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Ohmic heating-based extraction of biocompounds from cocoa bean shell

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ABSTRACT

Cocoa bean shell (CBS), a by-product of the chocolate industry, was employed as substrate for the sustainable recovery of bioactive compounds using ohmic heating (OH). Total phenolic content and antioxidant activity of the treated CBS were optimized by experimental design. Maximum extraction of antioxidant phenolic compounds (23 mg GAE/g CBS) was obtained at 67 °C, 50 min and 44% ethanol (v/v). The antioxidant activity of the extracts obtained under of the central point conditions was 284.5 μM Fe^{2+}/g extract (FRAP) and 36.4 μM TE/g extract (DPPH). The use of OH increased the extraction of bioactive phenolic compounds when compared to the conventional process (CH) (approximately 40%). An increased on the chemical antioxidant activity was also observed, ranging from 4 to 20%. The metabolic activity of the extracts obtained by the two methods (OH and CH) was evaluated in non-tumoral (HEK293T and L929) and tumoral cell lines (Caco-2, HT-29, and HeLa). The CBS extracts presented low toxicity in non-tumoral cells and ROS preventive effects. These characteristics make them ideal to be used in food processing and formulation, as well as nutraceutical products due to their antioxidant protection. The use of OH results in an extract with higher phenolic content and higher antioxidant activity and low environmental impact.

1. Introduction

Annually, the agri-food industry generates large amounts of residues derived from the production and processing activities, which entails notable environmental and economic issues (Nogueira et al., 2022; Sánchez, Laca, Laca, & Díaz, 2022). In line with the Sustainable Development Goals (Directive 2008/98/CE), minimizing the generation of residues and by-products by transforming them into valuable resources is an important action for the Circular Economy Action Plan that the EU should embrace in next years (Sánchez, Laca, Laca, & Díaz, 2023; Stahel, 2016, pp. 6–9).

Nearly 700 thousand tons of waste are generated worldwide each year by the cocoa industry, fact that poses a significant environmental concern (Acosta et al., 2018; Rojo-Poveda et al., 2020). Cocoa bean shell (CBS), the external part that covers the cocoa bean, is the main by-product generated during the cocoa roasting process. CBS is an important source of compounds of interest, including proteins, carbohydrates, and phenolic compounds, which accounts for approximately 10% of CBS dry weight (Mellinas, Jiménez, & Garrigós, 2020). The main phenolic compounds found in cocoa bean shells are flavanols such as catechin and epicatechin. These phenols are associated to the health benefits related with the consumption of cocoa products, i.e., anti-inflammatory, antioxidant, and free radical scavenging properties. Additionally, these compounds provide astringency and bitterness to cocoa (Barbosa-Pereira, Guglielmetti, & Zeppa, 2018; Hernández-Hernández et al., 2019; Okiyama, Navarro, & Rodrigues, 2017).

CBS polyphenols have great potential in industrial applications as high nutritional value additives and supplements. Thus, the development of new sustainable strategies for obtaining high-added value products from this by-product could be an interesting alternative of valorisation (Barbosa-Pereira et al., 2018). Conventional extraction methods employed to recover phenolic compounds from plant-based materials are time-consuming, require high amounts of solvents and energy consumptions. The conventional process can lead to the denaturation and oxidation of the polyphenols, leading to low extraction yields (Ferreira-Santos, Genisheva, Pereira, Teixeira, & Rocha, 2019;

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Jesus et al., 2020). Therefore, it is necessary to investigate new extraction methods capable of recovering high concentrations of bioactive compounds, while using environmentally friendly conditions. Recently, green technologies including high voltage electrical discharges (HVED), pulsed electric fields (PEF), microwave-assisted extraction (MAE), ultrasonic-assisted extraction (UAE), supercritical fluid extraction (SFE) and ohmic heating (OH) have been used to extract compounds of interest from plant-based matrices (Barišić et al., 2020; Pereira et al., 2016; Plazzotta & Manzocco, 2018; Rocha et al., 2018; Valadez-Carmona, Ortiz-Moreno, Ceballos-Reyes, Mendiola, & Ibáñez, 2018). Among the electrotechnologies, OH has been described as an attractive approach for the eco-extraction of bioactive compounds from different food matrices such as pomegranate peel, pineapple or lemon peel (Sharifi, Hamidi-Esfahani, Ahmadi Gavlighi, & Saberian, 2022; Gavahian & Chu, 2022; Çilingir, Goksu, & Sabanci, 2021). OH is an emerging technology in which electrical energy is turned into heat and whose effectiveness resides in a fast and uniform heating. In addition, this technique has high energetic efficiency, thus entailing fewer running costs (Coelho, Pereira, Rodrigues, Teixeira, & Pintado, 2019; Ferreira-Santos et al., 2019; Markhali, Teixeira, & Rocha, 2022). Furthermore, OH can favour the heat and electro-permeation of the cell membranes, an essential process for the extraction of bioactive molecules.

The aim of this work was to optimize the phenolic compound extraction from CBS using OH, in comparison with the extraction with conventional heating (CH). An experimental design (2^3) considering temperature, time, and ethanol concentration (using ethanol/water mixtures as solvent) was carried out in order to determine the best extraction conditions, taking into account the antioxidant phenolic compounds' recovery. To the best of our knowledge, this is the first research on OH-assisted extraction employed to obtain bioactive compounds from CBS.

2. Material and methods

2.1. Raw material

CBS was supplied by a local chocolate factory sited in Asturias (Spain). The material was milled in a professional blender (Braun 4041) at room temperature (20 °C).

2.2. Extraction of phenolic compounds from CBS

All reagents were supplied by Sigma Aldrich (St. Louis, MO, USA), and ultra-pure water was used throughout the experiments.

2.2.1. Ohmic heating extraction (OH)

Extractions were carried out in a cylindrical glass reactor of 30 cm total length with double-walled water-jacketed (100 mm height and 3 mm of internal diameter) and two inox electrodes isolated with Teflon caps. The distance between electrodes was kept constant (3.4 cm) in all assays tested. The power source worked with a sinusoidal wave at 25 kHz (Agilent 33220A; Penang, Malasya), which allowed to modify the voltage during the OH treatment (2–15 V/cm). The temperature was registered by a type-K thermocouple (temperature precision of ±1 °C; Omega Engineering, Inc. Stamford, CT, USA), placed in the centre of the extractor's system. The thermocouple was connected to a data logger (USB-9161, National Instruments Corporaton, Austin, TX, USA) and a commercial software (Lab view 7 Express software; National Instruments, NI Data logger) was employed to obtain the data. To measure electrical frequency, intensity, and voltage during the process, a portable oscilloscope (ScopeMeter 125/S, Fluke, WA, USA) was used.

For extractions, 1 g of CBS powder was mixed with 20 mL of solvent in a reactor. The extractions were performed at different temperatures, times, and concentrations of ethanol according to the experimental design (see Table 1). After the treatment, the hydroethanolic extracts were filtered through a Whatman filter paper (20 μm) and the extracts were stored at 4 °C until analytical experiments. NaCl was employed to adjust the electrical conductivity of the extraction medium (~2 mS/cm) before the process.

The pH and electrical conductivity were measured at room temperature (20 °C) using a pH meter (HANNA Instruments Inc.) and a conductivity/TSD/Salinity meter (HANNA Instruments Inc., USA), respectively.

Table 1
Experimental runs using coded levels of time (x_1, min), temperature (x_2, °C) and ethanol (x_3, %) according to the 2^3 full factorial central composite design and extraction results of phenolic compounds and antioxidant activity (FRAP and DPPH) from CBS obtained under those conditions.

