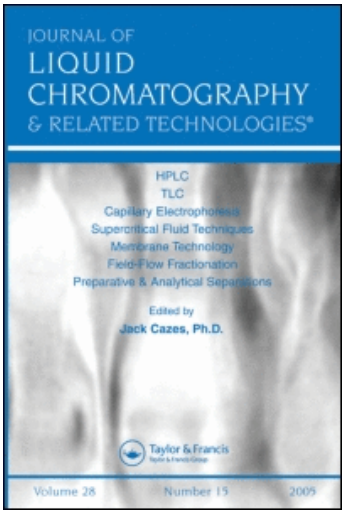

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Study of New Extraction Methods for Separation of Anthocyanins from Red Grape Skins: Analysis by HPLC and LC-MS/MS

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[a] Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran [b] Department of Chemistry, Faculty of Sciences, Shahid Beheshti University, Tehran, Iran [c] Department of Chemistry, Faculty of SciencesAlzahra University, Tehran, Iran [d] Department of Physiology and Pharmacology, Faculty of Veterinary Medicine, Tehran University, Tehran, Iran [e] Central Science Laboratory, University of Tasmania, Tasmania, Australia [f] Pharmaceutical and Medicinal Chemistry Department, National Research Centre, Cairo, Egypt

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Study of New Extraction Methods for Separation of Anthocyanins from Red Grape Skins: Analysis by HPLC and LC-MS/MS

Alireza Ghassempour,[1] Rouhollah Heydari,[2] Zahra Talebpour,[3] Ali Reza Fakhari,[2] Ali Rassouli,[4] Noel Davies,[5] and Hassan Y. Aboul-Enein[6]

[1]Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Tehran, Iran
[2]Department of Chemistry, Faculty of Sciences, Shahid Beheshti University, Tehran, Iran
[3]Department of Chemistry, Faculty of Sciences, Alzahra University, Tehran, Iran
[4]Department of Physiology and Pharmacology, Faculty of Veterinary Medicine, Tehran University, Tehran, Iran
[5]Central Science Laboratory, University of Tasmania, Tasmania, Australia
[6]Pharmaceutical and Medicinal Chemistry Department, National Research Centre, Cairo, Egypt

Abstract: Microwave assisted extraction (MAE) and ultrasound assisted extraction (UAE) methods were applied for the first time for the extraction of anthocyanins from red grape skins, and the results of these methods were compared with the maceration method. The extracted samples were analyzed by high performance liquid chromatography (HPLC) and nine anthocyanins were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). The effects

Correspondence: Hassan Y. Aboul-Enein, Pharmaceutical and Medicinal Chemistry Department, National Research Centre, Dokki, Cairo 12311, Egypt. E-mail: enein@gawab.com. Dr. A. Ghassempour, Department of Phytochemistry, Medicinal Plants and Drugs Research Institute, Shahid Beheshti University, Evin, P.O. Box 19835-389, Tehran, Iran. E-mail: aghassempour@scientist.com and a-ghasempour@cc.sbu.ac.ir

of sample weight to solvent volume ratio, temperature, and time of extraction on the efficiency of extraction in MAE were investigated by two full factorial designs at three levels. The results of two designs show that sample weight to solvent volume ratio and temperature were more important than the time of extraction in MAE. UAE has no variable parameters similar to MAE. The suitable solvent and required time for the UAE method were described. The recoveries of MAE, UAE, and maceration methods were 90%, 82%, and 98%, respectively.

Keywords: Anthocyanin, Grape skin, High performance liquid chromatography (HPLC), Microwave-assisted extraction (MAE), Tandem mass spectrometry (MS/MS), Ultrasound assisted extraction (UAE)

INTRODUCTION

Anthocyanins are naturally occurring colorants belonging to the flavonoid family. They are glycosylated polyhydroxy and polymethoxy derivatives of flavylium salts.[1] Different types and numbers of sugars are conjugated to the aglycones and form numerous structures of anthocyanins. The most prevalent glycosylation in anthocyanins is glucose. However, rhamnose, galactose, xylose, and arabinose are also present in anthocyanins.[2] In addition, many anthocyanins have sugar residues acylated with aromatic or aliphatic acids such as *p*-coumaric, caffeic, ferulic, and acetic substituents. However, Malvidin-3-glucoside and derivatives are main constituents in red grapes (Figure 1). Recently, anthocyanines has attracted considerable attention because of their antioxidant activity.[2–6]

Traditional methods including maceration and turbo extraction (high speed mixing) are very often time consuming and require relatively large

