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Extraction of methylxanthines by pressurized hot water extraction from cocoa shell by-product as natural source of functional ingredient

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ABSTRACT

Cocoa beans are one of the largest cultivated crops all over the world, producing large amount of by-products. For this reason, it is necessary to valorise cocoa by-products to obtain valuable source of bioactive compounds. In this paper, a pressurized hot water extraction process for recovery of theobromine and caffeine from cocoa by-product was developed and optimized. The extraction was carried out on ASE and all parameters affected by extraction efficiency and antioxidant capacity were optimized by a chemometric approach. Theobromine and caffeine were quantified by UPLC-UV (283 nm), whereas antioxidant capacity was evaluated by in vitro assays (ABTS and DPPH). By applying the PHWE under optimized conditions (ethanol 15%, temperature 90 °C, 5 cycles and static time 6 min), the extraction efficiency increased by 156% for theobromine and 160% for caffeine in comparison with the results obtained using ultrasound assisted liquid extraction. The effect of PHWE extract on cell viability of colorectal and breast cancer cell lines was also tested. The application of PHWE to selective recovery of theobromine and caffeine from cocoa shell by-products is a green, automatic, and rapid method, representing a valid alternative to conventional extraction methods to obtain ingredients for food industries.

1. Introduction

Nowadays food industry produces a large amount of waste during all production phases, and it often consists of inedible parts (Oreopoulou & Russ, 2007). Since their high contents of bioactive compounds, these food wastes should be considered as a food by-product. The huge by-product production causes an enormous economic loss and several environmental problems. In this context, there is an increasing interest in finding specific solutions in order to use food by-products as natural source of bioactive ingredients (Panak Balentić et al., 2018). Cocoa beans (*Theobroma cacao* L.) are widely used in food, pharmaceutical and cosmetic industries (Mazzutti, Rodrigues, Mezzomo, Venturi, & Ferreira, 2018). It is estimated that the world production of cocoa beans was about 4 million tons in the 2010 (FAO), and just a part of this production is exploited, whereas other parts such as cocoa pod husk, bean shell, and mucilage are usually considered wastes. Cocoa shells are about 12–20%

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of the cocoa beans and represent one of the main by-products during the pre-roasting/roasting process (Panak Balentić et al., 2018). It can be estimated that the annual production of coca shells reaches approximatively 700 thousand tons, and most of this material is usually disposed as waste (Okiyama, Navarro, & Rodrigues, 2017). Several studies have demonstrated the antioxidant properties of cocoa shell extract due to high content of phenolic compounds, such as catechins, procyanidins (Lee, Kim, Lee, & Lee, 2003; Othman, Ismail, Ghani, & Adenan, 2007), and its high content of alkaloids as caffeine (1,3,7-trimethylxanthine), theophylline (1,3-dimethylxanthine), and theobromine (3,7-dimethylxanthine) (Arlorio et al., 2005; Okiyama et al., 2018; Visioli, Bernardini, Poli, & Paoletti, 2012). Due to the interesting biological properties, cocoa shell extract has attracted the interest of scientific community and attention by food, pharmaceutical and cosmetic industries as natural and cheap source of bioactive compounds. Therefore, the valorisation of this residue may at same time reduce the environmental problems and create economic benefits. In this context, to obtain a good quality of extract and increase extraction yield, the extraction procedure used must be carefully selected. The conventional extraction methods (CSE) such as solid-liquid extraction (SLE), Soxhlet extraction (SE), and maceration extraction (ME), commonly used for the extraction of bioactive compounds from solid material, have a lot of disadvantages that render these applications quite uneconomical and highly polluting, due to excessive consumption of time, energy and toxic solvents (Ameer, Shahbaz, & Kwon, 2017). In the last decade, in order to overcome the limitations of CSE several emerging technologies, such as ultrasound assisted extraction (USAE), microwave-assisted extraction (MWE), supercritical fluid extraction (SFE), and pressurize liquid extraction (PLE), have been developed and successfully applied for the extraction of food and food by-products (Charis M Galanakis, 2013; Charis Michel Galanakis, 2015). Among these extraction techniques, pressurized liquid extraction has been successfully used to produce functional ingredients from different raw materials, since it allows the extraction of several bioactive compounds in a short time frame (Mustafa & Turner, 2011). The use of liquid solvents at high temperature enhances the solubility and the solvation of analytes, whereas the high pressure facilitates the penetration of solvents into the sample matrix, reducing extraction time and solvent consumption. In particular, when water is used as unique solvent in PLE, this technique is called pressurized hot water extraction (PHWE). At first PLE has been widely used for the extraction of pollutants (Björklund, Sporring, Wiberg, Haglund, & von Holst, 2006) and contaminants (L. Campone, Piccinelli, Aliberti, & Rastrelli, 2009; L. Campone et al., 2015) in food and environmental samples and subsequently has been also widely applied to extract natural bioactive compounds, such as polyphenol (Alonso-Salces et al., 2001; Erdogan, Ates, Durmaz, Yilmaz, & Seckin, 2011; Pagano, Piccinelli, et al., 2018; Pagano, Sánchez-Camargo et al., 2018) alkaloids (Hossain, Rawson, Aguiló-Aguayo, Brunton, & Rai, 2015; Mroczek & Mazurek, 2009; Okiyama et al., 2018) from food and food by-products. The objective of the present work was to develop a green, rapid, and inexpensive procedure for the extraction of dried cocoa bean shell, to obtain a valuable ingredient to be used in nutraceutical, cosmetics, and food industry. The extraction conditions of PHWE method have been carefully optimized by using a Box-Behnken design to improve the recovery of caffeine and theobromine, increase antioxidant capacity, minimizing the number of experiments. Under optimized extraction conditions of PHWE process, the extraction efficiency increased drastically, while the antioxidant remains roughly unchanged, compared with a conventional extraction method (ultrasound assisted extraction). Furthermore, the PHWE extract did not show cytotoxic effects on human cell cultures and displayed an interesting sensitization activity to a known anti-cancer agent targeting EGFR in cancer cell lines expressing *KRAS* oncogenes.

