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Optimization of Extraction of Apple Pomace Phenolics with Water by Response Surface Methodology

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Response surface methodology was employed to optimize the extraction of apple pomace phenolics with water. The constructed models were adequate to explain the behavior of the extraction system and predict the responses, total phenolics, and 5-hydroxymethylfurfural. Among the studied factors, temperature, extraction time, and solvent to solid ratio had the greatest influence on the responses. Water extraction using a combination of 100 °C for temperature, 37 min for extraction time, and 100 mL/g for solvent to solid ratio provided an opportunity to extract the antioxidants of apple pomace by limiting the formation of 5-hydroxymethylfurfural. Twenty-nine phenolic compounds were characterized in apple pomace by HPLC-MS. Phenolic content of apple pomace was 8341 mg/kg of dry matter at optimized conditions, which offer an alternative, safer way to extract antioxidants than by use of organic solvents.

KEYWORDS: Apple pomace; optimization; water extraction; phenolic compounds; 5-hydroxymethylfurfural; HPLC-MS

INTRODUCTION

Apples, a wide variety of which are available in many countries throughout the year, are a good source of phenolics (*1*). Conventional apple juice production results in a juice poor in phenolic compounds and with only 3−10% of the antioxidant activity of the fruit from which they are produced because most of the phenolic compounds remain in the apple pomace (*2*). Apple pomace, produced in large quantities worldwide, is a heterogeneous mixture consisting of peel, core, seed, calyx, stem, and soft tissue (*3*, *4*). Numerous studies have been devoted to find practical uses for this vast waste source, estimated to be about several million tonnes per annum globally (*4*−*6*). Disposal of apple pomace may present an added cost to processors (*7*), and its direct disposal presents many problems. Fortunately, many opportunities exist for converting it to marketable byproducts such as production of animal feed, ethanol, natural gas, citric acid, charcoal, pectin, and fiber (*8*).

Extraction of phenolics from agricultural and industrial wastes has gathered great attention because they could represent cheap and safe sources of strong antioxidants (*9*). Apple pomace has been investigated as a potential source of phenolic compounds during recent years as a result of its abundance and owing to the increasing interests in new natural sources of antioxidant products (*10*). Water has been studied as an extractant for recovery of apple pomace phenolics in a study with a limited temperature range of 25−50 °C (*11*). However, most of the extractions aiming to evaluate the phenolics of apple pomace/apple skin have been carried out by using organic or aqueous organic solvent mixtures such as aqueous methanol (*12*), aqueous acetone (*4*), and ethanol (*10*). Recently, solid−liquid extraction (*13*), pressurized liquid extraction (*14*), and subcritical extraction (*2*) of apple pomace phenolics have been optimized by response surface methodology (RSM) using aqueous acetone, ethanol, and carbon dioxide−ethanol as extractant, respectively.

Aspects of the extraction process regarding safety, effects of solvents on both operator and environment, residual limits of used solvents, recycling, and financial sustainability should be taken into consideration before a final decision is made about the superiority of an extraction process. Water, as an extractant, is an environmentally friendly, nontoxic, inexpensive, and easily accessible solvent for the extraction of organic analytes from food matrices.

5-Hydroxymethylfurfural (HMF), a common product of the Maillard reaction, occurs in many foods in high concentrations (*15*). Although the toxicological status of HMF has not been fully clarified, certain agencies have made regulations to limit the content of HMF in some foods (*16*, *17*). Therefore, manufacturers must control and limit the levels of HMF in their final products.

The aim of present study was to optimize the extraction of apple pomace antioxidants with water by using RSM and to characterize and quantify the compounds extracted.

MATERIALS AND METHODS

Chemicals. Benzoic acid, (+)-catechin, caffeic acid, chlorogenic acid, (−)- epicatechin, Folin−Ciocalteu phenol reagent, gallic acid, 5-(hydroxymethyl)furfural, *p*-coumaric acid, phloretin, phloridzin, rutin, and quercetin were purchased from Sigma-Aldrich Chemie Inc. (Steinheim, Germany). Procyanidins B1 and B2 were obtained from Extrasynthese (Lyon, France). Water (18.2 MΩ-cm) was purified on a Millipore Direct-Q system (Millipore Corp., Bedford, MA). All other chemicals were of analytical grade obtained from Merck (Darmstadt, Germany). All chromatographic solvents were of HPLC grade.

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Table 1. Three-Factor Three-Level Face-Centered Design and Experimental Data for the Responses[a]

	standard order	run order	X_1	X_2	X_3	Y_1	Y_2 (transformed)
block 1	5	1	60 (0)	65 (0)	60 (0)	3.60	6.41 (2.532)
	3	2	20 (−1)	120 (+1)	100 (+1)	3.02	0.07 (0.265)[b]
	4	3	100 (+1)	120 (+1)	20 (−1)	3.42	39.12 (6.255)
	6	4	60 (0)	65 (0)	60 (0)	3.32	6.65 (2.579)
	1	5	20 (−1)	10 (−1)	20 (−1)	2.36	0.07 (0.265)[b]
	2	6	100 (+1)	10 (−1)	100 (+1)	5.37	16.67 (4.082)
block 2	11	7	60 (0)	65 (0)	60 (0)	3.46	5.37 (2.317)
	9	8	20 (−1)	120 (+1)	20 (−1)	3.07	0.07 (0.265)[b]
	8	9	100 (+1)	10 (−1)	20 (−1)	3.19	11.20 (3.347)
	7	10	20 (−1)	10 (−1)	100 (+1)	2.29	0.07 (0.265)[b]
	12	11	60 (0)	65 (0)	60 (0)	3.62	6.41 (2.532)
	10	12	100 (+1)	120 (+1)	100 (+1)	6.02	109.02 (10.441)
block 3	19	13	60 (0)	65 (0)	60 (0)	3.55	6.54 (2.557)
	20	14	60 (0)	65 (0)	60 (0)	3.78	6.38 (2.526)
	15	15	60 (0)	10 (−1)	60 (0)	3.58	0.07 (0.265)[b]
	13	16	20 (−1)	65 (0)	60 (0)	2.92	0.07 (0.265)[b]
	14	17	100 (+1)	65 (0)	60 (0)	5.38	48.02 (6.930)
	16	18	60 (0)	120 (+1)	60 (0)	3.80	8.49 (2.914)
	18	19	60 (0)	65 (0)	100 (+1)	3.69	9.50 (3.082)
	17	20	60 (0)	65 (0)	20 (−1)	2.77	4.81 (2.193)
replicates[c]	21	21	100 (+1)	120 (+1)	100 (+1)	6.18	93.60 (9.675)
	22	22	20 (−1)	10 (−1)	100 (+1)	2.23	0.07 (0.265)[b]

[a] X_1, temperature (°C); X_2, extraction time (min); X_3, solvent to solid ratio (mL/g); Y_1, TPC (mg of GAE/g of DM); Y_2, HMF (mg/L). [b] Detection limit for HMF. [c] Experimental points 21 and 22 are the replicated points of 10 and 7 in standard order.

