DOI:10.1021/jf103839j

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Optimization of Conditions for Anthocyanin Hydrolysis from Red Wine Using Response Surface Methodology (RSM)

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Optimization of conditions for anthocyanin hydrolysis from red wine was investigated using response surface methodology. The aglycon forms of the anthocyanins were quantified by high-performance liquid chromatography with diode array detection. The combined effects of three independent variables, HCl amount, heating temperature, and hydrolysis time, were studied using a 2^3 full-factorial central composite design. Anthocyanin hydrolysis yield depended mainly on the heating temperature and time of hydrolysis. HCl amount was the factor that least influenced the hydrolysis of anthocyanins. From experimental results, the maximum yield of anthocyanidins was reached with 9.8 mL of HCl (32% v/v), a heating temperature of 166.2 °C, and a hydrolysis time of 46.6 min. Five anthocyanidins, namely, delphinidin, cyanidin, petunidin, peonidin, and malvidin, were quantified in red wine. The reliability of the method was confirmed by recovery experiments, performed under optimal conditions. Recoveries indicated that anthocyanidins resisted the hydrolysis conditions.

KEYWORDS: Anthocyanidins; hydrolysis; red wine; response surface methodology; optimization

INTRODUCTION

Anthocyanins constitute the major flavonoid group of grapes (*Vitis vinifera*) and are responsible for the characteristic color of red wine (*1*). Recent and renewed interest in the study of anthocyanins is due to not only their bright colors as natural colorants but also their potential health benefits as antioxidants and anti-inflammatory agents, in metal chelation, in the stimulation of the immune system, in the prevention of tyrosine nitration, and for their antiproliferative, antiallergic, antibacterial, anticarcinogenic, and antiviral effects (*1*, *2*).

The anthocyanins are derived from the 2-phenylbenzopyrylium cation, more commonly known as the flavylium cation (*1*,*2*). Due to the attached sugars and acids often acylating these sugars, the number of anthocyanins is greater than the number of aglycon forms (anthocyanidins). This results in a large number of peaks on the chromatogram and difficulties in identifying individual anthocyanins. The major challenge for HPLC quantification of individual anthocyanins is often the difficulty in obtaining anthocyanin reference compounds. Fortunately, the complex pattern of anthocyanins can be reduced to six major anthocyanidins by acid hydrolysis. These major aglycones are delphinidin, cyanidin, pelargonidin, petunidin, peonidin, and malvidin. Differences between the aglycons are due to the number of hydroxyl groups and the degree of methylation of these groups. Acid hydrolysis, with hydrochloric acid performed in a sealed vial (*2*−*5*) or using a refluxing condenser (*6*−*9*), has become an accepted practice for the quantification of anthocyanidins.

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Anthocyanidins are usually detected and quantified at 520 nm after high-performance liquid chromatography (HPLC) separation; meanwhile, other compounds and impurities are not seen (*10*). Due to the instability of anthocyanidins, all of the analytical steps should be carried out in subdued light and under controlled conditions (*4*,*11*). Anthocyanin hydrolysis requires selection of several variables (namely, HCl amount, heating temperature, and time of hydrolysis) influencing the process, and it is essential to determine optimum conditions to guarantee maximum yield and feasible quantification. The literature describes different conditions for the hydrolysis of anthocyanins extracted from several food matrices. Addition of concentrated HCl and heating in a water bath (100 °C) during 60−120 min with or without refluxing are among the most usual methods (*2*, *4*, *5*, *7*−*9*). For a reliable quantification of anthocyanidins it is important to guarantee not only a complete hydrolysis of anthocyanins but also that no degradation of anthocyanidins occurs during the hydrolysis process. Merken et al. (*6*) described HCl concentrations of <1.8 M as being unsatisfactory because complete hydrolysis of glycosides from anthocyanin in food samples requires ≥ 4 h using a refluxing condenser. On the other hand, concentrations of HCl >2.4 M increased anthocyanidin degradation after hydrolysis.

