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Extraction and Neoformation of Antioxidant Compounds by Pressurized Hot Water Extraction from Apple Byproducts

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ABSTRACT: There is a great interest in searching for new environmentally sustainable techniques to enhance the use of agricultural byproducts. In this work, a response surface methodology was used to study the influence of the two independent variables, temperature (25−200 °C) and extraction time (3−17 min), in the extraction of antioxidants by pressurized hot water extraction (PHWE) from industrial apple byproducts. The optimized extraction method for determination of flavonols was at 120 °C and 3 min, giving a predicted total yield of flavonols of 1.3 μmol/g dry apple byproduct. Results obtained suggest that new antioxidant compounds were formed at the higher extraction temperatures. A desirability function response surface, considering maximum antioxidant capacity and minimal formation of brown color, was calculated and gave an optimum of 125 °C and 3 min. This latter PHWE method correlates well with the obtained results for flavonols; thus, a desirability function is a simpler alternative method for finding optimal conditions.

KEYWORDS: *antioxidant capacity, apple byproduct, caramelization, desirability function, flavonols, Maillard reaction, polyphenols, PHWE, response surface methodology*

INTRODUCTION

Apples are one of the most widely consumed fruits, due in part to their wide-ranging positive health effects. Being a rich source of carbohydrate, pectin, crude fiber, and minerals, they are a good source of nutrients. In addition, apples have been identified as one of the main dietary sources of antioxidants, mainly phenolic compounds, such as flavonoids and phenolic acids, and they also possess high antioxidant capacity.[1,2] The main groups of flavonoid compounds present in apple fruit are flavonols such as quercetin glycosides, flavanols such as catechin, epicatechin, and procyanidins, dihydrochalcones such as phloridzin, and anthocyanins such as cyanidin glycosides.

Food processing activities in Europe produce large amounts of byproducts and waste (about 2.5×10^8 ton/year).[3] The removal and environmental problems relating to agro-food industry waste management are heterogeneous due to the large variety of different waste materials produced by different sources. Such waste is only partially valorized at different value-added levels (spread on land, animal feed, composting).[4]

Vegetable and fruit processing byproducts and waste typically consist of high amounts of proteins, sugars, and lipids along with particular volatile and aliphatic compounds and, therefore, they could be cheap and abundant sources of fine chemicals.[5] They might provide value-added natural antioxidants, antimicrobial agents, vitamins, etc., along with macromolecules (such as, cellulose, starch, lipids, protein, among others) of vast interest to the pharmaceutical, cosmetic, and food industries. Several million tonnes of apple pomace, which consist of the leftovers after apple pressing for the production of apple juice and cider, is currently generated per year in the world.[6] Moreover, numerous research studies have focused on the extraction and recovery of high-value compounds from apple byproducts.[7−11] Apple byproduct contains a large amount of phenolic compounds with antiradical activity. Extracts of apple could therefore potentially be used for the production of nutraceutical compounds or functional foods. In the literature, there are several examples demonstrating the recovery of antioxidants using different nonconventional extraction technologies such as microwave- and ultrasound-assisted extraction, supercritical fluid extraction, and pressurized liquid extraction.[12−15]

Pressurized hot water extraction (PHWE) has been shown to be a powerful approach to isolate valuable components from plants.[15,16] PHWE is based on the application of temperatures ranging from 100 to 374 °C and pressures high enough to maintain the water in liquid state during the whole extraction procedure. This extraction technique is gaining increasing attention due to the advantages it can provide compared to other traditional extraction approaches. Among them, PHWE is widely considered as an environmentally sustainable extraction technique, because it avoids the use of organic solvents. Besides, PHWE is able to typically produce higher extraction yields and faster extraction procedures than the more common extraction techniques. The applicability of this technique for the extraction of bioactive and other interesting compounds from natural and food products has been investigated.[15,16] Under PHWE conditions, the cellular structure of plant tissues can be disrupted, releasing compounds of interest, which then may dissolve in the hot liquid water. There is, however, a concern that the released compounds may react during the extraction process, forming new compounds exhibiting different structures and chemical properties compared to the native compounds. For instance, chemical reactions such as Maillard and caramelization reactions involving major components found

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in vegetables might be favored under PHWE at elevated temperatures.[17,18]

The primary goal of this research was to develop an efficient and accurate analytical extraction method based on PHWE to determine antioxidant compounds, here polyphenols, in apple byproducts from the cider industry. The second goal of this study was to optimize a PHWE method that maximizes the polyphenol extraction yield and at the same time minimizes the formation of undesirable compounds from Maillard and caramelization reactions. This paper presents for the first time the use of a desirability function response surface to find best extraction conditions taking into account the positive responses of antioxidant capacity assays (trolox equivalent antioxidant capacity (TEAC), DPPH radical scavenging assay (DPPH), Folin−Ciocalteu (FC)) and the negative responses of melanoidins, furfural, and HMF concentrations.

MATERIALS AND METHODS

Chemicals and Reagents. All of the chemicals were of analytical grade. 2,2′-Azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) was purchased from Fluka (Buchs, Switzerland). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), potassium persulfate, furfural, hydroxymethylfurfural (HMF), 5-caffeoylquinic acid, phloridzin, glucose, arabinose, rhamnose, galactose, fructose, sorbitol, and sucrose were supplied by Sigma-Aldrich (Steinheim, Germany). Formic acid was from Merck (Darmstadt, Germany). Methanol was provided by Scharlau (Barcelona, Spain). The flavonoid standards were purchased from Extrasynthese (Lyon, France). The ultrapure water used was obtained from a Milli-Q (Millipore, Billerica, MA, USA) instrument.

Sample. The apple byproduct was obtained from Kiviks Musteri in Kivik, Sweden. This press cake was used to process the apple juice and was composed of seeds, cores, stems, skin, and parenchyma.

Design of Experiments. To investigate the influence of the two independent variables temperature and time on the extraction of antioxidant compounds by PHWE from apple byproduct, a response surface methodology (RSM) was employed. A circumscribed central composite design, namely, an augmented and rotatable 2^2 design with additional axial runs from the design center ($\alpha = 1.414$), was used. The total region investigated covered a temperature range of 25−200 °C, whereas the extraction time was between 3 and 17 min. The RSM design included nine unique runs where each experiment was performed in duplicate except the center point, where four replicates were made, resulting in a total of 20 experiments. The sample amount was 5 g in fresh weight. The pressure during PHWE might slightly influence the extraction yield, and, for that reason, the pressure (1500 psi = 103 bar) was consistently kept constant.[19]

The design of experiments was based on several responses including the TEAC (mmol/g dry apple byproduct), DPPH ($1/EC_{50}$), and FC (mg GAE/g dry apple byproduct) antioxidant assay values, the extraction yield (% dry extract weight obtained/initial dry sample weight), and concentration of selected polyphenols (μmol/g dry apple byproduct) and mono- and disaccharides (mmol/g dry apple byproduct), as well as the formation of new compounds such as browning compounds (absorbance at 360 and 420 nm) and furfural and HMF (μmol/g dry apple byproduct).

