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Extraction of phenolic compounds from cocoa shell: Modeling using response surface methodology and artificial neural networks

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ABSTRACT

This work's objective was to model and optimize a green extraction method of phenolic compounds from the cocoa shell as a strategy to revalorize this by-product, obtaining novel high-value products. According to a Box-Behnken design, 27 extractions were carried out at different conditions of temperature, time, acidity, and solid-to-liquid ratio. Total phenolic compounds, flavonoids, flavanols, proanthocyanidins, phenolic acids, *o*-diphenols, and *in vitro* antioxidant capacity were assessed in each extract. Response surface methodology (RSM) and artificial neural networks (ANN) were used to model the effect of the different parameters on the green aqueous extraction of phenolic compounds from the cocoa shell. The obtained mathematical models fitted well for all the responses. RSM and ANN exhibited high estimation capabilities. The main factors affecting phenolic extraction were temperature, followed by solid-to-liquid ratio, and acidity. The optimal extraction conditions were 100 °C, 90 min, 0% citric acid, and 0.02 g cocoa shell mL^{-1} water. Under these conditions, experimental values for the response variables matched those predicted, therefore, validating the model. UPLC-ESI-MS/MS revealed the presence of 15 phenolic compounds, being protocatechuic acid, procyanidin B2, (−)-epicatechin, and (+)-catechin, the major ones. Spectrophotometric results showed a significant correlation with the UPLC results, confirming their potential use for screening and optimization purposes. Aqueous phenolic extracts from the cocoa shell would have potential use as sustainable food-grade ingredients and nutraceutical products.

1. Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), there is a clear need for food chain optimization regarding sustainability and efficiency [1]. In this context, by-products' valorization, through their conversion into food-grade ingredients and novel foods, could be an effective strategy [2]. Plant by-products are very abundant and contain different bioactive compounds with potential health benefits. Moreover, by-products exhibit a low risk of toxicity and have excellent consumer acceptance. There is a definite interest from companies in the primary sector for their valorization [3].

As stated by the International Cocoa Organization [4], thousands of cocoa seeds are processed each year globally. Because of this enormous production, the generation of cocoa by-products is very high [5]. Cocoa shell is considered an industrial by-product of cocoa production since it represents up to 20% of cocoa seeds, and it is usually underutilized and mainly used as fuel. However, there are applications in feedstuff and fertilizer preparation [6]. This green material might be attractive as food ingredients due to the presence of a wide variety of bioactive compounds with potential health-promoting properties. The cocoa shell's chemical composition depends on the maturity, genotypic variations of cocoa beans, plants' stress and climatic, and soil conditions. In general, this by-product exhibits low moisture (7%) and considerable variability of lipids (2–15%) comparing with cocoa seeds, being the protein content similar in both materials (12–15%). Likewise, previous studies have described the cocoa shell as a raw material rich in dietary fiber and phenolic compounds [7,8]. The cocoa shell could have enhanced preference against other dietary fiber sources due to the presence of associated phytochemicals (phenolic compounds) with antioxidant properties, which entails additional health benefits [9,10]. These compounds, along with other phytochemicals and bioactive compounds found in the cocoa shell, prove the cocoa shell's industrial applicability as a food

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ingredient. Hence, obtaining phenolic compound-rich extracts as novel ingredients from the food industry by-products has gained attention in the last years.

In this sense, the conventional extraction of phenolic compounds is carried out using organic solvents. However, more eco-friendly methodologies are required by a sustainable food industry that enables the use of phenolic extracts as added-value ingredients for different applications. Heat-assisted extraction (HAE) is the most traditional and prevalent extraction method to separate phenolic compounds from plant, food, and by-product sources. It is considered a straightforward process that conduces to efficient yields [11]. Evaluating and identifying the conditions that improve these compounds' solid–liquid extraction from the cocoa shell is necessary. The extraction process's efficiency is influenced by the solid-to-liquid (S/L) ratio, time, temperature, and medium pH, among others. Therefore, it is essential to optimize the extraction conditions to maximize the efficiency of extraction. Response surface methodology (RSM) has been reported as a useful tool for process optimization when the independent variables had non-linear and interactive effects on the desired response [12]. RSM is presently being applied in process optimization in the chemical, pharmaceutical, and food industries [13]. Several elements affecting the extraction can be optimized in this procedure, primarily to minimize the process's energy costs. Nowadays, RSM successfully models, improves and optimizes extraction processes [14–16]. Likewise, artificial neural networks (ANN) have gained popularity to model and optimize processes [17]. This computational and mathematical methodology allows the study of relationships between the input parameters and the outputs or response variables of the processes using a limited number of experimental measurements, enhancing problem-solving ability. ANN can predict results based on previous data owing to its capacity to learn from observations and create conclusions via the generalization and modeling of complex non-linear processes' behavior. Thus, ANN emerges as a more robust and preferable modeling technique.

We hypothesized that modifying a combination of extraction parameters would increase the yield of green extraction of phenolic compounds from the cocoa shell and allow us to establish a method for their extraction. Thus, the present study aimed to model and optimize the extraction conditions to enhance phenolic compounds' recovery from the cocoa shell using a green sustainable method consuming water as an eco-friendly solvent. Furthermore, it aimed to characterize the obtained extracts comprehensibly. Experimental conditions comprised the use of different temperatures, times, S/L ratios, and acidity. RSM and ANN were used to model and optimize extraction. The optimum conditions that generated phenolic compounds aqueous extracts with the highest *in vitro* antioxidant activity were validated. The phenolic profile of these extracts was investigated by UPLC-ESI-MS/MS, and multivariate statistics were used to gain insight into the effects of extraction conditions on the phenolic composition and its relationship with the *in vitro* antioxidant capacity.

2. Material and methods

2.1. Material and sample preparation

The cocoa shell was supplied by Chocolates Santocildes (Castrocontrigo, León). Milling of the cocoa shell was carried out in a pilot-scale ball mill over three days. Ground cocoa shell was stored at –20 °C in sealed bags in dark and dry conditions to avoid components oxidation.

2.2. Experimental design

Box–Behnken, being a spherical experimental design, consists of a central point and several middle points on the edges of a cube superimposed on the sphere, which requires fewer experiments than other statistical designs. We used a four-factor, three-level Box–Behnken design coupled to RSM and ANN to find the optimal extraction conditions to obtain the highest extraction of phenolic compounds. The experimental conditions for the aqueous extraction of bioactive compounds from the cocoa shell are presented in Table 1. The design consisted of 27 experimental runs with three levels (−1, 0, 1) for each of the variables: temperature (°C) (X_1), time (min) (X_2), acidity as the percentage of citric acid in water (%) (X_3), and S/L ratio (g mL^{-1}) (X_4). Those parameters were selected based on previous studies found in the literature and tested on preliminary experiments to assure they had effects on the extraction of phenolics from cocoa shell [18]. The influence of extraction temperature was investigated in the range from 30 to 100 °C, time from 5 to 90 min, S/L ratio, 0.02–0.05 g mL^{-1}, and acidity, 0–2% citric acid.

2.3. Heat-assisted extraction (HAE)

The HAE was performed in closed vessels in a temperature-controlled water bath with continuous stirring. According to the experimental design, the milled cocoa shell was mixed with water in the different S/L ratios shown in Table 1. Extraction was carried out at various temperatures and times. Once HAE was completed, the samples were centrifuged (4000*g*, 4 °C, 15 min) and the supernatants were freeze-dried. The samples were dissolved in 10 mL of Milli-Q water after neutralization and stored at −20 °C until analysis.

2.4. Organic solvent extraction of free and bound phenolic compound fractions

Phenolic compounds present in the cocoa shell were extracted using conventional organic conditions to compare these conventional conditions to the extractability of water in the optimized methodology. Free and bound phenolic compounds were extracted according to the procedure described by Rebollo-Hernanz et al.[19] The total content of phenolic compounds was obtained as the sum of free and bound phenolics.

2.4.1. Extraction of free phenolic compounds

Cocoa shell flour (1.0 g) was macerated with 50 mL of a solution of methanol–HCl (1‰)/water (80:20, v/v) using an ultrasonic bath for 30 min and an orbital shaker for 16 h at 40 °C. The supernatants were separated by centrifugation (4000*g*, 4 °C, 15 min) and then collected, and the extraction was repeated twice. All the supernatants were pooled and evaporated to dryness under vacuum. The soluble phenolic extracts were brought to 10 mL in methanol and were maintained at −20 °C until analysis.

2.4.2. Extraction of bound phenolic compounds

The residues from the above extraction of soluble free phenolics were flushed with N_2 and hydrolyzed with 20 mL of 4 mol L^{-1} NaOH (1 h, 25 °C, with continuous shaking). The samples were acidified to pH 2 with concentrated HCl, centrifuged (4000*g*, 4 °C, 15 min), and three times extracted with diethyl ether:ethyl acetate (50:50, v/v). Organic fractions were evaporated to dryness, reconstituted into 10 mL of methanol, and stored at −20 °C until analysis.

2.5. Determination of phenolic compounds

2.5.1. Total phenolic compounds (TPC)

Total phenolic compounds were determined by the Folin-Ciocalteu colorimetric method, according to Singleton, Orthofer, & Lamuela-Raventós [20] adapted to micromethod format. Samples (10 μL) were added to 150 μL volume of Folin-Ciocalteu reagent (diluted 1:14, v/v in MilliQ water). After 3 min, 50 μL of Na_2CO_3 20% were added to each well. The absorbance was measured at 750 nm using a microplate reader after 2 h at room temperature. Calibration curves were constructed using standard solutions of gallic acid, and results were expressed as mg

Table 1
Box–Behnken design for the independent variables and corresponding response values.