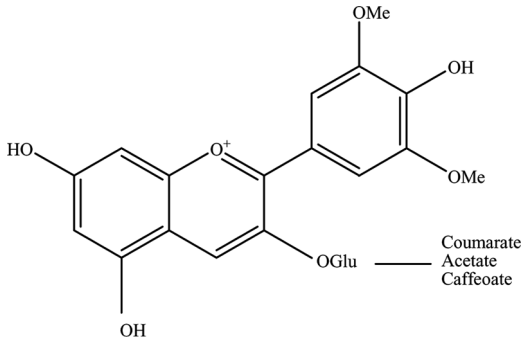

Figure 1. Structure of major anthocyanins in red grapes.

quantities of polluting solvents. Recently, pressurized liquid extraction (PLE) was used to extract anthocyanins from the red grape skins.[7]

Ultrasound assisted extraction (UAE) is often used for the extraction of plant material using liquid solvent. This extraction process is faster and more complete than maceration, due to the greater surface area in contact between the disrupted solid particles and liquid phase.[8]

Several studies show that microwave assisted extraction (MAE) has many advantages over the conventional extraction methods including shorter time, less solvent, and higher extraction efficiency.[9–11] One major challenge in the utilization of the MAE procedure for the extraction of anthocyanins from the real sample is the selection of experimental conditions that can provide the optimum recovery. Chemometric techniques have been frequently used for optimization of analytical methods.[12,13] Among these, factorial design is used mainly for preliminary evaluation of the significance of the variables.[14] This multivariate approach reduces the number of experiments and provides much more information than a one at a time method on the effects of different variables and their interactions, and also determines optimization of analytical procedures.[15]

The aim of this research was to develop UAE and MAE as rapid extraction methods for anthocyanins. To achieve this purpose, the efficiency of each extraction method was evaluated by high performance liquid chromatography (HPLC). On the other hand, identification of anthocyanins was performed using liquid chromatography tandem mass spectrometry (LC-MS/MS).

EXPERIMENTAL

Chemicals

Methanol and water (HPLC grade) were purchased from Caledon (Caledon, Canada). Ethanol (96%) was purchased from Persian Company (Shiraz, Iran). Formic acid was obtained from Merck (Taufkirchen, Germany). Malvidin-3-glucoside chloride standard was obtained from Extrasynthese (Genay, France).

Sample

The berries of grape (Vitis vinifera, cv Shahani) used in this study were obtained from the grape collection of Faculty of Agriculture, Tehran University (Karaj, Iran). Grape skins were manually separated from

the flesh, then transferred into plastic bags and stored at $-20^{\circ}C$ until analysis.

Instrumentation

HPLC analyses were carried out on a Knauer pump (K-1001) and a Knauer diode array detector (DAD, K-2800). The HPLC method proposed by Revilla et al.[16] was modified and used. The separation of anthocyanins was accomplished on a Eurospher column C_{18} (5 μm, 4×250 mm). The solvents were (A) 10% aqueous formic acid (v/v) and (B) water/methanol/formic acid, 45:45:10 (v/v/v). The solvent gradient was from 35 to 95% solvent B in 25 min and holding for 30 min. The DAD scan range was 200–800 nm and the detection wavelength was 530 nm. Flow rate was 1 mL min^{-1}. Column temperature was ambient and injection volume 20 μL.

LC-MS/MS analyses were performed by a Waters Alliance 2690 HPLC equipped with Waters 996 Diode Array and Finnigan LCQ ion trap mass spectrometer. The column was a Supelco Discovery C_{18} (5 μm, 4.6×250 mm). Mobile phase was (A) 10% formic acid in water and (B) methanol. The elution gradient was from 25% B for 3 min, and then linear ramp to 55% B at 30 min. Flow rate was 0.9 mL min^{-1}. The MS instrument was operated at the following settings: capillary temperature, 275°C; source voltage, 3.7 kV; full scan spectra were recorded over the range m/z 300–800 and scanned at 30 cycles per minute. Alternating with full scans were data dependent MS/MS scans of the strongest ion detected. Product ions of the selected ion were then scanned and monitored.

UAE was carried out using a Fritsch (220 V, 17.002 type, Germany) system. Microwave assisted extraction was carried out using a MarsX (1200 W, 2450 MHz) Microwave Accelerated Reaction System from CEM Corp. (USA).

Methods

Maceration Method

In this method, 2 g of ground grape skins were macerated with 50 mL methanol containing 0.1% of 12 N HCl for 48 h. After 24 h, the colored liquid was separated from the solid matrix and replaced with fresh solvent. The two extracts obtained were mixed and centrifuged at 3000 rpm for 10 min. The supernatant was then transferred into a round bottomed flask and evaporated down to 25 mL volume in a rotary evaporator. Distillated water was added to the sample up to 50 mL and

kept at 4°C until analysis. The samples were filtered and then analyzed by HPLC.

Microwave Assisted Extraction

Several solvents such as methanol, ethanol, and various proportions of methanol/water and ethanol/water containing 0.1% HCl were tested to find the best extraction solvent. Other parameters such as sample weight-to-solvent volume ratio, temperature, and time of extraction were optimized by a full factorial design in two separate steps. Extracted samples were centrifuged and the supernatant was evaporated to half of the initial volume in a rotary evaporator. Distillated water was added to the sample up to initial volume and kept at 4°C until analysis.