Unfortunately, few studies deal with method development and validation using statistical designs and response surface techniques to determine the optimum operational conditions for the hydrolysis. The conventional approach for the optimization of a multivariable system is usually one variable at a time (*2*, *7*). This can be very time-consuming and, when interactions exist between the variables, it is unlikely to find the true optimum. Response surface methodology (RSM) is a very useful tool for this purpose as it

 Published on Web 12/15/2010

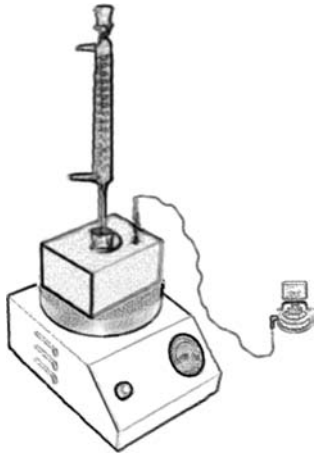

Figure 1. Scheme of hydrolysis of anthocyanins to anthocyanidins using a homemade aluminum block and hot plate. The homemade apparatus has the following characteristics: dimensions of block, 12 × 12 × 8.1 cm; dimensions of well for vial, ∅ = 4.8 and depth = 6.5 cm; reflux condenser size, 40 cm.

provides statistical models that help in understanding the interactions among the parameters that should be optimized (*1*, *12*−*14*). For example, Ghafoor et al. (*1*) optimized ultrasound-assisted extraction of compounds from grape seeds, including anthocyanins, using a five-level, three-variable central composite rotative design, and Fan et al. (*14*) investigated the combined effect of heating temperature, time, and solid−liquid ratio on the extraction of anthocyanins from purple sweet potato using RSM to select the optimum extraction conditions.

The aim of the present work was to select and validate the optimum hydrolysis conditions for a quantitative analysis of delphinidin, cyanidin, petunidin, peonidin, and malvidin (the aglycon forms of the anthocyanins) from red wine by liquid chromatography with diode array detection (HPLC-DAD) and understand the combined effect of hydrolysis parameters, namely, HCl amount, hydrolysis time, and heating temperature, on hydrolysis yield. RSM based on a central composite design (CCD) was employed for optimization of experimental conditions.

MATERIALS AND METHODS

Materials. Methanol (LiChrosolv) and formic acid (purity = 98−100%) were provided by Merck (Darmstadt, Germany). Hydrochloric acid 32% PA-ISO was purchased from Panreac (Barcelona, Spain). Delphinidin, cyanidin, malvidin, and cyanidin 3-*O*-glucoside standards were purchased from Sigma-Aldrich (Steinheim, Germany). Ultrapure water (0.055 μS/cm) was obtained by using a Seral Milli-Q system from Millipore. Standard solutions were prepared in methanol and kept at low temperature in the dark. Red wine with 13% alcohol, from the Douro valley region, produced with Tinta Roriz, Touriga Nacional, and Touriga Franca grape varieties was used.

Extraction and Hydrolysis of Anthocyanins from Red Wine. Red wine samples (25 mL) were added to 25 mL of 0.1% (v/v) hydrochloric acid in methanol and shaken for 10 min on a magnetic stirrer with vacuum. Twenty-five milliliters of the methanolic anthocyanin extract was used for the hydrolysis experiments. This extract was added to different amounts of concentrated HCl to perform hydrolysis in strong acidic medium (between 1.5 and 2.9 M HCl).

Experiments with three independent variables, HCl amount (5−10 mL), heating temperature (80−180 °C), and hydrolysis time (20−60 min), were conducted to optimize the best conditions for quantitative analyses of anthocyanidins from red wine. An appropriate device for the hydrolysis of anthocyanins was needed to carry out experiments at high temperatures, in some cases > 200 °C. Thus, a 50 mL vial (Schott Duran, U.K.) containing the extract to hydrolyze was adapted to a reflux condenser (40 cm) to prevent the escape of vapors and placed in a homemade aluminum block (12 × 12 × 8.1 cm) presenting a well for vial insertion (∅ = 4.8 and depth = 6.5 cm) with a heating plate (Falc model F60, Italy) (**Figure 1**). A sensor was used for temperature control (Lacor LC 62498, Spain; temperature maximum, 300 °C, and minimum, 0 °C). The hydrolyzed sample was cooled in the dark, filtered through a 0.45 μm filter, and diluted to 50 mL with methanol. A 20 μL aliquot was injected into the HPLC for analysis.