Materials. Apple pomace consisting of pressed skins and pulp residue was obtained from Findus Ltd. (Lier, Norway) and stored at −40 °C. Approximately 1 kg of apple pomace was lyophilized for 7 days in a Gamma 1-16 LSC freeze-dryer (Martin Christ GmbH, Germany). Freeze-dried apple pomace (200 g) was milled by a Mortar Grinder RM 200 (Retsch GmbH & Co., Haan, Germany) to obtain fine particles, vacuum packed in 10 g portions, and stored at −40 °C until analysis.

Extraction of Antioxidants. Extraction of antioxidants from the sample, that is, milled freeze-dried apple pomace, was investigated first by screening experiments and then by RSM. Details of the extraction experiments are specified below.

At basic conditions samples (250 mg) were extracted by deionized water (15 mL) in a screw-capped vial by using a magnetic stirrer (IKA, Staufen, Germany), an ultrasonic bath (VWR, Bridgeport, NJ), and a water bath shaker (Labortechnik, Burgwedel, Germany) at 50 °C for 30 min. After centrifugation (3500*g* for 10 min; Heraus Multifuge 4 KR, Kendro Laboratory Products GmbH, Hanau, Germany), the supernatant was collected and filtered through a 0.45 μm PVDF filter (Millipore, Cork, Ireland) and stored at −80 °C until analyzed. For comparison, apple pomace was extracted with 65% acetone at 25 °C (*13*) and with 80% methanol at 30 °C (*12*). Three extraction cycles were performed for aqueous organic solvent extractions, whereas one cycle was performed for water extraction of apple pomace antioxidants. When concentration was needed for some of the experiments, the extracts were concentrated under vacuum at 40 °C.

Screening Experiments. The aim of the screening experiments was to determine the effective and applicable factors for the extraction of phenolics from apple pomace by eliminating the ineffective or nonapplicable factors. The effects of the independent factors on response such as TPC were determined by changing the level of each factor and keeping the others constant. Water was assigned as a fixed factor because our aim was to extract antioxidants using only water. Particle size was evaluated as a factor at first because it would affect the extraction results. Nevertheless, after milling, the particle size distribution of the dried apple pomace (< 0.5 mm) was already homogeneous. Therefore, particle size was assigned as a fixed factor. Extraction technique (sonication, stirring, shaking, and soaking), temperature (25, 50, 75, and 100 °C), extraction time (10 min, 30 min, 1 h, and 4 h), solvent to solid ratio (5, 10, 50, 60, 100, and 200 mL/g), and effects of citric acid (0.1, 1.0, and 2%) were investigated by applying the aforementioned basic conditions and changing the level of the factors at every stage. Every extraction was replicated four times.

Experimental Design and Data Analysis. With the aim of evaluating the influence of important factors as determined in the screening experiments, that is, temperature, extraction time, and solvent to solid ratio, on TPC by FCR and HMF content, statistical optimization experiments were carried out according to a three-factor face-centered central composite design using important factors followed by eliminating the unimportant factor such as extraction procedure and inapplicable factors such as citric acid. Face-centered design was chosen because it was more appropriate to the used experimental domain than central composite rotatable design. Face-centered central composite design, a cubic design, contains axial points that are situated at a distance α from the center of the design. α takes a value of ±1 in the design (*18*). The total number of experimental points (N) in a central composite design (*19*) can be calculated by the equation

$$N = 2^k(\text{factorial points}) + 2k(\text{axial points}) + n_0(\text{center points})$$

where N is the number of experimental points, k is the number of independent factors, and n_0 is the number of center points. Thus, for this design the total number of experimental points will be 20 ($k = 3$; $n_0 = 6$). The original experimental design had 20 experimental points; however, 2 additional points were replicated at outlier points and added in the design (**Table 1**). The minimum and maximum values were set at 20 and 100 °C for temperature, 10 and 120 min for extraction time, and 20 and 100 mL/g for solvent to solid ratio, respectively.

Designation of experimental points, randomization, blocking, analysis of variance, graphical representations, and fitting of the second-order polynomial models were carried out by Design Expert 6.0.7 (Stat-Ease Inc., Minneapolis, MN). One-way analysis of variance (ANOVA) and Tukey's HSD test were performed using the SPSS 10.0.1 statistical package for Windows (SPSS Inc., Chicago, IL).

Total Phenolic Content (TPC) by Folin−Ciocalteu Phenol Reagent. TPC of the extracts was determined according to the modified Folin−Ciocalteu procedure (*20*). Appropriately diluted extract (0.2 mL) was mixed with 1.0 mL of FCR (1:10, v/v diluted with water) and incubated for 1 min before 0.8 mL of sodium carbonate was added. The mixture was incubated for 1 h at room temperature in the dark ,and then absorbances were measured at 765 nm (Agilent 8453 spectrophotometer, Agilent Technologies, Waldbronn, Germany). Blank was prepared in the same way using the same amount of water instead of extract. Total phenolics of the

extracts were expressed as gallic acid equivalents (GAE) in milligrams per gram of dry weight of apple pomace. All extracts were analyzed at least in three replicates.

HPLC with DAD and MS Detection. Chromatographic separations of phenolics and HMF in the extracts were performed as described previously (*21*, *22*) with minor modifications. The separation was carried out on a reversed phase C18 Betasil column (Thermo-Hypersil-Keystone, Bellefonte, PA; 250 mm × 2.1 mm i.d., 5 μm particle size) with a 5 μm C18 guard column operated at a temperature of 30 °C. The mobile phase consisted of 2% (v/v) acetic acid in water (solvent A) and 2% (v/v) acetic acid in water and acetonitrile (50:50, v/v, solvent B). The initial mobile phase composition was 10% B, and the flow rate was 0.25 mL/min. A volume of 20 μL of the extract was injected, and the phenolic compounds were eluted by a linear gradient from 10 to 55% B in 50 min. The column was then washed with 100% B for 5 min, followed by 10 min of re-equilibration of the column with 10% B before the next run.