Run	Independent variables				Responses						
	Temperature (X_1)	Time (X_2)	Acidity (X_3)	Ratio (X_4)	TPC (mg g^{-1})	TF (mg g^{-1})	TFL (mg g^{-1})	PAC (mg g^{-1})	TPA (mg g^{-1})	TOD (mg g^{-1})	AC (mg g^{-1})
1	30 (-1)	5 (-1)	1 (0)	0.035 (0)	4.62 ± 0.13	6.52 ± 0.01	0.77 ± 0.03	1.87 ± 0.18	2.05 ± 0.01	1.04 ± 0.02	11.83 ± 0.62
2	100 (1)	5 (-1)	1 (0)	0.035 (0)	4.38 ± 0.22	7.04 ± 0.47	0.67 ± 0.02	1.88 ± 0.14	2.31 ± 0.16	1.15 ± 0.01	11.59 ± 1.04
3	30 (-1)	90 (1)	1 (0)	0.035 (0)	3.00 ± 0.54	5.68 ± 0.04	0.53 ± 0.07	1.57 ± 0.28	2.17 ± 0.06	0.90 ± 0.03	9.37 ± 1.08
4	100 (1)	90 (1)	1 (0)	0.035 (0)	5.69 ± 0.39	9.91 ± 0.43	0.92 ± 0.01	2.19 ± 0.29	3.49 ± 0.02	1.29 ± 0.00	14.05 ± 1.09
5	65 (0)	47.5 (0)	0 (-1)	0.020 (1)	4.52 ± 0.28	7.69 ± 0.89	0.91 ± 0.05	2.18 ± 0.20	3.10 ± 0.50	1.53 ± 0.01	14.60 ± 1.07
6	65 (0)	47.5 (0)	2 (1)	0.020 (1)	4.24 ± 0.20	7.21 ± 0.88	0.64 ± 0.06	1.73 ± 0.08	2.80 ± 0.14	1.41 ± 0.02	9.22 ± 0.81
7	65 (0)	47.5 (0)	0 (-1)	0.050 (-1)	3.79 ± 0.11	6.64 ± 0.27	0.71 ± 0.06	1.85 ± 0.22	2.48 ± 0.56	1.12 ± 0.04	11.74 ± 1.05
8	65 (0)	47.5 (0)	2 (1)	0.050 (-1)	2.21 ± 0.00	5.21 ± 0.00	0.37 ± 0.00	1.57 ± 0.00	1.64 ± 0.00	0.64 ± 0.00	7.83 ± 0.90
9	65 (0)	47.5 (0)	1 (0)	0.035 (0)	4.19 ± 0.24	7.30 ± 0.23	0.47 ± 0.01	1.60 ± 0.03	1.98 ± 0.81	0.81 ± 0.01	10.12 ± 1.16
10	30 (-1)	47.5 (0)	1 (0)	0.020 (1)	3.39 ± 0.12	6.41 ± 0.78	0.50 ± 0.01	1.58 ± 0.06	1.90 ± 0.13	0.84 ± 0.02	9.33 ± 0.83
11	100 (1)	47.5 (0)	1 (0)	0.020 (1)	7.33 ± 0.33	12.69 ± 1.34	1.55 ± 0.29	3.10 ± 0.20	4.17 ± 0.63	1.68 ± 0.09	19.45 ± 1.12
12	30 (-1)	47.5 (0)	1 (0)	0.050 (-1)	2.41 ± 0.15	4.93 ± 0.23	0.37 ± 0.02	1.54 ± 0.01	1.23 ± 0.18	0.63 ± 0.04	7.05 ± 0.69
13	100 (1)	47.5 (0)	1 (0)	0.050 (-1)	4.51 ± 0.62	8.73 ± 0.05	0.89 ± 0.06	2.24 ± 0.23	3.03 ± 0.27	1.10 ± 0.07	14.53 ± 1.01
14	65 (0)	5 (-1)	0 (-1)	0.035 (0)	4.60 ± 0.05	7.51 ± 0.10	0.63 ± 0.02	1.85 ± 0.19	2.64 ± 0.00	1.08 ± 0.06	11.67 ± 1.19
15	65 (0)	90 (1)	0 (-1)	0.035 (0)	4.32 ± 0.21	8.62 ± 0.80	0.69 ± 0.00	2.14 ± 0.33	2.98 ± 0.31	1.13 ± 0.01	12.26 ± 1.20
16	65 (0)	5 (-1)	2 (1)	0.035 (0)	3.63 ± 0.43	6.22 ± 0.55	0.45 ± 0.09	1.77 ± 0.04	1.95 ± 0.21	0.71 ± 0.05	9.01 ± 0.91
17	65 (0)	90 (1)	2 (1)	0.035 (0)	3.59 ± 0.46	7.46 ± 0.06	0.56 ± 0.02	1.98 ± 0.13	2.26 ± 0.28	0.77 ± 0.03	10.54 ± 1.16
18	65 (0)	47.5 (0)	1 (0)	0.035 (0)	4.13 ± 0.39	7.29 ± 0.03	0.55 ± 0.00	1.81 ± 0.10	2.19 ± 0.18	0.78 ± 0.00	10.88 ± 1.24
19	30 (-1)	47.5 (0)	0 (-1)	0.035 (0)	4.20 ± 0.30	7.56 ± 0.13	0.64 ± 0.01	1.77 ± 0.17	2.53 ± 0.16	0.88 ± 0.05	12.48 ± 1.20
20	100 (1)	47.5 (0)	0 (-1)	0.035 (0)	7.10 ± 0.74	12.38 ± 0.79	1.28 ± 0.18	2.94 ± 0.00	3.99 ± 0.70	1.53 ± 0.00	18.31 ± 2.14
21	30 (-1)	47.5 (0)	2 (1)	0.035 (0)	3.63 ± 0.09	6.89 ± 0.61	0.53 ± 0.16	1.68 ± 0.19	2.07 ± 0.37	0.73 ± 0.04	9.24 ± 0.85
22	100 (1)	47.5 (0)	2 (1)	0.035 (0)	6.21 ± 0.39	11.64 ± 0.04	1.00 ± 0.10	2.70 ± 0.01	3.70 ± 0.45	1.19 ± 0.03	15.92 ± 1.23
23	65 (0)	5 (-1)	1 (0)	0.020 (1)	3.46 ± 0.37	6.83 ± 0.73	0.50 ± 0.02	1.68 ± 0.11	1.91 ± 0.93	0.65 ± 0.08	9.61 ± 0.80
24	65 (0)	90 (1)	1 (0)	0.020 (1)	4.06 ± 0.26	8.50 ± 0.46	0.66 ± 0.14	1.93 ± 0.30	2.38 ± 0.49	0.95 ± 0.06	11.41 ± 1.24
25	65 (0)	5 (-1)	1 (0)	0.050 (-1)	3.37 ± 0.27	6.32 ± 0.31	0.57 ± 0.20	1.46 ± 0.09	1.55 ± 1.05	0.58 ± 0.03	9.23 ± 0.71
26	65 (0)	90 (1)	1 (0)	0.050 (-1)	3.01 ± 0.22	5.96 ± 1.24	0.37 ± 0.17	1.39 ± 0.06	1.45 ± 0.00	0.55 ± 0.00	7.80 ± 0.81
27	65 (0)	47.5 (0)	1 (0)	0.035 (0)	4.21 ± 0.04	7.58 ± 0.14	0.53 ± 0.01	1.76 ± 0.09	2.37 ± 0.22	0.76 ± 0.03	10.32 ± 1.28

TPC: total phenolic compounds; TF: total flavonoids; TFL: total flavanols; TPA: total phenolic acids; PAC: total proanthocyanidins; TOD: total *ortho*-diphenols; AC: antioxidant capacity.

of gallic acid equivalents per gram (mg GAE g^{-1}) of dry cocoa shell.

2.5.2. Total flavonoids (TF)

The content was quantified using the aluminum chloride method adapted to micromethod format [21]. Briefly, 100 μL of the sample were mixed with 30 μL of 5% Na_2NO_2, and samples were incubated at room temperature for 5 min. Subsequently, 30 μL of 10% $AlCl_3$ were added, and incubation continued for 6 min. Then, 100 μL of NaOH 2 mol L^{-1} were added, and the solution was homogenized. The absorbance was read at 510 nm. Total flavonoid content was calculated with a calibration curve of quercetin, and the results were expressed as mg of quercetin equivalents per gram (mg QE g^{-1}) of dry cocoa shell.

2.5.3. Total flavanols (TFL)

Total flavanols were determined using the vanillin method adapted to micromethod format [22]. Samples (10 μL) were added to each well, and 50 μL of 8.4 mol L^{-1} vanillin 1% HCl and 250 μL of concentrated HCl were added and incubated at room temperature for 15 min. The absorbance was read at 500 nm, and the concentration of total flavanols was estimated using a standard curve of catechin. The results were expressed as mg of catechin equivalents per gram (mg CE g^{-1}) of dry cocoa shell.

2.5.4. Total proanthocyanidins (PAC)

Total proanthocyanidin content was determined using a

modification of the Bate-Smith method [23]. Briefly, 10 μL of each extract and 1 mL of 0.54 mmol L^{-1} $FeSO_4$ in butanol/ HCl (50:50) were incubated at 90 °C for 1 h. After cooling, the absorbance was measured at 550 nm against a blank prepared in the same way but without heating. Cyanidin chloride was used as a standard to construct the calibration curve. Results were expressed as mg of cyanidin chloride equivalents per gram of dry cocoa shell (mg CCE g^{-1}).

2.5.5. Total phenolic acids (TPA)

For the determination of total phenolic acids, according to Vukic et al.[24], samples (10 μL) were diluted with 50 μL of MilliQ water. 0.1 mol L^{-1} HCl (50 μL) was added before the addition of 50 μL of a sodium molybdate solution. Finally, 100 μL of 0.1 mol L^{-1} NaOH was added. The absorbance was read at 490 nm. Total phenolic acid content was calculated with a calibration curve of caffeic acid, and the results were expressed as mg of caffeic acid equivalents per gram (mg CAE g^{-1}) of dry cocoa shell.

2.5.6. Total ortho-diphenols (TOD)

Ortho-diphenols content was measured using a colorimetric method based on the formation of a metallic complex between *o*-diphenols and sodium molybdate dihydrate in a solution of water:ethanol (1:1 v/v) according to the method proposed by Granato et al. [25]. Samples (50 μL) were mixed with 200 μL of a 0.05 g mL^{-1} $Na_2MoO_4{\cdot}2H_2O$ (EtOH:H_20 1:1 v/v) and let to react for 25 min. The absorbance was recorded at 370 nm, and the concentration of *o*-diphenols was estimated using a standard curve of caffeic acid. The results were expressed as mg of caffeic acid equivalents per gram (mg CAE g^{-1}) of dry cocoa shell.