Independent variables	Symbol	Range and levels				
		−1.78	−1	0	1	1.78
Extraction time (min)	x_1	4.22	20	40	60	75.77
Extraction temperature (°C)	x_2	10.27	30	55	80	99.72
Concentration of ethanol (% v/v)	x_3	0.28	20	45	70	89.72

Runs	Coded variables levels			TPC (mg GAE/g CBS)	FRAP		DPPH		Extraction yield (%)
	x_1 (min)	x_2 (°C)	x_3 (EtOH%)		μM Fe^{2+}/g CBS	μM Fe^{2+}/g extract	μM TE/g CBS	μM TE/g extract	
1	40	55	0.28	6.3	27.8	102.5	3.65	13.4	27.2 ± 0.4
2	20	30	20	16.9	27.2	100.4	5.54	20.4	27.1 ± 0.5
3	60	30	20	11.9	32.2	113.9	4.48	15.8	28.3 ± 0.5
4	20	80	20	17.7	60.8	193.8	7.03	22.4	31.4 ± 0.4
5	60	80	20	22.9	48.2	144.9	8.47	25.4	33.3 ± 0.2
6	4.22	55	45	8.2	18.7	84.2	3.72	16.8	22.2 ± 0.8
7	40	99.72	45	9.6	29.8	90.0	3.13	9.4	22.5 ± 0.3
8	75.77	55	45	13.8	59.5	186.6	8.05	25.2	29.2 ± 0.8
9	40	10.27	45	13.6	31.4	98.8	4.40	13.8	24.6 ± 0.6
10	20	30	70	9.5	18.7	60.7	2.95	9.6	21.7 ± 0.1
11	60	30	70	13.5	30.5	135.5	1.46	6.5	26.8 ± 0.4
12	20	80	70	14.3	43.3	148.3	6.32	21.6	26.2 ± 0.2
13	60	80	70	20.2	49.8	202.8	7.42	30.2	36.7 ± 0.7
14	40	55	89.72	7.1	15.7	81.0	2.42	11.2	22.4 ± 0.8
15	40	55	45	21.1	58.7	219.0	8.56	31.9	33.2 ± 0.0
16	40	55	45	21.3	60.3	230.3	8.70	33.2	31.9 ± 0.1
17	40	55	45	20.5	57.7	157.1	7.77	21.2	31.8 ± 0.4
18	40	55	45	21.4	63.7	284.5	8.15	36.4	30.8 ± 0.2

2.2.2. Conventional heating extraction (CH)

To evaluate the influence of electric fields by OH, a conventional thermal extraction (0 V/cm) was performed at the optimal conditions obtained from the experimental design. The CH extraction was carried out under the same system components employed in the OH treatment. Temperature was controlled with a thermostatic circulator water system (F25-ED, Julabo, Seelbach, Germany). Extracts were filtered through a Whatman filter paper (20 μm) and stored until analysis.

2.3. Analytical methods

2.3.1. Total phenolic content

The concentration of TPC in the samples was determined using the Folin-Ciocalteu's method, adapted to 96-well microplate, as described in Jesus et al. (2020). TPC was determined using gallic acid as standard (R^2 = 0.998) and results were expressed as mg gallic acid equivalents (GAE) per gram of dry CBS (mg GAE/g CBS).

2.3.2. Total flavonoid content

The total flavonoid content (TFC) of samples was determined using the aluminium chloride method as previously described in Ferreira-Santos et al. (2020). TFC was calculated with a calibration curve of catechin (R^2 = 0.997), and results were expressed as mg catechin equivalent (CE) per gram of dry CBS (mg CE/g CBS).

2.3.3. Identification and quantification of individual phenolic compounds by UHPLC

Identification and quantification analysis of the phenolic compounds present in the optimized CBS extracts (obtained by OH and CH) were performed as described previously by Ferreira-Santos et al. (2019), using a Shimatzu Nexpera X2 UPLC chromatograph equipped with Diode Array Detector (DAD) (Shimadzu, SPD-M20A, Columbia, MA, USA) and a reversed-phase Aquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm particle size from Waters, Milford, MA, USA) at 40 °C. The HPLC grade solvents used were water/formic acid (0.1%) and acetonitrile and the flow rate was 0.4 ml/min. Quantification was carried out using calibration curves for each compound analysed using concentrations between 250 and 2.5 mg/ml (R^2 > 0.98). Phenolic compounds were identified by comparing their UV spectra and retention times with that of corresponding standards.

2.3.4. Antioxidant activity

Three different methods were employed to assess different mechanisms of the antioxidant action: DPPH and ABTS as radical scavenging capacity and FRAP as reducing antioxidant capacity. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH assay) of samples from CBS was determined using the method described in Ballesteros, Cerqueira, Teixeira, and Mussatto (2015). The antioxidant activity values were expressed as micromoles of Trolox equivalent (TE) per g of dry CBS or extract (μM TE/g).

The radical cation decolorization (ABTS) assay of samples was determined as described in Ferreira-Santos et al. (2019). The antioxidant activity values were expressed as micromoles of Trolox equivalent (TE) per g of dry CBS or extract (μM TE/g).

The antioxidant activity of the extracts by the ferric reducing antioxidant power (FRAP) assay was determined as described by Meneses, Martins, Teixeira, and Mussatto (2013). The absorbance was measured at 593 nm and an aqueous solution of ferrous sulphate was employed as standard (R^2 = 0.998). FRAP results were expressed as micromoles of ferrous equivalent (FE) per gram of dry CBS or extract (μmol Fe^{2+}/g).

2.4. Cell viability

In vitro cell metabolic activity of the CBS extracts from OH and CH at optimal conditions, was assessed in different cell lines: normal mouse fibroblast (L929 - ATCC CCL-185), human embryonic kidney (HEK293t – ATCC CRL-11268), human cervical adenocarcinoma (HeLa – ATCC CCL-2™), human colorectal adenocarcinoma (Caco-2 – ATCC HTB-37™ and HT-29 – ATCC HTB-38™). The metabolic activity of each cell line was evaluated by the resazurin reduction assay (Ferreira-Santos et al., 2020). Each experiment was performed in triplicate.

The percentage of cell metabolic activity was calculated correcting blank values (cell-free medium) and related to untreated controls (0.5% dimethyl sulfoxide (DMSO)). IC_{50} values were calculated by a dose response curve using GraphPad software.

2.5. Measurement of intracellular ROS levels

A commercial kit (ab 113851, DCFDA/H2DCFDA – Cellular ROS Assay Kit by Abcam plc®, Cambridge, UK) was used to determine intracellular ROS levels. L929 cells and HeLa cells were grown in 96-well plates at a density of 2.5 × 10^4 cells per well and were incubated overnight at 37 °C under a humidified atmosphere with 5% CO_2. For the treatment, CBS extracts were dissolved in cell culture medium at a concentration of 500 μg/ml. Cells were incubated with the extracts for 8 h. Then, cells were incubated for 45 min with 25 μM 2′,7′-dichlorofluorescein diacetate (DCFDA) at 37 °C under a humidified atmosphere with 5% CO_2 and protected from light. For *tert*-butyl hydrogen peroxide (tbHP) ROS induction, the cell culture medium was replaced by 100 μM tbHP (dissolved in PBS) except for the negative control cells, which were incubated with PBS. After 1 h of incubation protected from light, the fluorescence intensity was measured using a microplate reader. Excitation and emission wavelength settings were 485 and 535 nm, respectively. The intensity of fluorescence is considered a reflection of the total intracellular ROS levels. Each experiment was performed in triplicate.

