fit	0.3411	10	0.0341	0.9561	0.5689
Pure Error	0.1427	4	0.0357		
Cor Total	2.27	28			
R^2	0.7870				

Table 5. Analysis of variance (ANOVA) for the response surface quadratic model for catechin

Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**
Model	0.3733	14	0.0267	8.03	0.0002*
X_1-Temperature	0.0155	1	0.0155	4.66	0.0486*
X_2-Time	0.0130	1	0.0130	3.90	0.0683
X_3-Liquid/solid ratio	0.0662	1	0.0662	19.93	0.0005*
X_4-Power	0.0196	1	0.0196	5.91	0.0291*
X_1X_2	0.0011	1	0.0011	0.3361	0.5713
X_1X_3	0.0040	1	0.0040	1.22	0.2885
X_1X_4	0.0003	1	0.0003	0.1021	0.7541
X_2X_3	0.0004	1	0.0004	0.1302	0.7236
X_2X_4	0.0163	1	0.0163	4.91	0.0437*
X_3X_4	0.0011	1	0.0011	0.3231	0.5788
X_1^2	0.0088	1	0.0088	2.64	0.1267
X_2^2	0.0071	1	0.0071	2.14	0.1656
X_3^2	0.2240	1	0.2240	67.46	< 0.0001*
X_4^2	0.0002	1	0.0002	0.0618	0.8073
Residual	0.0465	14	0.0033		
Lack of Fit	0.0408	10	0.0041	2.85	0.1624
Pure Error	0.0057	4	0.0014		
Cor Total	0.4197	28			
R^2	0.8893				

Table 6. Analysis of variance (ANOVA) for the response surface quadratic model for TPC

Source	**Sum of Squares**	**df**	**Mean Square**	**F-Value**	***p*-Value**
Model	33668.56	14	2404.90	14.83	< 0.0001*
X_1-Temperature	2555.82	1	2555.82	15.76	0.0014*
X_2-Time	32.48	1	32.48	0.2003	0.6613
X_3-Liquid/solid ratio	23363.05	1	23363.05	144.06	< 0.0001*
X_4-Power	408.33	1	408.33	2.52	0.1349
X_1X_2	191.72	1	191.72	1.18	0.2953
X_1X_3	240.65	1	240.65	1.48	0.2433
X_1X_4	55.29	1	55.29	0.3409	0.5686
X_2X_3	63.18	1	63.18	0.3896	0.5425
X_2X_4	27.63	1	27.63	0.1704	0.6860
X_3X_4	145.23	1	145.23	0.8956	0.3600
X_1^2	168.18	1	168.18	1.04	0.3258
X_2^2	31.60	1	31.60	0.1949	0.6656
X_3^2	6280.86	1	6280.86	38.73	< 0.0001*
X_4^2	0.3391	1	0.3391	0.0021	0.9642
Residual	2270.42	14	162.17		
Lack of Fit	1876.16	10	187.62	1.90	0.2801
Pure Error	394.27	4	98.57		
Cor Total	35938.99	28			
R^2	0.9368				

Table 7. Analysis of variance (ANOVA) for the response surface quadratic model for % scavenging activity

Source	Sum of Squares	df	Mean Square	F-Value	*p*-Value
Model	16501.16	14	1178.65	5.44	0.0016*
X_1-Temperature	1105.42	1	1105.42	5.10	0.0404*
X_2-Time	229.85	1	229.85	1.06	0.3205
X_3-Liquid/solid ratio	7812.42	1	7812.42	36.06	< 0.0001*
X_4-Power	54.55	1	54.55	0.2518	0.6236
X_1X_2	8.51	1	8.51	0.0393	0.8458
X_1X_3	15.85	1	15.85	0.0732	0.7907
X_1X_4	201.12	1	201.12	0.9284	0.3516
X_2X_3	1066.11	1	1066.11	4.92	0.0436*
X_2X_4	115.97	1	115.97	0.5353	0.4765
X_3X_4	81.66	1	81.66	0.3769	0.5491
X_1^2	43.62	1	43.62	0.2014	0.6605
X_2^2	309.58	1	309.58	1.43	0.2518
X_3^2	5602.18	1	5602.18	25.86	0.0002*
X_4^2	8.07	1	8.07	0.0372	0.8498
Residual	3032.98	14	216.64		
Lack of Fit	2813.55	10	281.36	5.13	0.0646
Pure Error	219.43	4	54.86		
Cor Total	19534.14	28			
R^2	0.8447				