Table 1. Independent Variables and Their Coded Values Used for Optimization of Condition for the Hydrolysis of Anthocyanins to Anthocyanidins

			coded levels				
independent variable	unit	symbol	−1.682	−1	0	+1	+1.682
HCl amount	mL	X_1	3.3	5	7.5	10	11.7
heating temperature	°C	X_2	45.9	80	130	180	214
hydrolysis time	min	X_3	6.4	20	40	60	73.6

HPLC-DAD Conditions. Separation and quantification of anthocyanidins was performed by HPLC-DAD. The chromatographic analysis was carried out in an analytical HPLC unit (Jasco, Tokyo, Japan) equipped with Jasco PU-2080 HPLC pumps, an MD-2010 Plus multiwavelength detector, and a type 7725i Rheodyne injector with a 20 μL loop. The column was an ACE C18 column (5 μm; 250 mm length; 4.6 mm internal diameter). Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used.

Chromatographic separation was performed using a mixture of two eluents: solvent A, 10% formic acid; and solvent B, formic acid/water/methanol (10:40:50). The linear gradient program used was 0−50 min, 40−80% B in A; 50−55 min, column rinse and re-equilibration. The flow rate was 1.0 mL/min, and separations were carried out at ambient temperature. Diode array detection was set at 520 nm, and peak identification in red wine samples was carried out by comparing retention times and spectra of unknown peaks with reference standards.

The concentrations of anthocyanidins in red wine were calculated using corresponding anthocyanidin standard calibration curves (3.8−75 mg/L), except for petunidin and peonidin because no standard was available. To overcome this problem cyanidin was chosen as reference for the calibration of petunidin and peonidin, because the chromatographic response of the compound is rather similar at wavelengths around 520−530 nm. The concentration of anthocyanidins was expressed as milligrams of anthocyanidin per liter of red wine.

Recovery tests were made by spiking samples with anthocyanin standard (cyanidin 3-*O*-glucoside) before hydrolysis. The experimental recovery was obtained from the difference between two measurements (sample and spiked samples).

Experimental Design. Optimization of conditions for anthocyanin hydrolysis from red wine was carried out using RSM. Experiments with three independent variables, HCl amount (X_1), heating temperature (X_2), and hydrolysis time (X_3), were conducted following the experimental design statistical analysis obtained by the full factorial CCD. In this work, the full CCD consisted of (i) a complete 2^3 factorial design, (ii) n_0, center point ($n_0 > 1$), and (iii) two axial points on the axis of each design variable at a distance of $\alpha = 1.682$ from the design center. Hence, a total number of design points of $N = 2^k + 2k + n_0$ was used (*15*, *16*). The complete design consisted of 20 combinations including 6 replicates of the center point with 5 degrees of freedom for calculation of errors in the experiments.

The optimal values of response Y (sum of anthocyanidins peak area) were obtained by solving the regression equation and by analyzing the response surface contour plots. The variables were coded according to eq 1

$$X_i = \frac{x_i - X_0}{\Delta X} \quad (1)$$

where X_i is the coded value of variable i, x_i the uncoded real value of an independent variable, X_0 the value of x_i at the center point, and ΔX the step change between levels 0 and 1. The minimum and maximum values for HCl amount (X_1), heating temperature (X_2), and hydrolysis time (X_3) are listed in **Table 1**.

Experimental data from the CCD were analyzed using response surface regression and fitted to a second-order polynomial model (eq 2):

$$Y = \beta_0 + \sum_{i=1}^{a} \beta_i X_i + \sum_{i=1}^{a} \beta_{ii} X_i^2 + \sum_{i,j=1(i\neq j)}^{a} \beta_{ij} X_i X_j \quad (2)$$

Y is the sum of the peak area of anthocyanidins, β_0 is the constant coefficient, β_i is the linear coefficient, β_{ii} is the coefficient of squared effect,

β_{ij} is the coefficient of interaction effect, and X_i and X_j are the coded values of variables i and j, respectively.

The goodness-of-fit of the regression model and the significance of parameter estimates were determined through appropriate statistical methods. Work on experimental design, data analysis, response surfaces, and contour diagrams was performed by Design Expert trial-version 7 (Stat-Ease Inc., Minneapolis, MN).