2.6. Morphological characterization of CBS

In order to compare the effects of the different treatments (temperature, solvents and electric fields) on the CBS structure, a morphological analysis of the treated CBS biomass was performed. The solid residue obtained after the treatment was recovery by filtration, and both treated and untreated biomasses were dried overnight at 60 °C prior to analysis. Samples were sputter-coated with a thin layer of gold in a Sputtering Balzers SCD 004 and were observed employing scanning electron microscopy (SEM; JEOL JMS-6610LV, Jeol, MA, USA) with a 0.3–30 kV voltage.

2.7. Experimental design

An experimental design (2^3) was conducted for the optimization of phenolic compounds recovery where the variables extraction time (x_1, 4.22–75.77 min), extraction temperature (x_2, 10.27–99.72 °C) and ethanol concentration (x_3, 0.28–89.72% (v/v)) were evaluated. A solid/liquid ratio of 5% of CBS was selected for OH experiments considering previous results by us (data not shown) and existing data in the literature (Pereira et al., 2016; Jesús et al., 2020). Table 1 shows the conditions of the experiments carried out. The independent variables were correlated with the dependent variables (TPC and antioxidant activity by FRAP and DPPH methods) considering a quadratic model (Eq. (1)):

$$Y_i = \beta_{0i} + \beta_{1i} x_1 + \beta_{2i} x_2 + \beta_{3i} x_3 + \beta_{11i} x_1^2 + \beta_{22i} x_2^2 + \beta_{33i} x_3^2 + \beta_{12i} x_1 x_2 + \beta_{13i} x_1 x_3 + \beta_{23i} x_2 x_3 \quad \text{(Eq.1)}$$

Where, Y_i are the dependent variables corresponding to the concentration of TPC, (expressed in mg GAE/g CBS) and antioxidant activity (FRAP in μM Fe^{2+}/g CBS and DPPH in μM TE/g CBS); x_1, x_2 and x_3 value of independent variables; β_{0i}, β_{1i}, β_{2i}, β_{3i}, β_{11i}, β_{22i}, β_{33i}, β_{12i}, β_{13i} and β_{23i} are regression coefficients calculated from experimental data by multiple regression employing the least-squares method. The experimental data were fitted to the model using Statistica software (version 12, StatSoft Inc, Oklahoma, USA).

2.8. Statistical analysis

All experiments were performed in triplicate and the data are presented as mean ± standard deviation (SD) values. GraphPad Prism software (version 6.0, GraphPad Software Inc., San Diego, CA, USA) was used for the statistical analyses. Student's *t*-test and ANOVA were used to determine statistical differences at a significant level of $p < 0.05$.

3. Results and discussion

3.1. Optimization of OH extraction of antioxidant phenolic compounds

3.1.1. Extraction yields

Extraction yields (%) differ depending on the concentration of ethanol, temperature and time employed. As demonstrated in Table 1, the extraction solvent with 70% ethanol showed the highest extraction yield, specifically 36.7% when CBS was processed at 80 °C for 60 min. The results indicate that when CBS is employed as raw material in OH, for the same percentage of ethanol used, extraction yields increased when the extraction was performed at the highest temperature and time, except for extractions at 45% of ethanol where the maximum values were obtained at the central point of the experimental design (55 °C for 40 min). In addition, it can be observed that employing higher ethanol concentrations (>80%) as solvent result in lower extraction yield. This can be explained by the fact that CBS components such as fibre, proteins or sugars are more water-soluble compounds (Choi, Kim, Kim, & Choi, 2019). Further, many phenolic compounds have intermediate polarity, with low affinity for higher ethanol concentrations. These results are in accordance with those reported in the literature for cocoa residues. For instance, Soares, Okiyama, and Rodrigues (2020), employing CBS, achieved a maximum extraction yield of 40% when using 90% ethanol and a treatment temperature of 90 °C. Further, Pagliari et al. (2022) reported that the extractive yield of CBS using pressurized hot water extraction with temperatures between 50 and 130 °C, ranged from 20 to 30% for ethanolic extractives. In another work (Mellinas et al., 2020) using MAE and different conditions for extraction of CBS compounds, the maximum extraction yield obtained was 37.2%, almost the same maximum value achieved in this work. The extraction yield rate is not only largely dependent on the extraction condition and method, but also on variations from different batch collections related to agro-food practices, environmental parameters, or storage conditions.

3.1.2. Electrical conductivity and pH

Values of pH and electrical conductivity for samples before and after extraction using OH are summarized in the Supplementary Materials Table S1 (Online Resource 1). As can be seen, results show that, in all cases, the electrical conductivity of the extracts increased after the extraction process. This is an important parameter to control in ohmic heating-based processes and its variation may also be an indicator of the degree of extraction of conductive compounds. The highest increase in the conductivity (28.2%) was observed when employing 45% ethanol, (v/v), 99 °C and 40 min of treatment while when CBS was submitted to OH using 89% ethanol (v/v) at 55 °C during 40 min, the conductivity of the process just increased 0.97% respect to before the extraction. This phenomenon could be explained by the release, from the matrix to the medium, of extracellular and intracellular minerals and other conductive compounds that can drive electrical energy (Loo-Loo Miranda, Chire Fajardo, & Ureña Peralta, 2020). This fact was also observed in the literature when electrical extraction techniques (as PEFs) were applied to different agri-food feedstock (Bhat, Morton, Mason, & Bekhit, 2019; Nowacka et al., 2019). Regarding the pH of extracts, differences were scarcely appreciable between untreated samples/solvent and the OH-assisted extractions, remaining in most cases around 5. It has been reported that during OH, due to high electrical power and salt content, a loss in the buffering capacity of some plant-based matrices could be noted (Darvisi, Khostaghaza & Najafi, 2013). However, according to pH results obtained in this work, OH does not appear to significantly affect the buffer capacity of CBS.

3.1.3. Total phenolic content and antioxidant activity

As can be seen in Table 1, the phenolic compounds extraction yield ranged from 6.3 to 23.9 mg of GAE/g CBS. The extracts obtained employing the solvents with 20% and 45% ethanol (v/v) presented the highest amount of TPC (22.9 and 21.4 mg GAE/g CBS dry weight, respectively), whereas aqueous and 89.7% (v/v) ethanolic extracts showed the lowest content of phenolic compounds (6.3 and 7.1 mg GAE/g of CBS, respectively). This is probably related to the type of phenolic compounds presents in CBS, which, have intermediate polarity and are more soluble in water/ethanol mixtures (Shi, Yu, Pohorly, & Kakuda, 2003). Similar values of TPC were found in the literature when OH was employed in other lignocellulosic substrates. For example, Barrón-García et al. (2022), obtained an amount of total phenolic content of 26–29 mg GAE/g when submitted mango wastes to OH extraction at optimal conditions: 3% (w/v), 72 °C, and ethanol 80% (v/v) as solvent. Darvishi, Salami, Fadavi, and Saba (2020), recovered between 4 and 30 mg GAE/g (dry weight) of total phenolic compounds from mulberry wastes treated by OH extraction at 90 °C, with a methanol-water solution (80:20 v/v) as solvent and different extraction times (0–20 min).

