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OPTIMIZATION OF ULTRASONIC-ASSISTED EXTRACTION OF QUERCETIN AND CYANIDIN FROM *PYRACANTHA COCCINEA* AND THEIR SCAVENGING EFFECT ON FREE RADICALS

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ABSTRACT

The response surface method was used to optimize the quercetin and cyanidin content experimental parameters for the ultrasonic-assisted extraction of phenolics from *Pyracantha coccinea*. The optimal conditions were an extraction time of 97 and 93 min, ethanol concentration of 71 and 79% (v/v), HCl concentration of 1.55 and 1.52 mol/L, liquid–solid ratio of 12.2 and 12.6 mL/g for determining the quercetin and cyanidin contents, respectively. The predicted quercetin and cyanidin contents were 2.30 and 4.01 mg/g, respectively. The experimental values agreed with those predicted at the 95% confidence level, which indicates the response surface method was suitable for optimizing the ultrasound-assisted extraction of phenolics from *P. coccinea*. The scavenging effects of cyanidin and quercetin in *P. coccinea* extract, which extracted at optimum conditions, on free radicals were investigated using the Fenton oxidation. It was also demonstrated that cyanidin had more effective free radical scavenging activity than quercetin.

PRACTICAL APPLICATIONS

The phenolic compounds differ in polarity, acidity, number of both hydroxyl groups and aromatic rings, concentration and matrix complexity, specific extractions techniques must be designed and optimized for each phenolic compound. Response surface methods have been used widely to produce and optimize different industrially important biotechnological and biochemical products. A central composite design was used to optimize the parameters for *P. coccinea* extractions based on the quercetin and cyanidin content. The study results indicate the ultrasonic-assisted extraction is an economical and efficient method for extracting quercetin and cyanidin from *P. coccinea*. The free radical scavenging effects of the two phenolic compounds investigated in this study. They act as free scavengers and inhibit free radical production. This study indicates *P. coccinea* can be considered a good source of naturally occurring antioxidant compounds.

INTRODUCTION

The importance of traditional medicine as a health care source has been recognized by the World Health Organisation. Thus, extracting and purifying bioactive components has become more important for utilizing phytochemicals to prepare dietary supplements, nutraceuticals, food ingredients and additives (Cacace and Mazza 2003). Investigations into bioactive components such as phytochemicals and certain organic plant components from natural sources has greatly increased in recent years.

Pyracantha coccinea M.J. Roemer (Rosaceae) is a cheap, readily available plant in many countries that is commonly planted as an ornamental in parks and gardens. It is used in traditional medicine for the diuretic, cardiac and tonic properties of its fruits (Kowaleuki and Mrugasiewicz 1971). *P. coccinea* exhibits antioxidant potential tightly connected to its flavonoid content (Fico *et al.* 2000). Since the 1990s, a number of epidemiological studies have been performed to correlate high dietary phenolic compounds and flavonoid

intake through the consumption of fruits and vegetables to reduced risks of degenerative diseases (Jaganath and Crozier 2010). Phenolic compounds and flavonoids have also been shown to possess anticarcinogenic, antimicrobial, antiviral, antimutagenic and antioxidant properties (Lampe 2003; Srinivasan 2005; Sariburun *et al.* 2010; Şahin *et al.*, 2011). Because phenolic compounds and flavonoids differ in polarity, acidity, number of both hydroxyl groups and aromatic rings, concentration and matrix complexity, specific extractions techniques must be designed and optimized for each phenolic compound. Many factors contribute to the extraction efficiency, such as the solvent type and concentration, pH, temperature, time, pressure, particle size (Juntachote *et al.* 2006) and liquid–solid ratio (Nayak and Rastogi 2013). Additionally, different variables impact the process differently, and there may be interactions between the analysed factors. Therefore, the pretreatment should be optimized according to the chemical structure and properties of the target compound.

Conventional extraction methods, such as heating, boiling or refluxing, can extract phenolic compounds. However, their disadvantages include the loss of phenolic compounds due to oxidation, ionization and hydrolysis during extraction and long extractions times (Li *et al.* 2005). New extraction techniques such as ultrasound- and microwave-assisted, supercritical fluid and accelerated solvent extractions have been recently developed to extract target compounds from different materials (Wang and Weller 2006). Ultrasound-assisted extractions improve the extraction efficiency for targeted compounds from plant materials by increasing the yield and shortening the extraction time relative to conventional extraction methods (Wang and Weller 2006; Vilkhu *et al.* 2008; Chua *et al.* 2009). The action mechanisms for ultrasound-assisted extractions are cavitation, mechanical forces and thermal impact, which disrupts the cells walls, reduces the particle size and enhances mass transfer across the cell membranes (Pan *et al.* 2011).