2. Materials and methods

2.1. Chemicals and standards

Methylxanthines (Theobromine TB, theophylline and caffeine CF) and polyphenols (Catechin Epicatechin and procyanidin B2) were purchased by Sigma-Aldrich (Milan, Italy). MS-grade solvents used for UPLC analysis, acetonitrile (MeCN), water (H_2O) and formic acid (HCOOH) were provided by Romil (Cambridge, UK); analytical-grade solvents, acetonitrile (MeCN), methanol (MeOH) and ethanol (EtOH) were supplied by Sigma-Aldrich (Milan, Italy). Water was purified by using a Milli-Q system (Millipore, Bedford, USA). The 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox), 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) ABTS, were purchased by Sigma-Aldrich (Milan, Italy).

2.2. Cocoa shell samples

Cocoa bean shell mainly belong to Trinitario variety was kindly supplied by a cocoa processor after roasting process carried out at 225 °C for about 20 min. Shell was blended using a knife mill Grindomix GM 200 (Retsh, Haan, Germany) and ground samples were sieved through a test sieve to obtain homogeneous particle size powders in three different range 150–300 μm, 300–600 μm, 600–900 μm. Preliminary experiments showed no qualitative and quantitative differences between different particles sizes, therefore the range from 300 to 600 μm was selected for all the experiments.

2.3. Ultrasound assisted extraction (USAE)

The chemical composition of cocoa bean shell was preliminarily evaluated by the exhaustive extraction carried out by ultrasound assisted solid liquid extraction (USAE). One g of sample was consecutively extracted for 15 min at room temperature for three time, with fresh solvents, (H_2O; EtOH 15%; EtOH 50% and EtOH). At the end of each extraction cycle, the extract was collected, filtered, and dried under vacuum at 40 °C in a rotary evaporator (Rotavapor R-200 Buchi Italia s.r.l, Cornaredo, Italy); the global yield was calculated as the mass ration between the total extract and sample weight (eq. (1)).

2.4. Pressurized hot water extraction (PHWE)

Pressurized hot water extractions were performed using a Dionex ASE200 (Dionex Sunnyvale, CA).

Extraction procedure was performed by using 1 g of dried sample (300 600 μm) packed into 5 mL stainless still extraction cell. The empty space of extraction cell was filled with solid-glass beads 4 mm (Sigma-Aldrich, Milan Italy), and a paper filter was placed at the bottom of the extraction cell (Whatman n°1). To select the extraction temperature range to employ into the experimental design, preliminary experiments were carried out at pressure 69 bar EtOH 7.5%, 3 cycles, static time 4 min. The extracts obtained were collected into a glass vial (60 mL) and solvent was evaporated by rotary evaporator (Rotavapor R-200 Buchi Italia s.r.l., Cornaredo, Italy) to calculate the extraction yield (eq. (1)).

2.5. Extraction yield

The extraction yield was calculated gravimetrically and expressed as percentage $Y_{(\%)}$ of mass of extract (m_{Ex}) relatively to the total mass of raw material (m_{RM}) according to Eq. (1)

$$Y_{(\%)} = m_{Ex}/m_{RM} * 100 \quad \text{Eq. (1)}$$

2.6. Experimental design

Chemometric approach was used to find the best PLE extraction

parameters using Statgraphic Centurion XVI 16.1 version (Rockville, USA.). A Box-Behnken design 2-factor interaction with 4 canter point, an error of 13 degree of freedom, for a total of 28 randomized run was used (Table 1). Four experimental factors: temperature (90–130 °C) number of cycles (1–5) modifier (EtOH 0–15%) and static time (2–6 min) were studied, whereas according to preliminary studies pressure flush volume and purge time were fixed at 69 bar 150% and 100 s respectively. Theobromine (TB) and caffeine (CF) content (g/100g DM), and ABTS antioxidant activity (TEAC μmol/mg) were used as response variables. In Table 1, it was reported the experimental matrix design, with the experimental levels of the independent variables (factors) and the results obtained for the analysed response variables. Results from the CCD were subjected to regression analysis using least squares regression methodology to obtain the parameters of the mathematical models. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of independent variable (A, B and C) contributions and their first order interaction. The effect of each factor on the response variables was analysed from the standardized Pareto chart, and response surfaces of the mathematical models were obtained. Finally, the optimized conditions extrapolated by Box-Behnken design were as follow: EtOH 15%, temperature 90 °C, 5 cycles and static time 6 min.

2.7. Quantitative (UPLC-UV) and qualitative analysis (UPLC-HRMS)

Quantitative and qualitative analysis of theobromine and caffeine of PHWE cocoa shell extract was performed on Waters ACQUITY UPLC system coupled with a Waters Xevo G2-XS QTof Mass Spectrometer and UV detector (Waters Corp., Milford, MA, USA). The extract was chromatographed on a column Kinetex Biphenyl (100 × 2.1 mm I.D., 2,6 μm) from Phenomenex (Torrance, CA, USA). Water (A) and acetonitrile (B), both acidified with 0.1% HCOOH, were used as mobile phases at flow rate of 0.4 mL min^{-1}. The gradient was programmed as follow: 0–2 min 5% B, 2–4 min 5–10% B, 4–6 min 10–15% B, held for 2 min 8–10 min 15–50% B, 10–13 min 50–98% B held for 3 min. Before injection, the initial conditions were held for 5 min as re-equilibration step. The injection volume was 5 μL, and the column was maintained at 30 °C. The UPLC system was coupled to a UV and three different wavelengths: 210, 283 and 325 nm were acquired, and external standard calibration method was used to quantify TB and CA in all samples. Stock solution of TB and CA at concentration of 1 mg mL^{-1} was properly diluted with H_2O to obtain calibration curves (six level) in the range of 1–200 μg mL^{-1}. The linearity of each calibration curve was tested by the analysis of variance (ANOVA), and linear model was found appropriate over the concentration used.

