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Deployment of response surface methodology to optimise recovery of grape (*Vitis vinifera*) stem polyphenols

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ABSTRACT

A 2^3-full factorial design and response surface methodology were deployed to assess some basic factors (time, % ethanol and pH) affecting profoundly the extractability of polyphenolic phytochemicals from grape (*Vitis vinifera*) stems. In an effort to obtain a thorough insight into the applicability of the models established, stem extracts from three different varieties were tested, by determining several indices of the polyphenolic composition, such as total polyphenol (TP), total flavanol (TFl), total flavone (TFn) and proanthocyanidin (PC) concentration. It was shown that the models generated can adequately predict the recovery levels for each polyphenol group, but the optimal conditions predicted for TP, TFl, TFn and PC recovery varied significantly. Notable differences were also seen among the different varieties. Correlation of the polyphenol indices with the antiradical activity and reducing power of the extracts indicated that the PC fraction might exert strong effects, while the influence of other groups was not apparent. Examination of the optimally obtained extracts using liquid chromatography–mass spectrometry revealed that the most prominent compounds were caftaric acid, flavanols and derivatives thereof, as well as dehydroflavonols and flavonols.

1. Introduction

Industrial wine production is accompanied by generation of large quantities of waste streams, including inorganic material (e.g. bentonite clay) but most importantly by-products composed of bioorganic substances (skins, seeds and stems). Owed to rapidly expanding global demand on manufacturing processes and final products exerting minimal or no environmental risks, the wine industry has begun to accept legislative pressure to become more efficient [1]. Thus the increasing demand for environmentally compatible production, coupled with rising operational and waste treatment cost, has started to move wine industry towards adoption of integrated waste preventive approaches. In Europe, it is estimated that 14.5 million tonnes of grape by-products are produced, on an annual basis, deriving from the winemaking industry [2]. This waste material has been a subject of significant research over the last few years, given the high richness in polyphenolic phytochemicals, which a wide spectrum of bioactivities has been attributed to [3]. The investigations carried out on the efficient retrieval of polyphenols from winery wastes have mainly been focused on red pomace, which is characterised by relatively high burden in phenolics and pigments [4–7].

Grape stems, which represent a fraction of the total grape waste generated during the vinification process, is a tissue that has been given relatively little attention, in spite of recent reports on its polyphenolic composition that appears to incorporate substances not encountered in other by-products, e.g. flavonols and stilbenes, in addition to monomeric and oligomeric flavanols [8,9]. Further, the polyphenolic content of stems was shown to be approximately 5.8% on a dry weight basis [10], and therefore stems may be a source of bioactive phenolics that should not be overlooked.

Ethanol is a bio-solvent, which is food compatible, reusable and cheap and it has become the solvent of preference for quite a few recent studies pertaining to the recovery of phenolics from various plant tissues. In the case of winery wastes, there is a scarcity of data concerning the use of ethanol-based solvents for extracting phenolic phytochemicals. This being the conceptual basis, the study presented herein provided some novel aspects pertaining to polyphenol retrieval from grape stems, using factorial design and response surface methodology.

Abbreviations: A_{AR}, antiradical activity; CTE, catechin equivalents; CyE, cyaniding equivalents; GAE, gallic acid equivalents; PC, proanthocyanidins; P_R, reducing power; RtE, rutin equivalents; S.D., standard deviation; TFl, total flavanols; TFn, total flavones; TP, total polyphenols.

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Table 1
Experimental values and coded levels of the independent variables used for the 2^3-full factorial design.

Independent variables	Code units	Coded variable level		
		−1	0	1
Ethanol content (%)	X_1	40	50	60
pH	X_2	2	4	6
Time (h)	X_3	1	3	5

2. Materials and methods

2.1. Chemicals

All solvents used for chromatographic purposes were HPLC grade. Absolute ethanol was of analytical grade. Folin–Ciocalteu phenol reagent and ascorbic acid were from Fluka (Steinheim, Germany). Gallic acid, catechin, rutin (quercetin 3-*O*-rutinoside), 2,4,6-tripyridyl-*s*-triazine (TPTZ), *p*-(dimethylamino)-cinnamaldehyde (DMACA), trolox® and 2,2′-diphenyl-picrylhydrazyl (DPPH•) stable radical were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Citric acid was from Merck (Darmstad, Germany).