Response surface methods have been used widely to produce and optimize different industrially important biotechnological and biochemical products (Bezerra *et al.* 2008; Aybastıer and Demir 2010). A central composite design (CCD) is suitable for response surface methods because the CCD optimization not only rapidly screens a wide range of conditions but also indicates the role of each factor (Aybastier *et al.* 2011). A CCD was used to describe the individual and cumulative effects the parameters had on the response. CCDs have been the most successful factorial design for optimising parameters using a limited number of experiments (Bezerra *et al.* 2008; Aybastıer and Demir 2010).

Plant phenolic compounds such as flavonoids have a variety of biological and pharmacological properties. Phenolic compounds are found in almost every plant, and can act as antioxidants or their modes of action include indirect action (Rice-Evans *et al.* 1996). Flavonoids are subcategorized into seven groups: flavonols, flavones, catechins, flavonones, chalcones, anthocyanidins and isoflavonoids (Rodriguez *et al.* 2001). Quercetin is a representative member of the flavonols and has displayed the ability to prevent the oxidation of low-density lipoproteins (LDL) by scavenging free radicals and chelating transition metal ions. Cyanidin is a particular type of anthocyanidin that has antioxidant and radical-scavenging effects. As a result, quercetin and cyanidin may aid in the prevention of certain diseases, such as cancer (Hollman and Katan 1997; Murota and Terao 2003).

The first aim of this work was to use response surface methods to optimize the parameters for *P. coccinea* extractions based on the quercetin and cyanidin content. The time, solvent, acid concentrations and liquid–solid ratio were optimized for the ultrasound-assisted extraction of antioxidant compounds from *P. coccinea*. A five level, four-variable central composite design was employed to simultaneously maximize the quercetin and cyanidin contents. The second aim of this work was to undertake a study of the reactivity of quercetin and cyanidin toward reactive oxygen species. The potency of these phytochemicals was also examined with respect to protecting Fenton-oxidation.

MATERIAL AND METHODS

Plant Material

P. coccinea is widely distributed across the University of Uludag campus in Görükle (Bursa, Turkey), and the mature berries of *P. coccinea* were collected. The samples were milled into uniform dry powder using a chopper and stored at 4C.

Chemicals and Reagents

Ethanol, methanol, acetonitrile, formic acid and iron(II) sulfate heptahydrate were obtained from Merck (Merck, Darmstadt, Germany). DPPH, cyanidin chloride, quercetin hydrate ($\geq$95%) and H_2O_2 were purchased from Sigma-Aldrich (St. Louis, MO). ABTS [2,2-azinobis(3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt] was purchased from Fluka (Buchs, Switzerland). The phenolic standard solutions were prepared in methanol.

Ultrasound-Assisted Extraction

The ultrasound-assisted extractions were performed in a temperature controlled ultrasonic cleaner. Furthermore, the temperature was monitored using a thermometer. *P. coccinea* powder (1.5–3 g) was put in a glass vial (45 mL), and acidic ethanol solvent (30 mL) was added before placing the vial in the ultrasonic cleaning bath (United) set to 40 kHz. A acidic ethanol solution was prepared across a range of HCl concentrations (0.8–1.6 mol/L) and ethanol volumes (20–80%, v/v). The extract was filtered and analyzed.

Experimental Design

The response surface method (RSM) is an empirical statistical technique used for multiple regression analyses using quantitative data. This technique uses multivariable data obtained from carefully designed experiments to simultaneously resolve multivariable scenarios (Tan *et al.* 2008). The total number of experiments (N) for a central composite design (CCD) can be calculated from the following equation (1):

$$N = 2^k + 2k + x_0 \quad (1)$$

where k is the number of variables, and x_0 is the number of central points. A five-level-four-factor CCD was performed in this study and required 30 ($k = 4$; $x_0 = 6$) experiments to optimize the extraction parameters. Of these experiments, six replications were at central points, while the axial points were $\sqrt{4} = 2$ (Tan *et al.* 2008). Thirty experiments were performed to optimize the parameters (Aybastıer and Demir 2010). Twenty-four experiments were augmented with six replications at the central point to evaluate the pure error (Yuan *et al.* 2008).

The parameters and their levels were time (40–100 min), ethanol volume (20–80%, v/v), HCl concentration (0.8–1.6 mol/L) and liquid-solid ratio (10–20 mL/g) (Table 1).

A second-order polynomial equation (2), which included all of the terms, was used to calculate the predicted responses:

$$y = b_0 + \sum_{i=1}^{4} b_i x_i + \sum_{i=1}^{4} b_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} b_{ij} x_i x_j \quad (2)$$

where y is the response, b_0 is the offset term, b_i is the linear effect, b_{ii} is the squared effect, b_{ij} is the interaction effect, and x_i and x_j are both independent variables.