Three-dimensional surfaces were plotted for each response based on multiple linear regressions. The linear functions were either a first-order or a second-order polynomial model, either with or without an interaction term. Coded variables were used when the adequacy of possible linear fits was first assessed based on the ordinary least-squares method. Each fit for every response was evaluated primarily on the basis of obtaining as low as possible P value of lack of fit until satisfactory ($P > 0.10$), where the pure error sum of squares was based on all replicate values through analysis of variance (ANOVA). The regressions were also evaluated on the basis of visual inspection of the residual plots to detect any possibly faulty conclusions. The standard deviation was used as a measure of variability and is presented as such unless stated otherwise.

To find a general optimum of extraction conditions that allows for the highest antioxidant capacity of the extract but with minimal concentrations of melanoidins, furfural, and HMF, a desirability function response surface approach was applied.[20] The individual desirability (d) was calculated using eq 1 where a maximum predicted response of the antioxidant assays TEAC, DPPH, and FC was desired. Equation 2 was used for the predicted responses of brown color and HMF and furfural for which a minimum value was desired. The upper limits (U) of brown color were set to 0.8 and 0.4 AU at 360 and 420 nm, respectively. For all others the target value (T), upper limit value, and the lower limit value (L) were set to the highest or lowest predicted response within the tested space of time and temperature, depending on whether a minimum or a maximum value was desired. Calculated predicted responses ($\hat{y}$) were derived from previous multiple linear regressions. All variables were weighted equally ($r = 1$).

$$d = \begin{cases} 0 & \text{if } \hat{y} < L \\ \left(\dfrac{\hat{y} - L}{T - L}\right)^r & \text{if } L \le \hat{y} \le T \\ 1 & \text{if } \hat{y} > T \end{cases} \quad (1)$$

$$d = \begin{cases} 0 & \text{if } \hat{y} < T \\ \left(\dfrac{U - \hat{y}}{U - T}\right)^r & \text{if } T \le \hat{y} \le U \\ 1 & \text{if } \hat{y} > U \end{cases} \quad (2)$$

The overall desirability function (D) was generated using eq 3, consisting of the geometric mean of the individual desirability functions (d_m). A total of seven responses ($m = 7$) were incorporated into the desirability surface response model. The overall desirability function was three-dimensionally plotted as a function of time and temperature.

$$D = \sqrt[m]{d_1 \times d_2 \times d_3 \ldots d_m} \quad (3)$$

All statistical data processing was carried out using MATLAB R2012b including the statistical toolbox (MathWorks Inc., Natick, MA, USA).

Pressurized Hot Water Extraction (PHWE). Pressurized hot water extractions were performed on a Dionex ASE-200 (Thermo Fisher, Germering, Germany) system. At the beginning of the day the water was sonicated for 10 min. Extractions were performed at five different extraction temperatures (25, 50, 112, 175, and 200 °C) and five different extraction times (3, 5, 10, 15, and 17 min) according to the above experimental design. Each extraction started with a heatup time of the extraction cell for a given time, between 5 and 9 min depending on the set temperature. All extractions were performed in 11 mL extraction cells, containing 5 g of fresh sample. Samples were prepared in duplicate.

The dry weight of apple byproduct was calculated through subtraction of water content to total weight. The fresh apple byproduct was taken to dryness in an oven at 125 °C for 24 h.[21] This process was carried out in triplicate. The percent of water content in the apple byproduct was 80.82 ± 0.90%.

The extraction procedure was as follows: (i) the extraction cell was loaded into the oven; (ii) the cell was filled with solvent up to a pressure of 1500 psi; (iii) heatup time was applied; (iv) a static extraction with all system valves closed was performed; (v) the cell was rinsed (with 60% of the cell volume using extraction solvent); (vi) solvent was purged from the cell with N_2 gas; and (vii) depressurization took place. Between the extractions, a rinse of the complete system was made to overcome any extract carry-over. The extracts obtained were protected from light, freeze-dried, and stored at −20 °C until analysis.

Evaluation of Antioxidant Capacity. *Trolox Equivalent Antioxidant Capacity (TEAC) Assay.* The TEAC assay described by

Re et al.[22] with some modifications was used to measure the antioxidant capacity of the extracts. ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting 7 mM ABTS with 2.45 mM potassium persulfate and allowing the mixture to stand in the dark at room temperature for 12−16 h before use. The aqueous $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate buffer (pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm. Ten microliters of sample (four different concentrations) was added to 1 mL of diluted $ABTS^{\bullet+}$ radical solution. After 50 min at 30 °C, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 734 nm on a microplate spectrophotometer reader (Multiskan GO, Thermo Fisher). Trolox was used as a reference standard, and results were expressed as TEAC values (mmol trolox/g dry apple byproduct). These values were obtained from at least four different concentrations of each extract tested in the assay giving a linear response between 20 and 80% of the initial absorbance. All analyses were done at least in triplicate for each extract.

DPPH Radical Scavenging Assay. The antioxidant capacity of all the obtained extracts was measured using the DPPH radical scavenging assay based on the protocol by Brand-Williams et al.[23] Briefly, a solution was prepared by dissolving 23.5 mg of DPPH in 100 mL of methanol. This stock solution was further diluted 1:10 with methanol. Both solutions were stored at 4 °C until use. Four different concentrations of extracts were tested. Twenty-five microliters of these solutions were added to 975 μL of DPPH diluted solution to complete the final reaction medium (1 mL). After 4 h at room temperature, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 516 nm in a microplate spectrophotometer reader (Multiskan GO, Thermo Fisher). DPPH−methanol solution was used as a reference sample. The DPPH concentration remaining in the reaction medium was calculated from a calibration curve. The percentage of remaining DPPH against the extract concentration was then plotted to obtain the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% or EC_{50}. Therefore, the lower the EC_{50}, the higher the antioxidant activity. For rational reasons of clarity, the antioxidant capacity was determined as the inverse value of the efficient concentration EC_{50} (mg/mL), representing a comparable term for the effectiveness of antioxidant and radical scavenging capacity ($1/EC_{50}$). The larger is the antioxidant capacity, the more efficient the antioxidant. Measurements were done at least in triplicate for each extract.

Folin−Ciocalteu Reducing Capacity (FC Assay). The Folin−Ciocalteu reducing capacity was estimated as gallic acid equivalents (GAE), expressed as mg gallic acid/g dm.[24] The total volume of the reaction mixture was miniaturized to 1 mL. Six hundred microliters of water and 10 μL of sample were mixed, to which 50 μL of undiluted Folin−Ciocalteu reagent was subsequently added. After 1 min, 150 μL of 2% (w/v) Na_2CO_3 and 790 μL of water were added. After 2 h of incubation at 25 °C, 300 μL of the mixture was transferred into a well of the microplate, and the absorbance was measured at 760 nm in a microplate spectrophotometer reader (Thermo Scientific) and compared to a gallic acid calibration curve (0.025−2.000 mg/mL) elaborated in the same manner. The data were presented as the average of triplicate analyses for each extract.