2.2. Vinification wastes

Stems from three widely cultivated wine grape varieties were chosen; one white (Savatiano), one red used for white wine production (Moschofilero) and one red used for red wine production (Agiorgitiko). All samples used were obtained from wineries within the prefecture of Attica (central Greece), located in the region of Megara. Stems were collected immediately after destemming of grapes, transferred within a few hours to the laboratory and stored at −40 °C.

2.3. Extraction procedure

A similar procedure of that described previously was used [11]. Briefly, stems were lyophilised, ground to a fine powder using a domestic blender and chlorophyll was removed with sequential extractions with dichloromethane. An amount of approximately 0.5 g of chlorophyll-free material was placed in a 30-mL glass vial with 10 mL of solvent, composed of varying amounts of aqueous ethanol. All solvent systems used contained citric acid ($1\,g\,L^{-1}$) and were adjusted to the desired pH using 1N NaOH. Extractions were carried out under magnetic stirring at 400 rpm, at room temperature (22 ± 2 °C) for predetermined time periods. Upon com-

Table 2
Measured and predicted total polyphenol values determined for individual design points.

Design point	Independent variables			Response (TP, mg GAE/100 g dw)					
				Moschofilero		Savatiano		Agiorgitiko	
	X_1	X_2	X_3	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	−1	−1	−1	12960	12807	6495	6436	9095	9504
2	−1	−1	1	10053	9649	5975	5962	10712	10400
3	−1	1	−1	9819	9742	6022	6113	8404	8225
4	−1	1	1	9883	9845	6667	6948	9408	9728
5	1	−1	−1	10659	10591	7140	6966	10035	9693
6	1	−1	1	9644	9615	7025	7042	10483	10640
7	1	1	−1	8529	8827	5640	5761	8932	9222
8	1	1	1	11065	11112	6979	7146	11207	10776
9	−1	0	0	8605	9278	7151	6852	8926	8688
10	1	0	0	9052	8804	7347	7216	8981	9307
11	0	−1	0	9258	9912	8565	8794	9270	9358
12	0	1	0	9357	9128	9344	8685	9567	8786
13	0	0	−1	12189	12565	5700	5721	10779	10601
14	0	0	1	11703	12128	6628	6177	11561	11826
15	0	0	0	10820	10317	6728	7588	9745	9755
16	0	0	0	10664	10317	6701	7588	9940	9755

Table 3
Measured and predicted total flavanol values determined for individual design points.

Design point	Independent variables			Response (TFl, mg CTE/100 g dw)					
				Moschofilero		Savatiano		Agiorgitiko	
	X_1	X_2	X_3	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	−1	−1	−1	3158	3139	2302	2303	3661	3712
2	−1	−1	1	2814	2927	2206	2248	3713	3646
3	−1	1	−1	2646	2709	1949	1903	3914	3902
4	−1	1	1	2566	2587	1959	1997	3280	3377
5	1	−1	−1	3318	3342	2863	2838	3646	3554
6	1	−1	1	3713	3696	2593	2652	3901	3918
7	1	1	−1	3149	3081	1957	1928	3564	3636
8	1	1	1	3460	3525	1880	1891	3588	3542
9	−1	0	0	3485	3308	1854	1820	4045	3976
10	1	0	0	3882	3878	2051	2034	3931	3980
11	0	−1	0	3531	3430	2395	2318	3933	4024
12	0	1	0	3210	3130	1711	1737	4042	3931
13	0	0	−1	3031	2988	1594	1693	3680	3660
14	0	0	1	3285	3104	1797	1647	3602	3579
15	0	0	0	3167	3397	1666	1702	3922	3957
16	0	0	0	3264	3397	1636	1702	3952	3957

pletion of extraction, the extracts were filtered through paper filter, and stored at −20 °C until analysed. All extracts were also filtered through 0.45-μm syringe filters prior to determinations.

2.4. Experimental design

A 2^3-full factorial experimental design was used to identify the relationship existing between the response functions and process variables as well as to determine those conditions that optimised the extraction process. The response factors considered were four characteristic indices pertaining to the polyphenolic composition, namely total polyphenols (TP), total flavanols (TFl), total flavones (TFn) and proanthocyanidins (PC). The three independent variables or factors studied were ethanol concentration [X_1, varying between 40 and 60% (v/v)], pH (X_2, varying between 2 and 6) and extraction time (X_3, varying between 1 and 5 h). Each variable to be optimised was coded at three levels, −1, 0 and 1 (Table 1).