The CCD experimental results were fit to a second-order polynomial via a multiple regression technique. The data were analyzed using the Design Expert program (7.0.0 version), and the coefficients were interpreted using the F test. Three main analytical steps, analysis of variance (ANOVA), regression analysis and plotting the response surface, were performed to establish the optimum conditions for the antioxidant capacity and total phenolic content.

HPLC-DAD Analysis

An Agilent 1200 HPLC system (Waldbronn, Germany) consisting of a vacuum degasser, binary pump, autosampler and a diode-array detector was used to determine the phenolic compounds in the fractions. Chromatographic separations were performed using an XBridge C18 (4.6 × 250 mm, 3.5 μm) column from Waters (Ireland). The mobile phase consisted of 1% formic acid in water (solvent A) and acetonitrile (solvent B). The gradient conditions were as follows: 0–10 min 13% B, 10–20 min 41.5% B, 20–25 min 70% B, 25–35 min 10% B for a total run time of 35 min. The column was equilibrated for 10 min prior to analysing at 25C. The flow rate was 0.5 mL/min, and the injection volume was 10 μL. A Chemstation (Agilent) was used to acquire and pre-process the liquid chromatography (LC) data. The monitoring wavelengths of interest were 360 and 530 nm for quercetin and cyanidin, respectively. The peaks were identified by comparing the retention times and UV-vis spectra to standards.

TABLE 1. RANGE OF CODED AND ACTUAL VALUES FOR CENTRAL COMPOSITE DESIGN

	Level				
Factor	−2	−1	0	1	2
Time (min)	40	55	70	85	100
Ethanol volume (%, v/v)	20	35	50	65	80
HCl concentration (mol/L)	0.8	1.0	1.2	1.4	1.6
Liquid-solid ratio (mL/g)	10	12.5	15	17.5	20

Fenton Oxidation

The extracts that prepared at optimum extraction conditions for quercetin and cyanidin were diluted to 250 μM. Fe (II) of 65 μM and H_2O_2 of 125 μM were added to solution. Fenton reaction was stopped taking samples each minute during 30 minute by catalase solution. Each sample was analyzed by HPLC and calculated rate constant and half life.

Antioxidant Capacity

The total antioxidant capacity of sample was determined with ABTS method, $ABTS^+$ was produced by reacting 20 mM ABTS solution with 2.45 mM potassium persulfate solution and allowing the mixture to stand in dark at room temperature for 12–16 h before use. The procedure for extracts was performed by adding 0.1 mL extract, 3.9 mL of ethanol, and 1 mL of the $ABTS^+$ radical cation solution, which was diluted with ethanol at a ratio of 1:10, and the absorbance at 734 nm was recorded against blank after 6 min. The results were expressed as milligram trolox equivalent (TE) per gram dried weight.

The free radical scavenging activity of sample was determined with DPPH reagent. An amount of 0.10 mL of extract and 0.18 mL DPPH (1×10^{-3} M stock solution) were mixed; then, methanol was added to bring the final volume to 3 mL of 6×10^{-5} M in test tube. After the reaction was allowed to take place in the dark for 30 min, the absorbance at 515 nm was recorded to determine the concentration of remaining DPPH. The results were expressed as milligram TE per gram dried weight.

TABLE 2. CENTRAL COMPOSITE DESIGN OF FACTORS WITH EXPERIMENTAL AND PREDICTED VALUES

	Amount of quercetin (mg/g dried plant)		Amount of cyanidin (mg/g dried plant)	
Treatment	Experimental	Predicted	Experimental	Predicted
1	0.19	0.24	0.09	0.02
2	0.21	0.27	0.24	0.42
3	0.33	0.27	0.82	0.85
4	0.82	0.72	1.81	1.68
5	0.24	0.15	0.11	0.29
6	0.42	0.32	1.02	0.85
7	0.63	0.69	1.52	1.43
8	1.35	1.29	2.55	2.43
9	0.27	0.29	0.41	0.61
10	0.21	0.12	0.61	0.57
11	0.30	0.37	1.09	1.13
12	0.57	0.62	1.62	1.51
13	0.25	0.32	0.77	0.77
14	0.27	0.29	0.85	0.89
15	1.02	0.92	1.71	1.60
16	1.39	1.31	2.21	2.15
17	0.29	0.25	0.81	0.69
18	0.55	0.67	1.47	1.64
19	0.12	0.12	0.15	0.04
20	1.08	1.16	1.81	2.05
21	0.28	0.25	0.65	0.57
22	0.75	0.85	1.35	1.48
23	0.27	0.36	0.88	0.94
24	0.44	0.43	1.26	1.25
25	0.22	0.30	0.65	0.87
26	0.39	0.30	1.03	0.87
27	0.34	0.30	1.07	0.87
28	0.28	0.30	0.82	0.87
29	0.26	0.30	0.78	0.87
30	0.32	0.30	0.86	0.87

RESULTS AND DISCUSSION

Fitting the Models

Investigating the extraction variables was necessary to determine the best variable combination. Preliminary trials determined the time (40–100 min), ethanol volume (20–80%, v/v), HCl concentration (0.8–1.6 mol/L) and liquid-solid ratio (10–20 mL/g). According to the literature, high temperatures might increase the diffusion and solubility rate for many compounds and increase the antioxidant compound extraction rate (Şahin *et al.* 2013). Therefore, an extraction temperature of 70C was chosen. The experimental and predicted data for the amount of quercetin and cyanidin in *P. coccinea* are shown in Table 2. Quercetin and cyanidin were selected as the response due to their high concentration in *P. coccinea*.