2.5.7. Assessment of in vitro antioxidant capacity (AC)

The antioxidant capacity of samples was estimated by the $ABTS^{\bullet+}$ assay, as previously described [26]. 2.2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic) acid radical cations ($ABTS^{\bullet+}$) were obtained by reacting ABTS solution with potassium persulfate and stirring it in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted in 5 mmol L^{-1} PBS pH 7.4 to an absorbance of 0.70 ± 0.02 at 734 nm. Samples (30 μL) and diluted $ABTS^{\bullet+}$ solution (270 μL) were mixed. The absorbance of the samples at 734 nm was measured before 10 min of reaction. Calibration curves were constructed using standard solutions of Trolox, and the results were expressed as mg Trolox equivalents per gram (mg TE g^{-1}) of dry cocoa shell.

2.6. Response surface methodology (RSM)

RSM model was constructed was used for modeling the extraction of phenolic compounds from the cocoa shell employing extraction variables [temperature (°C) (X_1), time (min) (X_2), acidity as the percentage of citric acid in water (%) (X_3), and S/L ratio (g mL^{-1}) (X_4)] and response variables (TPC, TF, TFL, PAC, TPA, TOD, AC). The extraction conditions variables were coded according to the following equation:

$$X = \frac{x_i - x_0}{\Delta x}$$

where X is the coded value; x_i, the corresponding actual value; x_0, the real value at the center of the domain; and Δx, the increment of x_i corresponding to a variation of 1 unit of x. The response variables were fitted to the following second-order polynomial model equation, which described the relationship between the responses and the independent variables.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_{ii}^2 + \sum_{i}^{k-1} \sum_{j}^{k} \beta_{ij} X_{ij}$$

where Y was the response variables; X_i and X_j were independent coded variables; β_0 was the constant coefficient; β_i was the linear coefficient; β_{ii} was the quadratic coefficient, and β_{ij} was the cross-product coefficients.

Based on the analysis of variance (ANOVA), the regression coefficients of individual linear, interaction, and quadratic terms were determined. The numerical magnitude of the standardized model coefficients evidenced their significance in the obtained model. Among standardized coefficients, the larger values are more effective. The polynomial equation's fitness to the responses was estimated using the coefficient of determination (R^2). The significance of all the polynomial equation terms was analyzed statistically by analyzing the F-value at $p <$ 0.05. Equations were constructed, selecting the significant ($p < 0.05$) non-standardized coefficients (including non-significant terms if needed to ensure that the model was hierarchical), and their statistical parameters (F-value and R^2) were calculated.

2.7. Artificial neural networks (ANN)

A multilayer perceptron (MLP) based feed-forward ANN was applied for modeling the extraction of phenolic compounds from the cocoa shell. MATLAB version R2020a was used to model the data using ANN. The experimental data was constructed using the regression-based network approach. The Broyden–Fletcher–Goldfarb–Shanno (BFGS) quasi-Newton back-propagation (TRAINBFG) method was selected since it is an efficient training function because it performs non-smooth optimizations and smaller networks [27]. The gradient descent method (LEARNGDM) as the adaptive learning function was used to minimize the mean squared error (MSE) between the network output and the actual error rate [28]. The hyperbolic tangent sigmoid transfer function (TANSIG) and linear transfer function (PURELIN) were used to calculate a layer's output from its net input [29]. All these functions were used to train the neural network and built the best ANN. Multiple feed-forward neural networks were trained and subsequently tested by determining the number of neurons in the hidden layer to select an optimized ANN topology with the lowest RMSE and highest R^2 values. However, the number of epochs (or cycles through the full training dataset) was restricted to a minimum to avoid over-fitting while establishing an optimal topology. Increased epoch numbers may cause model over-fitting issues. The network architecture consisted of an input layer with four neurons (temperature (T), time, (t), acidity, and S/L ratio), one hidden layer with ten neurons, and an output layer with one neuron, which represented each of the response variables (Total Phenolic Compounds, TPC, in Fig. 1**A**). The experimental dataset utilized to create the RSM model was also employed to build the ANN model: 70% (19 points) for network training, 15% (4 points) for validation, and the remaining 15% (4 points) for network testing (Fig. 1**B**). The output responses were generated by passing the weighted sum of input variables to each neuron via an activation function that was generally non-linear and was represented by the hidden layer in the ANN architecture. The interconnected weights were randomly initialized and adjusted to minimize residual errors between the target and the models' actual outputs (Fig. 1**A**). ANN are a complex optimization and simulation instrument that displays great potential due to their robust prediction and estimation proficiency. We identified the optimal number of neurons in the hidden layer through a systematic trial-and-error method using the TPC input. The efficiency of the models was based on the R^2 values. Based on this principle, the best results were obtained with feed-forward network topologies, with three layers: input, output, and one hidden layer, with ten neurons, trained with the back-propagation algorithm. Adding more neurons/layers did not increased the prediction ability of the models significantly, therefore these architectures were then used for all the response variables.

2.8. Comparison of the prediction ability of RSM and ANN

Several statistical parameters, including the coefficient of determination (R^2), the root mean square error (RMSE), and the absolute average deviation (AAD) were calculated for the comparison of estimation capabilities of RSM and ANN, according to the following

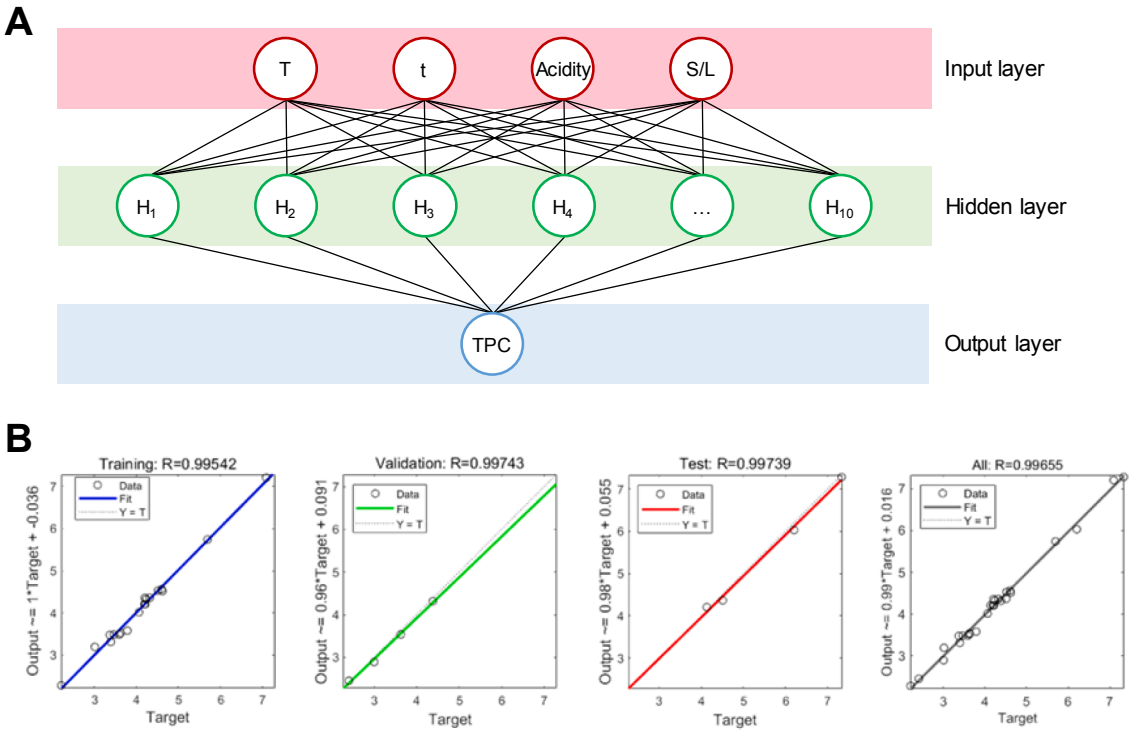

Fig. 1. The topology of the multilayer feed-forward neural network for total phenolic compounds (TPC) (**A**), and scatter plot between experimental and predicted yield by artificial neural networks (ANN) for training, validation, testing, and overall data fitting for TPC (**B**).

equations.

$$R^2 = 1 - \frac{\sum_{i=1}^{n}(Y_{pre} - Y_{exp})^2}{\sum_{i=1}^{n}(Y_m - Y_{exp})^2}$$

$$\mathrm{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}(Y_{pre} - Y_{exp})^2}{n}}$$

$$\mathrm{ADD}(\%) = \left(\frac{\sum_{i=1}^{n}\left(\left|Y_{pre} - Y_{exp}\right|/Y_{pre}\right)^2}{n}\right)\hat{\mathrm{A}}\cdot 100$$

where Y_{pre} is the predicted response variable (by either RSM or ANN), Y_{exp} is the observed response variable, Y_m is the average response variable, and n is the number of experiments.

2.9. Validation of the model

The extraction conditions were optimized for the maximum yield of phenolic compounds [total phenolic compounds (TPC), total flavonoids (TF), total flavanols (TFL), total proanthocyanidins (PAC), total phenolic acid (TPA), and total *ortho*-diphenols (TOD)] and the antioxidant capacity (AC) by employing RSM. Then, the responses were determined under the optimal and suboptimal extraction conditions. Finally, the experimental values were compared with predicted values (from RSM and ANN) based on the coefficient of variation, CV (%), to determine the validity of the model. The UPLC-ESI-MS/MS profiles of phenolic compounds were also determined at the optimized conditions.

2.10. UPLC-ESI-MS/MS analysis of phenolic compounds

The targeted phenolic compounds were analyzed using UPLC-ESI–MS/MS following the method described by Sanchez-Patán et al. [30]. Dissolved extracts were filtered (0.22 μm), and internal standard 4-hydroxybenzoic-2,3,5,6-d4 acid solution (Sigma-Aldrich, St. Louis, MO) was added to the samples in a proportion 1:5 (v/v). Data were collected under the multiple reaction monitoring mode for the quantification, tracking the specific transition of parent and product ions for each compound. The ESI was operated in negative ionization mode. All phenolics were quantified using the calibration curves of their corresponding standards. Injections were carried out in triplicate ($n = 3$).