The three independent variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X_i}, \quad x_i = 1, 2, 3$$

where x_i and X_i are the dimensionless and the actual value of the independent variable i, X_0 the actual value of the independent variable i at the central point, and ΔX_i the step change of X_i corresponding to a unit variation of the dimensionless value. Responses (polyphenol indices) at each design point were recorded (Tables 2–5). Data from the central composite experimental design were subjected to regression analysis using least square regression methodology to obtain the parameters of the mathematical models. The Student's t-test permitted checking of the statistical significance of the regression coefficients deriving from the model. Analysis of variance (ANOVA) was applied to evaluate the statistical significance of the model. Response surface plots were obtained using the fitted model, by keeping the independent variables simultaneous.

2.5. Determinations

2.5.1. Total polyphenols

Measurements were carried out according to a previously published protocol [12], employing the Folin–Ciocalteu methodology. Gallic acid was used as the reference standard, and results were expressed as mg gallic acid equivalents (GAE) per 100 g of dry weight.

2.5.2. Total flavanols

Flavanols were determined after derivatisation with *p*-(dimethylamino)-cinnamaldehyde, using an optimised methodol-

Table 4
Measured and predicted total flavone values determined for individual design points.

Design point	Independent variables			Response (TFn, mg RtE/100 g dw)					
				Moschofilero		Savatiano		Agiorgitiko	
	X_1	X_2	X_3	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	−1	−1	−1	332.9	305.8	155.1	136.8	399.5	420.1
2	−1	−1	1	374.4	385.9	197.9	214.4	382.9	380.1
3	−1	1	−1	455.8	441.6	326.4	329.7	569.1	537.4
4	−1	1	1	676.1	671.7	514.8	502.8	494.2	505.8
5	1	−1	−1	229.3	222.5	144.9	153.7	340.5	330.9
6	1	−1	1	247.5	250.5	159.0	152.5	409.9	443.6
7	1	1	−1	262.5	239.7	156.4	136.8	355.6	360.5
8	1	1	1	401.9	417.7	216.0	231.1	500.2	481.6
9	−1	0	0	463.5	497.8	362.8	373.3	546.3	548.7
10	1	0	0	318.4	329.2	243.7	245.9	502.4	492.0
11	0	−1	0	236.2	255.6	146.3	145.8	398.5	356.6
12	0	1	0	381.5	407.1	268.5	281.6	400.4	434.2
13	0	0	−1	328.3	399.2	193.1	218.9	492.5	508.3
14	0	0	1	554.2	528.3	318.0	304.9	572.8	548.9
15	0	0	0	481.9	420.8	294.9	276.5	474.7	505.9
16	0	0	0	449.8	420.8	283.4	276.5	521.0	505.9

Table 5
Measured and predicted proanthocyanidin values determined for individual design points.

Design point	Independent variables			Response (PC, mg CyE/100 g dw)					
				Moschofilero		Savatiano		Agiorgitiko	
	X_1	X_2	X_3	Observed	Predicted	Observed	Predicted	Observed	Predicted
1	−1	−1	−1	237.2	241.3	156.8	153.1	272.7	283.7
2	−1	−1	1	259.5	254.2	172.1	169.0	298.5	289.1
3	−1	1	−1	149.6	141.5	144.7	135.4	256.6	248.2
4	−1	1	1	160.5	173.7	159.3	157.5	223.5	226.9
5	1	−1	−1	238.8	222.1	202.5	203.3	285.3	281.0
6	1	−1	1	223.9	228.4	191.6	199.9	311.8	319.4
7	1	1	−1	194.9	196.7	161.9	164.0	241.2	249.8
8	1	1	1	230.1	222.4	164.2	166.8	273.4	261.5
9	−1	0	0	211.7	207.8	115.3	133.2	222.8	226.2
10	1	0	0	204.4	222.5	176.7	163.0	264.1	242.2
11	0	−1	0	229.3	242.7	207.1	204.8	304.9	300.0
12	0	1	0	188.9	189.8	173.0	179.4	245.0	253.3
13	0	0	−1	202.8	221.7	159.2	169.4	295.1	288.3
14	0	0	1	245.6	241.0	184.7	178.7	286.6	296.9
15	0	0	0	242.1	226.3	177.0	172.8	264.9	266.8
16	0	0	0	239.1	226.3	177.0	172.8	275.6	266.8

Table 6
Polynomial equations and statistical parameters describing the effect of the independent variables considered on the recovery of various polyphenol classes, calculated after implementation of a 2^3-full factorial, central composite experimental design.