These results highlight that the use of higher concentrations of ethanol (70% and 89.7% v/v) in the extractive process using CBS, results in extracts with lower amounts of these secondary metabolites. Other works have also reported a correlation between higher values of phenolic compounds and the use of intermediate concentrations of ethanol as a solvent for lignocellulosic matrices. For example, Rajha, Boussetta, Louka, Maroun, and Vorobiev (2014) studied different extraction conditions to obtain polyphenols from vine residues and reported that the extraction of these compounds was maximized when 50% ethanol was used. However, when 75% and 100% alcoholic solutions were employed, the concentration of phenolic compounds notably decreased. In addition, Ferreira-santos et al. (2021), using OH to obtain eggplant extracts, observed that the amount of TPC increased by increasing the percentage of ethanol used as solvent (up to 50% (v/v)), while when higher concentrations were tested (70 and 90% (v/v)), the amount of phenolics obtained decreased. This phenomenon was also reported by Ferreira-Santos et al. (2020), who observed a greater content of TFC in intermediate hydroalcoholic extracts (50% ethanol (v/v) of pine bark treated by OH when compared to 30, 70 and 90% ethanol extracts. The DPPH assay is based on the electron-transfer mechanism and the ferric reducing antioxidant power (FRAP) relies on the reduction of an iron complex (Fe^{3+} to Fe^{2+}). The results of antioxidant activities of all extracts evaluated are presented in Table 1 and varied between 81 and 284.5 μM Fe^{2+}/g extract for FRAP and 6.5–36.34 μM TE/g extract for DPPH. Maximum antioxidant activity for FRAP (284.5 μM Fe/g extract) and DPPH (36.4 TE/g extract) was quantified under conditions of the central points (40 min, 55 °C and 45% ethanol concentration (v/v)). The antioxidant capacity of CBS extracts is in accordance with those obtained in the literature when other lignocellulosic materials were employed. For instance, Delgado-Ospina et al. (2021) obtained an amount of 56–181 μM Fe^{2+}/g extract for FRAP from cocoa residues employing methanol (80% v/v) as solvent.

3.1.4. Experimental design

The experimental variables were correlated by the polynomial Eq. (1), of second order (see section 2.2.1.). The proposed mathematical models defining the extraction time (x_1), extraction temperature (x_2) and ethanol concentration (x_3) as functions are described in Eqs. (2)–(4) for TPC (mg GAE/g CBS) (Y_1), FRAP (μM Fe^{2+}/g CBS) (Y_2) and DPPH (μM TE/g CBS) (Y_3), respectively. The optimization of the model was carried out considering the results in g per g of CBS.

$$Y_1 = -2.25 + 0.13x_1 - 0.08x_2 + 0.19x_3 - 0.001x_1^2 + 0.0002x_2^2 - 0.001x_3^2 + 0.001x_1x_2 - 0.0008x_1x_3 + 0.001\ x_2x_3 \quad \text{Eq. 2}$$

$$Y_2 = -58.43 + 1.21x_1 + 1.38x_2 + 1.95x_3 - 0.01x_1^2 - 0.01x_2^2 - 0.01x_3^2 + 0.006x_1x_2 - 0.001x_1x_3 + 0.005\ x_2x_3 \quad \text{Eq. 3}$$

$$Y_3 = -2.18 + 0.15x_1 + 0.08x_2 + 0.16x_3 - 0.002x_1^2 + 0.001x_2^2 - 0.002x_3^2 + 0.0002x_1x_2 - 0.0007x_1x_3 + 0.001\ x_2x_3 \quad \text{Eq. 4}$$

The R^2 of the models performed in eqs. (2)–(4) were 0.76, 0.76 and 0.71, respectively. The calculated *F-value* were 1.37 (Eq. (2)), 2.61 (Eq. (3)) and 1.98 (Eq. (4)). The models were fitted by ANOVA analysis and *F*-test at 95% of confidence level before creating the response surface graphs depicted in Fig. 1A–C. All models were statistically significant since the calculated *F-values* were higher than the listed *F-value* at 95% of confidence level. Fig. 1 represent the effect of the concentration of ethanol and extraction temperature, with a fixed extraction time of 40 min, on the recovery of TPC and antioxidant activity determined by FRAP and DPPH. As time did not seem to have a significant contribution to the extraction process, level 0 of this independent variable was chosen to illustrate this relation. As can be seen in Fig. 1A, the ethanol concentration has a notable positive effect on the concentration of TPC when intermediate proportions are employed. Fig. 1B shows the response surface and the Pareto plot of the effect of extraction temperature and ethanol concentration for FRAP assays. The FRAP Pareto graph (Fig. 1B) showed that the most efficient factor influencing the antioxidant activity of CBS was the concentration of ethanol, and this factor certainly affected it. The quadratic level of extraction temperature and concentration of ethanol also significantly affected the antioxidant