2.11. Statistical analysis

Statistical analysis of the experimental results was performed using the statistical programs Design Expert 11, MATLAB version R2020a, and SPSS 24.0. All data are presented as the mean ± standard deviation (SD) of at least three independent experiments (n = 3), where each experiment had a minimum of three replicates for each sample. For comparisons between samples, data were analyzed by one-way analysis of variance (ANOVA) and *post hoc* Tukey test. Differences were considered significant at $p < 0.05$. The experiments and optimization's statistical design following the RSM were performed with Design Expert, obtaining the regression equations for the evaluated responses, the contribution and significance of each parameter, the response surface plots, and the optimal and suboptimal for extraction. ANN were constructed, tested, and validated using MATLAB. The chemometric analysis was carried out to describe the phenolic extracts better. Principal component analysis (PCA) was used to classify samples according to their phenolic composition. Partial Least Squares Analysis (PLSA) was used to rank the spectrophotometric and chromatographic parameters according to their importance (Variable importance in projection (VIP) scores) on the variability among extracts. An agglomerative hierarchical cluster analysis coupled to heatmap was generated to depict the variability among extracts. Principal Components Regression (PCR) and Principal Least Squares Regression (PLS-R) were constructed to evaluate individual phenolic compounds' influence on the *in vitro* antioxidant capacity. Pearson's linear correlations were performed to study the association between spectrophotometric results and individual phenolic compounds' concentration.

3. Results and discussion

3.1. Fitting of the RSM and ANN models

The experimental parameters and results of 27 extraction conditions are presented in Table 1. The RSM optimization of the aqueous extraction was carried out by using quadratic polynomial equations. Response surface 3D plots were generated for each response variable (TPC, TF, TFL, PAC, TPA, TOD, and AC) (Fig. 2), whose regression equations and statistical parameters (ANOVA) are presented in Table 2. All the response variables were adjusted to second-order polynomial equations, which explained the variation in the different responses as a function of the extraction parameters. The non-significant terms ($p > 0.05$) were not considered to improve the models' fitting and prediction. The *p*-values were used to evaluate the significance of each coefficient. Low *p*-values, below 0.05, 0.01, and 0.001, indicated that the model terms were significant, highly significant, and remarkably significant, respectively, and *p*-values greater than 0.05 indicate that the model terms were not significant [31].

The equations obtained for each response are shown in the following sections. The temperature was the only parameter affecting all the responses significantly both linearly ($X1$) and quadratically X_1^2 (Table 2). S/L ratio (X_4) significantly affected all response variable linearly. Extraction acidity (X_3) had significant ($p < 0.05$) linear effects on most of responses. Extraction time (X_2) presented no effects ($p > 0.05$) but for TPA. The interactive effects temperature-time (X_{12}) was only significant for the responses of TPC. Determination coefficients (R^2) values were between 0.8327 and 0.9256, which implied that the fitted model could explain at least 84.2% of the variations. The adjusted determination coefficients (Adj. R^2) proved the high correlation between the experimental and predicted values. ANOVA result for each response variable indicated that at least one of the model parameters could explain the experimental variation for response variables (Table 2).

Mathematical RSM models could be validated as they exhibited significant *F*-values. The model was remarkably significant ($p < 0.001$) for TPC and TPA, and highly significant ($p < 0.01$) for TF, TFL, PAC, TOD, and AC. Table 3 shows the three statistical parameters measuring the predictive ability of RSM models. The lower RSME and ADD are, the better is the fit between experimental and prettied values. Hence, it is observed that the complete RSM models exhibited higher R^2 values and lower RSME and ADD than the RSM $_{ST}$ models for all the response variables.

ANN was used to predict non-linear associations between the extraction parameters (X_1, X_2, X_3, and X_4) and response variables (TPC, TF, TFL, PAC, TPA, TOD, and AC). The correlation coefficient between experimental response variables and the ANN's predicted values was higher than 0.9 for training, validation, testing, and overall fitting for all variables. The ANN models presented higher R^2 values than RSM and RSM $_{ST}$ (without non-significant terms), proving the ANN model's superiority in terms of predictive and estimation capabilities (Table 3).

The results showed that ANN had a significantly higher predictive ability than RSM since it can approximate non-linear systems. In contrast, RSM is useful whenever the extraction nature follows a second-order polynomial regression. Besides, ANN do not depend on the experimental design and are more effective at calculating multiple responses in a single run. In contrast, RSM needs several runs under a standard experimental design for multi-response optimization. ANN models' generation requires many iterative calculations, whereas RSM only needs a single step for analysis. The ANN model may need a high computational time to create a design and is more costly than RSM. ANN is also useful as it is flexible toward the addition of new experimental data for model generation.

3.2. Effect of HAE parameters on total phenolic compounds

The TPC in the cocoa shell extracts varied from 2.21 to 7.33 mg g^{-1} (Table 1). The highest TPC values were found in extractions with high temperature (100 °C) and low acidity (1%) combinations. The model for

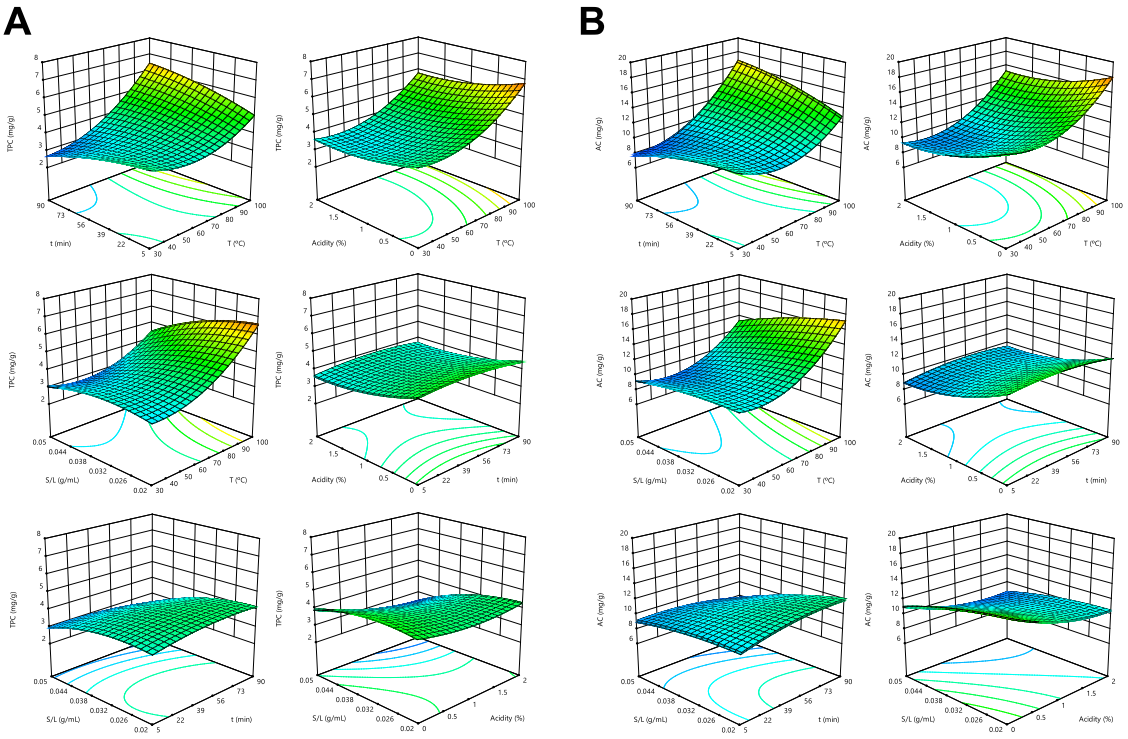

Fig. 2. Representative 3D plots depicting the behavior of response variables (**A**) Total phenolic compounds (TPC) and (**B**) antioxidant capacity (AC) under different conditions of extraction. Responses (mg g^{-1}) are plotted against two paired variables: T (temperature in °C), acidity (% citric acid), t (time in min), and S/L (solid-to-liquid ratio in g mL^{-1}).

Table 2
Regression coefficient (β), contribution, coefficient of determination (R^2 and Adj. R^2), and *F*-test value of the predicted second-order polynomial models for the different families of phenolic compounds and the antioxidant capacity.

	TPC		TF		TFL		PAC		TPA		TOD		AC	
	β	Contrib. (%)	β	Contrib. (%)	β	Contrib. (%)	β	Contrib. (%)	β	Contrib. (%)	β	Contrib. (%)	β	Contrib. (%)
Constant (X_0)	4.179***		7.390***		0.516***		1.724***		2.181***		0.783***		10.439***	
Linear		61.2***		65.2***		54.7**		57.2**		66.1***		59.2**		62.33***
X_1	1.165***	42.7***	2.034***	48.9***	0.247***	37.7***	0.419***	45.0***	0.729***	43.2***	0.2246***	28.2**	2.878***	41.1***
X_2	−0.032	0.0	0.474	2.7	0.012	0.1	0.058	0.9	0.193	3.0*	0.033	0.5	0.207	0.2
X_3	−0.419*	5.5*	−0.481	2.7	−0.109*	7.3*	−0.108	3.0	−0.276	6.2*	−0.153**	10.9**	−1.609**	12.8**
X_4	−0.642**	13.0**	−0.963**	11.0**	−0.124*	9.6*	−0.181*	8.7**	−0.408**	13.6**	−0.204**	19.5**	−1.287*	8.2*
Quadratic		20.7**		16.5**		22.3*		22.8*		23.1**		19.4**		21.0*
X_1^2	0.866**	13.7**	1.163**	10.7**	0.250**	17.8**	0.330**	13.6**	0.499**	10.2**	0.223**	8.4**	2.338**	15.2**
X_2^2	−0.225	1.1	−0.561	2.0	−0.039	1.9	−0.092	2.7	−0.188	3.1	−0.045	3.0	−0.759	2.3
X_3^2	0.311	2.1	0.494	2.6	0.096	1.9	0.217	6.4*	0.435*	9.3*	0.196**	7.1**	1.046	3.3
X_4^2	−0.405*	3.8*	−0.494	1.3	0.052	0.7	0.021	0.1	−0.127	0.6	0.065	0.9	−0.329	0.2
Interaction		9.6		6.1		8.7		6.3		3.4		4.7		4.7
X_{12}	0.733*	5.6*	0.929	3.4	0.121	3.0	0.151	2.0	0.268	2.0	0.071	0.8	1.232	2.5
X_{13}	−0.078	0.1	−0.018	0.0	−0.041	0.3	−0.036	0.1	0.040	0.0	−0.047	0.3	0.212	0.1
X_{14}	−0.460	2.2	−0.621	1.5	−0.132	3.6	−0.204	3.6	−0.118	0.4	−0.090	1.3	−0.659	0.7
X_{23}	0.061	0.0	0.035	0.0	0.016	0.1	−0.021	0.0	−0.006	0.0	0.004	0.0	0.237	0.1
X_{24}	−0.238	0.6	−0.508	1.0	−0.091	1.7	−0.077	0.5	−0.141	0.5	−0.081	1.0	−0.807	1.1
X_{34}	−0.323	1.1	−0.239	0.2	−0.019	0.1	0.042	0.2	−0.134	0.5	−0.090	1.3	0.369	0.2
Model		91.5***		87.9**		85.8*		86.3**		92.6**		83.3**		88.1**
R^2	0.9148		0.8787		0.8577		0.8629		0.9256		0.8327		0.8806	
Adj. R^2	0.8153		0.7372		0.6916		0.7030		0.8387		0.6375		0.7414	
F value	9.20***		6.21**		5.17**		5.40**		10.66***		4.27**		6.32**	