Of the 30 experiments, which included six replicates (Table 2), experiment 16 (time 85 min, ethanol volume 65% (v/v), HCl concentration 1.4 mol/L and liquid-solid ratio 17.5 mL/g) yielded the most quercetin (1.39 mg/g dried plant), and experiment 19 (time 70 min, ethanol volume 20% [v/v], HCl concentration 1.2 mol/L and liquid-solid ratio 15 mL/g) produced the least quercetin (0.12 mg/g dried plant). Experiment 8 (time 85 min, ethanol volume 65% (v/v), HCl concentration 1.4 mol/L and liquid-solid ratio 12.5 mL/g) yielded the most cyanidin (2.55 mg/g dried plant). Experiment 1 (time 55 min, ethanol volume 35% (v/v), HCl concentration 1.0 mol/L and liquid-solid ratio 12.5 mL/g) yielded the least cyanidin (0.09 mg/g dried plant).

The effects and interactions for each factor were calculated using the Design Expert program (version 7.0.0). Fitting the data to various models and the subsequent analysis of variance (ANOVA) showed that the quercetin and cyanidin yields were best described using a quadratic polynomial model. The quadratic polynomial model was significant and sufficiently represented the actual relationship between the response and the parameters with low *P*-values (<0.0001) from the ANOVA (Table 3). The *F*-values for the model (24.03 and 21.82 for quercetin and cyanidin, respectively) implied it was significant at the 95% confidence level. The model also showed a statistically insignificant lack of fit based on the computed *F*-values, 0.0930 and 0.3426 for quercetin and cyanidin, respectively, at the 95% confidence level. Furthermore, the pure error was low, which indicates a good reproducibility for data with a small *p*-value from the ANOVA and satisfactory coefficient of determination (Table 3). The coefficient of determination also indicated excellent correlation between the independent variables.

TABLE 3. ANALYSIS OF VARIANCE (ANOVA) FOR THE FITTED QUADRATIC POLYNOMIAL MODEL FOR OPTIMIZATION OF EXTRACTION PARAMETERS

	Quercetin ($R^2 = 0.9573$)					Cyanidin ($R^2 = 0.9532$)				
Source	DF	SS	MS	*F* value	*P* value	DF	SS	MS	*F* value	*P* value
Model	14	3.26	0.230	24.03	<0.0001	14	10.15	0.730	21.82	<0.0001
Lack of fit	10	0.13	0.013	3.43	0.0930	10	0.37	0.037	1.50	0.3426
Pure error	5	0.18	0.004			5	0.12	0.025		

DF, degree of freedom; SS, sum of squares; MS mean square.

TABLE 4. SECOND ORDER POLYNOMIAL EQUATIONS AND REGRESSION COEFFICIENTS OF THE RESPONSE VARIABLES (THE TIME; x_1, THE ETHANOL VOLUME; x_2, THE HCL CONCENTRATION; x_3, THE LIQUID-SOLID RATIO; x_4)

Responses	Second order polynomial equations
Amount of quercetin (mg/g dried plant)	$y = 0.30 + 0.11x_1 + 0.26x_2 + 0.15x_3 + 0.11x_1x_2 + 0.13x_2x_3 - 0.084\ x_2^2 + 0.063\ x_3^2$
Amount of cyanidin (mg/g dried plant)	$y = 0.87 + 0.24x_1 + 0.52x_2 + 0.23x_3 + 0.11x_1x_2 - 0.11x_1x_4 - 0.075\ x_1^2$

Response Surface Analysis of Quercetin

The effects of extraction parameters such as time, ethanol volume, HCl concentration, and liquid–solid ratio on the ultrasonic-assisted extraction of quercetin from *P. coccinea* were investigated. The significance was determined for each coefficient using the *F*-values and *p*-values listed in Table 3. A response surface analysis of the data in Table 4