Analysis of Polyphenols by HPLC-DAD. An UltiMate-3000 HPLC system from Dionex (Thermo Fisher) consisting of an online degasser, a quaternary solvent pump, an autosampler with cooler, a column oven, and a diode array detector (DAD), all controlled by Chromeleon 6.80 (Thermo Fisher) software, was used.

A Gemini 3 μm C6 phenyl 110 Å (100 × 2.0 mm i.d.) from Phenomenex (Torrance, CA, USA) was used as an analytical column for LC separation. The mobile phase consisted of (A) water with 0.5 vol % of formic acid and (B) methanol with 0.5 vol % of formic acid in a gradient elution programmed as follows: 0 min, 25% B; 0−2 min, 25% B; 2−40 min, 40% B; 40−45 min, 45% B; with 10 min of post-time at a flow rate of 200 μL/min. The column temperature was set at 25 °C, and the injection volume was 5 μL. The detection wavelengths were set at 200, 280, 350, 370, and 520 nm.

Quantification of polyphenols was carried out by an external standard method using a mixture containing quercetin, quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-rhamnoside (quercitrin), 5-caffeoylquinic acid, and phloretin-2′-glucoside (phloridzin) in concentrations from 0.1 to 40 μg/mL each, corresponding to around 0.2−132 μmol/L. Quercetin-3-*O*-arabinoside (avicularin) was quantified using the quercitrin calibration curve and quercetin-3-*O*-xyloside (reinutrin) using the isoquercitrin calibration curve due to the lack of commercial standards.[8]

All analyses were carried out in triplicate for each extract.

Analysis of Mono- and Disaccharides by High-Performance Anion Exchange Chromatography (HPAEC-PAD). The free sugars profiles of apple byproduct samples were analyzed using a Dionex ICS-5000 ion chromatograph (Thermo Fisher) equipment containing a gradient pump and an eluent degas module. Separation of carbohydrates was carried out on a CarboPac PA-20 guard column (3 × 30 mm) and a CarboPac PA-20 anion-exchange column (3 × 150 mm), and 5 μL was injected. The flow rate was 0.5 mL/min, and carbohydrates were detected by pulsed amperometric detection (PAD) (Thermo Fisher) with a gold working electrode and a combination pH−Ag/AgCl reference electrode using quadruple pulsed amperometry with the following potentials: E_1 = +0.10 V (t_1 = 200 ms), E_2 = +2.00 V (t_2 = 410 ms), E_3 = +0.60 V (t_3= 430 ms), and E_4 = −0.10 V (t_4 = 440 ms). Sampling time (t) was 20 ms. Carbohydrates were eluted by a gradient prepared from ultrapure water (eluent A), 20 mM sodium hydroxide (eluent B), and 200 mM sodium hydroxide (eluent C). Eluent A (60%) and eluent B (40%) were constant during the first 12 min, changing to 75% of eluent A and 25% of eluent C in 0.1 min. This proportion was kept constant until 30 min. The column was equilibrated at 200 mM sodium hydroxide for 5 min, followed by 8 mM sodium hydroxide for 5 min before injection. Both the column and detector were maintained at 30 °C. System control and data acquisition were carried out with Chromeleon 7 software (Thermo Fisher).

Quantification of sugars was carried out by an external standard method using a mixture containing glucose, fructose, arabinose, rhamnose, galactose, sorbitol, and sucrose in concentrations from 0.01 to 10 μg/mL each, corresponding to around 0.01−67 μmol/L.

All analyses were carried out in triplicate for each extract.

Final Maillard Reaction Product (Melanoidins) Determination. Melanoidins were estimated by means of browning intensity of the extracted samples. Browning intensity was measured at 360 and 420 nm wavelengths using a microplate spectrophotometer reader Multiskan GO (Thermo Fisher). When necessary, samples were diluted to obtain an absorbance reading of <1.5 absorbance units. The data were presented as the average of triplicate analyses for each extract.

Analysis of Hydroxymethylfurfural and Furfural by HPLC-DAD. The formation of HMF and furfural was analyzed with an UltiMate-3000 HPLC system from Dionex (Thermo Fisher). This system is described under Analysis of Polyphenols by HPLC-DAD. A Gemini 3 μm C6 phenyl 110 Å (100 × 2.0 mm i.d.) from Phenomenex (Torrance, CA, USA) was used as an analytical column for LC separation. The mobile phases consisted of (A) water with 0.5 vol % of formic acid and (B) methanol with 0.5 vol % of formic acid in a gradient elution analysis programmed as follows: 0 min, 0% B; 0−5 min, 0% B; 5−15 min, 5% B; 15−16 min, 0% B; with 5 min of post-time at a flow rate of 200 μL/min. The column temperature was set at 25 °C, and the injection volume was 5 μL. The detection wavelengths were set at 280 nm.

Quantification of furfural compounds was carried out by an external standard method using a mixture containing furfural and HMF in concentrations from 0.1 to 100 μg/mL each, corresponding to around 0.8−1042 μmol/L.

RESULTS AND DISCUSSION

As mentioned in the Introduction, PHWE has been suggested as a green alternative to obtain functional food ingredients from plants. The objective of this research was to develop an efficient extraction method to recover antioxidants from apple by-

Table 1. Response Surface Design of PHWE of Bioactive Compounds from Apple Byproduct, Including Extraction Yield of Solid Solutes, TEAC, DPPH, and FC Antioxidant Capacity, and Total Polyphenols Concentration, Total Sugar Concentration, and Formation of Brown Color (Melanoidins) Measured at 360 and 420 nm, HMF, and Furfural Concentrations[a]

				antioxidant capacity					brown color			
run order	temp (°C)	time (min)	yield (%)	TEAC (mmol/g)	DPPH ($1/EC_{50}$)	FC (mg GAE/g)	total polyphenols (μmol/g)	total sugars (mmol/g)	360 nm (UA/g)	420 nm (UA/g)	HMF (μmol/g)	furfural (μmol/g)
6	25	10	28.22	0.01	0.26	0.50	0.72	1.23	0.09	0.02	nd	nd
4	50	5	32.90	0.02	0.44	0.78	1.10	1.34	0.14	0.03	nd	nd
2	50	15	34.92	0.01	0.31	0.73	1.04	1.37	0.14	0.03	nd	nd
1	112	3	39.00	0.03	0.57	0.97	1.59	1.48	0.23	0.06	0.03	nd
7	112	10	40.95	0.04	0.72	1.46	1.54	1.56	0.29	0.09	0.09	0.01
8	112	10	41.11	0.03	0.60	1.40	1.25	1.59	0.24	0.07	0.14	0.03
5	112	17	45.91	0.03	0.64	1.54	1.04	1.69	0.26	0.08	0.09	0.01
9	175	5	62.51	0.27	5.65	9.34	1.96	1.84	2.43	0.85	71.65	3.39
3	175	15	59.58	0.37	6.67	11.45	1.65	1.53	3.63	1.32	121.97	7.98
10	200	10	49.19	0.47	8.51	13.66	1.18	1.05	4.85	1.76	211.63	25.83

[a]All measurements were performed in triplicate for each extraction. The data are presented as the average of duplicated extractions. RSD values for determinations made in triplicate ranged between 0.25 and 10.10%. nd, not detected.