Ultrasound Assisted Extraction

Two grams of ground grape skins were suspended in 50 mL of acidic ethanol (0.1% HCl) and then was subjected to ultrasound for 3 h at room temperature. The obtained extract was then centrifuged at 3000 rpm for 10 min. The subsequent steps were followed according to the maceration method.

Experimental Designs

The optimization process for extraction of anthocyanins from red grape skins by MAE was carried out using sequential experimental designs. To ascertain the influences of the sample weight-to-solvent volume ratio, temperature, and the extraction time on the extraction of anthocyanins, at first a full factorial design for three variables at three levels was applied. Maximum and minimum levels of each factor were established according to the data from previous experiments (Table 1). The significant factors in MAE were optimized using the second factorial design. The choice of the studied factors and their levels in the second design was made considering the first factorial design. The design matrices for each factorial design were generated and the results evaluated by using Statgraphics@Plus software.

Standard and Calibration Curve

A commercially available standard of malvidin-3-glucoside chloride (1.7 mg) was dissolved in 1% HCl/MeOH (v/v) (5 mL), and used as standard stock solution. The stock solution was diluted with a 1:1 water-/methanol solution containing 1% HCl to afford 68, 34, 17, 5, 2, and 0.5 mg/L solutions of malvidin-3-glucoside chloride.

Table 1. Design matrix and the results for the first factorial design

Factor	Symbol	Key	Level		
			−1	0	+1
Sample weight to solvent volume ratio (g/ml)	ss	A	0.1	0.25	0.4
Extraction temperature (°C)	temp	B	60	70	80
Extraction time (min)	time	C	2	6	10

Run	Block	A	B	C	Response (mg)	[a]Run	Block	A	B	C	Response (mg)
1	1	0	−1	0	0.78	15	2	0	0	+1	0.31
2	1	+1	−1	+1	0.25	16	2	+1	−1	0	0.50
3	1	0	+1	+1	0.14	17	2	−1	+1	−1	0.44
4	1	−1	0	+1	0.51	18	2	−1	−1	+1	1.55
5	1	0	0	−1	0.30	19	3	+1	+1	0	0.36
6	1	−1	+1	0	0.48	20	3	−1	−1	0	1.63
7	1	−1	−1	−1	1.00	21	3	−1	0	−1	0.58
8	1	+1	0	0	0.04	22	3	0	+1	−1	0.34
9	1	+1	+1	−1	0.22	23	3	+1	0	+1	0.13
10	2	0	+1	0	0.34	24	3	0	−1	+1	0.93
11	2	+1	+1	+1	0.13	25	3	0	0	0	0.33
12	2	+1	0	−1	0.09	26	3	−1	+1	+1	0.63
13	2	0	−1	−1	0.90	27	3	+1	−1	−1	0.47
14	2	−1	0	0	0.61						

[a]Responses were calculated for main compounds.

The quantification of the different substances was carried out in the chromatograms registered at 530 nm by an external standard procedure, using a series of solutions of malvidin-3-glucoside chloride. Results are expressed as malvidin-3-glucoside equivalents.

Recovery Test

For every extraction method, three samples of grape skins were spiked with malvidin-3-glucoside chloride (10 mg/L) in related optimized conditions, and were extracted and analyzed as for real samples. Injection was repeated twice for each sample solution. The recoveries were calculated via comparison of peak area of malvidin-3-glucoside in spiked and unspiked samples.

RESULTS AND DISCUSSION

LC-MS/MS Identification

The anthocyanins were characterised by MS, MS/MS spectra, and relative retention time (R_t) comparison with previous findings.[17–19,22] The anthocyanins were also recognized through the fragmentation signals of the aglycone moiety at m/z 331 (Malvidin, Mv), 287 (Cyanidin, Cy), 301 (Peonidin, Pn), 317 (Petunidin, Pt), and 303 (Delphinidin, De). Using LC/MS analysis in the single ion monitoring (SIM) mode, interested ions have been detected specifically at the expected retention times (Figure 2).

The polarity of the aglycone (anthocyanidin) is the most important factor affecting the LC retention time of anthocyanins. Under reversed phase conditions, the retention decreased with increasing polarity (increased number of hydroxyl groups in the flavylium nucleus).[22] The anthocyanin-3-glucoside eluted in the order De < Cy < Pt < Pn < Mv, similar to the previous reports.[21] The elution order for acylated anthocyanins is acetyl < caffeoyl < coumaroyl.[16]

Mass spectra contained the molecular ion $[M]^+$ as base peak and MS/MS spectra presented the fragmentations of $[M\text{-}162]^+$, $[M\text{-}324]^+$, $[M\text{-}308]^+$, corresponding to the loss of the glucose, caffeoyl-glucoside, and the coumaroyl-glucoside moieties, respectively. MS and MS/MS spectra for the main anthocyanins in red grapes are shown in Figure 3.