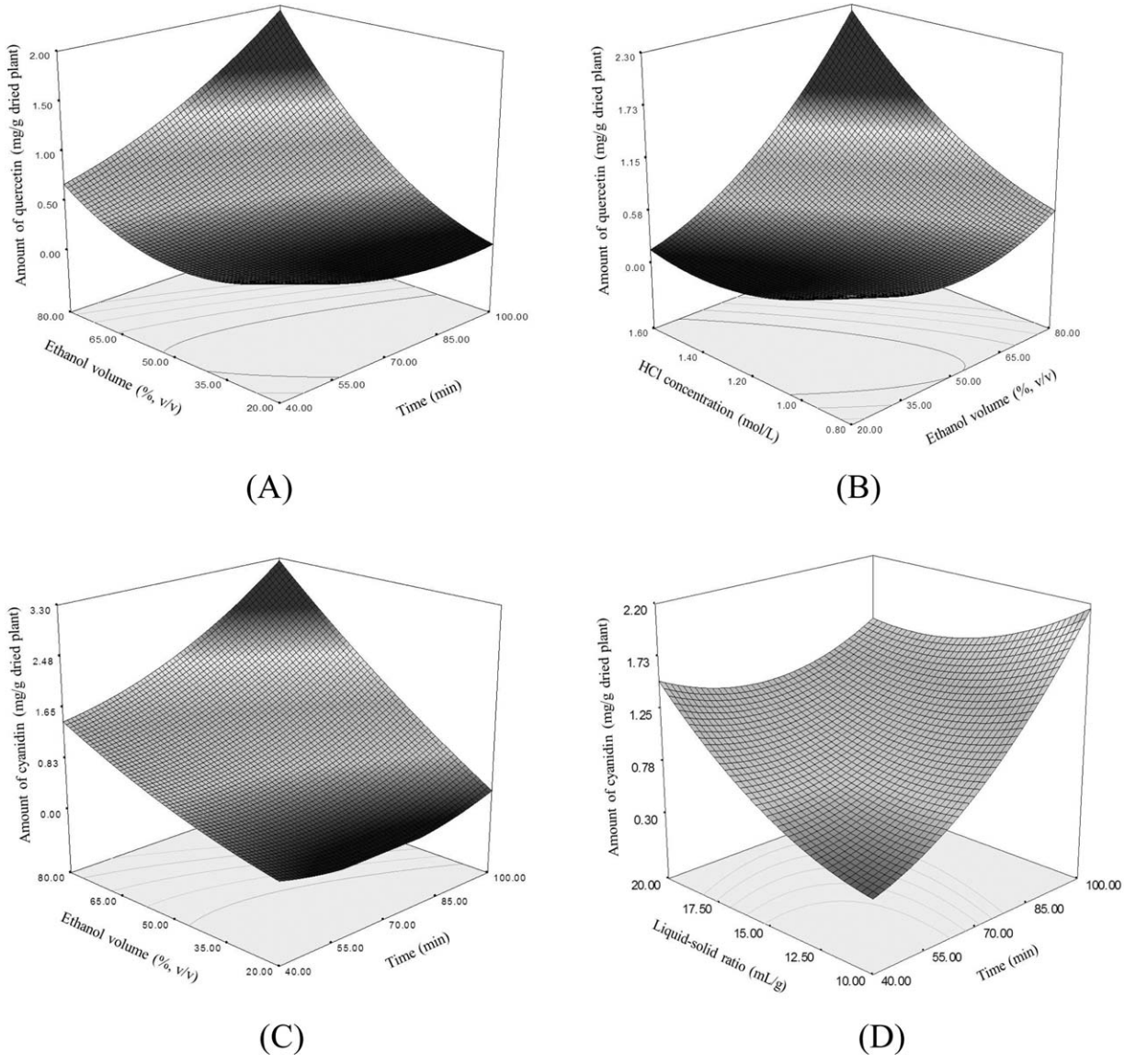

FIG. 1. RESPONSE SURFACE PLOTS OF *P. COCCINEA* SHOWING THE EFFECT OF
(A) Time and ethanol volume. (B) Ethanol volume and HCl concentration on the amount of quercetin. (C) time and ethanol volume. (D) time and liquid-solid ratio on the amount of cyanidin.

TABLE 5. OPTIMUM CONDITIONS, PREDICTED AND EXPERIMENTAL VALUES OF RESPONSES

	Optimum ultrasonic-assisted extraction conditions				Maximum values	
Responses	Time (min)	Ethanol concentration (%, v/v)	HCl concentration (mol/L)	Liquid–solid ratio (mL/g)	Predicted	Experimental
Amount of quercetin (mg/g dried plant)	97	71	1.55	12.2	2.30	2.21 ± 0.12
Amount of cyanidin (mg/g dried plant)	93	79	1.52	12.6	4.01	3.94 ± 0.15

demonstrated the relationship between the quercetin amount and the extraction parameters was quadratic with a good regression coefficient ($R^2 = 0.9573$). A larger F value and smaller p value indicated a more significant coefficient. The parameters x_1 (time), x_2 (ethanol volume), x_3 (HCl concentration), x_1x_2, x_2x_3, ${x_2}^2$ and ${x_3}^2$ were the most significant (P values below 0.05). However, x_4 (liquid-solid ratio), x_1x_3, x_1x_4, x_2x_4, x_3x_4, ${x_1}^2$ and ${x_4}^2$ had lesser effects (P values more than 0.05) on the ultrasonic-assisted extraction of quercetin.