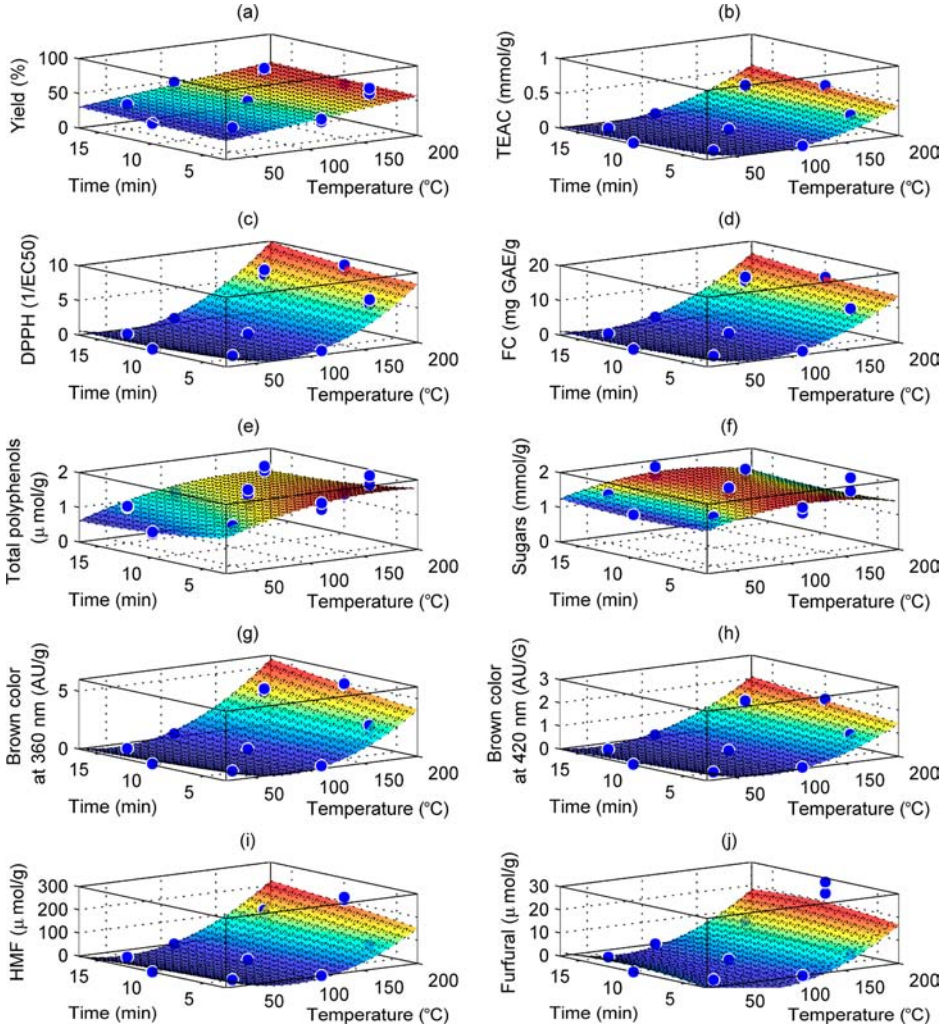

Figure 1. Response surface plots of PHWE of apple byproduct showing the effects of temperature and time on yield (% dry weight) (a), antioxidant capacity as measured by TEAC (mmol/g) (b), DPPH ($1/EC_{50}$) (c), and FC (mg GAE/g) (d), total polyphenols concentration (μmol/g) (e), total sugar concentration (mmol/g) (f), formation of brown color (melanoidins) measured at 360 nm (UA) (g) and 420 nm (UA) (h), HMF concentration (μmol/g) (i), and furfural concentration (μmol/g) (j).

products from the cider industry. In this study, the extraction of antioxidants from apple byproduct was optimized. For this purpose, the RSM was used to study the influence of two independent variables, temperature (25−200 °C) and time (3−

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17 min), in the extraction method (see Table 1). Ten response variables were evaluated: extraction yield of solid solutes; antioxidant capacity as measured by TEAC, DPPH, and FC assays; total concentration of polyphenols and mono- and disaccharides; formation of brown color as measured at 360 and 420 nm; and concentrations of formed HMF and furfural. Response values for each set of variable combinations from PHWE samples are presented in Table 1. ANOVA was used to assess the main terms affecting responses; among them, temperature had a significant effect ($P < 0.01$) on all responses. The extraction time, however, did not have a significant effect ($P > 0.05$) on the yield or the antioxidant capacity measured by any of the three assays, whereas the effect on extracted polyphenols, total sugar, formation of brown color, HMF, and furfural was significant ($P < 0.05$). In the establishment of the response surface models the chosen terms were included on the basis of minimizing the lack of fit and by visual inspection of the residual plots (see Figure 1 and Table 2). The predictive empirical models of the antioxidant capacity and the formation of brown color, HMF, and furfural were included in a desirability function response surface. The results of the experiments are further discussed below.

Table 2. Predictive Multiple Linear Regression Based on the RSM of Yield, Antioxidant Capacity As Measured by TEAC, DPPH, and Phenol Content, Total Flavonoids Concentration, Total Sugar Concentration, Formation of Brown Color (Melanoidins) Measured at 360 and 420 nm, and HMF and Furfural for Pressurized Hot Water Extracts of Apple Byproduct

response		predictive model[a]	R^2
yield (%)	pure linear	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t$	0.768
TEAC (mmol/g)	quadratic with interaction	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T \times t + \beta_4 \times T^2 + \beta_5 \times t^2$	0.988
DPPH ($1/EC_{50}$)	quadratic with interaction	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T \times t + \beta_4 \times T^2 + \beta_5 \times t^2$	0.984
FC (mg GAE/g)	quadratic with interaction	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T \times t + \beta_4 \times T^2 + \beta_5 \times t^2$	0.982
polyphenols (mg GAE/g)	pure quadratic	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T^2 + \beta_4 \times t^2$	0.688
sugars (mmol/g)	pure quadratic	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T^2 + \beta_4 \times t^2$	0.974
brown color (360 nm) (UA/g)	quadratic with interaction	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T \times t + \beta_4 \times T^2 + \beta_5 \times t^2$	0.985
brown color (420 nm) (UA/g)	quadratic with interaction	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T \times t + \beta_4 \times T^2 + \beta_5 \times t^2$	0.985
HMF (μmol/g)	quadratic with interaction	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T \times t + \beta_4 \times T^2 + \beta_5 \times t^2$	0.960
furfural (μmol/g)	pure quadratic	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T^2 + \beta_4 \times t^2$	0.799
flavonols (μmol/g)	quadratic with interaction	$\hat{y}(T,t) = \beta + \beta_1 \times T + \beta_2 \times t + \beta_3 \times T \times t + \beta_4 \times T^2 + \beta_5 \times t^2$	0.801

[a] T = temperature; t = time.