RESULTS AND DISCUSSION

Preliminary Assays. Hydrolysis conditions similar to those described by Merken et al. (*6*) for anthocyanidin quantification in foods were tested for red wine. For this purpose four assays were performed using HCl concentrations of 1.5 M (4.2 mL of HCl 32%)/2.5 M (8 mL of HCl 32%) and hydrolysis time of 30 or 90 min at 100 °C. Delphinidin, cyanidin, petunidin, peonidin, and malvidin peak areas were monitored at 520 nm to evaluate the amount of anthocyanidins released. For the same hydrolysis time 2.5 M HCl provided a higher area of each anthocyanidin peak when compared with respective peak areas obtained with 1.5 M HCl. The anthocyanidin hydrolysis yield was established as a comparison between the peak area of each anthocyanidin and the respective maximum area obtained in the four assays. Maximum yield was obtained for 2.5 M and 90 min hydrolysis. The anthocyanidin yields were similar for delphinidin, cyanidin, petunidin, peonidin, and malvidin. Thus, in further studies the area of all anthocyanidins was summed and reported as a single entity, named response Y (sum of anthocyanidins peak area). The results of preliminary assays were in agreement with those obtained by Merken et al. (*6*) as summarized in **Table 2**. However, it should be pointed out that it is unlikely that true optimum conditions will be found without investigating if interactions between the variables exist.

Table 2. Experimental Data of Acid Hydrolysis of Anthocyanins at 100 °C: Comparison with the Literature

experimental data			data from the literature			
HCl concn (M)	time (min)	% anthocyanidin yield	HCl concn (M)	time (min)	% anthocyanidin yield	ref
1.5	30	43	1.8	30	50	*6*
1.5	90	88	1.8	90	97	*6*
2.5	30	78	2.4	30	77	*6*
2.5	90	98	2.4	90	97	*6*
			2.8	90	93	*7*

Table 3. CCD and Actual and Predicted Results for Hydrolysis of Anthocyanins from Red Wine

	coded values of the independent variables			sum of peak area (Y)	
standard order	X_1	X_2	X_3	observed	predicted
1	−1	−1	−1	2.57×10^5	1.18×10^5
2	1	−1	−1	9.06×10^5	8.39×10^5
3	−1	1	−1	1.75×10^6	1.92×10^6
4	1	1	−1	2.73×10^6	2.87×10^6
5	−1	−1	1	1.64×10^6	1.55×10^6
6	1	−1	1	2.28×10^6	2.17×10^6
7	−1	1	1	2.32×10^6	2.44×10^6
8	1	1	1	3.10×10^6	3.29×10^6
9	−1.682	0	0	1.80×10^6	1.80×10^6
10	1.682	0	0	3.18×10^6	3.12×10^6
11	0	−1.682	0	6.58×10^4	3.33×10^5
12	0	1.682	0	3.12×10^6	2.79×10^6
13	0	0	−1.682	1.03×10^6	9.99×10^5
14	0	0	1.682	2.59×10^6	2.56×10^6
15	0	0	0	2.73×10^6	2.46×10^6
16	0	0	0	2.72×10^6	2.46×10^6
17	0	0	0	2.50×10^6	2.46×10^6
18	0	0	0	2.26×10^6	2.46×10^6
19	0	0	0	2.11×10^6	2.46×10^6
20	0	0	0	2.44×10^6	2.46×10^6

Model Prediction and Fitting. Table 3 presents a full-factorial CCD with 20 experiments (including 6 replicates of the center point to verify any change in the estimation procedure as a measure of the precision property) as well as the experimental and predicted response function (measured as anthocyanidins peak area).

Regression analysis was performed to fit the response function (Y). The second-order model expressed by eq 2, where the variables take their coded values, represents the sum of the peak area of anthocyanidins in study (Y) as a function of X_1, X_2, and X_3 and is given in eq 3. This equation was used to predict the sum of peak area shown in **Table 3**.

$$Y = 2461073 + 393439X_1 + 729062X_2 + 463356X_3 + 58983X_1X_2 - 25644X_1X_3 - 228569X_2X_3 - 1057X_1^2 - 318965X_2^2 - 241491X_3^2 \quad (3)$$

ANOVA is important in determining the adequacy and significance of the quadratic model. The analyses were done by means of Fisher's F test, and the results are shown in **Table 4**.