The Xevo G2-XS QTof Mass Spectrometer equipped with an ESI source, was used in positive and negative ionisation mode to acquire full-scan MS, and MS/MS, and the spectra were recorded in the range of m/z 100–1200. The source parameters were as follows: electrospray capillary voltage 3.0 kV, source temperature 150 °C and desolvation temperature 500 °C. The cone and desolvation gas flows were 10 and 1000 L/h, respectively. A scan time of 0.1 s was employed. The cone voltage was set to 60 V, and ramping collision energies for MS/MS analysis was set up at 20 V to produce abundant product ions before detection at the TOF. The mass spectrometer was calibrated with 0.5 M sodium formate, and leucine-enkephalin (100 pg/μL) was used as LockMass at m/z 557.2771 in positive and 554.2615 in negative ion mode and 2 kV ionisation voltage) which was infused simultaneously with the flow of column at 5 μL/min and acquired for 1 s each 15 s. The full mass base peak chromatograms (BPI) were acquired, and the molecular ion mass [M+H]+ and [M-H]- were obtained, from which the elementary composition was calculated (mass error <5 ppm). From the peak identification of the molecular ion mass whereas, from MS/MS spectra acquired at 20V collision energy the fragmentation pattern was obtained, and the information was used for the identification. TB and CF were identified comparing their retention time (t_R) accurate mass (positive) calculated molecular formula error ppm (between detected mass and calculated accurate mass), MS/MS fragmentation with pure standard. MassLynx software (version 4.2) was used for mass spectrometer control, data acquisition and data analysis.

Table 1
Experimental conditions of the response surface design (Box-Behnken design 2-factor interactions) and experimental values of the response variables (theobromine, caffeine and AOC).

Run	Independent variables				Response Variables		
	Static (min)	T (°C)	C (n°)	Solvent[a] (%)	TB (g/100g DM)	CF (g/100g DM)	ABTS (μmol/mg)
DOE1	4	110	3	7.5	1.82	0.42	1.45
DOE2	4	110	3	7.5	1.84	0.44	1.46
DOE3	4	110	5	15	1.91	0.45	1.52
DOE4	4	90	1	7.5	1.41	0.32	1.22
DOE5	4	130	1	7.5	1.68	0.38	1.49
DOE6	4	90	5	7.5	1.78	0.41	1.31
DOE7	4	130	5	7.5	1.90	0.45	1.53
DOE8	2	90	3	7.5	1.76	0.41	1.28
DOE9	2	130	3	7.5	1.86	0.43	1.53
DOE10	2	110	5	7.5	2.03	0.47	1.48
DOE11	4	110	1	15	1.65	0.37	1.35
DOE12	2	110	3	0	1.86	0.43	1.29
DOE13	2	110	3	15	1.85	0.42	1.42
DOE14	6	130	3	7.5	1.85	0.43	1.44
DOE15	6	110	5	7.5	1.97	0.47	1.51
DOE16	4	130	3	15	1.77	0.41	1.44
DOE17	6	90	3	7.5	1.86	0.43	1.34
DOE18	4	90	3	15	1.95	0.45	1.48
DOE19	6	110	3	15	1.94	0.46	1.50
DOE20	6	110	3	0	1.96	0.46	1.35
DOE21	4	110	3	7.5	1.81	0.42	1.40
DOE22	4	110	5	0	1.90	0.44	1.28
DOE23	2	110	1	7.5	1.61	0.35	1.23
DOE24	4	110	3	7.5	1.75	0.41	1.35
DOE25	4	90	3	0	1.81	0.41	1.19
DOE26	6	110	1	7.5	1.58	0.35	1.32
DOE27	4	130	3	0	1.75	0.40	1.29
DOE28	4	110	1	0	1.59	0.33	1.05

[a] Refers to % ethanol in water.

2.8. Evaluation of antioxidant activity (ABTS)

ABTS assay was used to evaluate the antioxidant activity of all sample extracts. The experimental conditions were reported by Campone et al. (Luca Campone et al., 2018). Briefly, 5 μL of PBS (control), Trolox (0.25–1 mg mL^{-1}) and extracts 1 mg mL^{-1} were mixed with 500 μL of ABTS standard solution at a concentration of 1 mM 300 μL of each mixture were transferred into a 96 well plate and were incubated, protected from light, and after 60 min the absorbance was read at 734 nm using a Multiskan Go spectrophotometer (Thermo Fischer Scientific). Results of ABTS assay were expressed as Trolox equivalent TEAC μmol/mg, and they were employed to quantify the antioxidant activity of the tested solution expressed as standard deviation (SD) of three measurements.

2.9. Cell culture and viability assay

Human breast adenocarcinoma cell line MDA-MB-231, and human colorectal adenocarcinoma cell lines Caco-2 and SW48 was routinely grown at 37 °C in a humidified atmosphere of 5% CO2 in RPMI 1640 (Sigma R0883) supplemented with 10% Fetal Bovin Serum (FBS), 2 mM glutamine, 100 units/ml penicillin and 100 mg ml^{-1} streptomycin. For MDA-MB-231, RMPI medium was also supplemented with 4 mM Sodium Pyruvate. SW48 (*KRAS WT/WT*) cell line and the isogenic SW48 expressing heterozygous KRasG13D (*KRAS WT/G13D*) or KRasG12V (*KRAS WT/G12V*) were obtained from Horizon Discovery Ltd. MDA-MB-231 and Caco-2 were obtained from the American Type Culture Collection. Cells were passaged using trypsin–EDTA.