The analyses were performed on an Agilent 1100 series HPLC system (Agilent Technologies) equipped with an autosampler cooled to 4 °C, a DAD scanning from 200 to 600 nm, and an MSD XCT ion trap mass spectrometer with an electrospray interface. The HPLC eluate was introduced directly into the ESI interface without splitting. HPLC/ESI-MS analyses were performed in both negative and positive modes. The nebulizer pressure was 40 psi; dry gas flow, 10 mL/min; dry temperature, 350 °C; and capillary voltage, 3.5 kV. Analysis was carried out using scan of m/z from 100 to 2200, with a scan speed of 27000 amu/s. Fragmentation (MS^{2-3}) was carried out in the automatic mode; that is, the two most abundant ions in MS^{1-2} were fragmented. Helium was the collision gas in the fragmentation experiments.

Individual phenolics and HMF were quantified on the basis of their UV absorbance and comparison with external standards. The wavelengths for quantification of components were as follows: 280 nm for flavanols, dihydrochalcones, and HMF, 320 nm for hydroxycinnamic acids; and 360 nm for flavonols. Phenolic compounds of which no standards were available were quantified using suitable standard compounds. Phenolic compounds were characterized by their UV−vis spectra, retention times relative to external standards, spiking with standards, and MS^n fragmentation patterns.

RESULTS AND DISCUSSION

Screening Experiments. Screening experiments showed that among the five tested extraction factors (i.e., extraction technique, temperature, extraction time, solvent to solid ratio, and citric acid), three of them (i.e., temperature, extraction time, and solvent to solid ratio) significantly affected the TPC in the extracts (results not shown). There were no statistically differences between sonication, stirring, shaking, and soaking as extraction techniques. Extraction by stirring gave the most precise results and was therefore chosen as extraction technique in subsequent experiments. In addition, this technique can easily be transferred to and employed in industrial scale. Citric acid (0.1−2.0%) did not influence TPC at 50 °C, whereas it increased TPC at 100 °C. However, when citric acid was used at 100 °C, it dramatically triggered the formation of HMF, and some quercetin glycosides began to hydrolyze to quercetin aglycone. It has been noted that several organic acids show catalytic effects on HMF accumulation, due to their destructive effects on sugars (*23*). In addition, the pH of the extracts obtained with citric acid was < 1.90, which limited the use of the extracts for human consumption. For these reasons, citric acid was excluded from further testing in the experimental design. The other three factors (temperature, extraction time, and solvent to solid ratio) significantly affected the TPC. These three factors are also easily applicable, controllable, and changeable both in the laboratory and in industry.

Model Fitting. The level of the factors was selected according to the results obtained from screening experiments and limits of factors. For temperature, 100 °C was the upper limit because of the boiling point of water. The lower limit of temperature was selected as 20 °C to observe the extraction results at around room temperature. The upper limit of time was chosen as 120 min because extraction times > 120 min did not affect TPC but affected HMF content significantly. Solvent to solid ratios of > 100 mL/g did not affect the TPC significantly. The lower and upper levels of solvent to solid ratios were selected as 20 and 100 to observe and determine the optimum conditions for this factor.

Table 1 shows the coded and uncoded levels of experimental factors and the resulting responses, that is, TPC (Y_1) and HMF (Y_2). Data transformations were needed for the response of HMF to meet the assumption of normality of the residuals that made the ANOVA valid because raw HMF data could not be fitted (*24*). Therefore, square root transformation was applied for HMF raw data. The extracts were too diluted in their present form and not appropriate for subsequent incorporation into any samples for enrichment purposes. For this reason all of the extracts were concentrated to a fixed level. The HMF results were calculated in concentrated forms of the extracts. For instance, HMF content of the extracts obtained with a solvent to solid ratio of 20 was multiplied by 20 by assuming that evaporation under vacuum would not result in additional HMF formation in the extracts. This assumption was also tested experimentally by evaporating the extracts at two different conditions. The results showed that evaporation at 40 or 55 °C did not result in additional HMF formation. The same procedure was applied by multiplying by 60 and 100 for the extracts obtained with solid to solvent ratios of 60 and 100, respectively. The HMF results calculated in this way are equal to the results of dry matter basis as well. At these concentration levels Brix values of the extracts were between 33.5 and 43.0. As can be seen from **Table 1**, HMF started forming at 60 °C with lengthened extraction time. The HPLC detection limit (0.07 mg/L) was replaced for extracts with HMF concentrations of < 0.07 mg/L.

The experiments were run in a random order to minimize the effect of uncontrolled variables. It was not possible to complete all 20 experiments in one working day; accordingly, they were divided into three blocks. The experiments were blocked to remove the expected variation caused by some change during the course of the experiment (*25*). The original face-centered design was based on 20 experimental points, but two experimental points were replicated and added to this design because, after data analysis, two experimental points were considered as outliers by the software. One possible solution was to exclude the outlier points when data were analyzed. However, to assign an experimental point as an outlier for when no replicates were available might cause useful information to be overlooked. For this reason, experiments were replicated at the outlier points.

The ANOVA data used to evaluate the significance of the constructed quadratic models, model terms after eliminating the insignificant ones without damaging model hierarchy, and the other statistical parameters related to the adequacy of the models have been summarized in **Tables 2** and **3**. There were no block effects for the two responses. This implies that there is no external factor on different days affecting the experimental values. The lack of fits for the two models were also insignificant, which implies that the fitted models could describe the variation of the data. The adequacy of the models was checked by residual analysis, R^2, adj-R^2, and pred-R^2 values. The plot of studentized residuals versus the run order for the responses of TPC and HMF (**Figure 1**) showed that the residuals scattered randomly, which indicated the adequacy of the models (*26*). In the present study, R^2 and adj-R^2 were close to each other, which indicated that all used terms in the models were necessary for constructing the correct models. The pred-R^2 statistic gives some indication of the predictive capability of the regression model (*19*). All R^2 terms were > 0.9, indicating that the models were adequate and had sufficient predicting capability. Adequate precision values of both quadratic models were > 30, indicating that these models could be used to navigate the design space.