X_1: extraction temperature (°C), X_2: extraction time (min), X_3: acidity (% citric acid), X_4: solid-to-liquid ratio (g mL^{-1}), R^2: Coefficient of determination. Level of significance: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. TPC: total phenolic compounds; TF: total flavonoids; TFL: total flavanols; TPA: total phenolic acids; PAC: total proanthocyanidins; TOD: total *ortho*-diphenols; AC: antioxidant capacity.

Table 3
Comparison of optimization and prediction capabilities of response surface methodology (RSM) and ANN for the extraction of total phenolic compounds (TPC), total flavonoids (TF), total flavanols (TFL), total proanthocyanidins (PAC), total phenolic acids (TPA), total *o*-diphenols (TOD), and the *in vitro* antioxidant capacity (AC) measured by the ABTS method.

Response	Modeling method	R^2	RSME	AAD (%)
TPC	RSM	0.9148	0.35	6.37
	RSM $_{ST}$	0.8436	0.47	7.73
	ANN	0.9930	0.10	2.17
TF	RSM	0.8787	0.68	6.56
	RSM $_{ST}$	0.7049	1.05	10.06
	ANN	0.9889	0.20	2.25
TFL	RSM	0.8577	0.10	1.07
	RSM $_{ST}$	0.7246	0.14	1.33
	ANN	0.9891	0.03	0.33
PAC	RSM	0.8629	0.15	5.98
	RSM $_{ST}$	0.7814	0.19	7.16
	ANN	0.9889	0.04	1.68
TPA	RSM	0.9256	0.20	6.75
	RSM $_{ST}$	0.8770	0.26	8.69
	ANN	0.9800	0.10	2.79
TOD	RSM	0.8327	0.13	10.25
	RSM $_{ST}$	0.7620	0.15	11.32
	ANN	0.9931	0.03	2.33
AC	RSM	0.8806	0.10	7.00
	RSM $_{ST}$	0.8187	0.13	8.31
	ANN	0.9887	0.03	2.15

RSM $_{ST}$: RSM simplified models including only significant ($p < 0.05$) terms.

TPC could be validated by displaying a remarkably significant ($p < 0.001$) *F*-value (9.20) (Table 2). The model was fitted to the spatial influence of the extraction variables with a good prediction ($R^2 = 0.9148$). ANOVA exhibited a significant ($p < 0.05$) linear (X_1, X_3, and X_4), quadratic (X_1^2 and X_4^2), and interactive (X_{12}) contribution on TPC content. Conforming to the regression coefficients (β) and contributions, extraction temperature (X_1) exhibited the highest positive effect, followed by the S/L ratio (X_4). The fitted second-order polynomial equation after removing the non-significant variables was described as follows:

$$Y_{TPC} = 5.5 - 7.4{\cdot}10^{-2}x_1 - 3.3{\cdot}10^{-2}x_2 - 4.2{\cdot}10^{-1}x_3 + 1.1{\cdot}10^{2}x_4 + 6.4{\cdot}10^{-4}x_1^2 - 2.2{\cdot}10^{3}x_4^2 + 4.9{\cdot}10^{-4}x_{12}$$

Thus, the significance of the model increased (*F*-value = 14.64, $p < 0.0001$, $R^2 = 0.8436$) by eliminating the variables with non-significant ($p > 0.05$) effects, despite the decrease on the R^2-value. Increasing temperatures, times, and volumes of solvent (Fig. 2**A**) promoted the extraction, therefore, reaching higher phenolic content recovery due to the enhancement of phytochemicals solubility and diffusion from the cocoa shell cell walls. Reductions in the S/L ratio promote phenolic extraction from plant samples by diminishing the saturation effects due to the high content of phenolic compounds. However, the increase of citric acid concentration did not show an improving effect on TPC extraction. Against expectations, increasing acidity lead to reduced extraction of TPC.

3.3. *Effect of HAE parameters on total flavonoids*

The TF in the cocoa shell extracts oscillated from 4.93 to 12.69 mg g^{-1} (Table 1). Temperature of 100 °C and 1% citric acid promoted a better extraction of flavonoids. The RSM model for TF extraction could be validated by displaying a highly significant ($p < 0.01$) *F*-value (6.21) and a good determination coefficient was ($R^2 = 0.8787$) (Table 2). ANOVA showed a significant ($p < 0.01$) linear (X_1 and X_4) and quadratic (X_1^2) effect on TF content. From regression coefficients (β) and contributions, extraction temperature (X_1) showed the highest positive impact, followed by its quadratic effect (X_1^2) and the S/L ratio (X_4). The equation obtained after removing the non-significant variables was:

$$Y_{TF} = 10.0 - 7.7{\cdot}10^{-2}x_1 + 6.4{\cdot}10^{1}x_4 + 1.0{\cdot}10^{-3}x_1^2$$

Then, the new statistical parameters for the model were *F*-value = 18.32, $p < 0.0001$, and $R^2 = 0.7049$. A similar trend to total phenolic compounds was shown for total flavonoids; maximum values were obtained by increasing temperatures that solubilize these components, accompanied by a low S/L ratio. Again, it was observed that acidity had no impact on TF extraction. Previous studies demonstrated a positive influence of this parameter on flavonoid extraction [32]. Differences in the raw material matrix and the selected acid might be responsible for the divergent effect observed.

3.4. Effect of HAE parameters on total flavanols

The highest concentrations of flavanols (1.55 mg g^{-1}) were observed when the temperature was set to 100 °C, and the extraction was performed at 1% citric acid addition (Table 1). The significant ($p < 0.01$) *F*-value (5.17) allowed to validate the model for TFL, showing a good fitting determined by the determination coefficient ($R^2 = 0.8577$). The content of TFL was significantly influenced ($p < 0.05$) linearly (X_1, X_3, and X_4) and quadratically (X_1^2) (Table 2). Extraction temperature (X_1) and the S/L ratio (X_4) showed the main contribution to flavanols extraction (37.7 and 9.6%, respectively), wielding acidity in a secondary role. The equation obtained after removing the non-significant variables was:

$$Y_{TFL} = 1.3 - 1.7{\cdot}10^{-2}x_1 - 1.1{\cdot}10^{-2}x_3 - 8.3x_4 + 1.9{\cdot}10^{-4}x_1^2$$

Then, the RSM model obtained eliminating the non-significant terms from the equation exhibited an improved and more significant *F*-value (14.47, $p < 0.0001$) and reduced $R^2 = 0.7246$. Hence, the recovery of total flavanols seemed to improve by increasing temperatures, which promote the solubility of TFL content. Okiyama et al. [33] evaluated the kinetic influence of temperature on the extraction of flavanols from the cocoa shell over time. In general, flavanols extraction increased over time at high temperatures, although some dimers may degrade after prolonged exposition to temperature. Additionally, lower citric acid concentrations and higher volumes of solvent were conducted to maximum TFL levels in extraction.

3.5. Effect of HAE parameters on total proanthocyanins

The extraction of PAC showed content between 1.39 and 3.10 mg g^{-1}. The best extractions were obtained using high temperature, medium acidity, and low S/L ratios (Table 1). A significant *F*-value (5.40, $p < 0.01$) and a high determination coefficient ($R^2 = 0.8629$) allowed us to validate the fitting of the mathematical model for PAC extraction, explaining 86.3% of the variability. PAC extraction was mainly influenced by temperature, with both linear (X_1) and quadratic (X_1^2) effects. After removing the non-significant variables, the significance of the RSM model increased (*F*-value = 15.02, $p < 0.0001$, $R^2 = 0.7814$) and the equation obtained was:

$$Y_{PAC} = 2.9 - 2.6{\cdot}10^{-2}x_1 - 6.0{\cdot}10^{-1}x_2 + 1.2{\cdot}10^{1}x_4 + 2.9{\cdot}10^{-4}x_1^2 + 2.4{\cdot}10^{-1}x_2^2$$

Increasing temperatures stimulated the extraction to reach total proanthocyanins content recovery. Previous research demonstrated that procyanidins could degrade under high-temperature exposition during extraction with pressurized liquids [33]. HAE did not exert these effects; solvent and pressure could be responsible for a higher procyanidin loss. Moreover, maximum PAC levels were mainly achieved by increasing volumes of solvent, which minimized saturation effects.