PHWE of Solutes from Apple Byproduct: Effect on Extraction Yield of Solids. The apple byproduct was extracted by PHWE using water at different temperatures (25–200 °C) and times (3–17 min). Table 1 and Figure 1a show the particular extraction conditions employed as well as extraction yields obtained (expressed as weight percent (dry weight)).

As can be seen in Table 1 and Figure 1a, the temperature directly influenced the extraction yield, whereas the time did not present any significant influence on the yield. The effect of increasing yield with increasing temperature has been extensively observed in PHWE processes,[15,17] and it is basically explained by the increasing mass transfer, lower surface tension, and higher solubility of numerous compounds. The optimum conditions of PHWE lie outside the range of variables investigated, although it can be concluded that higher temperature is highly beneficial. However, to run the extraction at 200 °C would not be the most selective extraction process because 60% of the original sample is recovered.

Antioxidant Capacity of the Extracts. The antioxidant capacity of PHWE extracts was measured using TEAC, FC, and DPPH in vitro assays. The FC assay is one of the oldest methods developed to determine the content of total phenols.[25] In this work, the FC assay was used to measure antioxidant capacity. In fact, the FC reagent is nonspecific to phenolic compounds, and for that reason, it should be considered not an accurate method for determination of total phenolic content, unless interfering species are considered or removed.[26] Therefore, the FC assay was recently proposed for the measurement of total reducing capacity of samples.[26] All three methods were, however, used in this study to take into account different antioxidative mechanisms present in complex apple extracts. Results obtained are summarized in Table 1 and Figure 1b–d.

All of the extracts were able to act against $ABTS^{\bullet+}$, DPPH radical, and FC reagent. As shown in Figure 1b–d, the three methods provided comparable results. The extraction time did not significantly influence the extraction of antioxidants. However, the temperature had a strong influence on the antioxidant capacity. The antioxidant capacity increased by 50–100% between 25 and 112 °C. However, the antioxidant capacity using any of the three assays increased suddenly when the temperature used was higher, between 175 and 200 °C. As expected, the antioxidant capacity of extracts obtained at 200 °C was 11–19 times higher when compared to the data obtained at 112 °C. This fact has been previously observed.[17,27] The best PHWE condition considering only antioxidant capacity was found using the highest achievable temperature (200 °C). However, this high extraction temperature is not necessarily the best to obtain polyphenols in the samples.

Determination of Polyphenols: Optimization of an Analytical Method. A conventional HPLC-DAD method was set up, with the aim to separate and identify the possible antioxidants present in the extracts. The chromatograms obtained (Figure 2) clearly demonstrated that several polyphenols could be separated in an analysis time of 45 min. The careful analysis of the separated compounds, using the information provided by the DAD together with information from the literature[28,29] and from commercial standards (when available) allowed the tentative identification of eight phenolic antioxidant compounds in all extracts (chemical structures in Figure 3).

Table 3 shows the determined concentration of phenolic compounds (nmol/g dry apple byproduct) for which standards were available. In the case of reinutrin and avicularin, isoquercitrin and quercitrin calibration curves were used, respectively. The predominant quercetin derivative was hyperoside (362 nmol/g), followed by avicularin (323 nmol/g), quercitrin (208 nmol/g), reinutrin (187 nmol/g), and isoquercitrin (160 nmol/g). The flavonoid found in lower concentration was quercetin (252 nmol/g). Other phenolic compounds that were detected in relatively high concentrations

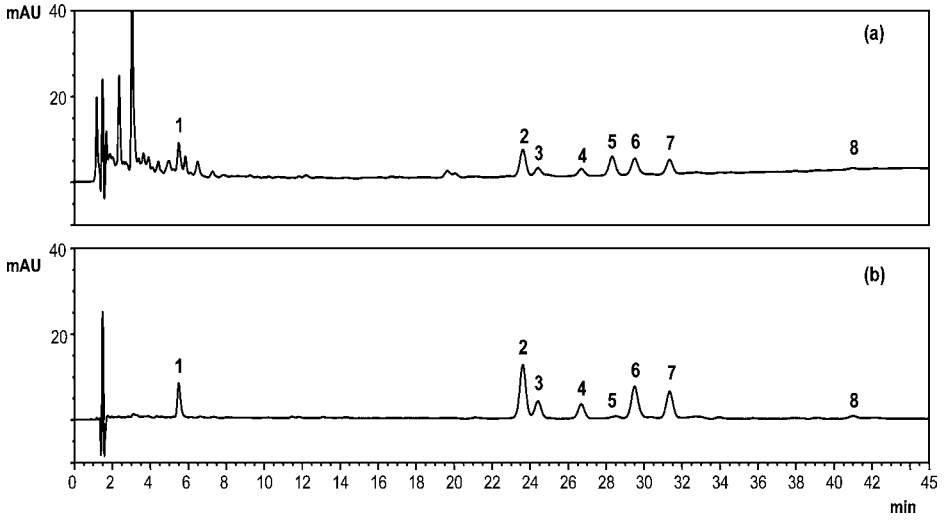

Figure 2. Chromatograms corresponding to the HPLC-DAD analysis of the PHWE extract obtained at 112 °C and 10 min at 280 nm (a) and 350 nm (b). Analytical conditions are given under Materials and Methods. Peak assignments: 1, 5-caffeoylquinic acid; 2, hyperoside; 3, isoquercitrin; 4, reinutrin; 5, phloridzin; 6, avicularin; 7, quercitrin; 8, quercetin.