Table 2. ANOVA Table for Response Surface Reduced Quadratic Model for TPC

source	sum of squares	degrees of freedom	mean square	*F* value	*p* value
block	0.20	2	0.10	1.91	0.188
model	25.37	6	4.23	136.0	<0.0001
A (temperature)	11.12	1	11.12	357.5	<0.0001
B (time)	0.84	1	0.84	26.9	0.0001
C (solvent to solid)	3.58	1	3.58	115.1	<0.0001
A^2	0.77	1	0.77	24.8	0.0002
C^2	0.54	1	0.54	17.3	0.0011
AC	3.76	1	3.76	121.0	<0.0001
residual	0.40	13	0.03		
lack of fit	0.31	8	0.04	2.2	0.2042
pure error	0.09	5	0.02		
total corrected	25.98	21			
R^2	0.9843				
adj-R^2	0.9771				
pred-R^2	0.9409				
adequate precision	33.53				

Table 3. ANOVA Table for Response Surface Reduced Quadratic Model for HMF[a]

source	sum of squares	degrees of freedom	mean square	*F* value	*p* value
block	5.27	2	2.64	3.09	0.086
model	174.94	8	21.87	152.5	<0.0001
A (temperature)	95.29	1	95.29	664.3	<0.0001
B (time)	14.48	1	14.48	101.0	<0.0001
C (solvent to solid)	3.58	1	3.58	25.0	0.0004
A^2	4.97	1	4.97	34.7	0.0001
B^2	1.66	1	1.66	11.5	0.0059
AB	11.98	1	11.98	83.5	<0.0001
AC	2.77	1	2.77	19.3	0.0011
BC	1.23	1	1.23	8.5	0.0138
residual	1.58	11	0.14		
lack of fit	1.26	6	0.21	3.3	0.1058
pure error	0.32	5	0.06		
total corrected	181.80	21			
R^2	0.9911				
adj-R^2	0.9846				
pred-R^2	0.9140				
adequate precision	37.25				

[a] Values are calculated for square root transformed responses.

The final quadratic equations in terms of actual factors for TPC and HMF (as square root transformed values) were as follows:

$$\text{TPC} = 2.524 - 0.036A + 0.005B + 0.022C + 0.0003A^2 - 0.0003C^2 + 0.0004AC$$

$$\text{sqrt HMF} = 1.687 - 0.077A + 0.011B - 0.017C + 0.0008A^2 - 0.0002B^2 - 0.0005AB + 0.0003AC + 0.0002BC$$

where *A*, *B*, and *C* were temperature, time, and solvent to solid ratio, respectively.

Three-dimensional plots for the predicted responses based on the quadratic equations to observe the change of TPC and HMF are shown in **Figures 2**−**4**. Because there were three individual factors, one of them was kept constant at the upper level and the responses were generated as a function of the other two factors. It is clear that the highest response values were obtained when the levels of the factors were at the highest levels. There was an almost 3-fold

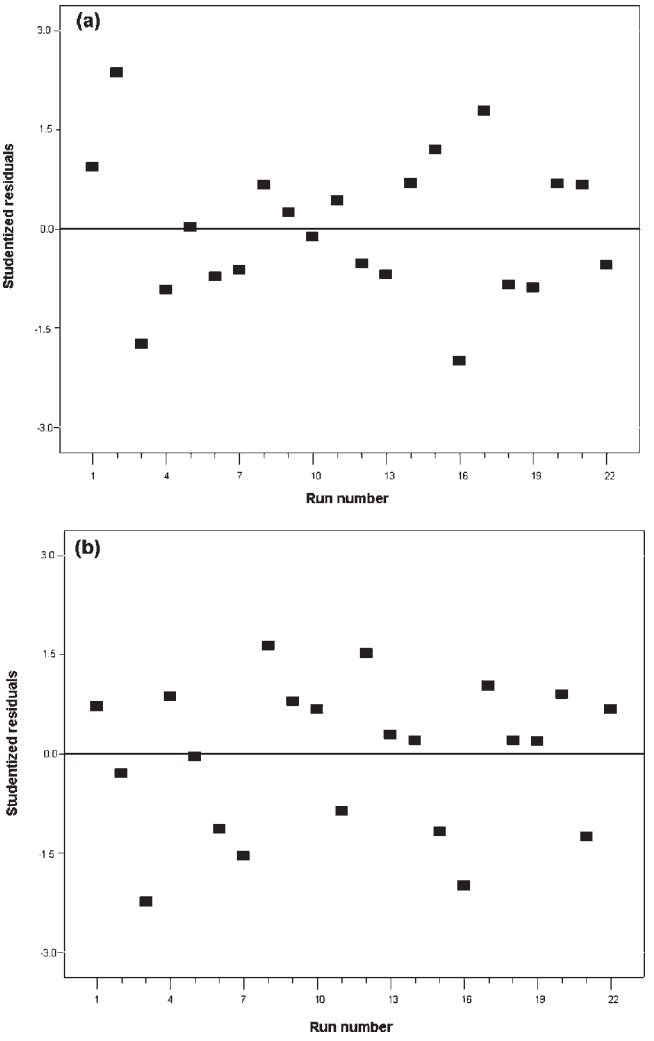

Figure 1. Studentized residual plots for TPC (**a**) and HMF (**b**).

increment in TPC when the highest levels of the factors were used in proportion to the lowest levels (**Table 1** and **Figure 2a**). HMF surfaces show that every increment in temperature together with time resulted in dramatic changes in HMF content (**Figure 2b**). It has been noted that the Maillard reaction rate is increased 4-fold by every 10 °C increment (*23*).

Optimization. It can be seen from **Figure 2a** that by increasing extraction time it is possible to obtain higher TPC, but the formation of HMF must be taken into consideration because concentration of this compound in certain types of foods is limited. HMF is the most common intermediate product of the Maillard reaction and occurs in many carbohydrate-rich foods (*27*). The European Union (EU Directive 2001/110/EC of 20 December 2001) has established maximum HMF concentration levels in honey (40 mg/kg) and in apple juice (50 mg/kg) as deterioration and heat-treatment indicators (*17*). Turkish Standards (TS 3686) permit a maximum HMF content of 60 mg/L in apple juice concentrate (*16*). The International Federation of Fruit Juice Processors recommends a maximum concentration of 5−10 mg/L of HMF in fruit juices and 25 mg/L in fruit concentrates (*28*). There is no established or recommended limit of HMF value in concentrated apple pomace extracts. On the basis of the limitations referred to above, however, we assigned the upper limit of HMF content as 40 mg/kg for this type of concentrated extracts. After entering the criteria for independent variables as in range, HMF upper level as 40 mg/kg, and TPC as maximum, several solutions were proposed by Design Expert software. We selected