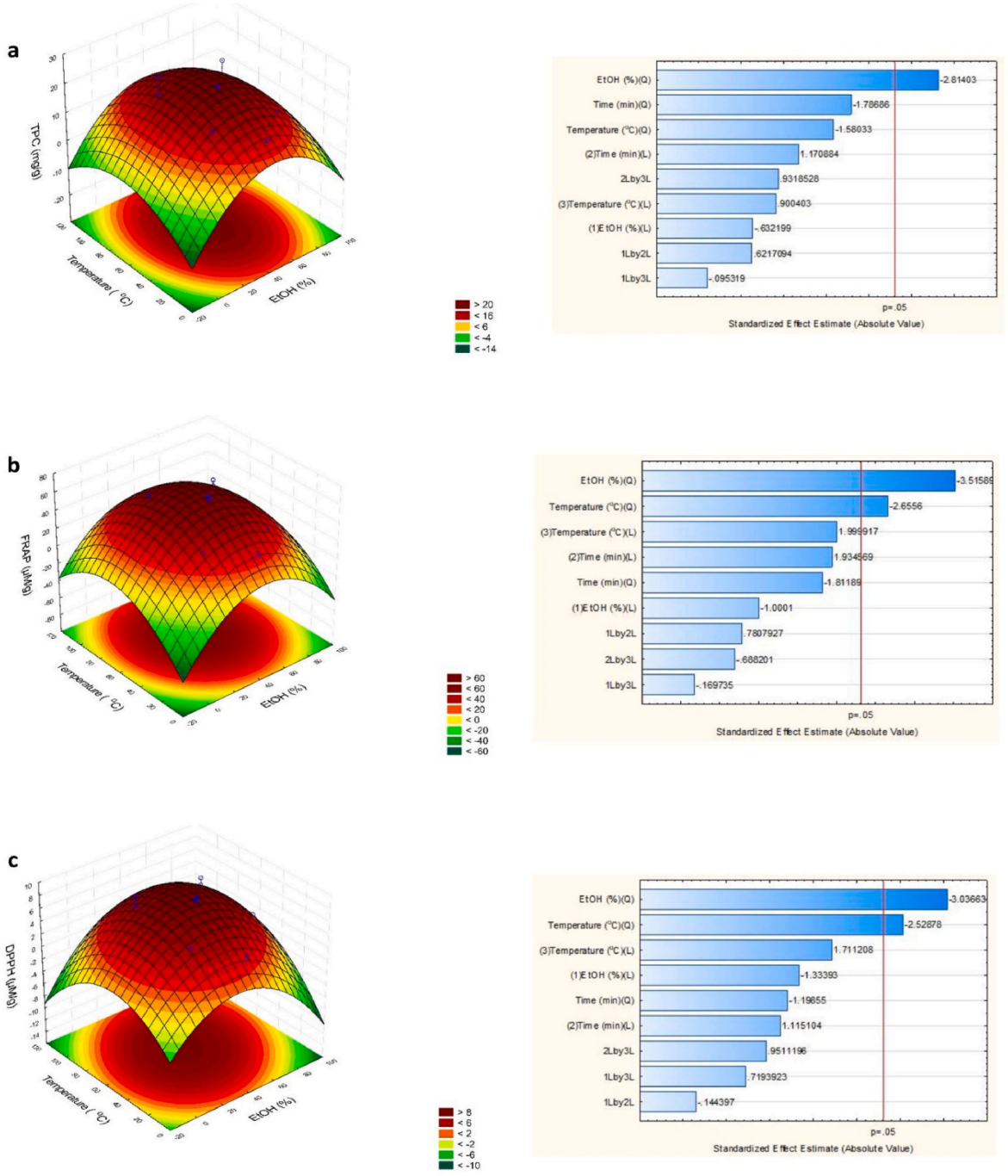

Fig. 1. Response Surface and Pareto diagram showing the effect of temperature and ethanol concentration (fixed extraction time at 40 min) of a) total phenolic content, expressed in mg GAE/g CBS, b) FRAP, expressed in µM Fe/g CBS and c) DPPH, expressed in µM TE/g CBS ($p > 0.05$).

capacity, while the linear temperature extraction, linear ethanol concentration, quadratic and linear extraction time and the interactions between time/temperature, time/ethanol and temperature/ethanol were insignificant. The shown 3D plot in Fig. 1C represents a response surface for DPPH results at an extraction time of 40 min. As in the case of DPPH, just quadratic extraction temperature and ethanol concentration positively affected the antioxidant activity of CBS extracts.

The regression coefficients and variance analysis of linear, quadratic and the interactions between variables are listed in the Supplementary Materials Table S2 (Online Resource 1). The total phenolic content, antioxidant activity of CBS extracts and the interactions between the variables presented different meanings according to the statistical analysis method employed ($p < 0.01$, $p < 0.05$ or $p > 0.05$).

In order to obtain the highest total polyphenolic content and the antioxidant activity, the extraction process was optimized. For all responses (TPC, FRAP and DPPH), the optimal extraction conditions selected in this work were those obtained for phenolic compounds based on that phenolics are responsible for antioxidant activity. Optimized values and parameters for TPC and antioxidants are shown in Table 2. As can be observed, 67 °C for 50 min with an ethanol concentration of 44% (v/v) are the best operating conditions to maximize the recovery of phenolic compounds with high antioxidant activity. The validation assays conducted at the predicted conditions resulted from the experimental design demonstrated that experimental values were quite similar to the predicted values, confirming the adequacy of the models. The error rate between the predicted and experimental values is less than 1% for TPC and FRAP and less than 6% for antioxidant activity determined by DPPH assay.

3.2. Comparison of conventional heating extraction with ohmic heating-assisted extraction

3.2.1. Characterization of optimal extracts

To evaluate the efficiency of the OH-assisted method, the optimized extraction procedure was applied to CBS and then compared with the results of the CH extraction method under optimal conditions. All extracts were analysed for TPC, TFC, FRAP, DPPH and ABTS and the results are shown in Table 3.

As can be observed in Table 3, the results demonstrate that OH is considerably more efficient to recover natural compounds from CBS compared to CH (extraction efficiency around 50% more in the case of OH) ($p < 0.05$). This is more noticeable for TPC, where OH allowed to extract almost twice the phenolic compounds in comparison to the amount extracted by the CH process (22 and 13 mg GAE/g CBS, respectively). The increase in the extraction of compounds by OH compared to CH treatment, was also detected in the TFC (9.2 and 6.5 mg CE/g CBS for OH and CH treatment, respectively) ($p < 0.05$) although differences are not so remarkable. The results achieved by comparing OH and CH are similar to those referred to in the literature for other raw materials. For example, M'hiri, Ioannou, Boudhrioua & Ghoul (2015), who focused on the effect of different conditions on the extraction of phenolic compounds from orange peel, described an increase of more than 30% in the phenolic compounds obtained with dependent voltage extraction methods compared to CH. Ferreira-Santos et al. reported that OH is capable of improving the extraction yield of phenolic compounds by approximately 30% in red eggplant and 50% in pine bark, using ethanol concentrations from 30 to 70% (Ferreira-Santos et al., 2019, 2021).

As in the case of phenolic and flavonoid content, the results showed that all antioxidant activities tested were always higher in the extracts from OH compared with those obtained from CBS treated by the CH procedure and significant differences were observed ($p < 0.05$). The mean values (see Table 3) of radical scavenging activity detected by ABTS assay were clearly greater than the corresponding values measured by DPPH method for OH and CH (25.3 and 86.2 μM TE/g extract for OH and 19.1 and 71.7 μM TE/g extract for CH). These results highlight the correlation between TPC and TFC of CBS extracts with the antioxidant capacity determined by ABTS, DPPH and FRAP methods, showing the contribution of these bioactive compounds to the antioxidant activities. These findings demonstrate the potential applications of CBS extracts from OH, as bioactive products in food, chemical or pharmaceutical industry sectors. Mannozzi et al. (2019), through their

Table 2
Optimized conditions for total phenolic content and antioxidant activity from cocoa bean shell extracts, including predicted and experimental values.

Responses	Process variables			Predicted value	Experimental value
	x_1 (min)	x_2 (°C)	x_3 (% v/v)		
TPC (mg GAE/g CBS)	50.3	67.4	43.8	22.1	22.3 ± 0.87
FRAP (μM Fe/g CBS)	49.0	62.7	42.8	62.6	62.7 ± 1.12
DPPH (μM TE/g CBS)	55.8	67.8	40.8	8.8	8.3 ± 0.41

Table 3
Extraction yield, total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity (FRAP, DPPH and ABTS), and individual phenolic compounds of CBS extracts obtained by ohmic heating (OH) and conventional heating (CH) extraction methods at optimal conditions.

Component	OH	CH
Extraction yield (%)	33.0 ± 0.4[a]	20.8 ± 0.2[b]
TPC (mg GAE/g CBS)	22.3 ± 0.8[a]	13.2 ± 0.8[b]
TPC (mg GAE/g extract)	67.7 ± 0.1[a]	57 ± 0.7[b]
TFC (mg CE/g CBS)	9.2 ± 0.4[a]	6.5 ± 0.2[b]
TFC (mg CE/g extract)	27.9 ± 1	26.1 ± 0.9[b]
FRAP (μM Fe^{2+}/g CBS)	62.7 ± 0.9[a]	39.3 ± 0.7[b]
FRAP (μM Fe^{2+}/g extract)	190.1 ± 1.2[a]	169.4 ± 0.1[b]
DPPH (μM TE/g CBS)	8.3 ± 0.7[a]	6.7 ± 0.2[b]
DPPH (μM TE/g extract)	25.3 ± 0.5[a]	19.1 ± 0.1[b]
ABTS (μM TE/g CBS)	28.5 ± 1.4[a]	16.5 ± 0.8[b]
ABTS (μM TE/g extract)	86.2 ± 0.5[a]	71.1 ± 0.9[b]