3.6. Effect of HAE parameters on total phenolic acids

TPA extracted the cocoa shell varied from 1.23 to 4.17 mg g^{-1}. The highest value was obtained using a S/L ratio of 0.02 g mL^{-1} at 100 °C (Table 1). The high significant ($p < 0.001$) *F*-value (10.66) allowed us to validate the model for TPA with a high determination coefficient ($R^2 = 0.9256$). ANOVA showed a remarkably significant ($p < 0.01$) linear (X_1 and X_4), quadratic (X_1^2 and X_3^2), but not interactive influence on TPA content (Table 2). The equation obtained after removing the non-significant variables was:

$$Y_{TPA} = 4.1 - 4.9{\cdot}10^{-2}x_1 - 6.3{\cdot}10^{-3}x_2 - 1.5x_3 + 2.2{\cdot}10^{1}x_4 + 5.3{\cdot}10^{-4}x_1^2 + 5.9{\cdot}10^{-1}x_3^2$$

Therefore, the new statistical parameter for the simplified model were: *F*-value = 15.58, $p < 0.0001$, $R^2 = 0.8238$. TPA extraction's main variable was the temperature (X_1), followed by the S/L ratio (X_4). Likewise, temperature and acidity affected extraction in a quadratic manner (X_1^2 and X_3^2) and significant contributions (10.2 and 9.3%).

3.7. Effect of HAE parameters on total ortho-diphenols

TOD's highest content extracted from the cocoa shell was 1.68 mg g^{-1}, using a S/L ratio of 0.02 g mL^{-1}, with 1% of citric acid at 100 °C, for 47.5 min. (Table 1). The content of TOD was influenced significantly ($p < 0.01$) linearly (X_1, X_3, and X_4), quadratically (X_1^2 and X_3^2) (Table 2). Analogously to TPA, the extraction of TOD was mainly influenced by the temperature (X_1), the S/L ratio (X_4), and acidity (X_3). In addition, temperature and acidity affected extraction in a quadratic manner. This model could explain 83.3% of the variability with a good ($R^2 = 0.8327$) and significant fitting (*F*-value model = 4.27; $p < 0.01$) (Table 1). Eliminating the non-significant terms diminished R^2 to 0.7620, increasing the significance of the model (*F*-value = 13.45, $p < 0.0001$). Thus, the equation obtained was:

$$Y_{TOD} = 1.9 - 1.1{\cdot}10^{-2}x_1 - 5.3{\cdot}10^{-1}x_2 - 1.6{\cdot}10^{-2}x_3 - 2.6x_4 + 1.8{\cdot}10^{-4}x_1^2 + 1.9{\cdot}10^{-1}x_3^2$$

The recovery of total *o*-diphenols seemed to improve by increasing temperatures accompanied by high volumes of solvent, which promote the solubility of TOD content.

3.8. Effect of HAE parameters on the in vitro antioxidant capacity

The AC values ranged from 7.05 to 19.45 mg g^{-1} (Table 1). ANOVA exhibited that the AC of the cocoa shell extract was influenced significantly ($p < 0.01$) linear (X_1, X_3, and X_4) and quadratically (X_1^2) (Table 2). In this case, temperature and acidity played a crucial role in the AC of the extract. The model could explain 88.1% of the variability with proper fitting ($R^2 = 0.8806$) and presented a significant value of lack of fit (*F*-value = 6.32). This model's good fitting could better explain the variability of extraction than previous optimization studies [34]. The equation obtained after removing the non-significant variables was:

$$Y_{AC} = 17.8 + 1.7{\cdot}10^{-1}x_1 - 1.6x_3 - 8.6{\cdot}10^{1}x_4 + 1.9{\cdot}10^{-3}x_1^2$$

The non-significant terms' removal lead to the following statistical parameters: *F*-value = 18.71 ($p < 0.0001$) and $R^2 = 0.7729$. From the model, the antioxidant capacity would rise with increasing temperatures, as shown in Fig. 2**B**.

3.9. Assessment and experimental validation of optimal conditions

The optimal conditions were obtained by maximizing the desirability of the responses. These optimal conditions, which produced a maximum yield of phenolic compounds, were subsequently employed for the experimental extraction of phenolics. Their responses were assessed and validated according to the procedure mentioned above. RSM and ANN-predicted values and experimental results obtained after extraction under optimal and suboptimal conditions are shown in Table 3. The optimal conditions for TPC, TF, TFL, PAC, TPA, TOD, and AC were

achieved at 100 °C, 90 min, 0% citric acid, and 0.02 g mL^{-1} S/L ratio. Furthermore, other suboptimal conditions were obtained, which reduced the extraction time (79, 51, and 39 min). Under these optimal and suboptimal conditions, the experimental values matched with the RSM-predicted ones (Table 3). The experimental values agreed with the RSM-theoretical values, with CV ranging from 0.1 to 10.6%.

The aqueous extracts produced using optimal and suboptimal conditions only significantly ($p < 0.05$) differed in their TPC, TF, and TFL contents; PAC, TPA, TOD, and AC of optimal and suboptimal (t ≥ 51 min) extracts were statistically ($p < 0.05$) similar. It should be more profitable for the food industry to apply those suboptimal conditions since bioactive compounds' content did not vary considerably. The time required for the aqueous extraction could be sharply reduced (79, 51, and 39 min). Extending the time over 90 min may also increase the extraction of phenolic compounds from the cocoa shell and use higher temperatures (by applying pressure to maintain the solvent's liquid state). Compared to the organic solvent extraction (i.e., methanolic extraction), aqueous extraction at optimal conditions could extract 36.8–89.5% of the free phenolic fraction of the cocoa shell. Nonetheless, this aqueous extraction could not separate the bound phenolic fraction tightly linked to the cell wall matrix of the cocoa shell [35]. This fraction could be of great interest, considering the content of total bound phenolic compounds is similar to free ones' content (Table 4). Thus, after the aqueous extraction, the residue could be used as a source of antioxidant dietary fiber or treated to obtain phenolic compounds, further applying more sophisticated extraction methods (such as utilizing enzymes to hydrolyze fibers or strong alkali medium) [35,36].

The highest TPC values obtained in aqueous extraction (7.99 mg g^{-1}) are lower than those obtained with supercritical CO_2 extraction [37]. It should be noted that other studies determined a higher content of total phenolic compounds in other cocoa by-products, such as the cocoa pod husk [38]. The maximum concentration found for total flavanols (1.79 mg/g) was similar to that obtained in other aqueous extractions from the cocoa shell; however, the extraction of proanthocyanidins was higher [39]. In contrast to previous studies, antioxidant capacity in the obtained extracts was enhanced by the use of water at high temperatures and low acidity; other authors pointed out that antioxidant capacity could be higher using temperatures below 50 °C and low pH values [40].

3.10. UPLC-ESI-MS/MS phenolic compound profile and chemometric analysis

The UPLC-ESI-MS/MS phenolic profile (Table 5) allowed a better understanding of the composition of the aqueous extracts from the cocoa shell. The optimum extract was mainly composed of protocatechuic acid (128.7 μg g^{-1}) followed by epicatechin, procyanidin B2, and catechin ranging from 33.9 to 37.5 μg g^{-1} (Fig. 3**A**). Reducing extraction times resulted in a decrease in all phenolic compounds concentration; some of them were under the limit of detection or not present. The content of these compounds in the extract obtained at optimal conditions (100 °C, 90 min, 0% citric acid, and 0.02 g mL^{-1} S/L ratio) was significantly different from those of the other extracts (79, 51, and 39 min). Interestingly, time (X_2), which had not a significant impact on the modeling of phenolic compounds extraction, exhibited a positive effect on the recovery of these compounds of great interest for health. Besides, most compounds were found in lower concentrations in the aqueous extracts obtained under optimal conditions than using organic solvents to extract the free phenolic fraction. However, the content of mono- and dimeric proanthocyanidins, catechin, epicatechin, procyanidin B1, and procyanidin B2 was higher in the aqueous extracts than in the methanolic one. These results suggested a possible degradation of oligo- and polymeric procyanidins into mono- and dimeric ones, as reported by De Taeye et al. [41] in cocoa, proposing the thermal degradation of procyanidin C1 leading to procyanidin B2 and (−)-epicatechin, which can epimerize yielding (−)-catechin and epimers of procyanidin B2. Protocatechuic acid was the primary hydroxybenzoic acid found in the cocoa shell and

Table 4
Validation of predicted values at optimal conditions of aqueous extraction and comparison with organic solvent extraction of the phenolic compounds from the cocoa shell. †

Response (mg g^{-1})	Optimal conditions aqueous extraction								Organic solvent extraction		
	100 °C, **90 min**, 0% acid, 0.02 g mL^{-1}			100 °C, **79 min**, 0% acid, 0.02 g mL^{-1}			100 °C, **51 min**, 0% acid, 0.02 g mL^{-1}			100 °C, **39 min**, 0% acid, 0.02 g mL^{-1}			MeOH: H_2O	NaOH-AcEt	Σ
	Predicted (CV, %)		Experimental	Predicted (CV, %)		Experimental	Predicted (CV, %)		Experimental	Predicted (CV, %)		Experimental	*Free phenolics*	*Bound phenolics*	*Total phenolics*
	RSM	ANN		RSM	ANN		RSM	ANN		RSM	ANN				
TPC	7.81 (1.6)	7.54 (4.1)	7.99 ± 0.39^{b}	7.68 (2.0)	7.58 (1.1)	7.47 ± 0.05^{a}	7.24 (3.1)	8.18 (5.6)	7.56 ± 0.29^{ab}	6.97 (7.2)	8.48 (6.6)	7.72 ± 0.51^{b}	16.52 ± 1.85^{c}	17.18 ± 1.70^{c}	33.70
TF	14.07 (1.5)	14.46 (3.4)	13.77 ± 0.35^{c}	13.77 (4.8)	13.48 (3.3)	12.87 ± 0.10^{a}	12.76 (0.8)	9.85 (19.0)	12.91 ± 0.57^{ab}	12.20 (5.2)	7.81 (36.0)	13.13 ± 1.11^{b}	37.41 ± 1.70^{d}	36.49 ± 1.98^{d}	73.90
TFL	1.72 (2.8)	1.34 (20.2)	1.79 ± 0.06^{b}	1.68 (0.8)	1.37 (13.3)	1.66 ± 0.06^{a}	1.56 (2.7)	1.44 (8.2)	1.62 ± 0.06^{a}	1.51 (2.8)	1.57 (5.1)	1.57 ± 0.06^{a}	2.00 ± 0.42^{b}	3.32 ± 0.50^{c}	5.33
PAC	3.45 (10.6)	3.49 (9.9)	4.01 ± 0.27^{ab}	3.42 (6.1)	3.71 (0.5)	3.73 ± 0.10^{a}	3.26 (7.4)	3.82 (3.9)	3.62 ± 0.02^{a}	3.17 (7.8)	3.59 (1.0)	3.54 ± 0.13^{a}	5.04 ± 0.51^{c}	4.56 ± 0.55^{bc}	9.60
TPA	4.77 (0.7)	3.48 (21.4)	4.72 ± 0.18^{b}	4.69 (4.4)	3.62 (21.0)	4.89 ± 0.32^{b}	4.40 (6.4)	3.81 (16.4)	4.81 ± 0.23^{b}	4.22 (2.3)	3.94 (7.2)	4.36 ± 0.13^{a}	6.95 ± 0.61^{c}	78.23 ± 9.2^{d}	85.18
TOD	2.02 (0.7)	1.35 (27.5)	2.00 ± 0.06^{a}	2.00 (4.8)	1.55 (13.3)	1.87 ± 0.08^{a}	1.90 (3.3)	2.03 (1.3)	1.99 ± 0.12^{a}	1.84 (4.8)	2.16 (6.5)	1.97 ± 0.03^{a}	4.54 ± 0.26^{b}	3.39 ± 0.50^{c}	7.93
AC	21.33 (0.4)	21.19 (0.8)	21.42 ± 1.53^{a}	21.46 (0.5)	21.65 (1.0)	21.36 ± 0.66	20.21 (3.2)	21.36 (0.5)	21.15 ± 1.09^{a}	19.62 (6.5)	20.63 (2.9)	21.48 ± 1.27^{a}	26.19 ± 1.19^{b}	26.94 ± 0.87^{b}	53.13