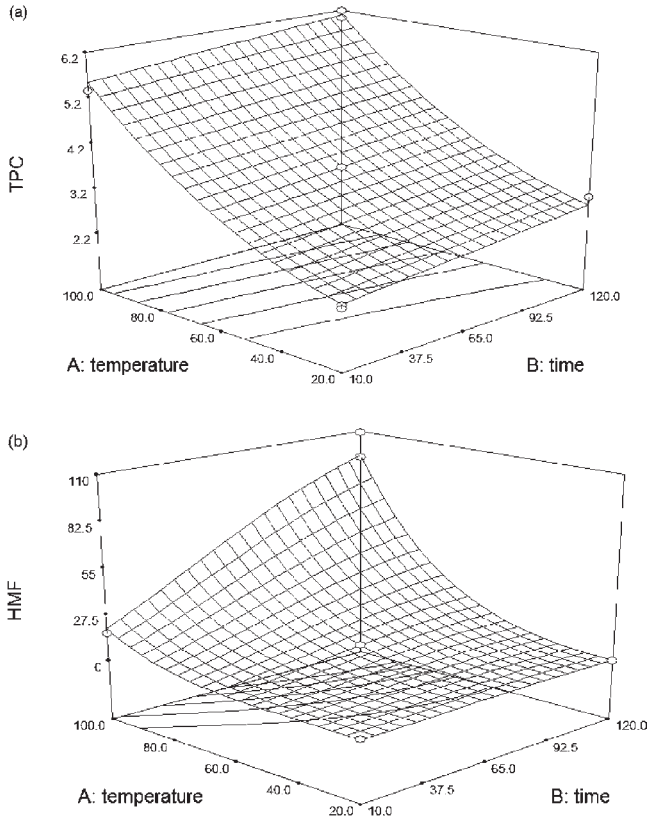

Figure 2. Response surface plots for the effects of temperature and time on TPC (**a**) and HMF (**b**) at a constant solvent to solid ratio of 100 (mL/g).

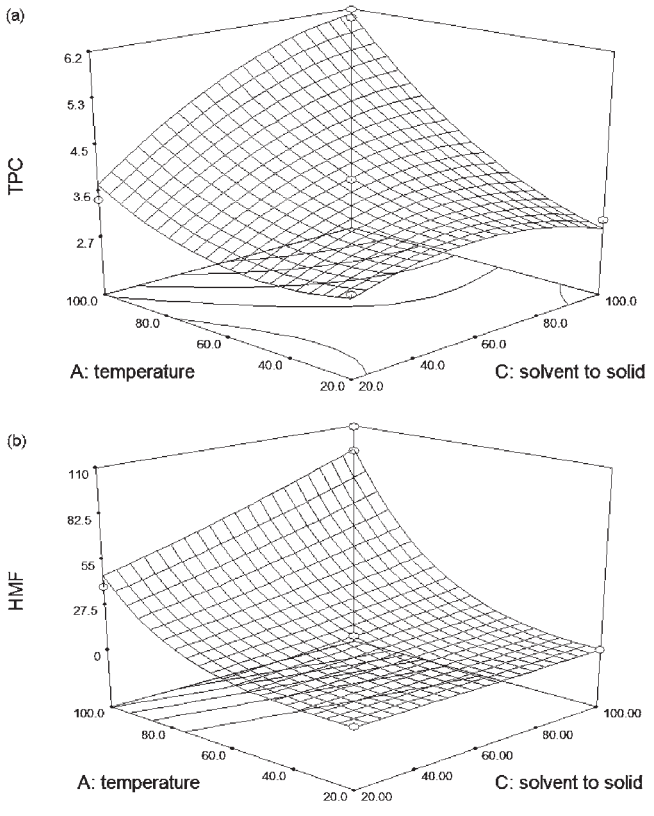

Figure 3. Response surface plots for the effects of temperature and solvent to solid ratio on TPC (**a**) and HMF (**b**) at a constant time of 120 min.

the point of 100 °C for temperature, 37 min for extraction time, and 100 mL/g for solvent to solid ratio as optimum because these

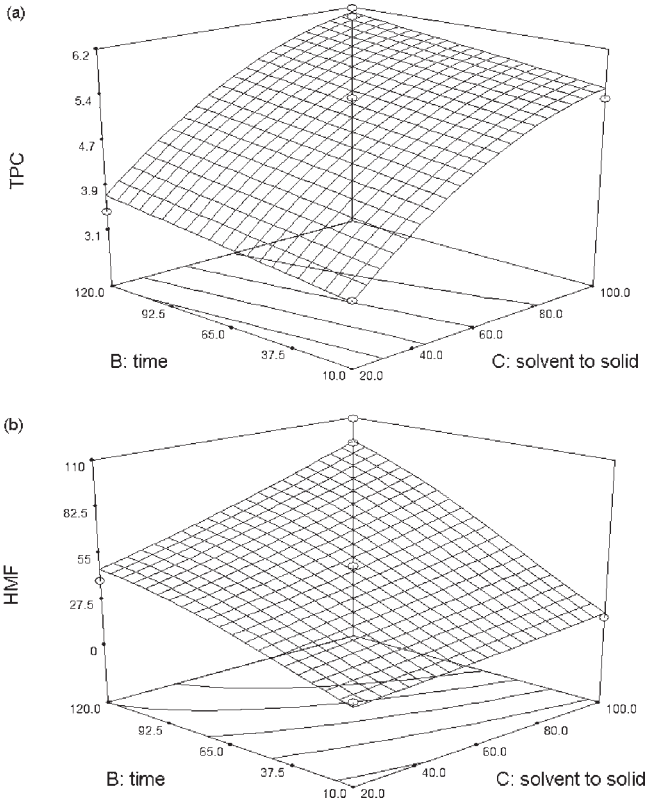

Figure 4. Response surface plots for the effects of time and solvent to solid ratio on TPC (**a**) and HMF (**b**) at a constant temperature of 100 °C.

Table 4. Verification Experiments at Two Optimum Points

conditions	responses	
	TPC (mg/g of DM)	HMF (mg/L)
optimum point 1[a]		
experimental value (±SD)	5.8 (±0.2)	42 (±3)
predicted value	5.68	39.63
optimum point 2[b]		
experimental value (±SD)	5.2 (±0.1)	39 (±5)
predicted value	5.01	39.99

[a] Optimum point 1: temperature, 100 °C; time, 37 min; solvent to solid ratio, 100 mL/g. [b] Optimum point 2: temperature, 100 °C; time, 57 min; solvent to solid ratio, 60 mL/g.

points had the highest desirability value (0.875), which can range from 0 to 1. Water content of the extracts is very important, and the extracts need to be processed in a suitable form for subsequent exploitation and/or storage. Every additional amount of water would result in increments in energy costs and workload. Therefore, it is sensible to perform the extraction with as little water as possible. With this aim a solvent to solid ratio of 60 was selected. The values of the remaining parameters were kept the same as previously stated. Thus, the second optimum point was found at 100 °C for temperature and 57 min for extraction time. At this point the desirability value was 0.838.