Phenolic compound	OH		CH	
	μg/g CBS	μg/extract	μg/g CBS	μg/g extract
Vanillic acid	120.3 ± 20[a]	364.5 ± 18[a]	120.2 ± 10[a]	277.9 ± 14[b]
Syringic acid	62.8 ± 17[a]	190.3 ± 19[a]	33.7 ± 8[a]	162 ± 6[b]
Cinnamic acid	7523 ± 454[a]	22797 ± 425[a]	5675 ± 99[b]	21283.7 ± 87[b]
Caffeic acid	250.8 ± 19[a]	760 ± 15[a]	178.8 ± 16[b]	659.6 ± 12[b]
Ferulic acid	18.3 ± 4[a]	55.5 ± 2.5[a]	6.7 ± 1[b]	32.2 ± 0.8[b]
o-Coumaric acid	31.6 ± 10[a]	95.8 ± 12[a]	10.3 ± 1[b]	49.5 ± 0.7[b]
Rosmarinic acid	138.1 ± 0.7[a]	418.5 ± 0.9[a]	68.9 ± 0.7[b]	331.3 ± 1[b]
Ellagic acid	447.9 ± 74[a]	1357.3 ± 73[a]	212 ± 46[b]	1019.2 ± 50[b]
4-HBA	7921 ± 128[a]	24003 ± 105[a]	4201 ± 493[b]	20197.1 ± 387[b]
2,5-HBA	342.6 ± 15[a]	1038.2 ± 10[a]	169.6 ± 7[a]	815.4 ± 4[b]
Taxifolin	498.1 ± 95[a]	1509.4 ± 87[a]	330.6 ± 22[b]	1589.4 ± 19[b]
Epicatechin	233.5 ± 26[a]	707.6 ± 31[a]	108.4 ± 18[b]	521.2 ± 21[b]
Kaempferol	5.6 ± 0.6	17 ± 0.8	n.d.	n.d.
Naringin	844.0 ± 181[a]	2557.6 ± 176[a]	334.8 ± 23[b]	1609.6 ± 20[b]
Resveratrol	24.4 ± 0.5[a]	73,9 ± 1[b]	12.1 ± 0.2[a]	58.2 ± 0.7[b]
TOTAL	18462	55945.5	11462	48806.3

* Values of phenolic compounds are expressed as concentration (μg/g CBS) mean ± SD of 3 experiments. 4-HBA: 4-Hydroxybenzoic acid; 2,5-HBA: 2,5-Dihydroxybenzoic acid; n.d.: not detected. The means followed by different letters within a file means statistic differences ($p < 0.05$) by Students t-test.

investigation on the extraction of antioxidants from carrots and apples using OH pre-treatment, obtained values of 2–4 μM TE/g and 10–14 μM TE/g for DPPH and ABTS, respectively, values notably lower than those obtained in this work for CBS.

For the three assays tested (FRAP, DPPH and ABTS), results confirmed the higher antioxidant potency of OH, increasing the antioxidant activity by 10–30%, compared to CH process. This fact was also reported in literature when the effectiveness of OH is compared to conventional extraction methods in different agri-food residues. For example, Barrón-García et al. (2022), reported an improvement in the antioxidant capacity of mango pulp treated by OH (in comparison with CH) of 9–74% and 2–7%, for DPPH and ABTS, respectively. In another study using OH and ethanol mixtures (ethanol content ranging from 40 to 60% (v/v)) to obtain olive leave extracts, the antioxidant capacity of ohmic extracts was approximately 30% higher than the activity measure in CH extracts (Markhali et al., 2022). The observed enhancement of extraction with OH treatment could be explained because these processes are usually linked to an electropermeabilization mechanism on cell walls which facilitates the extraction of intracellular compounds. The flow of electric current through the tissue can cause temperature rise and membrane deterioration resulting in the diffusion of solutes to the medium (Aamir & Jittanit, 2017; Rocha et al., 2018). In addition, its heating mechanism allows a rapid and uniform temperature increase, which ensures that OH reduces processing energy and time consumption and preserves (or impairs less) the sensorial, nutritional, and structural characteristics of agri-food products when compared to CH (Bhat et al., 2019; Rodrigues et al., 2019).

Regarding the extraction yield (Table 3), for OH-assisted extraction the yield values are significantly higher than those achieved for the CH treatment ($p < 0.05$) (33 and 20,8% for OH and CH, respectively). As commented above, ohmic technology generates a cell permeabilization effect on the matrix which allows the release of intracellular components, boosting the extraction efficiency (Pereira & Vicente, 2010; Rocha et al., 2018).

3.2.2. Identification and quantification of phenolic compounds

In total, 16 phenolic compounds were identified and quantified in the CBS extracts obtained by OH and CH at optimized conditions by HPLC (Table 3). Phenolic compounds are found in cocoa residues such as CBS, and its phenolic content and concentration are usually influenced by the variety, degree of ripeness, processing, and storage (Mazor, Radojčic, Marković, Ivanec & Delonga, 2011). As far as we know, there are few studies on the analysis of phenolic profile of CBS extracts (Rojo-Poveda, Zeppa, Ferrocino, Stévigny, & Barbosa-Pereira, 2021).

All individual phenolic compounds tested were detected in OH extracts while it was not possible to quantify kaempferol in samples from CH extraction. The main compounds found in all extracts were cinnamic acid and 4-HBA. Cinnamic acid was found in concentrations of 7523 ± 454 μg/g CBS for OH and 5675 ± 99 μg/g CBS for CH that accounts for between 40.7 and 49.5% of the total phenolic compounds. 4-HBA was found in concentrations of 7921 ± 128 μg/g CBS for extracts obtained from OH and 4201 ± 493 μg/g CBS for CH samples, values that accounted for between 36.6 and 43% of the total phenolic compounds. Extracts from cocoa residues obtained with ultrasonic methods had 934 μg/g of cinnamic acid and 52 μg/g of epicatechin, concentrations four to eight times lower than the ones obtained in this work for ohmic extracts (Muhammad et al., 2021). Additionally, Rebollo-Hernanz et al. (2021) obtained an amount of 9–12 μg/g of HBA in CBS, values much lower than those detected here for ohmic and conventional samples. Caffeic acid, coumaric acid, vanillic acid and rosmarinic acid were also reported in the literature as individual phenolics detected in cocoa by-products (Boungo Teboukeu et al., 2018; Irondi et al., 2019; Karim et al., 2016; Valadez-Carmona et al., 2017).

In terms of the effect of ohmic-assisted extraction, extracts made with OH had the highest total amount of phenolics when compared with CH method. These results are in accordance with those obtained for the TPC, antioxidant activities and extraction yields, of the resulting broths. Thus, the extraction methods and conditions studied in this work could an advantageous alternative to recover these compounds at an industrial level.

3.2.3. Cell metabolic activity and oxidative stress

It is well known that, due to its high content in polyphenols, CBS has great potential as a bioactive ingredient or additive, entailing biofunctionalities against cancer, diabetes, or neurodegenerative disorders (Martín & Ramos, 2016; Martin, Goya, & Ramos, 2016; Martin, Fernández-Millán, Ramos, Bravo & Goya, 2014; Rojo-Poveda et al., 2020; Vauzour, Rodriguez-Mateos, Corona, Oruna-Concha, & Spencer, 2010). However, and despite their potential nutraceutical effects, it is also necessary to assess the biological effect of the of obtained OH and CH CBS extracts, as they are for human consumption. So, five different cell lines, one normal mouse cell line (L929) and four human cell lines (normal: HEK239t, and three derived from cancer tissues: Caco-2, HT-29 and HeLa) were used to determine the CBS extracts' biological effect in terms of metabolic activity. Similar cell lines have been used in the literature to evaluate the toxicity of cocoa and other natural extracts (Ferreira-Santos et al., 2022; Kosińska & Andlauer, 2012; Zainal, Abdah, Taufiq-Yap, Roslida, & Rosmin, 2014). It is important to mention that metabolic activity can be used as a viability indicator.