As can be seen, the model F value of 26.04 with a low probability P value indicates high significance of the model. There is only a 0.01% chance that a model F value so large could occur due to noise. On the other hand, values of prob > F of < 0.05 indicate that model terms are significant, for X_1, X_2, X_3, X_2X_3, X_2^2, and X_3^2. The lack of fit for an F value of 1.082 means that this term is not significantly relative to the pure error. The

Table 4. Analysis of Variance (ANOVA) for Response Surface Quadratic Model for the Sum of Peak Area

source	sum of squares	degrees of freedom	mean square	F value	prob > F	remarks
model	1.49×10^{13}	9	1.66×10^{12}	26.04	<0.0001	significant
X_1	2.11×10^{12}	1	2.11×10^{12}	33.25	0.0002	significant
X_2	7.26×10^{12}	1	7.26×10^{12}	114.2	<0.0001	significant
X_3	2.93×10^{12}	1	2.93×10^{12}	46.12	<0.0001	significant
X_1X_2	2.78×10^{10}	1	2.78×10^{10}	0.438	0.5231	
X_1X_3	5.26×10^{9}	1	5.26×10^{9}	0.083	0.7795	
X_2X_3	4.18×10^{11}	1	4.18×10^{11}	6.574	0.0282	significant
X_1^2	1.61×10^{7}	1	1.61×10^{7}	0.0003	0.9876	
X_2^2	1.47×10^{12}	1	1.47×10^{12}	23.06	0.0007	significant
X_3^2	8.4×10^{11}	1	8.40×10^{11}	13.22	0.0046	significant
residual	6.36×10^{11}	10	6.36×10^{10}			
lack of fit	3.3×10^{11}	5	6.61×10^{10}	1.082	0.4668	not significant
pure error	3.05×10^{11}	5	6.11×10^{10}			
total	1.55×10^{13}	19				

nonsignificant value of lack-of-fit (> 0.05) shows that the quadratic model is valid for the present study (*17*). Thus, the sum of peak area of anthocyanidins is adequately explained by the model equation (eq 3).

The relationship between the experimental values and predicted values are represented in **Figure 2**. Results showed that the plotted points cluster around the diagonal line, indicating good fitness of the model because the value of R^2_{pred} of 0.8098 is in reasonable agreement with the R^2_{adj} of 0.9222. Le Man et al. (*15*) and Chauhan and Gupta (*18*) have emphasized the acceptance of any model with $R^2 > 0.75$.

Response Surface Plots and Optimization Conditions. Surface and contour plots demonstrating the effects of different variables, two variables varied at time while the third is kept constant, on the response function (sum of anthocyanidins peak area) are shown in **Figure 3**.

Figure 3a shows the contour map of the combined effect of HCl amount and heating temperature in the efficiency of hydrolysis, whereas the combined effect of HCl amount and hydrolysis time is shown in **Figure 3b**. As can be seen, within the ranges tested the anthocyanidin yield is little affected by the amount of acid added. **Figure 3c** shows the contour map of the effect of heating temperature and time of hydrolysis on anthocyanidin yield. The convex response surface suggested well-defined optimum variables (heating temperature and time of hydrolysis) and indicates that the sum of the peak area of anthocyanidins increased with the increase of temperature and time up to maximum values of 166.2 °C and 46.6 min, respectively. Therefore, the anthocyanidin yield mainly depends on the heating temperature and time of hydrolysis, and the HCl amount was the factor that less influenced the total hydrolysis of anthocyanins to anthocyanidins. Several authors refer to the use of a water bath (100 °C) and different hydrolysis times (between 60 and 120 min) performed in a sealed vial (*2*−*5*) or using a refluxing condenser (*6*−*9*), making it difficult to select the optimum conditions to obtain a reliable hydrolysis. However, our results indicate that lower hydrolysis times can be used with a refluxing system if higher temperatures are used. The selection of temperatures higher than those usually described coupled with lower hydrolysis time enabled higher hydrolysis yield. The homemade aluminum block with a heating plate was an appropriate device to perform hydrolysis at constant high temperatures that enable rapid rigorous boiling. As far as we know, no other work describes the use of a heating source at 166.2 °C to obtain a maximum yield of hydrolysis of anthocyanins. The combined effect of the three variables (HCl amount, heating temperature, and time of hydrolysis) evaluated by RSM was successful in selecting the optimum conditions, saving analysis time. As reviewed by Escarpa and Gonzalez, the ensuing stability of anthocyanidins depends in great measure on the species in which it is found, on the pH (due to their acid−base properties), and on other environmental parameters (*19*).