Factorial Design, Evaluation of the Response Surface for MAE Procedure

After selection of methanol as the extraction solvent, in order to optimize other parameters in MAE, a three level full factorial design involving 27

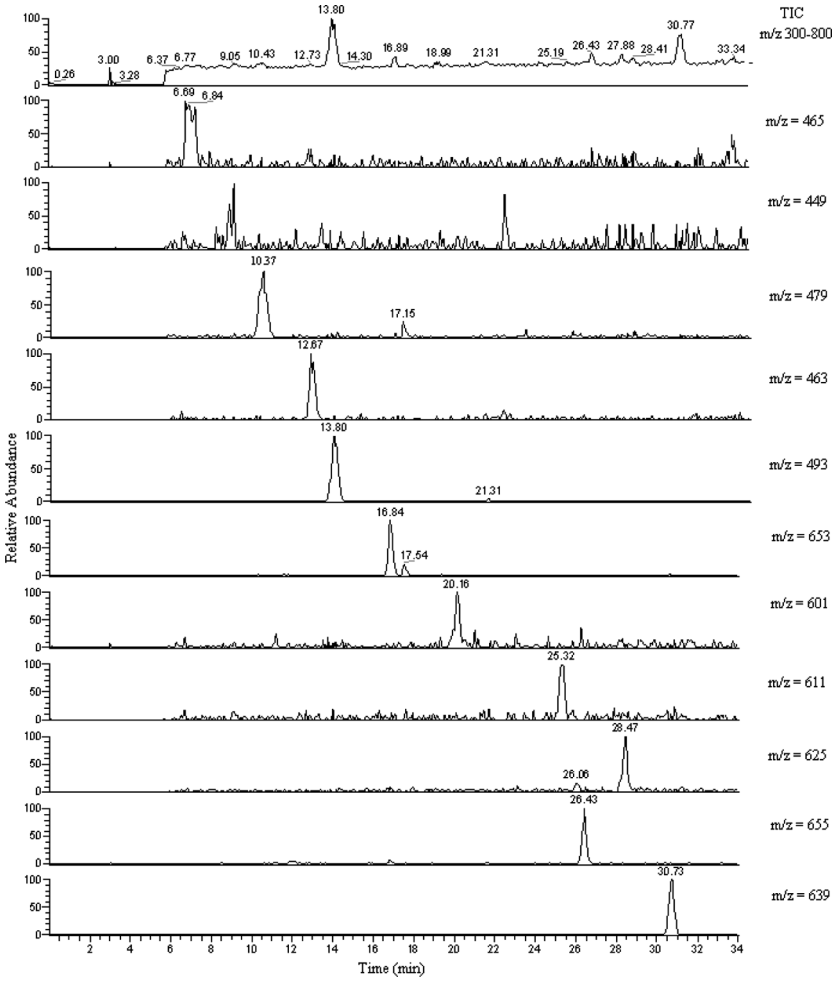

Figure 2. Total ion chromatogram (TIC) and LC/MS chromatograms in various m/z for compounds in red grape skin.

runs and two additional central points was chosen. Table 1 list the low and high levels given to each factor and shows the experimental design matrix and the results derived from each run. The significance of the effects was checked by analysis of the variance (ANOVA) and using P-value significance levels.

The ANOVA results produced the Pareto chart of main effects shown in Figure 4. Bar lengths are proportional to the absolute value of the estimated effects, which helps in comparing the relative importance of the effects. The interpretation of this chart demonstrated that the

factors of sample weight-to-solvent volume ratio and temperature were statistically significant, being affected by the negative signs (with increase of these factors decreasing the amount of extraction). Also, interaction between sample weight to solvent volume ratio temperature and sample weight-to-solvent volume ratio time were less significant, but the former