Verification of the Models. Two additional experiments were conducted by applying the conditions for the selected optimum points. As shown in **Table 4**, experimental values and predicted values were in close agreement for the two optimum points. It is possible to gain 11.6% higher yield at optimum point 1 than at optimum point 2 in terms of TPC. Extracts obtained at these conditions contained almost equal amounts of HMF.

Characterization of Components in Extracts of Apple Pomace by HPLC-DAD-ESI-MS^n. The phenolic compounds in extracts of

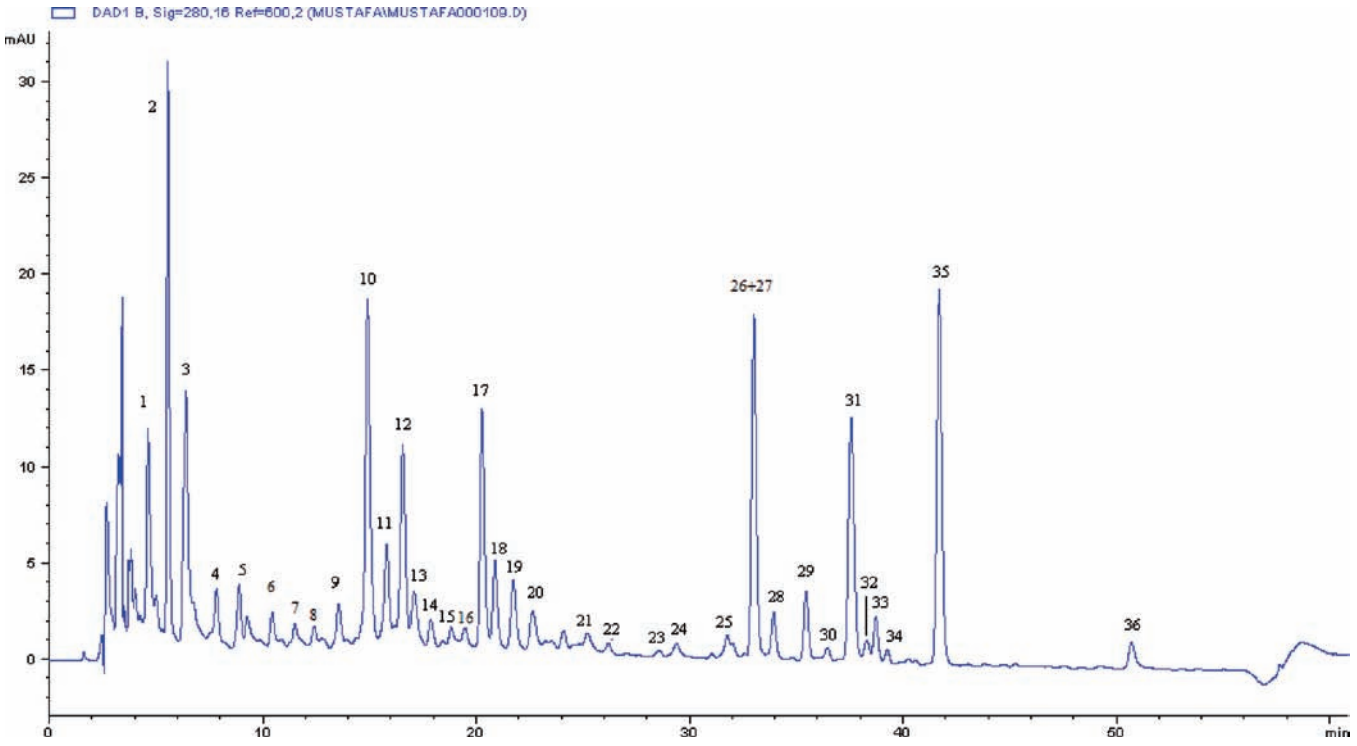

Figure 5. HPLC chromatograms of apple pomace extracts at 280 nm. (Peak assignments are listed in **Table 5**.)

apple pomace were identified by comparison of their chromatographic behavior and UV−vis and mass spectral characteristics with reported studies (*29*−*41*). The occurrence of HMF, procyanidin B1, catechin, chlorogenic acid, procyanidin B2, epicatechin, rutin, phloridzin, and quercetin in the extracts was, in addition, confirmed by comparing retention times and MS and UV−vis spectra and by spiking samples with authentic standards. HMF and quercetin were found in some of the extracts. HMF occurred in extracts obtained with a combination of extraction temperature of ≥60 °C and extraction time ≥65 min (**Table 1**; **Figure 5**). Quercetin was determined only in the extracts obtained at 100 °C.

The identity of phenolic compounds detected in apple pomace in the present study was in accordance with previous findings in apples and apple pomace (*30*, *35*). Details of peak assignment of the 36 compounds found in the extracts of apple pomace are summarized in **Table 5**. Six of the 36 components in the extracts were not identified. Two early eluting compounds, **1** and **2**, were formed under the same conditions as HMF and had UV−vis spectra similar to those of the Maillard reaction products 2-furaldehyde and 5-methylfurfural, supposed to mainly be produced from pentoses (*42*). Identification of compounds **1** and **2**, however, was not possible, because these compounds, like HMF, were not ionized in either positive or negative mode ESI-MS. The low sensitivity of HMF when using ESI as the ionization source is also reported in previous studies (*27*, *32*). In a study of apple pomace consisting of peel, core, seed, calyx, stem, and soft tissues, 60 phenolic compounds were identified by HPLC-MS/MS (*35*). In another study, 30 phenolic compounds in apple peel were identified by HPLC-DAD/MS (*30*). Different phenolic compounds identified may be due to differences between the methodologies used, and the differences between the fractions of analyzed materials, that is, peel, seed, etc.