In general, the liquid/solid ratio showed the largest statistically significant effect for all tested responses (p <0.05), while the extraction time did not show statistically significant effect on any response. Temperature showed statistically significant effect for catechin (p = 0.0486), TPC (p = 0.0014) and % scavenging activity (p = 0.0404), while ultrasound power had statistically significant effect only on catechin concentration (p = 0.0291). Regression models for all examined responses showed a statistically significant effect (p-values from <0.0001 to 0.0263) with satisfactory coefficients of determination (R^2) ranging from 0.75 to 0.94. Given that p-value for all regression models were below 0.05 means that there is a statistically significant influence between the independent variables and the variables of the observed responses. The Lack of Fit in all cases was not statistically significant (p>0.05), which means that the obtained second order polynominal equation is adequate for accurate estimation of experimental values and can be used to make predictions about the response for given levels of each factor. The high levels of the factors were coded as +1 and the low levels as -1, by default. The coded equations are useful for identifying the relative impact of the factors by comparing the factor coefficients (Table 8).

Table 8. Polynominal equations calculated after implementation of BBD (in terms of coded factors)

Regresion Coefficient	2nd Order Polynominal Equation
Gallic acid (Y_1)	$0.6599 - 0.0113X_1 + 0.0091X_2 + 0.2283X_3 - 0.0075X_4 - 0.0109X_1^2 + 0.0118X_2^2 + 0.3272X_3^2 - 0.0830X_4^2 + 0.0476X_1X_2 + 0.0248X_1X_3 + 0.0035X_1X_4 + 0.0072X_2X_3 - 0.0294X_2X_4 + 0.2685X_3X_4$
Theobromine (Y_2)	$3.97 + 0.0440X_1 + 0.1216X_2 + 0.9120X_3 + 0.1590X_4 - 0.6720X_1^2 - 0.3760X_2^2 + 0.8792X_3^2 - 0.4620X_4^2 + 0.2714X_1X_2 - 0.3138X_1X_3 + 0.1216X_1X_4 + 0.0183X_2X_3 - 0.2055X_2X_4 + 0.1282X_3X_4$
Caffeine (Y_3)	$0.5095 + 0.0436X_1 + 0.0117X_2 + 0.0846X_3 + 0.0503X_4 - 0.0804X_1^2 - 0.0602X_2^2 + 0.1123X_3^2 + 0.0168X_4^2 + 0.0333X_1X_2 + 0.0434X_1X_3 + 0.0030X_1X_4 - 0.0307X_2X_3 - 0.0155X_2X_4 - 0.0499X_3X_4$
Catechin (Y_4)	$0.3454 + 0.0359X_1 + 0.0329X_2 + 0.0743X_3 + 0.0404X_4 - 0.0367X_1^2 - 0.0331X_2^2 - 0.1858X_3^2 + 0.0056X_4^2 - 0.0167X_1X_2 + 0.0318X_1X_3 + 0.0092X_1X_4 - 0.0104X_2X_3 - 0.0639X_2X_4 + 0.0164X_3X_4$
TPC (Y_5)	$101.56 + 14.59X_1 + 1.65X_2 + 44.12X_3 + 5.83X_4 - 5.09X_1^2 - 2.21X_2^2 - 31.12X_3^2 + 0.2286X_4^2 + 6.92X_1X_2 + 7.76X_1X_3 - 3.72X_1X_4 + 3.97X_2X_3 - 2.63X_2X_4 - 6.03X_3X_4$
% scavenging activity (Y_6)	$75.80 + 9.60X_1 - 4.38X_2 + 25.52X_3 + 2.13X_4 - 2.59X_1^2 - 6.91X_2^2 - 29.39X_3^2 - 1.12X_4^2 + 1.46X_1X_2 + 1.99X_1X_3 - 7.09X_1X_4 - 16.33X_2X_3 - 5.38X_2X_4 - 4.52X_3X_4$

X_1: temperature; X_2: time; X_3: liquid/solid ratio; X_4: ultrasaund power

Three-dimensional plots for two most abundant methylxanthines in CBS, theobromine and caffeine (Figs. 1 and 2) showed very similar shapes. The plots show that by increasing the liquid/solid ratio,

concentration of those two compounds significantly increase. By increasing the temperature to 60 °C, the ultrasonic power up to 50% as well as the longer extraction time up to 60 minutes, the concentration of theobromine first slightly increases and then decreases (Fig. 1). In caffeine, also by increasing the temperature to 60 °C and extracting to 60 minutes, the concentration first slightly increases and then decreases while the increase in ultrasound power had no effect on the concentration of this compound (Fig. 2). The increase in the liquid/solid ratio affected the increase in gallic acid concentration while the increase in temperature, ultrasound power as well as the length of the extraction time had no significant effect on the concentration of this compound (Fig. 3). By increasing the liquid/solid ratio from 10 to 30 mL/g the catechin concentration also increased, while by further increasing of this parameter, catechin concentration decreased. The graph also showed the slight increase of catechin concentration with increase in temperature, ultrasound power and extraction time (Fig. 4). Three-dimensional plots for TPC (Fig. 5) and % scavenging activity (Fig. 6) showed similar shapes, which was expected due to their already proven correlation. Both observed responds showed the tendency to increase by increasing liquid/solid ratio and the slight increase with temperature increase. The increase in ultrasound power as well as the extraction time showed no significant effect for either response (Figs. 5 and 6).