The relationship between the extraction parameters and quercetin amount were investigated using response surface plots. Fig. 1A represents the affect varying the time and ethanol volume had on the quercetin amount. High ethanol volumes (80% v/v) increased the amount of quercetin at higher extraction times (100 min). A stronger interaction was observed between time and the ethanol volume than any other interaction according to the ANOVA results and response surface graphs. The increased mass transfer rate, solubility and extraction time could be primarily responsible for these changes. These findings suggest there is an optimum aqueous ethanol solution for effectively extracting quercetin, which is most likely due to the solvent polarity. Meanwhile, the degree of breakage in the cell membrane of the raw material was also enhanced by increasing the ethanol concentration (Vatai *et al.* 2009).

Fig. 1B shows the effect the ethanol volume and HCl concentration and their mutual interaction had on the quercetin amount. More quercetin was observed with increased ethanol volume and HCl concentration. Less quercetin was initially observed upon increasing the HCl concentration; however, this trend reversed once the HCl concentration reached 1.2 mol/L, and the extracted quercetin increased thereafter. Ethanol was a controlling factor with a positive linear impact on the quercetin amount. According to the literature (Yang *et al.* 2009), the highest phenolic compound recovery for most fruits uses over 50% (v/v) ethanol. We found similar results for extracting quercetin from *P. coccinea*.

TABLE 6. THIRD-ORDER RATE CONSTANTS AND HALF LIFE FOR THE REACTIONS OF HYDROXYL RADICALS WITH CYANIDIN AND QUERCETIN FOR 10 MIN.

	k (10^{-6} $L^2mol^{-2}min^{-1}$)	$t_{1/2}$ (min)
Cyanidin	212	0.11
Quercetin	4.66	5.16

Temperature = 25C; $[\text{phenolic compound}]_o = 250$ μM)

Response Surface Analysis of Cyanidin

The response surface analysis in Table 3 shows a good regression value ($R^2 = 0.9532$) for the relationship between the cyanidin and extraction parameters such as time, ethanol volume, HCl concentration and liquid–solid ratio. A significant quadratic polynomial equation for the cyanidin amount is given in Table 4. The x_1 (time), x_2 (ethanol volume), x_3 (HCl concentration), x_1x_2, x_1x_4, ${x_1}^2$ parameters were the most significant for the ultrasonic-assisted extraction of cyanidin from *P. coccinea*. x_4 (liquid–solid ratio), x_1x_3, x_2x_3, x_2x_4, x_3x_4, ${x_2}^2$, ${x_3}^2$ and ${x_4}^2$ had less effect on cyanidin yield of the ultrasonic-assisted extraction.

The relationship between the extraction parameters and cyanidin yield were investigated using the response surface plots shown in Fig. 1. As shown in Fig. 1C, increasing ethanol volume at a fixed time rapidly increased the cyanidin yield, while increasing the time at a fixed ethanol volume also markedly increased the cyanidin amount. The most cyanidin (3.30 mg/g dried plant) was observed using the highest time and ethanol volume. Adding a certain amount of water to the ethanol might improve the extracting efficiency (Yu *et al.* 2002). As shown in Table 4, the time and liquid–solid ratio had significant mutual effects. At the lowest liquid–solid ratio (10 mL/g), the cyanidin recovery increased as the time approached a certain value (approximately 100 min). Increasing the liquid–solid ratio increased

TABLE 7. THE ANTIOXIDANT CAPACITIES OF EXTRACTS DETERMINED WITH ABTS AND DPPH METHODS (MILLIGRAM TE PER GRAM DRIED WEIGHT)

Sample	ABTS	DPPH
Quercetin extract	168.08 ± 0.01	55.13 ± 0.03
Cyanidin extract	168.30 ± 0.02	55.89 ± 0.01

the cyanidin recovery at shorter times. However, the cyanidin recovery decreased with increasing liquid–solid ratio at longer times.

Extraction Parameter Optimisation and Model Validation

The optimum ultrasonic-assisted extraction conditions were determined to be 97 and 93 min for the extraction time, 71 and 79% (v/v) for the ethanol concentration, 1.55 and 1.52 mol/L for the HCl concentration, and 12.2 and 12.6 mL/g for the liquid–solid ratio for quercetin and cyanidin, respectively, within the studied region. Although other responses, such as the antioxidant activity, total phenol content and presence of other phenolic compounds can also be used to optimize the extraction factors, we considered the quercetin and cyanidin yields as the response due to their high content in *P. coccinea* as shown in Table 5. The model accuracy was validated using the optimum conditions obtained from the CCD. The theoretical quercetin and cyanidin yields were calculated as 2.30 and 4.01 mg/g dried plant, respectively. The extraction was repeated under the optimum conditions and the experimental amount of quercetin and cyanidin was found to be 2.21 ± 0.12 and 3.94 ± 0.15 mg/g dried plant. This verification experiment confirmed the model validity. Therefore, the CCD model was considered accurate and reliable for predicting the quercetin and cyanidin yield for ultrasonic assisted extractions.