As a result, the optimum combination of factors that provided the maximum sum of peak anthocyanidin area from red wine is represented in **Table 5**. Experimental and predicted values under these conditions are shown. Response function (sum of anthocyanidin peak area) is adequately explained by the model equation. The sum of the peak area of anthocyanidins at the optimum

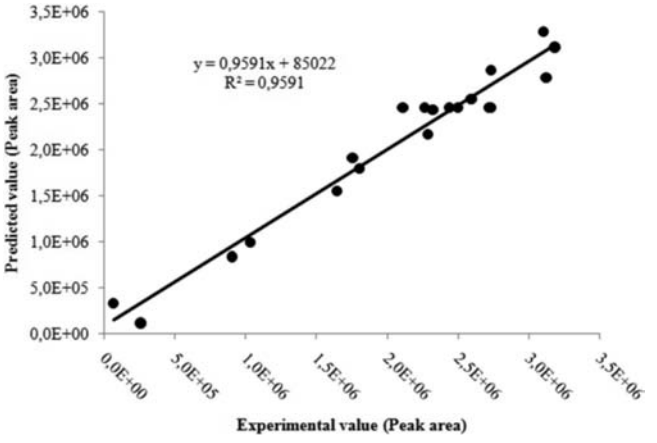

Figure 2. Experimental and predicted plot of peak area of anthocyanidins.

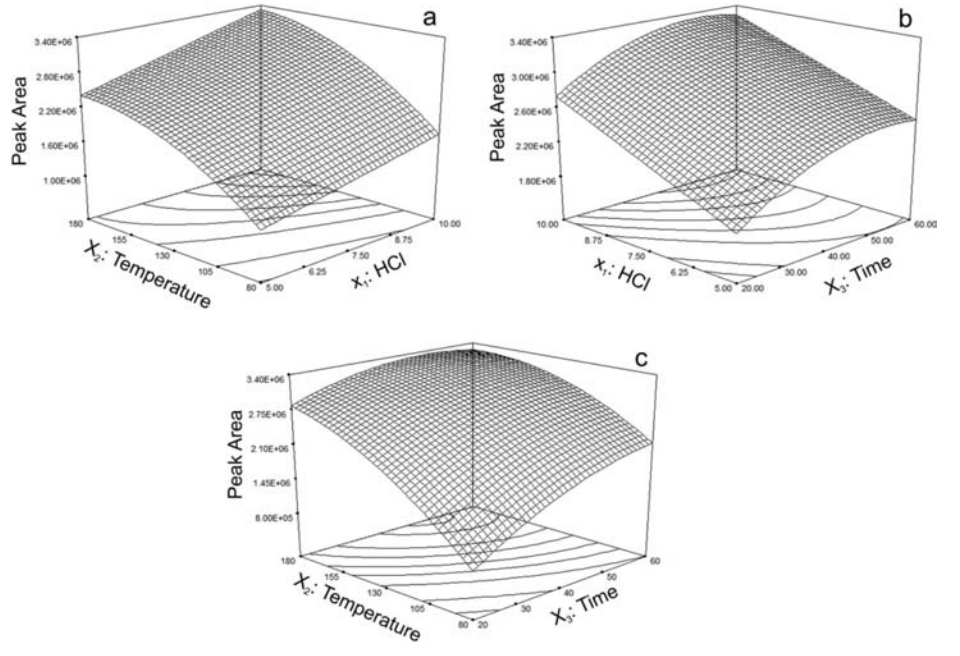

Figure 3. Response surface plots on the sum of peak area of anthocyanidins in red wine as affected by HCl amount, heating temperature, and time of hydrolysis: (**a**) HCl amount and heating temperature at constant hydrolysis time (min); (**b**) HCl amount and hydrolysis time at constant heating temperature (°C); (**c**) temperature and time of hydrolysis at constant HCl amount (mL).