† Results are reported as mean ± SD ($n = 3$). Mean values followed by different superscript letters significantly differ (among columns) when subjected to ANOVA analysis and Tukey multiple range *post hoc* test ($p < 0.05$).
TPC: total phenolic compounds; TF: total flavonoids; TFL: total flavanols; TPA: total phenolic acids; PAC: total proanthocyanidins; TOD: total *ortho*-diphenols; AC: antioxidant capacity.

Table 5
UPLC-ESI-MS/MS phenolic compounds profile of the cocoa shell extracts obtained by the aqueous extraction using optimal conditions and the organic solvent extraction of the free and bound phenolic fractions. †

Compound (μg g^{-1})	R_t (min)	Mass spectral data		Optimal conditions aqueous extraction				Organic solvent extraction		
		$[M-H]^-$ (*m/z*)	MS^2 (*m/z*)	100 °C, 0% citric acid, 0.02 g mL^{-1}				MeOH: H_2O *Free phenolics*	NaOH-AcEt *Bound phenolics*	Σ *Total phenolics*
				90 min	79 min	51 min	39 min			
Hydroxybenzoic acids										
Gallic acid	1.73	169	125	3.24 ± 0.07^{b}	–	–	–	6.03 ± 0.94^{c}	2.32 ± 0.32^{a}	8.35
Protocatechuic acid	3.34	153	109	128.70 ± 12.61^{b}	94.34 ± 11.11^{a}	90.24 ± 5.47^{a}	88.50 ± 5.05^{a}	137.88 ± 11.36^{b}	141.49 ± 10.65^{b}	279.36
3-*O*-metylgallic acid	4.22	183	168	–	–	–	–	1.20 ± 0.01	–	1.20
4-hydroxybenzoic acid	4.43	137	93	11.84 ± 1.57^{b}	9.03 ± 0.89^{b}	10.34 ± 1.70^{b}	9.75 ± 0.33^{b}	20.97 ± 1.40^{c}	3.67 ± 0.91^{a}	24.65
Vanillic acid	5.43	167	152	–	–	–	–	14.64 ± 1.79	–	14.64
Syringic acid	5.96	197	182	–	–	–	–	1.73 ± 0.07	–	1.73
Salicylic acid	8.96	137	93	0.55 ± 0.07^{a}	–	–	–	0.73 ± 0.05^{b}	–	0.73
Hydroxycinnamic acids										
Caffeic acid	5.48	179	135	0.32 ± 0.10^{a}	–	–	–	0.84 ± 0.12^{b}	1.02 ± 0.09^{b}	1.86
p-coumaric acid	6.81	163	119	0.71 ± 0.11^{a}	–	–	–	0.78 ± 0.15^{a}	2.38 ± 0.40^{b}	3.16
Ferulic acid	7.81	193	134	–	–	–	–	–	1.57 ± 0.38	1.57
Mandelic acids										
Mandelic acid	4.63	151	107	1.89 ± 0.28^{a}	2.15 ± 0.43^{a}	2.08 ± 0.32^{a}	1.83 ± 0.18^{a}	–	–	–
Phenylacetic acids										
3,4-dihydroxyphenylacetic acid	4.18	167	123	4.38 ± 0.83^{a}	3.04 ± 0.84^{a}	3.15 ± 0.40^{a}	3.17 ± 0.25^{a}	–	–	–
4-hydroxyphenylacetic acid	5.22	151	107	8.20 ± 0.73^{c}	6.89 ± 0.49^{b}	5.26 ± 0.10^{a}	5.57 ± 0.92^{ab}	–	–	–
Flavan-3-ols: monomers										
(+)-catechin	5.80	289	245	33.94 ± 2.71^{d}	3.09 ± 0.27^{c}	1.78 ± 0.63^{b}	2.44 ± 0.08^{b}	0.24 ± 0.04^{a}	31.76 ± 3.37^{d}	32.00
(-)-epicatechin	6.27	289	245	37.54 ± 2.33^{d}	8.37 ± 0.34^{a}	7.82 ± 0.18^{a}	7.70 ± 0.51^{a}	14.15 ± 0.93^{b}	29.96 ± 2.05^{c}	44.12
Flavan-3-ols: dimers										
Procyanidin B1	4.90	577	289	14.13 ± 1.31^{c}	4.74 ± 0.65^{b}	1.63 ± 0.08^{a}	–	–	12.51 ± 1.41^{c}	12.51
Procyanidin B2	5.93	577	289	37.16 ± 1.93^{d}	10.72 ± 1.22^{c}	10.21 ± 0.87^{c}	9.99 ± 0.79^{bc}	6.34 ± 0.54^{a}	8.54 ± 0.71^{b}	14.88
Procyanidin A2	9.07	577	289	–	–	–	–	–	4.64 ± 0.91	4.64
Flavonols										
Quercetin-3-*O*-galactoside	8.34	463	301	1.57 ± 0.06^{c}	0.64 ± 0.02^{a}	0.64 ± 0.01^{a}	0.82 ± 0.06^{b}	2.08 ± 0.18^{d}	–	2.08
Quercetin-3-*O*-glucoside	8.65	463	301	1.88 ± 0.13^{d}	0.89 ± 0.12^{b}	0.78 ± 0.09^{a}	1.18 ± 0.07^{c}	1.63 ± 0.16^{d}	–	1.63

† Results are reported as mean ± SD ($n = 3$). Mean values followed by different superscript letters significantly differ (among columns) when subjected to ANOVA analysis and Tukey multiple range *post hoc* test ($p < 0.05$).

bean [42,43]. The high content of protocatechuic acid found under optimal extraction conditions was well correlated with the previous determinations using colorimetric methods ($r = 0.9742, p < 0.001$) since TPA content was higher than that of other families (Table 4). The UPLC-MS/MS profile also revealed that mono- and dimeric flavanols were found in significant concentrations in the extracts, being procyanidin B2, (-)-epicatechin, and (+)-catechin the main ones (Fig. 3**A**).

Cocoa bean primary polyphenols are, similarly, monomeric flavanols such as (−)-epicatechin, (+)-catechin, their dimers procyanidins B2 and B1, polymeric flavanols, and phenolic acids, mainly protocatechuic acid [42]. PCA (Fig. 3**B**) revealed the intrinsic grouping among samples. PCA extracted six factors or principal components (PCs) to explain the phytochemical variability among samples. The three first PCs explained 96.9% of the variability; PC1 and PC2 represented 64.0 and 18.2% of the whole variability. PC1 positively correlated with all the *in vitro* determinations (TPC, TF, TFL, PAC, TPA, TOD, and AC) and most compounds measured by UPLC-MS/MS, except for mandelic acid and the phenylacetic acids. PC2 showed association with the content of procyanidins and PC3 with the concentration of flavonols. Upon clustering, the optimum condition was grouped with the sample of bound phenolics due to their similar content in the main components (protocatechuic acid and flavanols). In contrast, the three other aqueous extraction conditions were grouped together. Free and total phenolics were depicted separately, demonstrating that neither the aqueous extraction nor the bound phenolics were similar to the free and total phenolics methanolic extracts. Fig. 3**C** depicts the VIP scores from the PLS analysis. Total phenolics measured by UPLC, hydroxybenzoic, and phenolic acids were the most variable compounds among the samples. Protocatechuic acid was the individual phenolic exhibiting the highest variation, whereas TPA and TF were the spectrophotometric variables