Quantification of Phenolic Compounds in Extracts of Apple Pomace. Table 6 shows the phenolic composition of seven selected extracts obtained with different experimental conditions. To evaluate and compare the efficiency of water extraction at optimum conditions, apple pomace was extracted with two different solvent systems, 65% acetone (*13*) and 80% methanol (*12*). The aqueous methanol extract gave the highest phenolic content, that is, 11421 mg/kg. The water extracts obtained at optimum conditions contained ∼25% lower concentrations of total phenolic compounds compared with acetone and methanol extracts. It should be noted that acetone and methanol extracts were obtained with three extraction cycles, whereas water extracts were obtained in one cycle. Theoretically, every additional extraction cycle with water would increase the amount of phenolics in the final extract, but it might not be practical to apply more than one cycle in large-scale extraction processes from an industrial point of view.

It should be noted that the amount of phenolics determined by HPLC-DAD at optima 1 and 2 were higher than TPCs calculated by FCR. This is probably due to different quantification procedures of the two methods. In the FCR method the phenolic compounds are calculated as equivalents of gallic acid, which is a low molecular weight phenolic compound not present in apples. Quantification of phenolics by HPLC, however, is not correct either, because standards are not available for all of the compounds; that is, all quercetin glycosides were quantified as rutin equivalents. Similarly, procyanidin dimer and trimers, caffeoylquinic acids, coumaroylquinic acids, and phloretin xyloglucoside were quantified using equivalent phenolic standards, and this might have resulted in overestimation of phenolics by HPLC. In accordance with our results, the amount of phenolics in the flesh and peel parts of eight apple cultivars were higher when calculated by HPLC than by FCR (*43*).

TPC of apple pomace was reported as 4.6 and 5.5 mg of GAE/g DM for water and methanol extraction, respectively (*44*). In a previous study subcritical extraction of apple pomace phenolics were optimized by RSM, and at optimized point TPC of apple pomace was reported as 0.47 mg of GAE/g of FW (*2*). TPCs of 11 apple pomace samples were reported as 2.3−15.1 mg of GAE/g of DM (*45*). In the present study, TPCs of the apple pomace samples for 22 experimental points were found as 2.23−6.18 mg of GAE/g of DM, which is in accordance with the above studies. The water

Table 5. Characterization of Compounds in Water Extracts of Apple Pomace by HPLC-DAD-ESI-MS^n Detection

peak	t_R	λ_{max}	MS (*m*/*z*); ID	MS^2 ions (*m*/*z*)[a]	MS^3 ions (*m*/*z*)[a]	identification	identification type (literature)
1	4.6	278	ND	ND	ND	unknown	
2	5.5	293	ND	ND	ND	unknown	
3	6.4	284	ND	ND	ND	HMF	UV, std
4	7.8	260, 283	153; $[M-H]^-$	153, 109		protocatechuic acid	UV, MS (*35*)
5	8.9	260, 292	353; $[M-H]^-$	191, 179, 135	173, 127	caffeoylquinic acid 1	UV, MS (*36*)
6	10.4	280	577; $[M-H]^-$	425, 451, 407, 559	407	procyanidin B1	UV, MS, std
7	11.5	245, 277	337; $[M-H]^-$	249, 267, 319	231, 175	unknown	-
8	12.4	280	353; $[M-H]^-$	173, 179, 191, 135	173	caffeoylquinic acid 2	UV, MS (*36*)
9	13.5	280	289; $[M-H]^-$	245,205	203, 227, 161	catechin	UV, MS, std
10	14.9	245, 326	353; $[M-H]^-$	191	173, 127, 85	chlorogenic acid	UV, MS, std
11	15.8	245, 326	353; $[M-H]^-$	173, 179, 191	111,155	caffeoylquinic acid 3	UV, MS (*36*)
12	16.5	280	577; $[M-H]^-$	425, 451, 407, 559	407	procyanidin B2	UV, MS, std
13	17.1	287, 305	337; $[M-H]^-$	173, 163, 191	111, 93, 155	coumaroylquinic acid 1	UV, MS (*36*)
14	17.8	285, 325	355; $[M-H]^-$	193		ferulic acid hexoside	UV, MS (*36*)
15	18.2	280, 520	449; $[M]^+$	287		cyanidin-3-galactoside	UV, MS (*30*, *31*)
16	18.7	282, 520	449; $[M]^+$	287		cyanidin-3-glucoside	UV, MS (*31*)
17	20.3	280	289; $[M-H]^-$	245, 205, 179	203, 187, 227	epicatechin	UV, MS, std
18	20.9	312	337; $[M-H]^-$	173, 191, 163	93, 111, 155	coumaroylquinic acid 2	UV, MS (*36*)
19	21.7	280	865; $[M-H]^-$	695, 577, 739,	451, 425, 407	procyanidin trimer 1	UV, MS (*37*)
20	22.6	280	865; $[M-H]^-$	695, 577, 713, 739	287, 407, 425, 451	procyanidin trimer 2	UV, MS (*37*)
21	24.5	285, 305	337; $[M-H]^-$	191, 163	173, 111	coumaroylquinic acid 3	UV, MS (*36*)
22	26.0	285, 305	455; $[M-H]^-$	395, 453	249, 161	unknown	
23	29.1	285, 320	425; $[M-H]^-$	263, 161	161, 97	unknown	
24	29.6	285, 320	579; $[M-H]^-$	285, 245	203, 115	unknown	
25	30.9	280	577; $[M-H]^-$	425, 407, 451, 559, 289	407, 273, 285	procyanidin dimer	UV, MS (*37*)
26	33.0	256, 354	611; $[M+H]^+$	303, 465, 449	165, 257, 229	rutin	UV, MS, std
			609; $[M-H]^-$	301, 343, 463	179, 151, 300		
27	33.0	256, 354	463; $[M-H]^-$	301		quercetin-3-*O*-galactoside	UV, MS (*40*)
28	33.9	256,354	463; $[M-H]^-$	301		quercetin-3-*O*-glucoside	UV, MS (*40*)
29	35.4	256, 354	433; $[M-H]^-$	301		quercetin 3-*O*-xylanoside	UV, MS (*35*)
30	36.2	256, 354	433; $[M-H]^-$	301		quercetin 3-*O*-arabinopyranoside	UV, MS (*35*)
31	37.5	256, 354	433; $[M-H]^-$	301		quercetin 3-*O*-arabinofuranoside	UV, MS (*35*)
32	37.5	285	567; $[M-H]^-$	273, 435, 167	273, 167	phloretin-2′-*O*-xyloglucoside	UV, MS (*41*)
33	38.7	256,354	447; $[M-H]^-$	301, 271	301, 179, 151	quercetin-3-rhamnoside	UV, MS (*39*)
34	39.0	256, 354	477; $[M-H]^-$	315, 357, 285, 271,	285, 300, 271	isorhamnetin 3-*O*-glucoside	UV, MS (*39*)
35	41.7	285	435; $[M-H]^-$	273	167, 273	phloridzin	UV, MS, std
36	50.7	255, 371	301; $[M-H]^-$	179, 151		quercetin	UV, MS, std

[a] Ions are given in abundancy order.

extraction procedure at first optimum point gave a higher TPC as 5.8 mg of GAE/g of DM than that at the second optimum point, 5.2 mg of GAE/g of DM. Moreover, optimum point 1 is preferable to optimum 2 because the latter needs a longer extraction time, which increases the cumulative cost of the procedure. However, these two optimum points are superior to any organic solvent extraction in terms of unit organic solvent prices, especially in large-scale extraction processes.