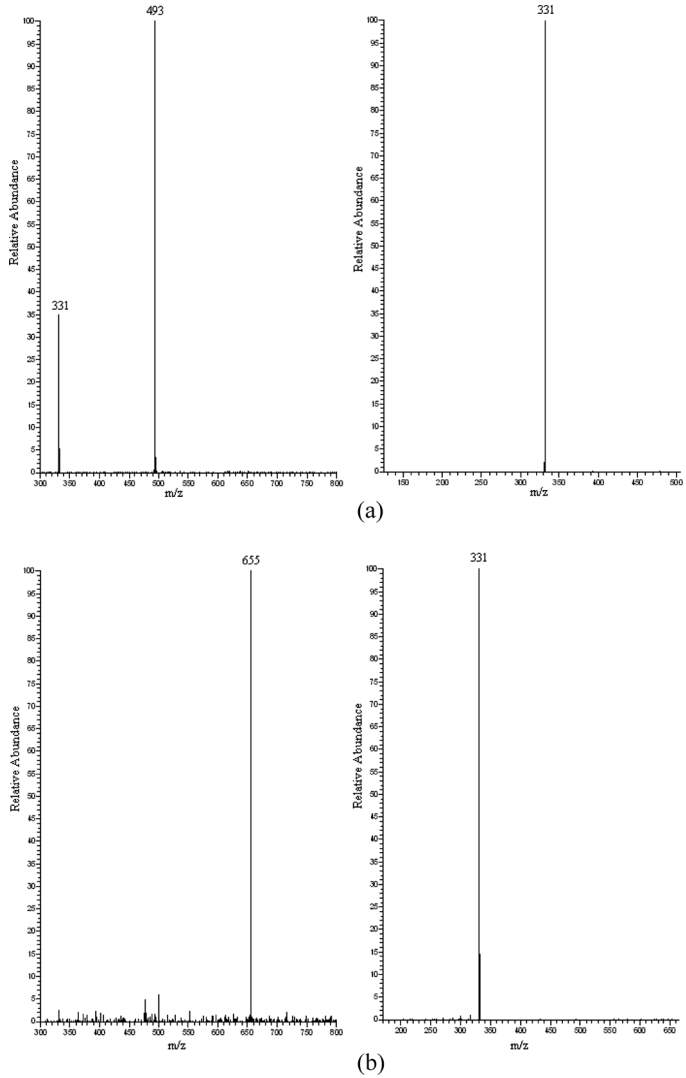

Figure 3. MS and MS/MS spectra obtained for the main compounds in red grape skins. (a) malvidin-3-glucoside, (b) malvidin-(6-caffeoyl)-3-glucoside, (c) malvidin-(6-coumaroyl)-3-glucoside (right MS/MS and left MS spectrum).

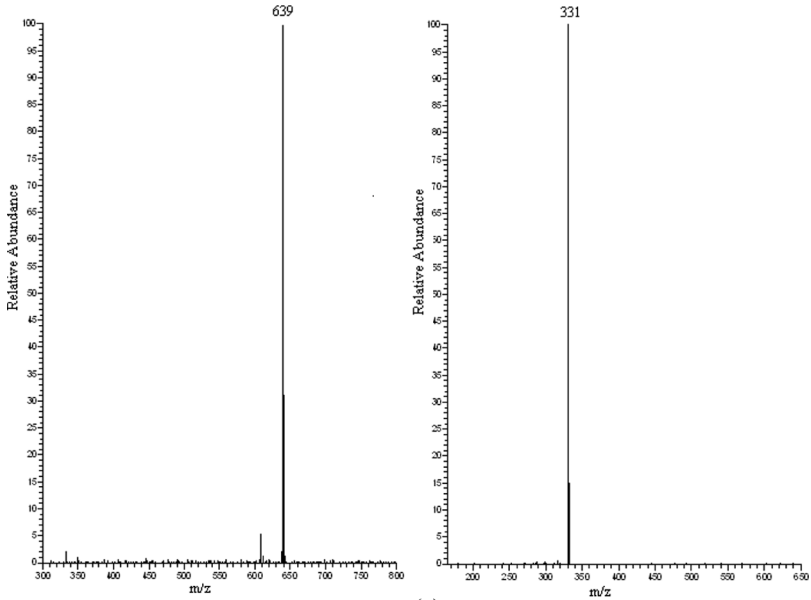

Figure 3. (Continued).

was affected by a positive sign and the latter by a negative sign. The time and the blocking factors were not significant.

Figure 5a and 5b show the response surface function developed by the model considering sample weight-to-solvent volume ratio temperature and temperature time, respectively. The extraction efficiency presents a minimum between 64–72°C and increased to its lowest level

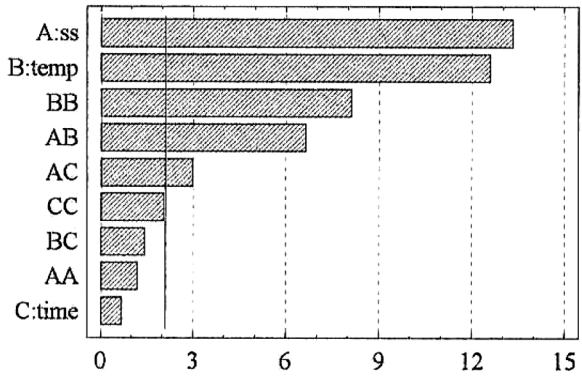

Figure 4. Pareto chart of standardized effects obtained from 3^3 full factorial design. The vertical line defines the 95% confidence interval.