Table 5. Model Validation

HCl amount (mL), X_1	heating temperature (°C), X_2	hydrolysis time (min), X_3	sum of peak area	
			experimental	predicted
9.8	166.2	46.6	2.91×10^6	3.29×10^6

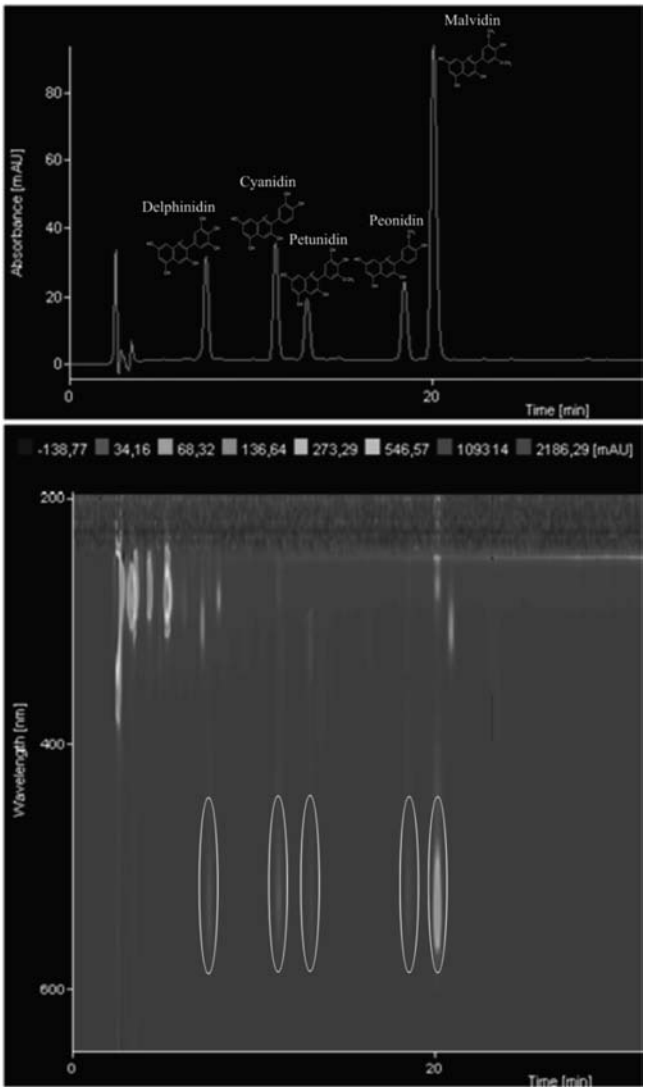

Figure 4. HPLC-DAD chromatogram of anthocyanidins in sample of red wine at 520 nm.

experimental condition (2.91×10^6) agreed well with the predicted one (3.29×10^6).

Model Validation and Confirmation. To confirm the validity of the statistical experimental strategies, a confirmation experiment with a duplicate set was performed at the selected optimum conditions. As expected, five peaks were identified in red wine after hydrolysis, namely, delphinidin, cyanidin, petunidin, peonidin, and malvidin. **Figure 4** shows a typical HPLC-DAD chromatogram of anthocyanidins in red wine at 520 nm and respective chemical structures.

The reliability of the method was confirmed by two recovery experiments, performed in optimal conditions. Recovery tests were made by spiking samples with glycoside forms of cyanidin, before hydrolysis. The concentration of the fortification was obtained by calculating the concentration of the corresponding aglycon after complete hydrolysis of the glycoside. Recoveries obtained varied between 86 and 95%, indicating that cyanidin resisted the hydrolysis conditions well. Thus, under appropriate pH the anthocyanin added to the red wine was converted into the corresponding aglycon without degradation after hydrolysis conditions as suggested by Merken et al. (*6*).

The levels of anthocyanidins found in red wine were 23.4 ± 0.9 mg delphinidin/L;, 35.9 ± 2.1 mg cyanidin/L, 21.8 ± 0.8 mg petunidin/L, 29.0 ± 1.1 mg peonidin/L, and 112.8 ± 2.4 mg malvidin/L. The anthocyanidin content in red wine can vary depending on climatic factors, grape ripeness, and winemaking process. However, these results were similar to those obtained by Nyman et al. (*3*). The RSM based on CCD was applied for optimization of anthocyanin hydrolysis for anthocyanidin quantification in red wine with advantages in terms of reducing hydrolysis time and maximizing of hydrolysis yield. In contrast to traditional techniques, this model takes into account the interactions among several independent variables.

ABBREVIATIONS USED

CCD, central composite design; HPLC-DAD, high-performance liquid chromatography with diode array detection; RSM, response surface methodology.

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Received for review July 12, 2010. Revised manuscript received November 23, 2010. Accepted November 28, 2010.