Response variables	2nd order polynomial equations	R^2	p
Moschofilero			
TP (mg GAE/100 g dw)	$10317 - 237X_1 - 392X_2 - 218X_3 + 325X_1X_2 + 546X_1X_3 + 815X_2X_3 - 1276{X_1}^2 - 797{X_2}^2 + 2029{X_3}^2$	0.91	0.0369
TFl (mg CTE/100 g dw)	$3397 + 285X_1 - 150X_2 + 58X_3 + 42X_1X_2 + 141X_1X_3 + 23X_2X_3 + 196{X_1}^2 - 117{X_2}^2 - 351{X_3}^2$	0.90	0.0421
TFn (mg RtE/100 g dw)	$420.8 - 84.3X_1 + 75.8X_2 + 64.5X_3 - 29.6X_1X_2 - 13.0X_1X_3 + 37.5X_2X_3 - 7.4{X_1}^2 - 89.5{X_2}^2 + 42.9{X_3}^2$	0.94	0.0057
PC (mg CyE/100 g dw)	$226.3 + 7.4X_1 - 26.5X_2 + 9.6X_3 + 18.6X_1X_2 - 1.6X_1X_3 + 4.8X_2X_3 - 11.2{X_1}^2 - 10.1{X_2}^2 + 5.0{X_3}^2$	0.86	0.0467
Savatiano			
TP (mg GAE/100 g dw)	$7588 + 182X_1 - 55X_2 + 228X_3 - 221X_1X_2 + 137X_1X_3 + 327X_2X_3 - 554{X_1}^2 + 1152{X_2}^2 - 1639{X_3}^2$	0.88	0.0693
TFl (mg CTE/100 g dw)	$1702 + 107X_1 - 290X_2 - 23X_3 - 127X_1X_2 - 33X_1X_3 + 37X_2X_3 + 225{X_1}^2 + 325{X_2}^2 - 32{X_3}^2$	0.97	0.0006
TFn (mg RtE/100 g dw)	$276.5 - 63.7X_1 + 67.9X_2 + 43X_3 - 52.5X_1X_2 - 19.7X_1X_3 + 23.9X_2X_3 + 33.1{X_1}^2 - 62.8{X_2}^2 - 14.6{X_3}^2$	0.98	0.0002
PC (mg CyE/100 g dw)	$172.8 + 14.9X_1 - 12.7X_2 + 4.7X_3 - 5.4X_1X_2 - 4.8X_1X_3 + 1.6X_2X_3 - 24.7{X_1}^2 + 19.3{X_2}^2 + 1.2{X_3}^2$	0.88	0.0370
Agiorgitiko			
TP (mg GAE/100 g dw)	$9755 + 309X_1 - 286X_2 + 613X_3 + 202X_1X_2 + 12.8X_1X_3 + 152X_2X_3 - 757{X_1}^2 - 683{X_2}^2 + 1459{X_3}^2$	0.91	0.0356
TFn (mg RtE/100 g dw)	$3957 + 2X_1 - 47X_2 - 40X_3 - 27X_1X_2 + 108X_1X_3 - 115X_2X_3 + 21{X_1}^2 + 20{X_2}^2 - 338{X_3}^2$	0.90	0.0417
TFl (mg CTE/100 g dw)	$505.9 - 28.3X_1 + 38.8X_2 + 20.3X_3 - 21.9X_1X_2 + 38.2X_1X_3 + 2.1X_2X_3 + 14.4{X_1}^2 - 111{X_2}^2 + 22.7{X_3}^2$	0.90	0.0179
PC (mg CyE/100 g dw)	$266.8 + 8.0X_1 - 23.3X_2 + 4.3X_3 + 1.1X_1X_2 + 8.3X_1X_3 - 6.7X_2X_3 - 32.6{X_1}^2 + 9.9{X_2}^2 + 25.8{X_3}^2$	0.92	0.0305

ogy [13], with modifications [14]. Results were expressed as mg catechin equivalents (CTE) per 100 g dry weight.

2.5.3. Total flavones

A previously established protocol was used [15], with modifications. An aliquot of 0.05 mL $AlCl_3$ (2% in 5% acetic acid in MeOH) was mixed with 0.5 mL sample and 0.5 mL 5% acetic acid in MeOH. The mixture was left for 30 min at room temperature and the absorbance was measured at 415 nm. Quantification was carried out using rutin (quercetin 3-*O*-rutinoside) as the calibrating standard. Results were reported as mg rutin equivalents (RtE) per 100 g dry weight.