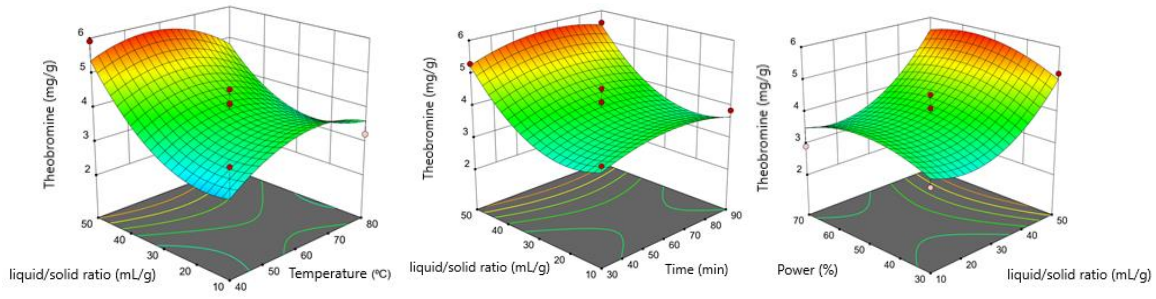

Fig. 1. Three-dimensional plots for theobromine content in CBS extracts obtained by UAE

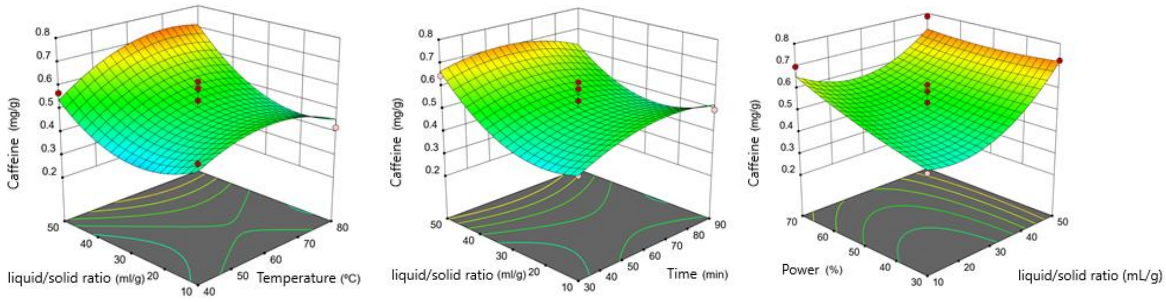

Fig. 2. Three-dimensional plots for caffeine content in CBS extracts obtained by UAE

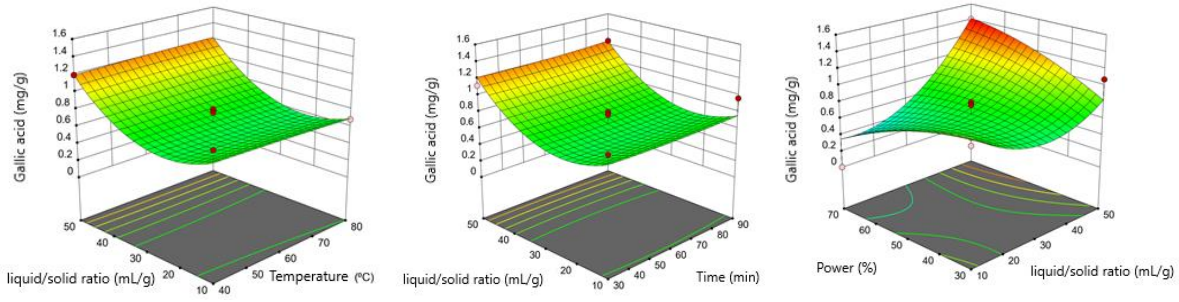

Fig. 3. Three-dimensional plots for gallic acid content in CBS extracts obtained by UAE