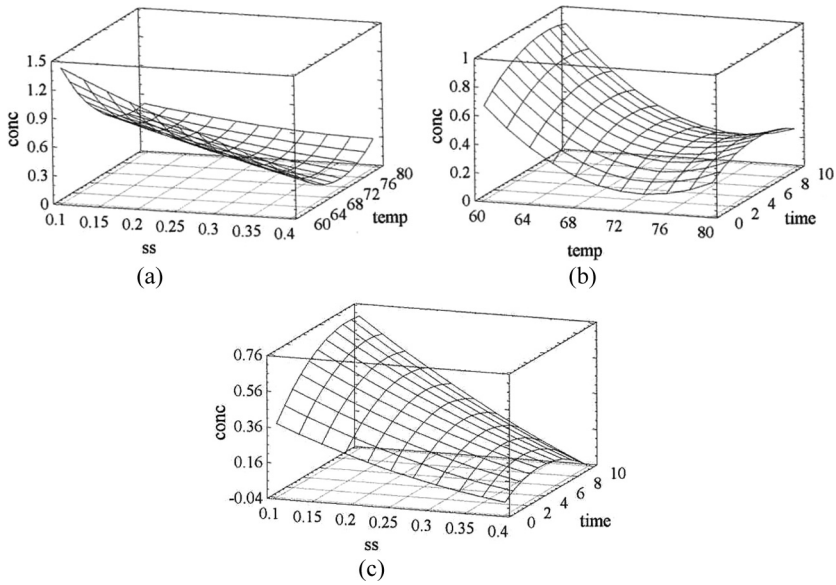

Figure 5. Response surface estimated from the factorial design by plotting (a) ss versus temperature, (b) temperature versus time and (c) ss versus time.

at all levels of sample weight-to-solvent volume ratio or time. Temperature elevation brings about thermal degradation of extracted compounds and a reduction in extraction efficiency takes place.

Figure 5c shows the response surface function developed by the model considering sample weight-to-solvent volume ratio time. The obtained response increased when sample weight-to-solvent volume ratio was at its lowest level in the experiments. This may be attributed to the limited capacity of extraction solvent, which ends up in back extraction.

The results of this design demonstrated for the final optimization the MAE method, the variables must be studied in the other levels. Thus, a new full factorial design involving the two most important variables (sample weight-to-solvent volume ratio and temperature) at new levels was developed. With respect to the observed results (Figure 5a and 5c), to obtain the optimum point, the levels of sample weight-to-solvent volume ratio and temperature in the second factorial design were reduced. The three levels of sample weight-to-solvent volume ratio and temperature with the new experiments, required by the second full factorial design, are described in Table 2. In all experiments, the time of extraction was fixed at 6 minutes.

The factorial design matrix at three levels yielded the relation among sample weight-to-solvent volume ratio, temperature, and response. The response surface considering the equation is shown in Figure 6. The

Table 2. Factor levels, design matrix and results for the final factorial design

Factor	Symbol	Key	Level		
			−1	0	+1
Sample weight to solvent volume ratio (g/ml)	ss	A	0.02	0.135	0.25
Extraction temperature (°C)	temp	B	30	50	70

Run	A	B	Response[a] (mg)	Run	A	B	Response (mg)
1	0	−1	2.49	6	+1	−1	1.53
2	+1	0	2.10	7	0	0	2.70
3	−1	−1	2.35	8	−1	+1	2.15
4	0	+1	3.01	9	−1	0	4.10
5	+1	0	0.59				

[a]Responses were calculated for main compounds.

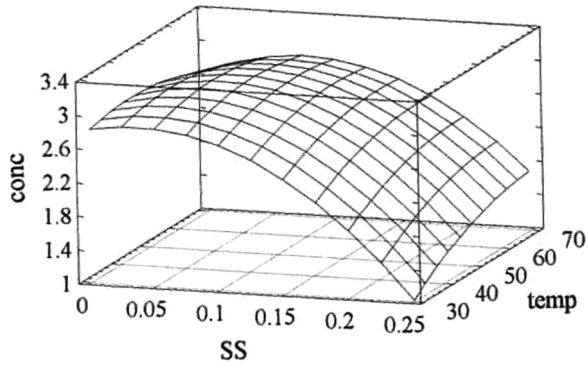

Figure 6. Response surface estimated from the last factorial design by plotting ss versus temperature.

dramatization of this equation as sample weight-to-solvent volume ratio and also as temperature was used to verify the presence of critical point, it was possible to deduce the coordinates of the maximum point of the surface, which corresponds to the point where the response achieves a more elevated volume. Therefore, solving the equations, a critical point is located at a sample weight-to-solvent volume ratio equal to 0.06 and temperature of 50°C. This point was observed in a contour plot (Figure 7).