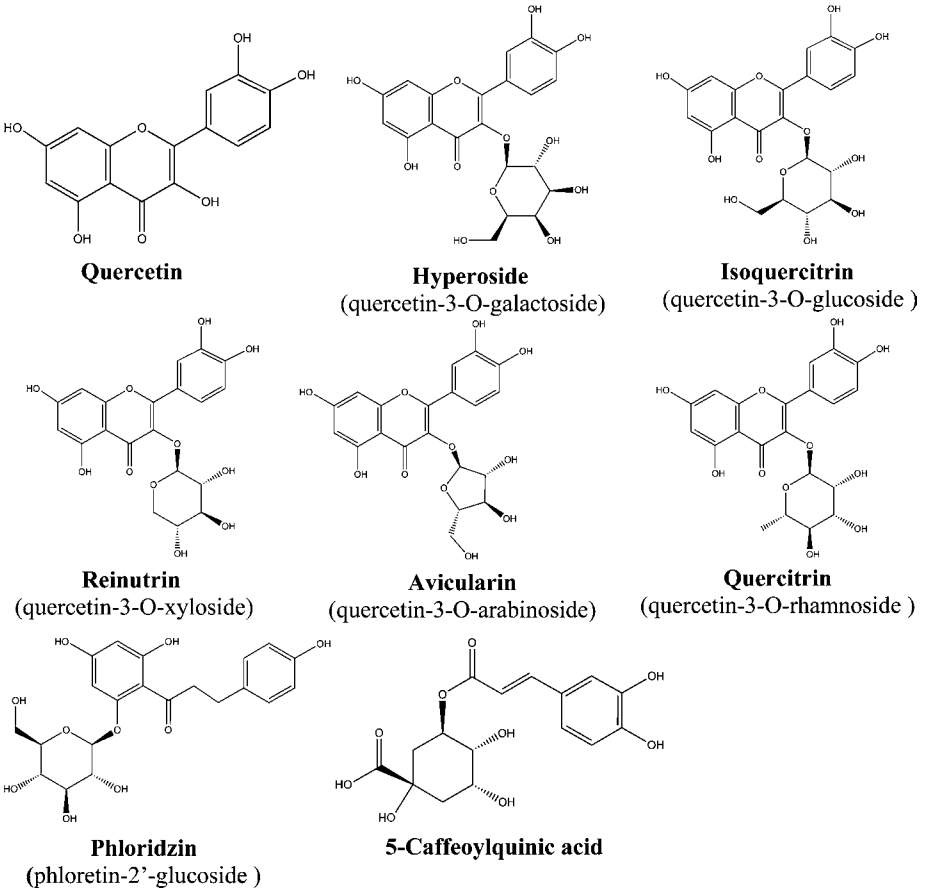

Figure 3. Molecular structures of phenolic compounds identified in apple byproduct extracts.

were 5-caffeoylquinic acid (683 nmol/g) and a dihydrochalcone called phloridzin (649 nmol/g).

As can be seen in Table 3, the optimum evaluated extraction conditions to extract quercetin derivatives were at short times (3 min) and at medium temperatures (around 105 °C). For quercetin, however, the highest concentration was found at 175 °C and 5 min extraction time. Quercetin is not very soluble in water at room temperature; hence, for this reason the quercetin yield could increase with higher temperature.[30] However, the increment in quercetin yield at higher temperature could also be due to hydrolysis of quercetin glycosides to quercetin. At the highest temperature, the quercetin level most likely decreased because flavonoids were degraded at these extraction conditions. On the other hand, the best extraction conditions to obtain 5-caffeoylquinic acid and phloridzin, the two

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Table 3. Quantification of the Polyphenols in the Apple Byproduct Extracts Indicated[a]

run order	temp (°C)	time (min)	5-caffeoylquinic acid	hyperoside	isoquercitrin	reinutrin	phloridzin	avicularin	quercitrin	quercetin
6	25	10	142 ± 4	98.8 ± 0.4	50.1 ± 0.2	49.4 ± 0.6	196 ± 7	82 ± 2	70 ± 2	nd
4	50	5	187 ± 10	236 ± 3	93 ± 7	108 ± 2	112 ± 1	168 ± 2	120 ± 1	17 ± 1
2	50	15	145 ± 4	209 ± 20	76.8 ± 0.8	110 ± 1	196 ± 6	178 ± 5	125 ± 5	21 ± 2
1	112	3	166 ± 1	362 ± 20	159.8 ± 0.7	184 ± 2	159 ± 1	323 ± 2	193 ± 2	31 ± 3
7	112	10	191 ± 8	402 ± 7	149 ± 2	187 ± 8	121 ± 1	279 ± 10	208 ± 1	52 ± 2
8	112	10	205 ± 2	269 ± 10	122.1 ± 0.7	149 ± 2	89 ± 4	218.4 ± 0.9	205 ± 1	51 ± 1
5	112	17	195 ± 3	281 ± 3	110.0 ± 0.6	22 ± 1	75.8 ± 0.9	208 ± 4	13.6 ± 0.6	62 ± 4
9	175	5	683 ± 20	238 ± 10	163 ± 2	63 ± 3	649 ± 8	124 ± 5	81 ± 4	252 ± 3
3	175	15	623 ± 10	82 ± 8	141 ± 2	69 ± 3	203 ± 2	24 ± 2	87 ± 2	94 ± 6
10	200	10	560 ± 2	73 ± 7	36 ± 3	8.9 ± 0.6	385.1 ± 0.8	62 ± 3	35 ± 3	48 ± 1

[a]Concentrations indicated as nmol/g dry apple byproduct ± standard deviation (sd). All measurements were done in triplicate for each extraction. The data are presented as the average of six measurements from two extracts. nd, not detected.

nonflavonol compounds, were high temperatures (175−200 °C).

The response surface showed that the optimum conditions to extract polyphenol compounds (total polyphenols based on total peak area of the identified polyphenols) by PHWE from apple byproduct were temperatures of approximately 170 °C and the shortest time studied, namely, 3 min (see Table 1 and Figure 1e). The optimum predicted extracted amount of polyphenols at these conditions was 1.8 μmol/g dry apple byproduct. However, the most accurate analytical PHWE method most likely differs for the different antioxidant compounds, depending on their solubility and stability in the extraction solvent, as well as matrix effects. It was therefore decided to exclude two of the phenolic compounds, phenolic acid (5-caffeoylquinic acid) and dihydrochalcone (phloridzin), because their response surfaces deviated, giving higher yield at the highest temperatures (Table 1). Hence, when considering only flavonols an optimal PHWE of 120 °C and 3 min was found with a predicted extraction of 1.3 ± 0.2 μmol flavonol/g dry apple byproduct (see Figure 4).

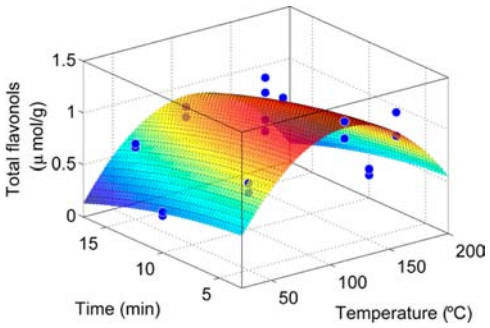

Figure 4. Response surface plots of PHWE of apple byproduct showing the effects of temperature and time on total flavonol concentration (μmol/g).