Cells were in contact with different concentrations of each extract (0–4000 μg/ml) for 36 h. Fig. 2 describes the results of cellular viability upon contact with extracts obtained at optimal conditions of CH and OH procedures. The five cell lines tested exhibited a clear dose-dependent response. The extracts did not induce significant metabolic activity changes or reduced cell viability. This result is even more clear in Fig. 2F, where it can be seen the IC_{50}. When comparing the effect of the extracts on non-tumoral and tumoral cells, it can be seen that non-tumour cell lines (Fig. 2 A and B) have the highest IC_{50} (lower cytotoxicity), particularly upon contact with OH extracts. Even though, L929 cells are more sensitive to the presence of the extracts. The higher sensitivity of L929 cells has also reported by other authors (Campoccia et al., 2021).

In line with our results, where the CBS extracts seem to have higher toxicity for tumoral cells, Bauer et al. (2016) reported that CBS extracts can influence proliferation and increase apoptosis in human lung carcinoma cells. Similarly, Rossin et al. (2021), demonstrated the antiproliferative effect in Caco-2 cell line, and high antioxidant activity of cocoa extracts.

These results show that CBS extracts exhibit low toxicity at the tested concentrations, and in addition, to some extent, they may have a slight ability to inhibit tumour cell growth. However, more research into the anticancer potential of the CBS extracts is needed.

It is well known that an imbalance between the ROS formation and antioxidant defences leads to oxidative stress, damaging biologically relevant molecules as DNA, membrane lipids or proteins and, thus, causing the oxidative destruction of cells (Andre, Larondelle, & Evers, 2010; Poljsak, ŠUPUT & Milisav, 2013). Excessive ROS production significantly contributes to the development of several chronic diseases such, as cardiovascular disorders, cancer, and diabetes (Martín & Ramos, 2016), as well as premature aging (Alfadda & Sallam, 2012). It has been reported that cocoa flavanols are able to avoid free radical-induced damage by controlling enzymes related to oxidative stress and modulating molecular signals associated with cell cycle, proliferative routes etc. (Martín, Goya & Ramos, 2016), which will positively contribute for the prevention of several diseases.

To assess the ability of the CBS to prevent an excessive ROS formation upon contact with an oxidant agent tBHP, cells were previously placed in contact with the extracts and ROS production evaluated.

HeLa line cell was selected because they were less susceptible to the extract compared to HT-29 and Caco-2. As can be seen in Fig. 3, all extracts were able to reduce the formation of ROS species by the two cell lines compared to the positive control (*t*BHP) ($p < 0.05$).

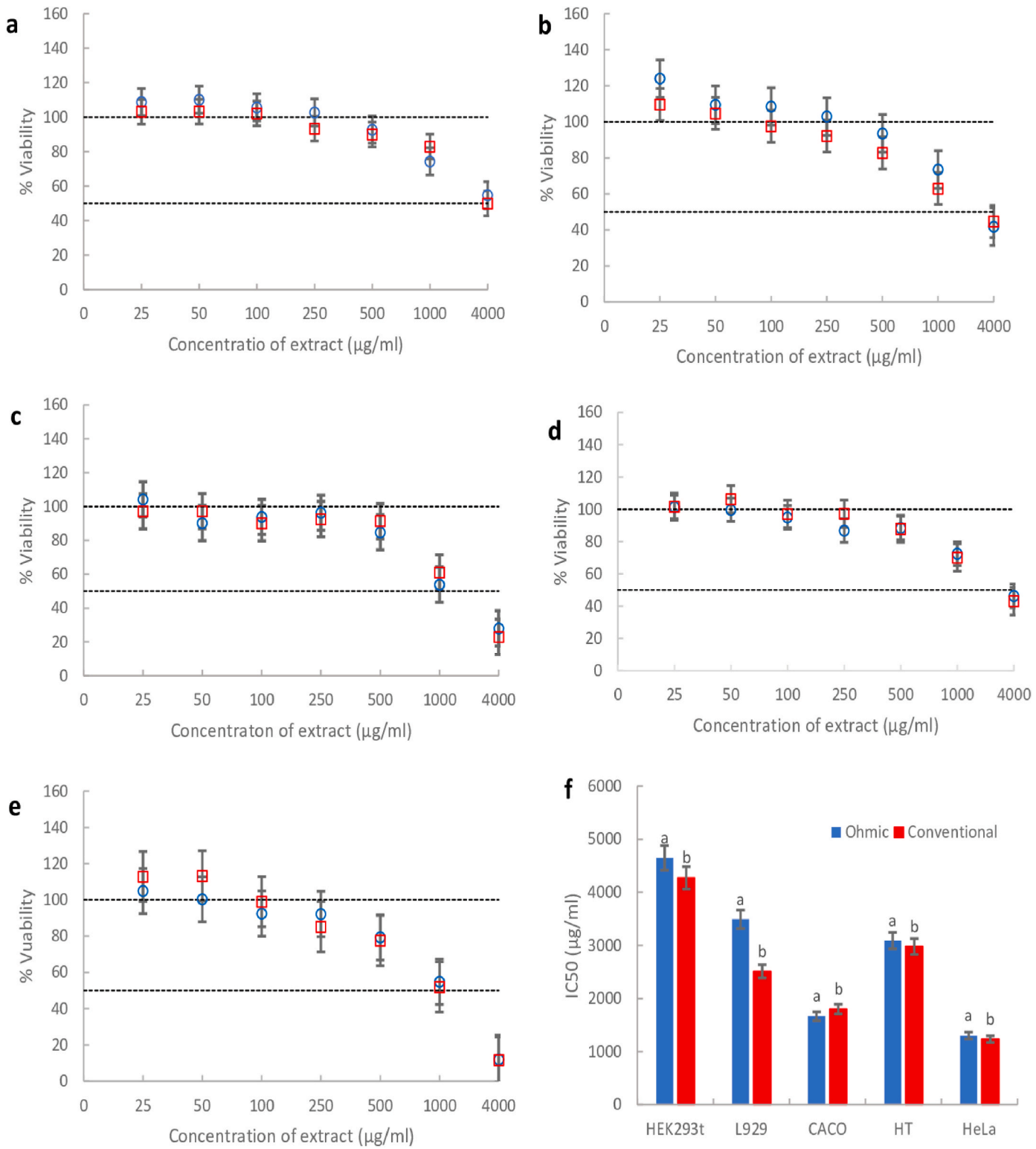

Fig. 2. Cell viability (%) employing ohmic (○) and conventional (□) heating extracts from cocoa bean shell against (a) HEK293t cells, (b) L929 cells, (c) Caco-2 cells, (d) HT-29 cells, (e) HeLa cells, and the respective IC_{50} values (f). Different letters show significant differences ($p < 0.05$) between groups for the same experiment by the Student's t-test.

In line with our results, Martín, Fernández-Millán, Ramos, Bravo, and Goya (2014) reported that epicatechin present in cocoa extracts inhibits oxidative metabolism of pancreatic cells in a dose-dependent mode and protects the cellular secretion mechanism of insulin against oxidative stress. In an interventional human study (Maskarinec, 2009), demonstrated that the intake of cocoa polyphenols reduced cancer progression improving biomarkers related to oxidative stress. In addition, Rivas-Chacón et al. (2022) described that methanolic extracts from cocoa beans reduced drastically the formation of ROS and Reactive Nitrogen Species (RNS) and inhibited the mitochondrial-apoptotic pathway thus reducing cell's apoptosis.