Optimization of Ultrasound Assisted Extraction Method

The following solvents were tested for their ability to extract anthocyanins from grape skins: (1) 0.1% HCl in ethanol (96%), (2) 0.1% HCl

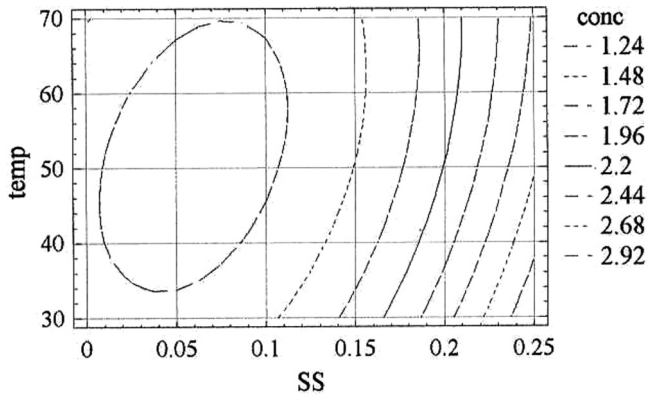

Figure 7. Contours of estimated response surface from the final factorial design by plotting ss versus temperature.

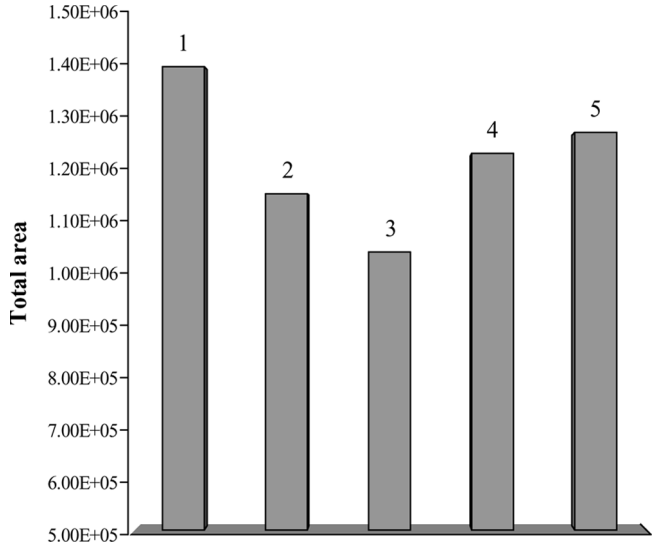

Figure 8. The effect of various solvents on efficiency of extraction by UAE. Extraction solvents: 1) ethanol (96%), 2) ethanol/water 50:50, 3) methanol, 4) methanol/water 50:50 and 5) methanol/water 80:20 (all solvents contained 0.1% HCl).

in 50% ethanol, (3) 0.1% HCl in methanol, (4) 0.1% HCl in 50% methanol, and (5) 0.1% HCl in 80% methanol. Hydrochloric acid was used at 0.1% because it provides a favorable medium for the formation of the stable flavylium ion and avoids the partial hydrolysis of acylated

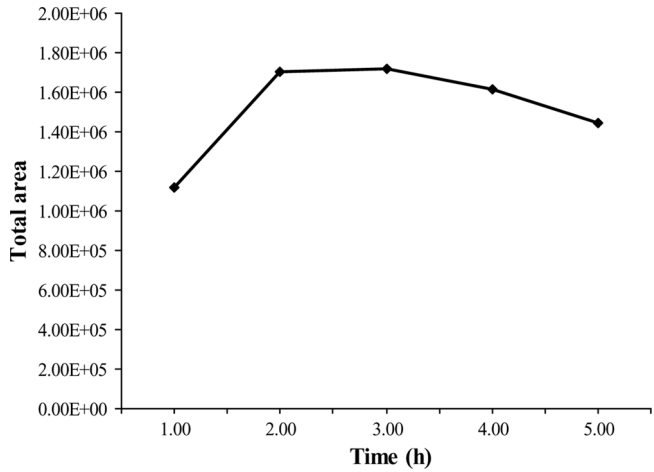

Figure 9. The effect of time on efficiency of extraction by UAE.

anthocyanins.[10] Figure 8 shows the effect of different solvents on the amount of extraction for total anthocyanins by UAE. As seen in Figure 8, in UAE, ethanol gives higher extraction of total anthocyanins than the other solvents. Therefore, ethanol was selected as extraction solvent in the UAE method. To determine the effect of UAE extraction time on the efficiency of anthocyanins extraction, times of 1, 2, 3, 4, and 5 h were tested using acidified ethanol as solvent. Figure 9 shows that the optimum time for UAE was 3 h.The results of HPLC analysis in optimum conditions of UAE (Table 3) showed that the extraction of compounds with less polarity and high stability (acylated compounds) increased when the ethanol was used as extracting solvent. The other reasons for this phenomenon can be attributed to the extraction method. In the UAE,

Table 3. Retention time, MS spectral details and concentration data of the anthocyanins obtained by different extraction methods