Procyanidins B1 and B2, catechin, epicatechin, and caffeoylquinic acids were extracted in higher amounts with water at optimum conditions than with acetone and methanol (**Table 6**). Thus, this may enrich the antioxidant value of water extracts because epicatechin and procyanidin B2 are the major contributors to the antioxidant activity of apples (*43*). The other phenolic compounds were extracted more effectively with aqueous organic solvents. The amounts of quercetin glycosides were especially higher, nearly 2 times higher, in acetone and methanol extracts compared with water extracts. The aglycone quercetin, probably due to hydrolysis of quercetin glycosides, was found only in water extracts obtained at 100 °C.

Effects of extended extraction time on phenolics are clearly seen from **Table 6**. Total phenolics decreased with elongated extraction time in the extracts obtained at identical conditions (100 °C), that is, optimum 1 compared with RSM 10. More specifically, an extended extraction time at 100 °C showed significant impact on total content of quercetin glycosides. The increment in the amount of quercetin with extended time, however, could not compensate the decrement of the quercetin glycosides.

Quercetin glycosides was found as predominant in apple pomace for optima 1 and 2 (**Table 6**), which is in accordance with the literature (*21*). HMF was formed only at high temperature treated samples, and it was not determined in the extracts obtained at 20 °C. This result is in accordance with a reported study (*21*).

In conclusion, this is the first study investigating the possibilities of using water as an extraction solvent for apple pomace antioxidants and optimizing the extraction conditions. It is obvious that water as an extraction solvent is superior to the organic solvents in terms of environmental and health advantages, accessibility, and price. Selecting methods giving the highest yield without taking into consideration environmental and health concerns has become old-school in recent years. The results of the present study show that a broad range of apple pomace antioxidants could be extracted effectively with water. Furthermore, after the extraction process, the extracts could be handled more easily than the extracts obtained by organic solvents, which require an additional evaporation process to remove organic solvents. The simplicity, applicability, and cost of this procedure suggest its potential use in all food waste management areas. The present study suggests a "green" extraction procedure preferable by both consumers and producers.

Table 6. Comparison of HMF and Phenolic Contents (Milligrams per Kilogram) of Selected Experimental Points[a,b]

	optimum 1[c]	optimum 2[c]	RSM 1[c]	RSM 5[c]	RSM 10[c]	65% acetone extraction[d]	80% methanol extraction[d]
HMF	42 b	42 b	ND	5.9 a	109 c	ND	ND
flavanols							
catechin	161 ab	188 b	93 a	125 ab	173 ab	181 ab	150 ab
epicatechin	800 bc	804 bc	412 a	551 a	937 c	765 b	739 b
procyanidin B1	171 cd	189 d	65 ab	108 bc	181 d	21 a	18 a
procyanidin B2	1347 b	1519 b	234 a	430 a	1346 b	664 a	604 a
procyanidin trimers[e]	776 bcd	756 bc	362 a	658 ab	581 ab	1024 cd	1097 d
procyanidin dimer[e]	171 a	167 a	175 a	111 a	101 a	152 a	172 a
hydroxycinnamic acids							
chlorogenic acid	673 ab	654 ab	496 a	594 a	622 a	923 c	890 bc
caffeoylquinic acids[f]	328 b	338 b	177 a	175 a	187 a	232 a	226 a
coumaroylquinic acids[g]	90 ab	98 ab	72 a	85 ab	94 ab	182 c	149 bc
dihydrochalcones							
ploridzin	551 b	528 b	340 a	530 b	515 b	942 c	999 c
phloretin xyloglucoside[h]	45 ab	39 ab	48 b	71 b	11 a	128 c	143 c
flavonols							
quercetin	64 a	70 a	ND	ND	174 b	ND	ND
quercetin glycosides[i]	3164 c	2887 bc	2190 a	3185 c	2588 b	6186 d	6235 d
total phenolics by HPLC	8341	8237	4663	6622	7511	11399	11421

[a] Values that are followed by different letters within each row are significantly different ($p < 0.05$). [b] Optimum 1: temperature, 100 °C; time, 37 min; solvent to solid ratio, 100 mL/g. Optimum 2: temperature, 100 °C; time, 57 min; solvent to solid ratio, 60 mL/g. RSM 1: temperature, 20 °C; time, 10 min; solvent to solid ratio, 20 mL/g. RSM 5: temperature, 60 °C; time, 65 min; solvent to solid ratio, 60 mL/g. RSM 10: temperature, 100 °C; time, 120 min; solvent to solid ratio, 100 mL/g. 65% acetone extraction: temperature, 25 °C; time, 60 min; solvent to solid ratio, 60 mL/g. 80% methanol extraction: temperature, 30 °C; time, 60 min; solvent to solid ratio, 60 mL/g. [c] One extraction cycle was applied. [d] Three extraction cycles were applied. [e] Quantified as procyanidin B2 equivalent. [f] Compounds **5**, **8**, and **11** were quantified as chlorogenic acid equivalents. [g] Compounds **13**, **18**, and **21** were quantified as *p*-coumaric acid equivalents. [h] Quantified as phloridzin equivalent. [i] Compounds **26**−**31** and **33** were quantified as rutin equivalents.

ABBREVIATIONS USED

amu, atomic mass unit; ANOVA, analysis of variance; DAD, diode array detector; DM, dry matter; FCR, Folin−Ciocalteu reagent; FW, fresh weight; HMF, 5-hydroxymethylfurfural; HPLC, high-performance liquid chromatography; MS, mass spectrometer; ND, not detectable; RSM, response surface methodology; R^2, coefficient of determination; adj-R^2, adjusted R^2; pred-R^2, predicted R^2; SD, standard deviation; sqrt, square root; std, authentic standard; TPC, total phenolic content; UV−vis, ultraviolet−visible light.

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