2.5.4. Proanthocyanidins

The method described by Waterman and Mole [16], with modifications [14], was used. Results were expressed as cyanidin equivalents (CyE) per 100 g dry weight using as ε = 26,900 and MW = 449.2.

2.5.5. Antiradical activity (A_{AR})

Determinations were performed as described previously [12], using the DPPH• assay. All samples were diluted 1:20 immediately before the analysis. Results were expressed as trolox® equivalents (mM TRE) per g of dry weight.

Table 7
Optimal, predicted conditions and theoretically calculated maximal values for the recovery of various polyphenol classes.

Response	Maximal predicted value	Optimal conditions		
		EtOH (%)	pH	t (h)
Moschofilero				
TP	13235 ± 1406	46.9	3.5	1
TFl	3928 ± 353	60	3	3.5
TFn	675.3 ± 99.1	40	5.6	5
PC	256.2 ± 36.0	44.2	2	5
Savatiano				
TP	8757 ± 1085	54.4	2	3
TFl	2838 ± 212	60	2	1
TFn	502.8 ± 48.9	40	6	5
PC	211.6 ± 25.3	53.1	2	5
Agiorgitiko				
TP	11864 ± 891	52.2	3.9	5
TFl	4098 ± 258	60	2	3.5
TFn	597.0 ± 75.8	40	4.5	1
PC	338.6 ± 31.8	52.3	2	5

2.5.6. Reducing power (P_R)

A protocol described elsewhere [10] was used. P_R was determined as mM ascorbic acid equivalents (mM AAE) per g of dry weight.

2.6. HPLC-DAD Analysis

The equipment utilized was an HP 1090, series II liquid chromatograph, coupled with an HP 1090 diode array detector and controlled by Agilent ChemStation software. The column was a Phenomenex Synergi Hydro RP18, 4 μm, 250 mm × 4.6 mm, protected by a guard volume packed with the same material. Both columns were maintained at 40 °C. Eluent (A) and eluent (B) were 0.05% aqueous trifluoroacetic acid (TFA) and MeCN containing 0.05% TFA, respectively. The flow rate was 1 mL min^{-1}, and the elution programme used was a linear gradient as follows: 5 min, 5% B; 65 min, 50% B. Monitoring of the eluate was performed at 275, 290, 320, and 360 nm.

2.7. Liquid chromatography–mass spectrometry

A Finnigan MAT Spectra System P4000 pump was used coupled with a UV6000LP diode array detector and a Finnigan AQA mass spectrometer. Analyses were carried out on a Superspher RP-18,

Table 8
Statistical parameters describing the correlation of various classes of polyphenols with the antioxidant parameters (A_{AR} and P_R), determined after implementation of simple linear regression.

Response variables	Antioxidant values			
	A_{AR}		P_R	
	R^2	p	R^2	p
Moschofilero				
TP	0.27	0.039	0.24	0.057
TFl	0.00	0.841	0.02	0.567
TFn	0.10	0.232	0.04	0.471
PC	0.21	0.078	0.62	<0.001
Savatiano				
TP	0.19	0.094	0.20	0.083
TFl	0.05	0.388	0.16	0.128
TFn	0.07	0.337	0.19	0.091
PC	0.14	0.155	0.48	0.003
Agiorgitiko				
TP	0.06	0.342	0.01	0.681
TFl	0.09	0.256	0.08	0.277
TFn	0.15	0.146	0.10	0.234
PC	0.32	0.023	0.14	0.151