For measuring cell viability, MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay was performed. 4800 cells per well were plated in a 96-well plate and were allowed to attach for 24 h in DMEM w/o phenol red and supplemented with 10% FBS, 10 mM glucose and 2 mM glutamine. Cells were then treated for 72h with different concentrations of PHWE extract and cetuximab (Erbitux®, Merck Serono). After the treatment, culture medium was removed and then MTT solution was added (1:11). After a 4h incubation, 100 μL of a solution of Isopropanol 0.1 N HCl and 10% Triton X100 was added to solubilize the formazan crystals formed. The plate was then read by spectrophotometer Victor X3 (PerkinElmer) at 570 nm wavelength.

2.10. Statistical analysis

All data were performed in triplicate and results were presented as average ± standard deviation. Analysis of variance (ANOVA) was used to compare the means while Tukey's test was used to assess the statistically significant differences among treatments. For MTT assay, results were presented as average ± standard deviation and data were obtained from two or three independent experiments, each performed with three technical replicates. ANOVA test was used to compare the statistically significant differences among treatments, using JMP 14 software. Welch's correction was applied in the case of data violating the assumption of homogeneity of variances, by Levene's test. A p-value of ≤0.05 was considered significant.

3. Results and discussion

3.1. Analysis of USAE extract

The chemical composition of cocoa shell is quite variable, as is that of cocoa beans, and will depend, among other factors, on its origin and the processing to which it has been subjected (Okiyama et al., 2017). For this reason, to preliminary investigate the chemical composition of cocoa shell by-products, an ultrasound assisted solid liquid extraction was performed and analysed by UPLC-UV-HRMS. The UV chromatograms (280 and 330 nm) showed the presence of only two main compounds eluted at 2.7 and 5.9 min (Fig. 1S A). However, to obtain a more detailed analysis useful to also identify the minor compounds in cocoa shell extract, the UHPLC-HRMS untarget analysis was performed in both positive and negative ionisation mode. The metabolites identification was carried out by using, HRMS data (accurate mass, isotopic distribution, and MS/MS characteristic fragmentation pathway) and using literature databases. Finally, the identified compounds were confirmed with standards. The UHPLC-MS/MS analysis in positive mode allowed the identification of 2 methylxanthines alkaloid, caffeine, and theobromine, corresponding at the two peaks shown in the UV chromatogram at 280 nm. The UHPLC-HRMS/MS analysis in negative ion mode allowed the identification of 3 flavanols, catechin epicatechin and procyanidin B1 which were not detectable in UV, perhaps due to poor concentration. The results of the qualitative analysis of cocoa shell were in accordance with the literature data on cocoa by-products (Okiyama et al., 2018). Once the main metabolites contained in the USA extract were identified, we carried out a quantitative analysis of the flavanols and alkaloids through the UPLC-UV-MS/MS analysis. The total extraction yield of USAE was 21.86 ± 1.3%, in particular theobromine and caffeine were the most abundant compounds in the extract at concentration of 35.9 ± 0.21 and 7.03 ± 0.15 μg/mg Ext (1.32 ± 0.01. g/100g DM and 0.30 ± 0.02 g/100g DM) respectively while catechin, epicatechin and procyanidin B2 were found at concertation of 0.34 ± 0.05; 1.77 ± 0.21 and 0.35 ± 0.05 μg/mg Ext respectively. In general methylxanthine content were comparable to the data reported in literature (Barbosa-Pereira, Guglielmetti, & Zeppa, 2018; Carrillo, Londoño-Londoño, & Gil, 2014), while they are slightly higher than the results obtained by Okiyama et al. (Okiyama et al., 2018). These differences in the alkaloids contents can be the consequence of a different migration of methylxanthines from bean to the shell during roasting process (Timbie, Sechrist, & Keeney, 1978) or due the fact that theobromine and caffeine levels depend on the origin of cocoa and cultivars (Arlorio et al., 2005). Regarding the concentration of flavonols the quantitative results were much lower comparing with the data reported in the literature (Nazaruddin, Seng, Hassan, & Said, 2006; Okiyama et al., 2018), such variations in the levels of catechin, epicatechin and procyanidin B2 present in cocoa shell, can be a consequence of several factors such as, climatic variations, genetic variability of the cultivars, harvest time, management, conditions which the cacao trees were grown (Oliveira, Mamede, Góes-Neto, & Koblitz, 2011). In addition, roasting processes could dramatically influence the polyphenol and flavanols content resulting in a reduction of portion of these compounds (Hurst et al., 2011)

3.2. Optimization of PHWE

3.2.1. Preliminary experiment for selection of PHWE temperature

After the qualitative and quantitative study of USA extract, which showed a low amount of phenolic substances, probably due to their degradation after roasting process our attention was focused mainly on methylxanthine being the majority compounds. Therefore the optimization of extraction conditions of PHWE process was carried out to primarily to improve extraction efficiency of caffeine and theobromine, but also trying to improve phenolic content. As commonly reported in literature, one of the most important parameters in PHWE process is the temperature (Pagano, Piccinelli, et al., 2018). For this reason, to select the temperature range to use in the experimental design, a preliminary experiment was performed increasing the temperature from 25 to 170 °C, and monitoring extraction yield (%), and TB and CF content. The other extraction parameters of PHWE system were set at central conditions of the experimental design (pressure 69 bar, cycles 3, EtOH 7,5% static time 4 min). As Fig. 1A shows, the extraction yield percentage increases within the entire temperature range with a maximum of 49% ± 0.3 at 170 °C, while the amount of TB and CF increases up to 110 °C and subsequently decreases till 170 °C, as shown in Fig. 1B and C respectively. This behaviour demonstrates that high values of extraction temperature correspond to an enhance of interfering compounds, without any benefit on extraction efficiency of target compounds. Based on these results, the temperature range between 90 and 130 °C was used in the next optimization performed by an experimental design.