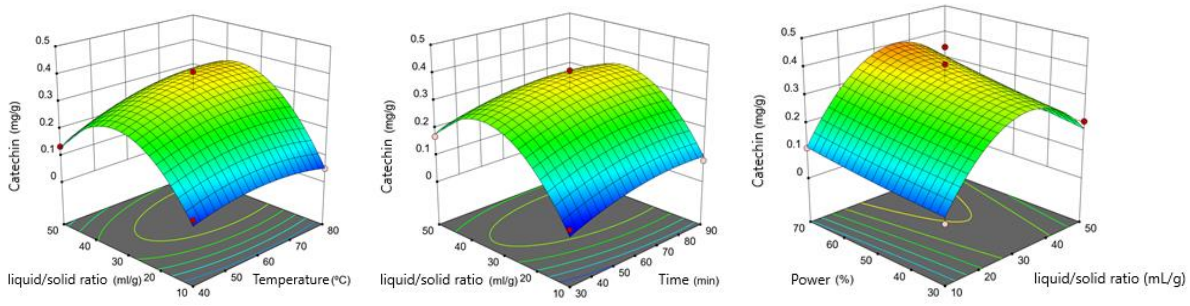

Fig. 4. Three-dimensional plots for catechin content in CBS extracts obtained by UAE

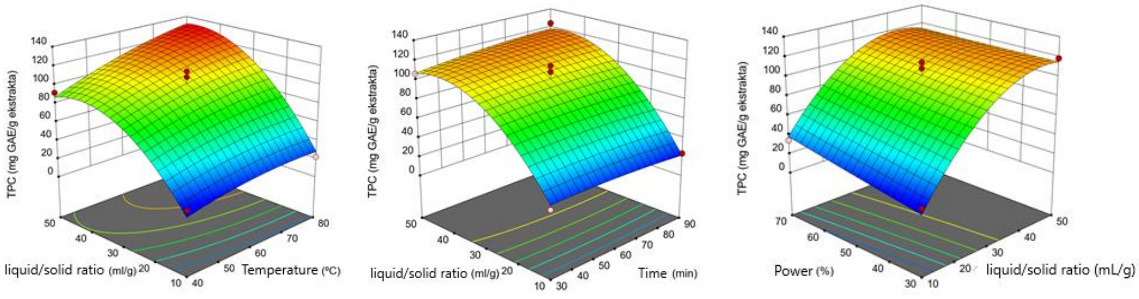

Fig. 5. Three-dimensional plots for TPC in CBS extracts obtained by UAE

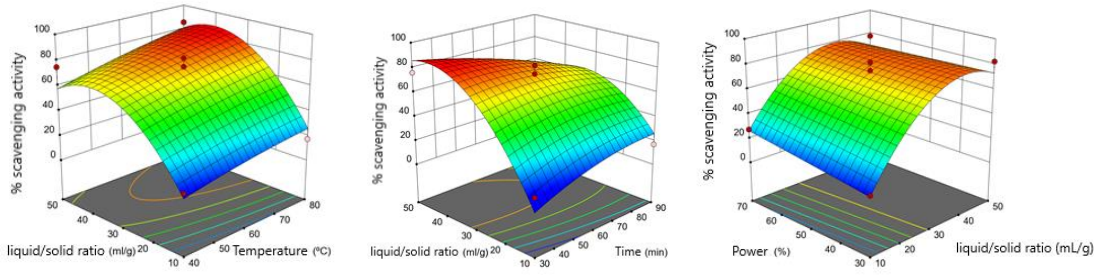

Fig. 6. Three-dimensional plots for % scavenging activity in CBS extracts obtained by UAE

The RSM in this study gave optimal conditions for the UAE extraction of bioactive compounds from CBS, taking into account their maximum: temperature of 69.45 °C, liquid/solid ratio 49.99 mL/g, ultrasonic power of 69.99% and an extraction time of 44.26 minutes. Under these conditions, the predicted concentration of theobromine was calculated to be 5.234 mg/g, caffeine 0.741 mg/g, catechin 0.341 mg/g, gallic acid 1.434 mg/g, TPC 118.380 mg GAE/$g_{extr.}$ and DPPH 81.846% which agrees with the experimentally obtained data.

Conclusions

Given today's popular zero-waste strategy, prevention of waste accumulation and its possible utilization, the study focused on investigating and proving how UAE, as the novel, green extraction method, is very successful for isolation of bioactive compounds from by-product CBS. According to the obtained results, the liquid/solid ratio had the greatest influence on the content of theobromine, caffeine, gallic acid and catechin in the obtained extracts. Although on a lab-scale level, the paper demonstrates how by optimizing

of UAE extraction, the desired composition of CBS extracts enriched in bioactive comounds can be achieved. In addition, the UAE has proven to be an excellent replacement for the conventional extraction techniques. High-valued extracts rich in bioactive compounds, obtained from by-products such as CBS, could eventually find purpose in the other industries such as the pharmaceutical, chemical, cosmetic or food industries.

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Declaration of interest:
None.

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