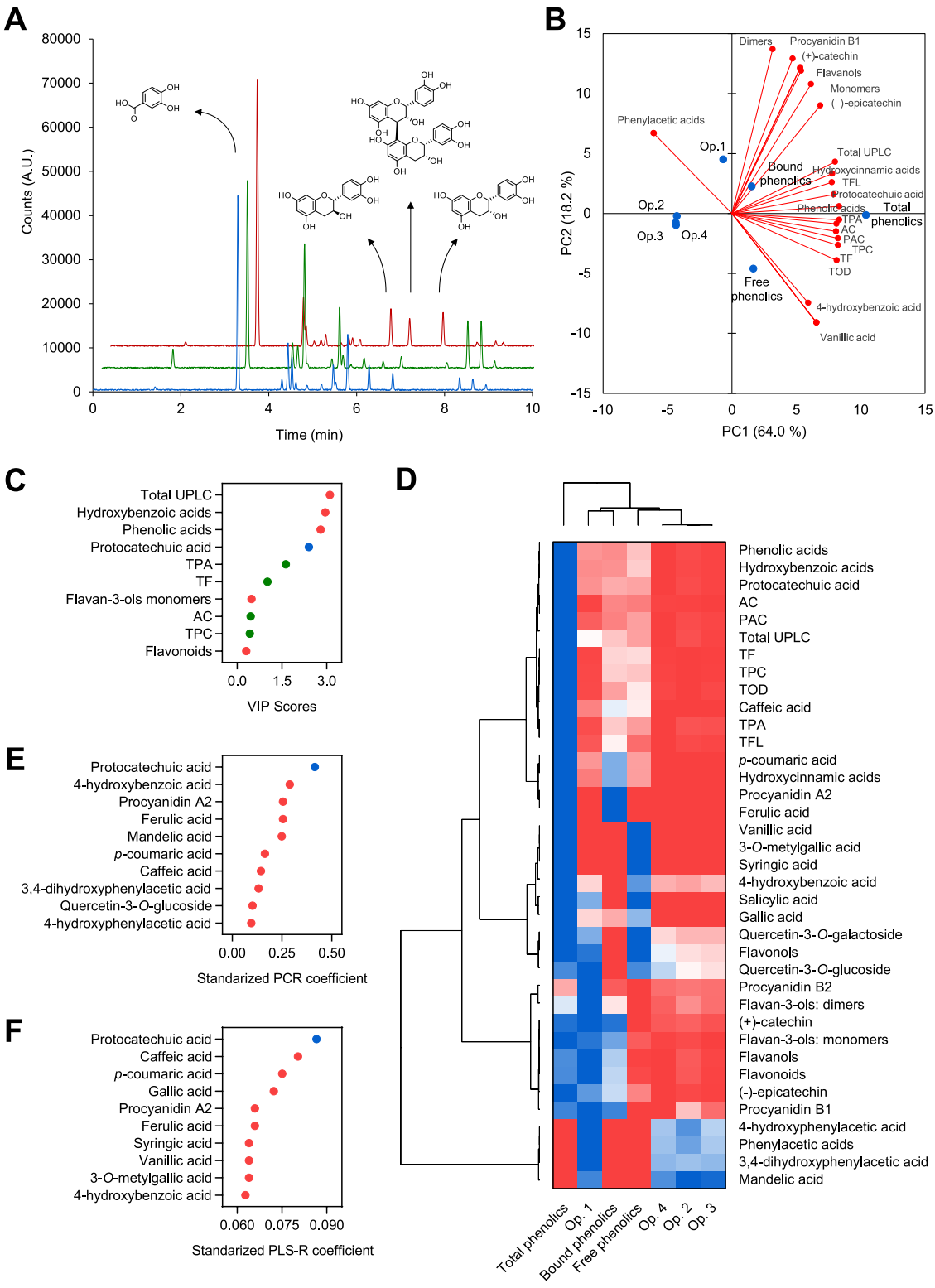

Fig. 3. Superimposed chromatograms of the optimum aqueous extract, free, and bound phenolic extracts and chemical structures of protocatechuic acid and the flavanols (+)-catechin, procyanidin-B2, and (−)-epicatechin, major phenolic compounds found in cocoa shell (**A**), biplot (scores of samples and load factors of each variable) of the principal component analysis (PCA) (**B**), VIP scores from Partial Least Squares Analysis (PLSA) (**C**), agglomerative hierarchical cluster analysis coupled to heatmap (from the lowest () to the highest () value for each parameter) (**D**) showing the associations among the measured parameters and classifying phenolic extracts from cocoa shell according to them, and the ten most significant coefficients from Principal Components Regression, PCR (**E**) and Principal Least Squares Regression, PLS-R (**F**). Circles in different colors indicate minor phenolic or phenolic family, red (), major phenolic, blue (), and spectrophotometric measurement, green (). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

displaying the most significant impact on sample classification. As observed in the heatmap assembled with the dendrogram of hierarchical clustering (Fig. 3**D**), the extract at optimum condition (Op.1) was significantly similar to the bound phenolics. In turn, free phenolics were grouped with the three other aqueous extracts (Op.2–4). Differences in extracts' phenolic composition pinpoint the extraction at optimal conditions (100 °C, 90 min, 0% citric acid, and 0.02 g mL^{-1} S/L ratio) as the best extraction, being similar to the conventional extraction of total phenolics (free + bound).

Phenolic compounds from the cocoa shell have proven antioxidant potential, as demonstrated in a previous work [44]. Identifying the compounds responsible for these properties generates a great interest. Extracts obtained using the proposed sustainable method could be further purified to strengthen their bioactivity and be used as ingredients in food, nutraceutical, or cosmeceutical products. Principal Component Regression (PCR) and Partial Least Squares Regression (PLS-R) ten most significant coefficients are depicted in Fig. 3**E, F**. From the coefficients, protocatechuic acid was the main phenolic compound responsible for the *in vitro* antioxidant capacity. Procyanidin A2, *p*-coumaric, and ferulic acids were also significant contributors to the cocoa shell extract's antioxidant properties. These compounds have been previously described as potent antioxidants both *in vitro* and *in vivo* [45,46].

Since strong associations were observed among *in vitro* parameters and some of the phenolic compounds were assessed chromatographically; therefore, Pearson's correlations were studied to analyze their relationship in more detail. Pearson's linear correlations depicted as a heatmap (Fig. 4) demonstrated that the concentration of several phenolic compounds correlated with the *in vitro* measurements. The concentration of protocatechuic acid in the cocoa shell strongly correlated with the content of TPC and TPA ($r = 0.9672$, $p < 0.001$ and $r = 0.9743$, $p < 0.001$, respectively). Epicatechin ($r = 0.7600$, $p < 0.05$) and procyanidin A2 ($r = 0.9046$, $p < 0.01$), and the sum of monomeric flavan-3-ols ($r = 0.7103$, $p < 0.05$) also exhibited strong association with TFL. Additionally, the sum of concentration of all individual phenolic compounds (Total UPLC) presented a significant ($p < 0.001$) correlation ($r = 0.849$–0.904) with the *in vitro* methods. Therefore, the use of these techniques to screen the best conditions of extraction could be validated [47]. *In vitro* methods are consistent during screening steps, provided that more specific and comprehensive techniques are then used for phytochemical profile analysis.

This study presents, up to date, the most comprehensive analysis of phenolic compounds in the cocoa shell. Its phenolic profile can be comparable to that of the cocoa bean, sharing the same main compounds. From the literature, these compounds have been widely reported to protect against oxidative stress and the development of chronic diseases [44,48]. Protocatechuic acid, the main phenolic compound of the cocoa shell, presented the highest PCR and PLS-R coefficients and positively correlated with the *in vitro* AC ($r = 0.9773$, $p < 0.001$). This compound has demonstrated being an excellent radical scavenger following different antioxidant mechanisms [49,50]. Moreover, protocatechuic acid possesses other health-promoting properties, including anti-diabetic, anti-obesity, and anti-cancer activities, which prompts the use of cocoa shell as a sustainable source of this phytochemical [51]. Thus, using these extracts as health-promoting ingredients could be a great strategy in valorizing cocoa by-products and producing novel sustainable and healthy products.

4. Conclusions

For the first time, a sustainable green aqueous extraction method of high-value phenolic compounds from the cocoa shell was established. From the results, the yield of extraction of polyphenols from the cocoa shell increased by modifying extraction parameters. The use of RSM and ANN allowed modeling and optimizing the study variables (temperature, time, acidity, and S/L ratio) to enhance total phenolic compounds, flavonoids, and flavanols proanthocyanidins, phenolic acids, and *o*-diphenols, and a high *in vitro* antioxidant capacity. UPLC-ESI-MS/MS results demonstrated the presence of protocatechuic acid, procyanidin B2, (-)-epicatechin, and (+)-catechin, phenolic compounds. Hence, we stated the optimal conditions (100 °C, 90 min, 0% citric acid, and 0.02 g mL^{-1} S/L ratio) to produce phenolic-rich extracts using water as the only extracting agent. This process might revalorize cocoa shell, a by-product of great interest and global production, as a new food ingredient to use due to their potential antioxidant and health-promoting properties.

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CRediT authorship contribution statement

Miguel Rebollo-Hernanz: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing - review & editing, Visualization. **Silvia Cañas:** Methodology, Investigation, Writing - review & editing. **Diego Taladrid:** Investigation. **Ángela Segovia:** Investigation. **Begoña Bartolomé:** Methodology, Writing - review & editing, Supervision. **Yolanda Aguilera:** Writing - review & editing, Supervision, Project administration, Funding acquisition. **María A. Martín-Cabrejas:** Writing - original draft, Writing - review & editing, Supervision, Project administration, Funding acquisition.

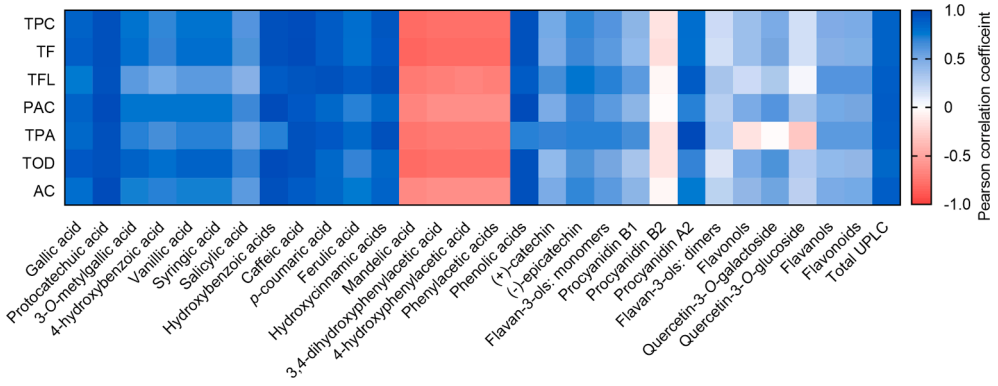

Fig. 4. Heatmap depicting the Pearson's correlation coefficients from the associations among *in vitro* determinations of phenolic families and the different phenolic compounds quantified using UPLC-ESI-MS/MS.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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