3.2.2. Response surface design of PHWE process

After the preliminary experiments performed to select the extraction temperature range, the optimization of the main extraction parameters that influence PHWE process was carried out by experimental design. As well known, pressurized liquid extraction is a technique affected by several parameters (temperature, static time, solvent composition, pressure, and cycles etc.) simultaneously (Pagano, Campone, Celano, Piccinelli, & Rastrelli, 2021), therefore to get the best extraction efficiency, it is extremely important to select the right parameters affecting the extraction process. The optimization of multivariate process such as PHWE can be carried out by one variable at time (OVAT) approach or using a chemometric analysis by an experimental design (DOE). The optimization performed by using OVAT approach requires many experiments, and it does not guarantee to find out the real optimum conditions, unless the variables are totally independent from each other. On the contrary, the use of an experimental design allows to evaluate the interaction among the variables, minimizing the experimental error and the number of experiments (Luca Campone et al., 2018). In this study, the optimization was performed considering four of the most important PHWE parameters (temperature, cycles, static time, and solvent composition) with three response variables: the two methylxanthines content and the antioxidant activity (ABTS). Regarding the extraction solvent, its selection was carried out considering the analytes affinity and the environmental impact, thus water containing small amount of organic solvent (maximum EtOH 15%) was selected to improve

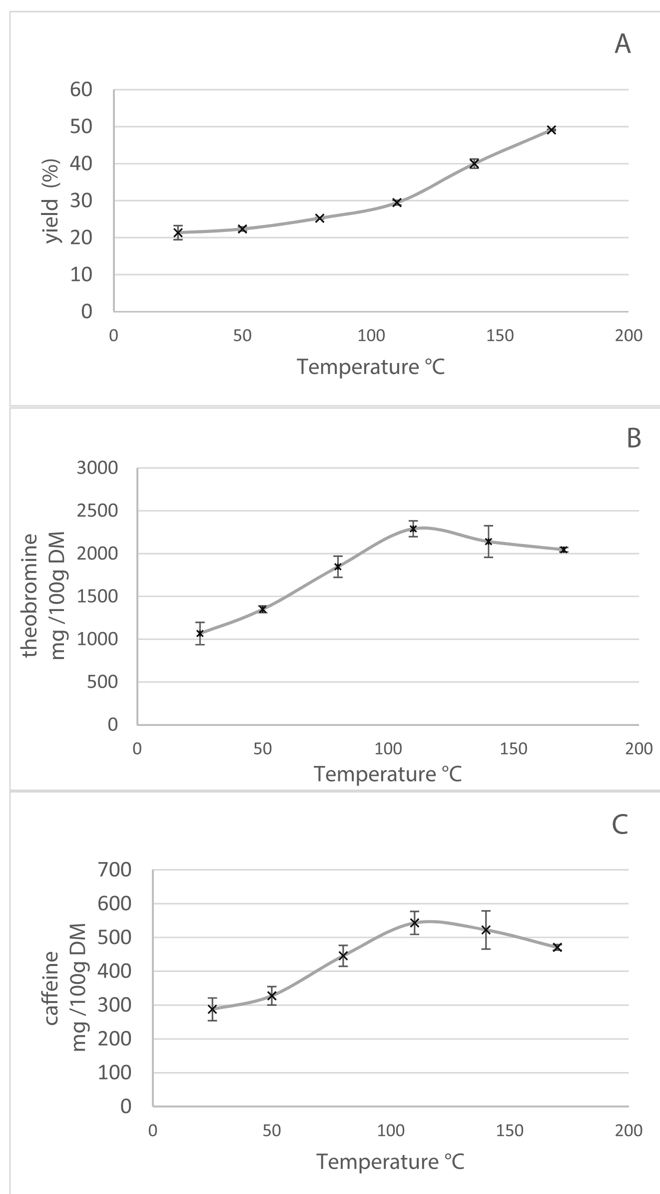

Fig. 1. Preliminary experiments of extraction yield (A), theobromine (B) and caffeine (C) versus temperature at the central point of the response surface design experimental conditions: pressure 69 bar; static time 4 min; cycles 3; and solvent EtOH 7,5%.

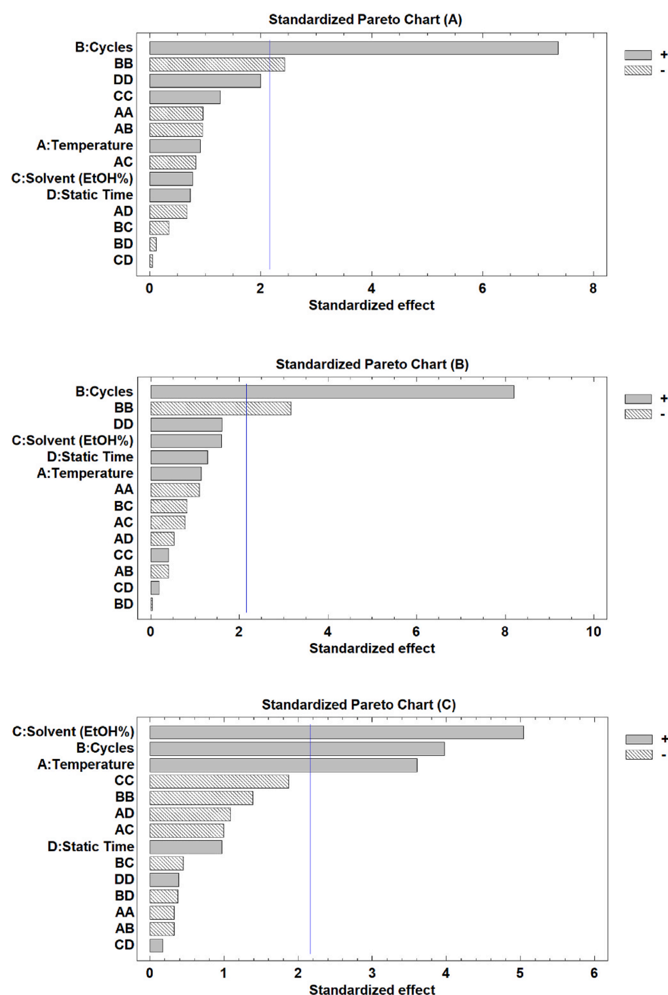

Fig. 2. Standardized Pareto Chart of Theobromine (A) Caffeine (B) and AOC-ABTS (C) shows the estimated effects and interactions of each term in the Box-Behnken design model in decreasing order of significance. Bars beyond the vertical line are statistically significant with confidence level of 95%.