Scavenging Ability of Quercetin and Cyanidin Against Reactive Oxygen Species

It is generally assumed that $HO^{\cdot}$ is produced in biological system from the Fenton-like reaction (Halliwell and Gutteridge 1992):

$$Fe(II) + H_2O_2 \rightarrow Fe(III) + HO^{\cdot} + HO^{-} \quad (3)$$

The rate of H_2O_2 decomposition increases with increasing pH and Fe (II) concentration. We used this Fenton-like reaction to generate HO radical and to study the ability of the phenolic compounds' reactivity towards the radical. The ability of both cyanidin and quercetin to scavenge the HO radical was examined in 30 minute. The samples taken in every 5 min were analyzed with HPLC-DAD. The remaining concentration of phenolic compound in each extract sample was calculated and also the rate constants and half life, calculated from these concentrations, are given in Table 6. We noted that the rate constants were in the order of 10^{-6} $L^2/mol^2/min$ and the k value for cyanidin was forty five times faster than quercetin. Cyanidin was found to be a more effective antioxidant than quercetin in *P. coccinea* extract. These compounds also inhibited reactive oxygen radicals about 90% inhibition were observed in the presence of 250 μM in 20 and 30 min for cyanidin and quercetin, respectively.

The antioxidant capacities of each extract sample were determined with ABTS and DPPH methods. The results were illustrated in Table 7. The antioxidant capacities using ABTS method were higher than the antioxidant capacities using DPPH method. The ABTS and DPPH methods differ in terms of their assay principle and experimental conditions. Because multiple reaction characteristics and mechanisms are usually involved, no single assay will accurately reflect all antioxidants in a mixed or complex system. Therefore, the use of different methods helps to identify variations in the response of the compounds extracted from the natural samples (Sarıburun *et al.* 2010).

CONCLUSIONS

The response surface method was successfully used to optimize the ultrasonic-assisted extraction parameters for *P. coccinea*. The central composite design proved to be a powerful tool for optimising the extraction parameters. The optimized conditions, including extraction time, ethanol volume, HCl concentration, and liquid–solid ratio, which were identified as the controlling factors, were determined for the quercetin and cyanidin extractions. The study results indicate the ultrasonic-assisted extraction is an economical and efficient method for extracting quercetin and cyanidin from *P. coccinea*. The free radical scavenging effects of the two phenolic compounds investigated in the present study. They act as free scavengers and inhibit free radical production. This study indicates *P. coccinea* can be considered a good source of naturally occurring antioxidant compounds.

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REFERENCES

AYBASTIER, Ö. and DEMIR, C. 2010. Optimization of immobilization conditions of *Thermomyces lanuginosus* lipase on styrene–divinylbenzene copolymer using response surface methodology. J. Mol. Catal. B: Enzym. *63*, 170–178.

AYBASTIER, Ö., ŞAHIN, S., IŞIK, E. and DEMIR, C. 2011. Determination of total phenolic content in *Prunella* L. by horseradish peroxidase immobilized onto chitosan beads. Anal. Methods *3*, 2289–2297.

BEZERRA, M.A., SANTELLI, R.E., OLIVEIRA, E.P., VILLAR, L.S. and ESCALEIRA, L.A. 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta, *76*, 965–977.

CACACE, J.E. and MAZZA, G. 2003. Mass transfer process during extraction of phenolic compounds from milled berries. J. Food Eng. *59*, 379–389.

CHUA, S.C., TAN, C.P., MIRHOSSEINI, H., LAI, O.M., LONG, K. and BAHARIN, B.S. 2009. Optimization of ultrasound extraction condition of phospholipids from palm-pressed fiber. J. Food Eng. *92*, 403−409.

FICO, G., BILIA, A.R., MORELLI, I. and TOME, F. 2000. Flavonoid distribution in *Pyracantha coccinea* plants at different growth phases. Biochem. Syst. Ecol. *28*, 673–678.

HALLIWELL, B. and GUTTERIDGE, J.M.C. (1992). Biologically relevant metal ion dependent hydroxyl radical generation. An update. FEBS Lett. *307*, 108–112.

HOLLMAN, P.C.H. and KATAN, M.B. 1997. Absorption, metabolism and health effects of dietary flavonoids in man. Biomed. Pharmacother. *51*, 305–310.