The presence of polyphenols cannot alone explain the antioxidant capacity observed in the extracts obtained at the highest temperatures, 175 and 200 °C (compare panels e and b−d of Figure 1). Several authors have related the increase in antioxidant capacity in extracts obtained at high temperature from natural matrices by PHWE with the formation of new compounds from Maillard and caramelization reactions.[15,17] Maillard reactions, also known as nonenzymatic browning or the amino−carbonyl reaction, initially occur between the carbonyl group of an open chain reducing sugar and the amino group of an amino acid.[31] The caramelization of sugars, which takes place at the same time, also contributes to nonenzymatic browning reactions. Therefore, to measure a reduction of sugar as a consequence of Maillard and caramelization reactions and what sugars are involved in these reactions, the sugars present in the extracts were analyzed with HPAEC-PAD. This technique is one of the most useful techniques for carbohydrate determination.[32,33]

Determination of Sugars as an Indicator of Chemical Deterioration during PHWE. The extraction yield of sugars, here mono- and disaccharides, was significantly influenced by temperature and time (see Figure 1f and Table 1). Chromatographic profiles of apple sugars obtained after PHWE at 25 and 200 °C are shown in Figure 5. The mono- and disaccharides present in the apple byproduct extracts were fructose, sucrose, glucose, galactose, rhamnose, arabinose, and sorbitol. By comparison of both sugar chromatograms, the concentrations of fructose and sucrose were higher at 25 °C, whereas rhamnose, arabinose, and galactose were found at higher concentration at 200 °C. Furthermore, at this high temperature, new peaks were observed, which were not possible to identify with this technique (see Figure 5).

As can be seen in Table 4, fructose (1127 μmol/g) was the most abundant sugar followed by glucose (450 μmol/g) and sucrose (156 μmol/g). However, when the temperature was increased at 200 °C, the sugar concentrations of these were decreased, being minimum (603, 314, and 32 μmol/g respectively). The decline in fructose and glucose from 100 to 200 °C could be explained by the fact that these sugars are sensitive toward Maillard and caramelization reactions.[34,35] The dramatic decrease in sucrose concentration can be explained by hydrolysis reactions, forming glucose and fructose.[36]

The sugars found at lower concentrations were rhamnose (11 μmol/g), arabinose (143 μmol/g), and galactose (46 μmol/g). In general, these minority sugar concentrations increased when the temperature was elevated (see Table 4). As can be seen in Figure 3, these monosaccharides are found linked to quercetin aglycone molecule in apple, forming the flavonoids hyperoside, avicularin, and quercitrin. Quercetin glycosides could be hydrolyzed at higher temperatures, thereby releasing free monosaccharides.

In addition, apples are a main source of pectins,[37] which are complex carbohydrates based on chains of linear regions of 1,4-α-D-galacturonosyl units and their methyl esters interrupted in places by 1,2-α-L-rhamnopyranosyl units.[38] The monosaccharide compositions of the pectins are rhamnose, arabinose,

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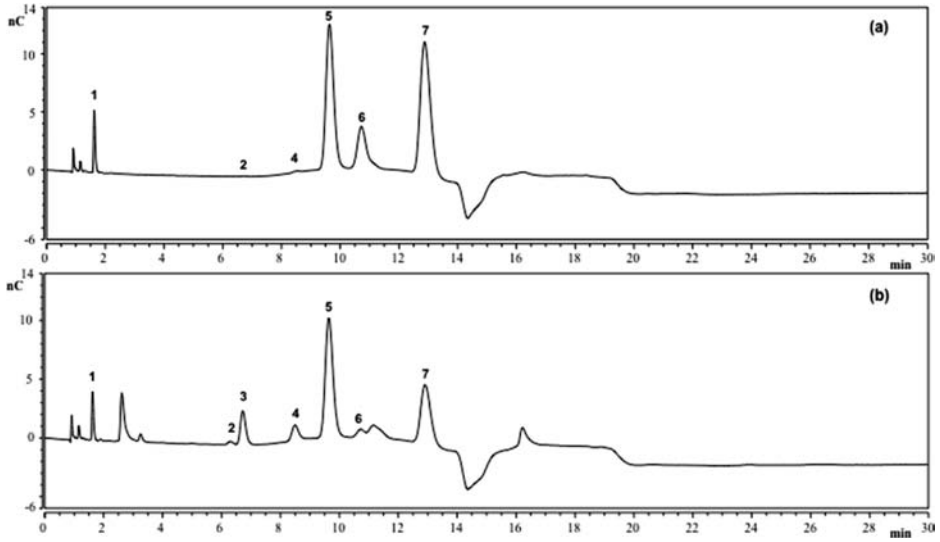

Figure 5. HPAEC-PAD chromatographic profiles of sugars extracted by PHWE from apple byproduct at 25 °C and 10 min (a) and at 200 °C and 10 min (b). Analytical conditions are given under Materials and Methods. Peak assignments: 1, sorbitol; 2, rhamnose; 3, arabinose; 4, galactose; 5, glucose; 6, sucrose; 7, fructose.

Table 4. Quantification of Sugars Found in the Apple Byproduct Extracts Obtained by PHWE[a]

run order	temp (°C)	time (min)	sorbitol	rhamnose	arabinose	galactose	glucose	sucrose	fructose
6	25	10	22.1 ± 0.1	0.71 ± 0.04	nd	18 ± 1	245 ± 6	141 ± 10	763 ± 30
4	50	5	24.4 ± 0.3	0.66 ± 0.01	nd	18 ± 1	260 ± 3	156 ± 5	818 ± 20
2	50	15	26.2 ± 0.4	1.1 ± 0.1	nd	2.0 ± 0.2	285 ± 7	154 ± 4	923 ± 20
1	112	3	24.8 ± 0.3	2.7 ± 0.2	nd	1.8 ± 0.1	316 ± 20	117 ± 6	937 ± 10
7	112	10	26.1 ± 0.3	2.9 ± 0.1	nd	1.7 ± 0.1	345 ± 7	118 ± 5	989 ± 9
8	112	10	29.0 ± 0.9	3.4 ± 0.1	nd	2.6 ± 0.1	378 ± 5	131 ± 8	1127 ± 20
5	112	17	26.5 ± 0.6	3.24 ± 0.03	nd	2.4 ± 0.2	369 ± 3	111 ± 4	1067 ± 30
9	175	5	32 ± 2	5.2 ± 0.5	60 ± 6	15.3 ± 0.2	450 ± 20	46 ± 3	1089 ± 60
3	175	15	29.3 ± 0.8	10.0 ± 0.3	143 ± 2	29 ± 1	375 ± 4	43 ± 3	826 ± 20
10	200	10	31.3 ± 0.4	10.5 ± 0.4	39 ± 1	46 ± 1	314 ± 3	32 ± 1	603 ± 20

[a]Concentrations indicated as mol/g dry apple byproduct ± standard deviation (sd). All measurements were done by triplicate for each extraction. The data are presented as the average of six measurements from two extracts. nd, not detected.

galactose, xylose, mannose, and glucose.[39] The hydrolysis of pectins during the PHWE at high temperature could be occurring.[40] Both events, hydrolysis of quercetin glycosides and pectin, would pertinently result in the increase of these monosaccharides observed during the extraction at higher temperatures (see Table 4). Besides, the alcohol sugar, sorbitol, also increased in concentration as the temperature was increased.