solubility of target analytes and at the same time keeping the extraction a green process (Pagano, Piccinelli, et al., 2018). Based on these considerations, a Box-Behnken design was selected to investigate the effect of extraction temperature, static time, numbers of cycles and EtOH % in water, on methylxanthines contents and antioxidant activity (ABTS) of PHWE extracts. Table 1 shows the experimental conditions for each run, and the experimental values of the responses at different experimental conditions. The statistical significance of the response variables studied can be observed from the standardized pareto chart for each experimental factor (Fig. 2A–C). The significance of the effects at 95% confidence level was highlighted by the vertical line in the chart whereas positive (grey) and negative (dashed) effect in the response variables were indicated by different bars colour. Cycles resulted to be the most important parameters in the model, as they make a positive contribution to the three response variables TB, CF, and AOC (Fig. 2 A, B and C). Besides, the quadratic effect of the cycles ($p < 0.05$) showed to be the second important term in two of the selected variables, making a negative contribution to TB and CF content (Fig. 2 A and B). At last, the other two experimental factors, temperature and static time, also showed a positive influence ($p < 0.05$) on the model of AOC. The influence of cycles and temperature were clearly highlighted for all the response variables in the response surface plots (Fig. 3A–C). They were also used for predicting and optimizing the responses. As it can be seen in Fig. 3 A and B, the two response variables, TB and CF show the same behaviour: the extraction efficiency improves as the number of cycles of the PHWE process increases; whereas an increase of the AOC (TEAC) is achieved by increasing both temperature and cycles. This behaviour was also observed in the desirability plot (Fig. 4), where cycles are the parameter that has the greater influence on the desired effect, indeed increasing the numbers of cycles from 1 to 5 the desirability increases. The temperature has instead a slight influence on the desirability. Finally, considering the three response variables (caffeine, theobromine and AOC), the chemometric analysis suggested the flowing parameters in the optimized conditions: Temperature 90 °C, Cycles 5, EtOH 15% and Static Time 6 min with an optimized desirability = 0.984166 (98.42%). Previous studies have evaluated the capability of PLE as green extraction techniques, Okiyama et al. (Okiyama et al., 2018) studied the kinetics of PLE at different temperature on the extraction of flavanols and alkaloids from cocoa bean shell using as solvent pure ethanol. Their results provided a comparable amount of caffeine and theobromine but a higher content of flavonoids. In the study conducted by Jokić et al.

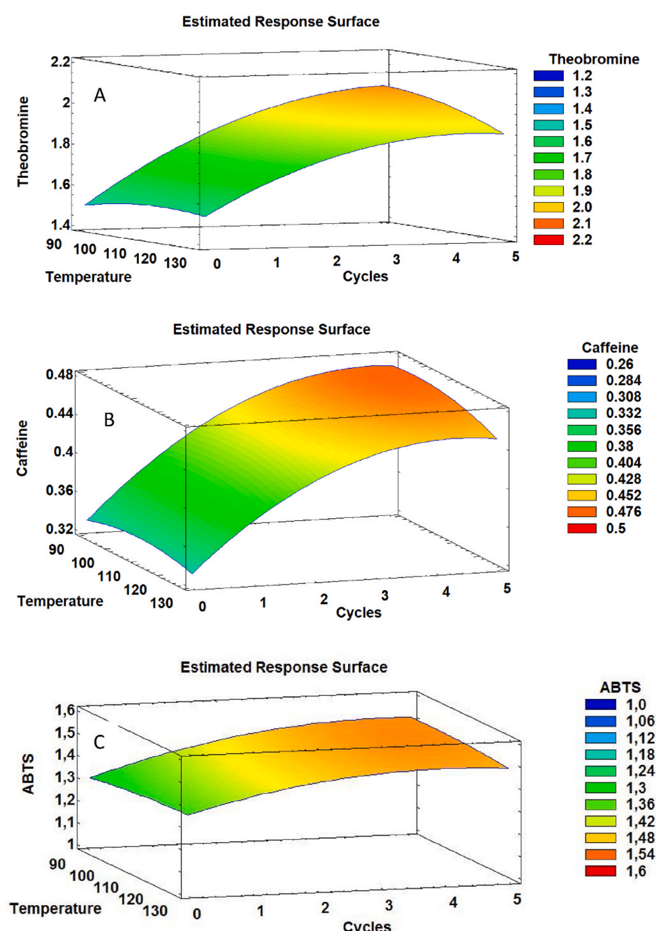

Fig. 3. Response surface plot representing the predicted value of each response variables (A) theobromine (g TB/100g DM), (B) caffeine (g CF/100g DM) and (C) AOC (μmol/mg) over the space of temperature (90–130 °C) and cycles (1–5). The other two factors were held constant at their higher values (optimized condition).

(Jokić, Gagić, Knez, Šubarić, & Škerget, 2018) regarding the optimization of subcritical water extraction of phenolic compounds and methylxanthine from coca shell. The authors demonstrated that the increase of the temperature in a range from 120 °C to 220 C° and reaction times from 15 min to 75 min resulted in a high content of target compounds in the same concertation range of those obtained in our study but they also highlighted, that high temperatures lead to the extraction of unwanted compounds.