No.	Rt	Compounds	m/z	Fragments (MS/MS)	MAE (mg)[a]	UAE (mg)	Maceration (mg)
1	7.1	Delphinidin-3-glucoside	465	303	0.20	0.04	0.03
2	9.21	Cyanidin-3-glucoside	449	287	0.01	0.01	0.01
3	10.8	Petunidin-3-glucoside	479	317	0.27	0.05	0.07
4	13.0	Peonidin-3-glucoside	463	301	0.08	0.03	0.07
5	14.0	Malvidin-3-glucoside	493	331	1.42	0.61	0.90
6	17.0	Unknown	653	635, 636, 509, 491, 447	0.01	0.08	0.06
7	17.5	Unknown	653	635, 636, 509, 491, 447	0.03	0.04	0.02
8	20.1	Unknown	601	439	0.03	0.35	0.02
9	25.2	Delphinidin-(6-coumaroyl)-3-glucoside	611	303	0.20	0.03	0.04
10	26.0	Unknown	625	449	0.02	0.02	0.01
11	26.4	Malvidin-(6-caffeoyl)-3-glucoside	655	331	0.18	0.18	0.10
12	28.5	Petunidin-(6-coumaroyl)-3-glucoside	625	317	0.31	0.08	00.04
13	30.8	Malvidin-(6-coumaroyl)-3-glucoside	639	331	1.40	0.65	00.37
		Total			4.16	2.17	01.74

[a]Concentration data are expressed as malvidin-3-glucoside equivalents in 1 g sample (grape skin).

Table 4. Percent Recovery of Anthocyanins extracted by different methods

Extraction method	Recovery (%)	RSD (%)
Maceration	98.0	1.7
UAE	82.0	2.5
MAE	90.0	8.0

mechanical destruction of the cell wall by ultrasound waves brings about heavy molecules to predominate in the extraction solvent.

Maceration Method

The maceration method was performed as described in literature.[22] According to the results presented in Table 3, the maceration method provided similar results, which were in agreement to previously reported works,[23,24] malvidin-3-glucoside was the major compound and other compounds were minor. Whereas, in UAE and MAE methods, the amount of other compounds such as malvidin-(6-coumaroyl)-3-glucoside, petunidin-(6-coumaroyl)-3-glucoside, and malvidin-(6-caffeoyl)-3-glucoside increased and, even in UAE, malvidin-(6-coumaroyl)-3-glucoside becomes a major compound. The observed behavior may be considered as a result of different mechanisms involved in extraction processes.

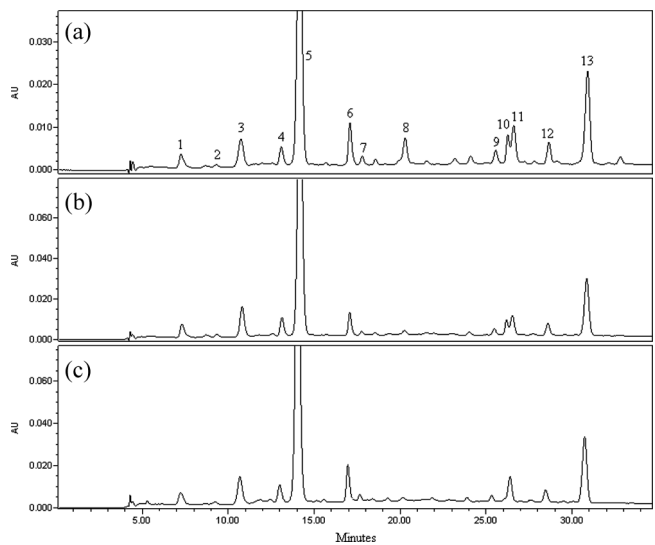

Figure 10. HPLC chromatograms for extraction of red grape skins by three extraction methods, (a) UAE, (b) MAE, (c) Maceration method.

In MAE and UAE the acylated compounds exhibited more tendencies toward the extraction in comparison with those which were not acylated (Table 3).

The precision of each method was performed under related optimized conditions by analyzing the samples in triplicate. The precision expressed as the percentage relative standard deviation (RSD), is included in Table 4. Because, exact control of all parameters that influenced the extraction process in the MAE and UAE is impossible, the values of RSD for these methods were higher than maceration. Also, the rough conditions of extraction in the MAE and UAE lead to a low recovery in comparison with the maceration method (Table 4).

The chromatograms obtained for extraction by MAE, UAE, and maceration are shown in Figure 10.

CONCLUSION

Conditions for MAE and UAE of anthocyanins from red grape skins have been studied. MAE and UAE have shown to be efficient methods for extraction of anthocyanins from red grape skins. Compared to the maceration method, the MAE and UAE procedures provided highly efficient extraction methods; require shorter time and less solvent, especially for MAE. Comparison of the three methods indicated that although there was little observable degradation under the optimum MAE, it was suitable for fast extraction of anthocyanins and more eco-friendly than maceration and UAE methods. This method would be useful for extraction of anthocyanins from the other plants.

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