The CBS extracts have antioxidant properties and exert a higher

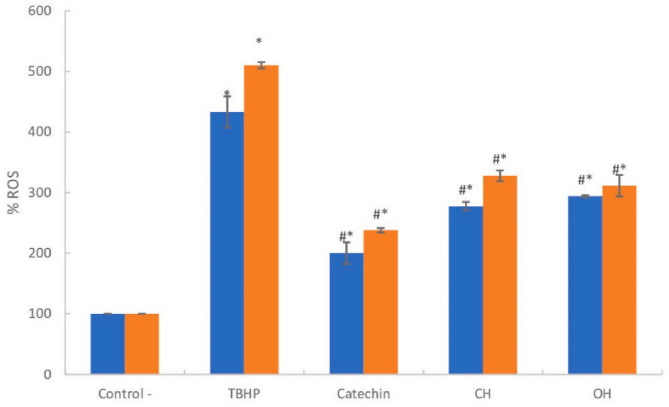

Fig. 3. Cellular antioxidant activity of extracts on L929 (■) and HeLa (■) cells. Measurement of reactive oxygen species (ROS) levels after 12 h of incubation with CH and OH extracts (500 $\mu g_{extract}$/mL), and catechin (100 μM) in the presence of *tert*-butyl hydrogen peroxide (tbHP, 100 μM). Values are expressed as mean ± SD of three experiments. ($p > 0.05$). *Significantly different *versus* negative control cells, and # significantly different *versus* positive control (*t*BHP) cells.

influence on the metabolic activity of the HeLa cells. These results are in accordance with the literature. Krstic and co-workers (Krstic, Stojadinovic, Smiljanic, Stanic-Vucinic & Cirkovic, 2015) concluded that cocoa extracts were found to possess antioxidant properties and specific cytotoxic effect on cervical carcinoma cell line (HeLa).

3.2.4. SEM analysis of CBS morphology

In order to evaluate the effect of OH and CH treatments on the structure of CBS, a morphological analysis of the CBS particles was performed by SEM. Obtained microphotographs are shown in Fig. 4, where it can be seen the structure of untreated, CH and OH treated samples of CBS.

The extraction method clearly influenced the morphology of CBS. In the untreated and CH treated samples (Fig. 4 A and B), the surface structure was found to be smooth, whereas in the samples treated by OH (Fig. 4C) a remarkable number of surface ruptures and pores can be observed. These changes could reflect an easier diffusion and extraction of phenolic compounds to the liquid phase, confirming the previous results in the extraction yields obtained with this work. In addition, several modifications and damage of tracheid (xylem conductive cells) in the treated samples by OH (Fig. 4E) were observed, compared to untreated samples (Fig. 4D). The SEM analysis indicated that when OH is employed, harsher morphological changes are generated in the membrane of the vegetable cells due to an electroporation effect, which favours the extraction of intracellular compounds (Ferreira-Santos et al., 2019).

4. Conclusions

In the present work, the extraction of phenolic compounds from CBS by means of OH treatments employing environmentally friendly solvents, i.e., ethanol and water, has been optimized. The antioxidant activity and TPC results obtained in the experimental procedure were accurately predicted by the linear and quadratic models developed in the optimization study (optimal conditions for TPC: 67 °C for 50 min and 44% ethanol). Extraction of total phenolic content and antioxidant activity from CBS was greater when using OH than CH methodology, increasing by 40% the amount of TPC and 4–20% the antioxidant capacity.

The CBS extracts obtained from OH and CH extraction at optimal conditions, have low cytotoxicity in non-tumoral cells and ROS preventive effects. These characteristics make them ideal to be used in food processing and formulation, as well as nutraceutical products due to their antioxidant protection.

OH technology is a potential green technique for the extraction of bioactive compounds, particularly polyphenols with significant enhancement in recovery content and reduced energy consumption. The results of this work highlight the great potential of CBS to be used as substrate with the aim to obtain value-added products, by embracing the circular economy concept based on waste minimization, recycling and recovery, leading to more sustainable and environmentally friendly

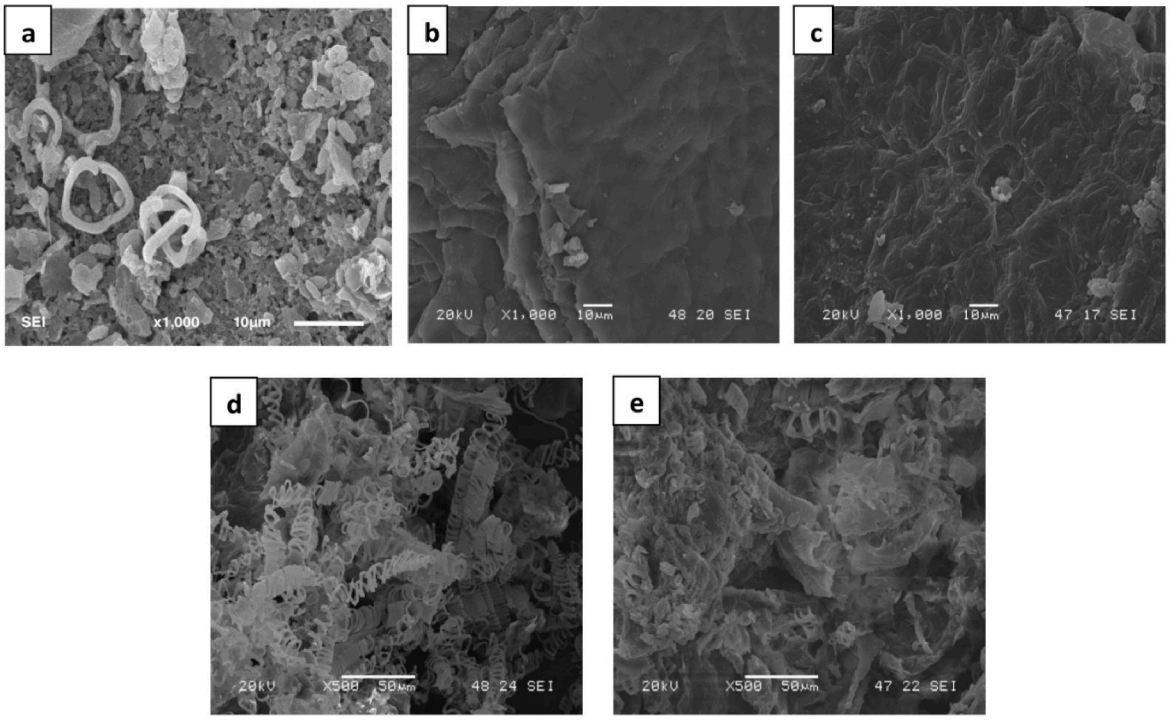

Fig. 4. SEM micrographs of CBS (a) untreated, (b and d) treated by CH and (c and e) treated by OH. Magnification indicated in each picture.

processes.

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Author contributions statement

Marta Sánchez wrote the main manuscript draft. Marta Sánchez, Pedro Ferreira-Santos, Joana S. Gomes-Dias, Cláudia Botelho, Amanda Laca and Cristina M.R. Rocha wrote, edited and reviewed the manuscript, and discussed the results. Marta Sánchez, Pedro Ferreira-Santos and Joana S. Gomes-Dias performed the main experimental work and data analysis. Pedro Ferreira-Santos, Cláudia Botelho, Cristina Rocha and Amanda Laca were responsible for the methodology and experimental design. Cristina Rocha and Amanda Laca were responsible for the supervision and resources.

Declaration of competing interest

The authors have no competing interests to declare.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fbio.2023.102886.

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