3.3. Quantitative analysis and antioxidant activity of PHWE extract

From a quantitative point of view optimized experimental conditions of response surface design provided an increase in total extraction yield of 10%. To evaluate the improvement of extraction efficiency of target compounds (TB and CF) obtained under optimized conditions, a recovery study was carried out by an UPLC-UV analysis. The recoveries were calculated by comparing the amount of methylxanthine compounds in PHWE extract with those obtained by the ultrasound extraction technique. The results were TB 156.4% ± 2.8 and CF 160.8% ± 3.5. This demonstrates the possibility of developing a method capable of providing an exhaustive extraction efficiency, even greater than conventional (USAE) extraction techniques. Finally, the quantitative analyses of methylxanthines in the PHWE extract under optimized condition were carried out by UPLC-UV using the external standard method. Theobromine and caffeine calibration curves in the concentration range of 0.1–10 μg mL^{-1} were used to quantify their content into PHWE extract. The external standard calibration curves for theobromine and caffeine provided food linearity within the investigated concertation range with correlation coefficients (R2) of 0.9993 and 0.9992 respectively. The quantitative analysis of PHWE extracts revealed TB and CF contents were 2.06 ± 0.06 and 0.48 ± 0.02 g/100g DM respectively (56.2 ± 0.16 and 11.3 ± 0.32 μg/mg Ext). In general, quantitative results concerning caffeine and theobromine were in accordance with the range of data reported in the literature for cocoa shell. In particular theobromine content was similar than that observed by Okiyama 2018 et all for the cocoa shell using pressurized liquid extraction (Okiyama et al., 2018), but it was much higher than that reported by Ortega et al. (Ortega et al., 2010) and Soares et al. (Soares, Okiyama, & da Costa Rodrigues, 2020). Regarding the caffeine content, it was so much higher than that observed by Soares et al. and Ortega et al. for the cocoa shell (Ortega et al., 2010; Soares et al., 2020), and it was slightly higher than that reported by Okiyama et al. and Ortega et al. (Okiyama et al., 2018; Soares et al., 2020). In addition, the antioxidant capacity (AOC) of PHWE extract under optimized conditions was evaluated. The AOC in the PHWE was 0.266 ± 0.12 (μmol TE/mg Ext), this result confirms the low radical scavenger activity of PHWE extract even after the optimization process. This data depends to the low content of catechin epicatechin and procyanidin B2 that remain comparable to the result obtained in preliminary experiment by using ultrasound assisted extraction. This result was close to those report by Soares et al. (Soares et al., 2020) but lower to the result reported by Hernanz et al., (Rebollo-Hernanz et al., 2021). This variability can be explained throughout different production process steps, such as fermentation, roasting, and alkalizing but also to the origin and variety of cacao. The improvement

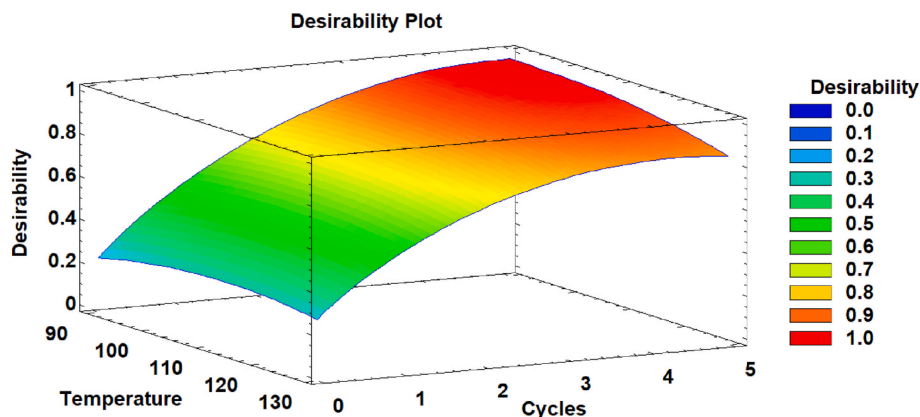

Fig. 4. Response surface plot displayed the overall desirability versus the space of two factors temperature (90–130 °C) and cycles (1–5) at same time, the other two factors, the other two factors were held constant at their higher values (optimized condition).

of extraction efficiency with a good contents of methylxanthine compounds suggest the potential use as additive into energy drinks and other supplements.

3.4. Cytotoxicity evaluation of PHWE extract, alone or in combination with cetuximab, on human cancer cell lines

To evaluate the presence of active ingredients capable of altering the viability of human cells in the PHWE extract, we treated with increasing doses of extract (0.016–250 ng/μl) colorectal cancer cell lines isogenic for *KRAS* gene, one of the proto-oncogenes more frequently mutated in human cancers, including the deadliest ones, and a breast cancer cell line. In detail, we used SW48 cells expressing wild type KRas (SW48 *KRAS* wt/wt) or the oncogenic mutant $KRas^{G13D}$ (SW48 *KRAS* wt/G13D), and the triple negative breast cancer cells expressing $KRas^{G13D}$, MDA-MB-231. Once verified that the extract alone had no cytotoxic effect, even at high doses (Fig. 5 A), we tried to test it in co-treatment with cetuximab, a known anti-cancer agent that is used against some cancers, including the colorectal one (Galizia et al., 2007). This drug is a monoclonal antibody directed against the EGF receptor, which acts by attenuating the mitogenic and pro-survival signaling pathway mediated by the Ras/Raf/MAPK axis (Molina & Adjei, 2006). Tumours harbouring specific oncogenic mutations in signaling elements downstream of the EGF receptor, such as KRas and BRAF, show intrinsic resistance to cetuximab and do not benefit from treatment (Bray et al., 2019). Unfortunately, these mutations accounts for ~40% of colorectal cancer cases. Here, by MTT assay, the viability of cells treated with the higher dose of PHWE extract (250 ng/μl) in combination with increasing doses of cetuximab (0.5–50 nM) was tested. In detail, we treated a cell lines group partially responsive to cetuximab, such as Caco-2 cells and SW48 cells expressing wild type KRas or $KRas^{G13D}$ (McFall & Stites, 2020; Tisi et al., 2021), and a cetuximab-insensitive cell line group, such as SW48 cells expressing $KRas^{G12V}$ (McFall & Stites, 2020; Tisi et al., 2021) and triple negative MDA-MB-231 cells, which express the oncoprotein Raf^{G464V} in addition to $KRas^{G13D}$. From the analysis, it emerged that the PHWE extract significantly sensitizes to cetuximab the cells expressing $KRas^{G13D}$, including partially responsive (SW48 $KRas^{G13D}$) and resistant (MDA-MB-231) cells (Fig. 5 B). In the cetuximab-insensitive SW48 cells, expressing the $KRas^{G12V}$, the combined treatment resulted in a reduction in cell viability if compared to cetuximab alone, albeit below the threshold of statistical significance, while no effect was detected in the Ras wt expressing cells, i.e. Caco2 and SW48 w.t. cells (Fig. 5 B). The observed effect could be due to the peculiar allele-specific signaling network generated by $KRas^{G13D}$ mutant in a context with over-expressed EGFR, as previously described (McFall et al., 2019; Tisi et al., 2021)