Overall, the total sugar (mono- and disaccharides) concentration decreased as the temperature was increased from 112 to 200 °C (see Table 1 and Figure 1f). The amount of total sugars in the apple as determined using 25 °C water was 88% (dry weight), whereas it was 39% of dry weight using 200 °C water for extraction (Table 1). These results suggest that the Maillard and caramelization reactions could happen at higher extraction temperatures.

Maillard and Caramelization Reactions. In the search for neoformed compounds from Maillard and caramelization reactions, it is widely accepted that the development of brown color can be effectively used to monitor the occurrence of nonenzymatic browning reactions.[34] In fact, this color is the simplest way to measure the existence of Maillard reaction products, through just a visual estimation. For this reason, this value has often been employed as an indicator of the extent of Maillard reaction advances in foods, as well as a marker for the occurrence of caramelization.[41] The increase in browning is directly associated with advanced phases of the reaction.[42] Table 1 and Figure 1g,h show the data collected concerning browning of the samples.

The results suggest that the browning compounds were formed from approximately 100 °C and continuously increased with higher temperatures, as can be deduced from measurements at both 360 and 420 nm (see Figure 1g,h). Table 1 shows that the rise in brown color was strong at 175 and 200 °C. The color appearance data supported the occurrence of Maillard and caramelization reactions during PHWE to high temperature and are in agreement with those of sugars. Furthermore, the formation of neoantioxidants derived from Maillard reaction and caramelization (like melanoidins) at these extractions conditions was previously demonstrated.[17,18] These newly formed compounds could be responsible for the high antioxidant capacity in the extracts obtained at the highest temperatures (see Table 1).

Another indicator of Maillard reactions is the formation of HMF and furfural.[42] Large quantities of furfural (several mg per kg) have been detected in many food commodities, including cereals, fruit juices, dried fruits, honey, milk, and coffee.[42] Although a low level of HMF has been used for years as a

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quality indicator of thermally processed foods, recently some toxicological concern has been raised. In fact, several studies show that HMF and related substances induce genotoxic and mutagenic effects in bacterial and human cells and promote colon cancer in rats.[43] Therefore, its presence in food has generated concerns of safety and toxicology.

The formation of HMF and furfural in the apple byproduct extracts was determined by HPLC-DAD (see Table 1 and Figure 1i,j). Both compounds were detected at temperatures of 112 °C and substantially increased along with higher temperatures.

The formation of brown color (melanoidins), HMF, and furfural suggested that Maillard and caramelization reactions appeared in the final stage. HMF and furfural are highly reactive compounds that take part in further reactions leading to the formation of melanoidins and other "brown" polymers and aromatic substances.[34] The sugars involved in the formation of furfural compounds are glucose and fructose, which increased their concentration at high temperatures during PHWE as discussed above (see Table 4).

Desirability Function Response Surface. As previously described, the extraction efficiency of compounds with antioxidant capacity is improved at higher temperatures. However, the formation of unwanted compounds is also accelerated at higher temperatures and is also significantly affected by longer extraction times. Thus, there is no given optimum extraction condition that satisfies the criteria of obtaining very high antioxidant capacity without acquiring large amounts of Maillard and caramelization products. Therefore, a desirability function was created to determine an overall optimum of extraction conditions. It was found that the highest desirability score was attained at about 125 °C and at the lowest investigated time of 3 min (see Figure 6). By increasing the extraction time, the ideal extraction temperature consequently decreased. The observed behavior is due to the formation of undesirable compounds that is influenced by the extraction time.

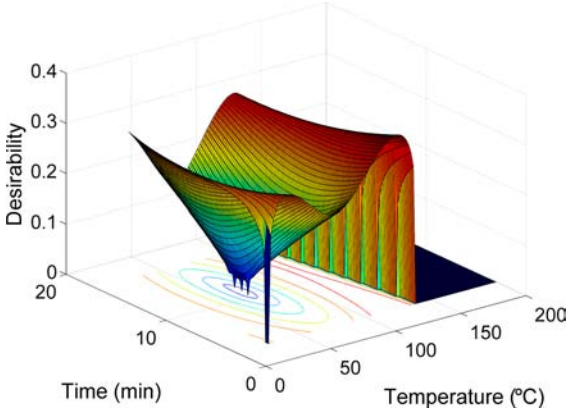

Figure 6. Desirability function response surface indicating the optimum extraction conditions based on maximizing the antioxidant capacity of the extract (based on TEAC, DPPH, and FC) while minimizing the formation of browning, HMF, and furfural. Upper limits of acceptable browning were set to 0.8 and 0.4 AU at 360 and 420 nm, respectively.

Due to the inability of a second-order polynomial to sufficiently describe all of the attained responses as a function of temperature and time, a systematic error is attained at the lowest extraction temperature. The curvature of the predicted responses leads to a consistent overshooting of the measured values. This gives rise to a second but somewhat lower optimum at lower temperatures, which is most likely incorrect.

In conclusion, results obtained on the basis of the RSM demonstrate the efficiency of using just water at 170 °C for 3 min extraction time, giving the highest content of polyphenols, 1.8 μmol/g dry apple byproducts. However, this extraction condition is not optimal for all polyphenols studied. For instance, the best PHWE condition for flavonols, excluding 5-caffeoylquinic acid and phloridzin, is 120 °C and 3 min, giving a predicted flavonol content of 1.3 μmol/g dry apple byproduct. Furthermore, the results from this work concerning the concentration of different sugars in the extracts as well as browning and furfural compounds, confirm the occurrence of Maillard and caramelization reactions in the extracts obtained by PHWE of apple byproducts at temperatures of 175 °C and above. These neoformed compounds present antioxidant capacity, therefore, being able to positively influence the overall antioxidant capacity obtained from apple byproducts under these particular extraction conditions. The calculated desirability function response surface maximized the antioxidant capacity and at the same time minimized the formation of undesirable compounds from Maillard and caramelization reactions, giving an optimum PHWE conditions of 125 °C and 3 min. Thus, the predicted extraction conditions of flavonols were found to be almost identical using the desirability function compared to the conventional RSM based on flavonol compounds, however, with the advantage of considering the minimization of unwanted compounds.

Finally, in cases when there are no expensive polyphenol standards available, the use of a desirability function is a good alternative for optimization of PHWE methods targeting polyphenols in plants. The methodology is simple and cheap, using antioxidant assays combined with the assessment of browning formation.

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Notes

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ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); DAD, diode array detector; FC, Folin−Ciocalteu; GAE, gallic acid equivalents; HMF, 5-(hydroxymethyl)furfural; HPAEC, high-performance anion exchange chromatography; HPLC, high-performance liquid chromatography; PAD, pulsed amperometric detection; PHWE, pressurized hot water extraction; RSM, response surface methodology; TEAC, trolox equivalent antioxidant capacity; trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid

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