JAGANATH, I.B. and CROZIER, A. 2010. Dietary flavonoids and phenolic compounds. In *Plant Phenolicss and Human Health*, (C.G. Fraga, ed.) pp. 1–50, John Wiley & Sons, Inc., Hoboken, New Jersey.

JUNTACHOTE, T., BERGHOFER, E., SIEBENHANDL, S. and BAUER, F. 2006. The antioxidative properties of holy basil and galangal in cooked ground pork. Meat Sci. *72*, 446–456.

KOWALEUKI, Z. and MRUGASIEWICZ, M. 1971. Neue flavanon heteroside in *Crataegus phenophyrum*. Planta Med. *19*, 311–313.

LAMPE, J.W. 2003. Spicing up a vegetarian diet: Chemopreventive effects of phytochemicals. Am. J. Clin. Nutr. *78*(3), 579–583.

LI, H., CHEN, B. and YAO, S. 2005. Application of ultrasonic technique for extracting chlorogenic acid from *Eucommia ulmodies* Oliv. (*E. ulmodies*). Ultrason. Sonochem. *12*, 295–300.

MUROTA, K and TERAO, J. 2003. Antioxidative flavonoid quercetin: implications of its intenstinal absorption and metabolism. Arch. Biochem. Biophys. *417*, 12–17.

NAYAK, C.A. and RASTOGI, N.K. 2013. Optimization of solid–liquid extraction of phytochemicals from Garcinia indica Choisy by response surface methodology. Food Res. Int. *50*, 550–556.

PAN, Z., QU, W., MA, H., ATUNGULU, G.G. and MCHUGH, T.H. 2011. Continuous and pulsed ultrasound-assisted extractions of antioxidants from pomegranate peel. Ultrason. Sonochem. *18*, 1249–1257.

RICE-EVANS, C.A., MILLER, N.J. and PAGANGA, G. 1996. Structure–antioxidant activity relationship of flavonoids and phenolic acids. Free Radic. Biol. Med. *20*, 933–956.

RODRIGUEZ, R.J., MIRANDA, C.L., STEVENS, J.F., DEINZER, M.L., BUHLER, D.R. 2001. Influence of prenylated and non-prenylated flavonoids on livermicrosomal lipid peroxidation and oxidative injury in rathepatocytes. Food Chem. Toxicol. *39*, 437–445.

SARIBURUN, E., ŞAHIN, S., DEMIR, C., TÜRKBEN, C. and UYLAŞER, V. 2010. Phenolic content and antioxidant activity of raspberry and blackberry cultivars. J. Food Sci. *75*(4), 328–335.

SRINIVASAN, K. 2005. Spices as influencers of body metabolism: an overview of three decades of research. Food Res. Int. *38*(1), 77–86.

ŞAHIN, S., AYBASTIER, Ö. and IŞIK., E. 2013. Optimisation of ultrasonic-assisted extraction of antioxidant compounds from *Artemisia absinthium* using response surface methodology. Food Chem. *141*, 1361–1368.

ŞAHIN, S., DEMIR, C. and MALYER, H. 2011. Determination of total phenolic content of Prunella L. by immobilized enzyme bioreactor. Anal. Methods *3*, 944–950.

TAN, I.A.W., AHMAD, A.L. and HAMEED, B.H. 2008. Optimization of preparation conditions for activated carbons from coconut husk using response surface methodology. Chem. Eng. J. *137*, 462–470.

VATAI, T., ŠKERGET, M. and KNEZ, Ž. 2009. Extraction of phenolic compounds from elderberry and different grape marc varieties using organic solvents and/or supercritical carbon dioxide. J. Food Eng. *90*, 246–254.

VILKHU, K., MAWSON, R., SIMONS, L. and BATES, D. 2008. Applications and opportunities for ultrasound assisted extraction in the food industry. Innov. Food Sci. Emerg. Tech. *9*, 161−169.

WANG, L. and WELLER, C.L. 2006. Recent advances in extraction of nutraceuticals from plants. Trends Food Sci. *17*, 300–312.

YANG, B., LIU, X. and GAO, Y. 2009. Extraction optimization of bioactive compounds (crocin, geniposide and total phenolic compounds) from Gardenia (Gardenia jasminoides Ellis) fruits with response surface methodology. Innov. Food Sci. Emerg. *10*, 610–615.

YU, L., HALEY, S., PERRET, J., HARRIS, M., WILSON, J. and QIAN. M. 2002. Free radical scavenging properties of wheat extracts. J. Agric. Food Chem. *50*, 1619–1624.

YUAN, X., LIU, J., ZENG, G., SHI, J., TONG, J. and HUANG, G. 2008. Optimization of conversion of waste rapeseed oil with high FFA to biodiesel using response surface methodology. Renew. Eng. *33*, 1678–1684.