4. Conclusions

The experimental results showed that pressurized hot water extraction is an efficient and sustainable approach to obtain a valuable source of theobromine and caffeine from cocoa shell by products. Static temperature and cycle number play a crucial role in the extraction of methylxanthines. Furthermore, temperature over 90 °C increase extraction yield without increasing the recovery of target compounds. These results indicate that cocoa shell by products can be considered an excellent source of value-added products, such as nutraceutical or functional foods with good perspective for an industrial scale up. Furthermore, the PHWE extract showed an interesting mechanism of sensitization of human cancer cells to the anti-cancer agent cetuximab, similarly to other natural products, such as lauric acid (Weng, Leung,

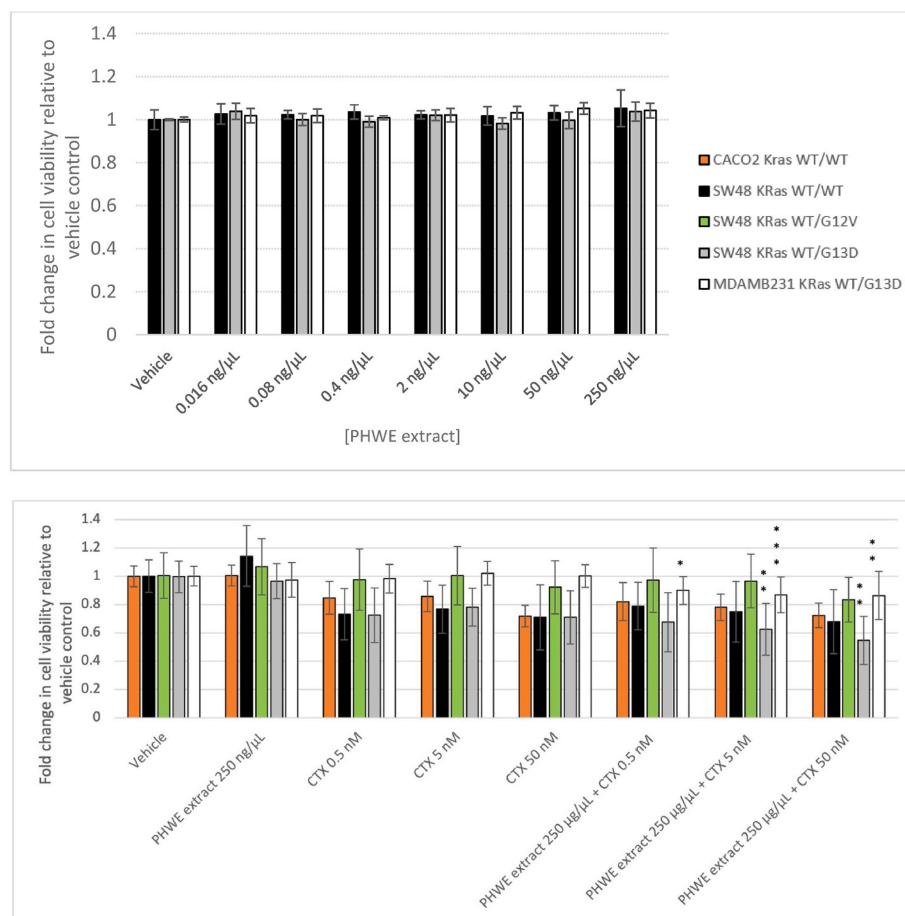

Fig. 5. Effect of 72h treatment with PHWE extract, alone (A) or in combination with cetuximab (CTX) (B), on cell viability of human colorectal cancer SW48 isogenic cell lines, colorectal cancer Caco2 cell line and breast cancer MDAMB231 cell line, as measured by MTT assay. Triple, double and single asterisks above histograms refer to the comparison to the same cell lines treated with cetuximab alone and indicate a statistical significance of 99,9, 99 and 95% respectively. Welch's correction was applied to the comparison between treatment with 50 nM CTX and the cotreatment with 50 nM CTX + PHWE in MDAMB231 cells since Levene's test did not confirm the homogeneity of variances.

Pang, & Hsu, 2016) and honokiol (Pearson et al., 2018). The PHW extract action mechanism, although limited to cancer cells expressing $KRas^{G13D}$ oncoprotein, in line with the KRas mutant allele–specific responses to targeted therapy (McFall et al., 2019), needs to be elucidated in the perspective of the development of novel sustainable treatments for cancer's patients refractory to conventional EGFR-targeted chemotherapy, in the context of precision medicine.

CRediT authorship contribution statement

Stefania Pagliari: Formal analysis, Investigation, Data curation. **Rita Celano:** Software, Review. **Luca Rastrelli:** Supervision, Writing. **Elena Sacco:** Writing, Data curation. **Federico Arlati:** Investigation, Formal analysis. **Massimo Labra:** Writing, Review, Funding acquisition. **Luca Campone:** Conceptualization, Writing – review & editing, Supervision.

Declaration of competing interest

All authors declare any conflicts of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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