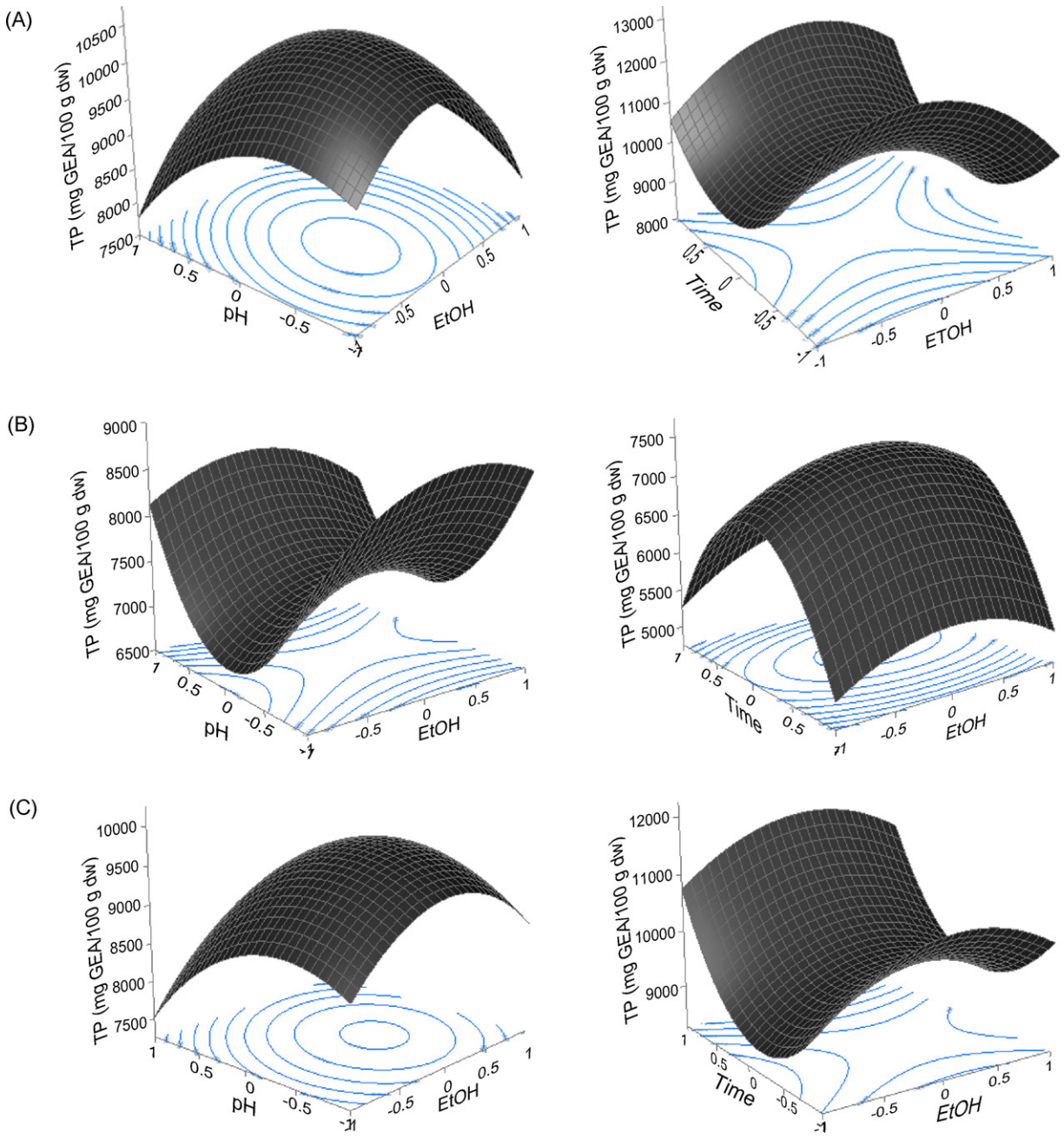

Fig. 1. Response-surface plot showing the effect of EtOH/pH (left) and EtOH/time (right) co-variance on the total polyphenol yield. (A) Moschofilero; (B) Savatiano; (C) Agiorgitiko.

125 mm × 2 mm, 4 μm, column (Macherey-Nagel, Germany), protected by a guard column packed with the same material, and maintained at 40 °C. Analyses were carried out employing electrospray ionization (ESI) at the positive ion mode, with acquisition set at 12 and 70 eV, capillary voltage 3.5 kV, source voltage 4 kV, detector voltage 650 V and probe temperature 400 °C. Eluent (A) and eluent (B) were 2.5% acetic acid and methanol, respectively. The flow rate was 0.33 mL min^{-1}, and the elution programme used was as follows: 0–5 min, 0% B; 5–30, 100% B; 30–35, 100% B.

2.8. Statistical analyses

All determinations were carried out at least in triplicate and values were averaged and given along the standard deviation (±S.D.). Linear regression analyses were performed on a 95% significance level. For all statistics, JMP™ 5.1 and Microsoft Excel™ 2000 were used.

3. Results

3.1. Optimisation of the extraction process

The experimental screening performed was designed to assess the influence of three factors, that is, the ethanol concentration, the pH and the extraction time. In Table 1 the experimental values and coded levels of the three independent process variables used for the 2^3-full factorial, central composite, experimental design implemented can be seen. Values of the independent process variables (X_1, X_2 and X_3) considered, as well as measured and predicted val-

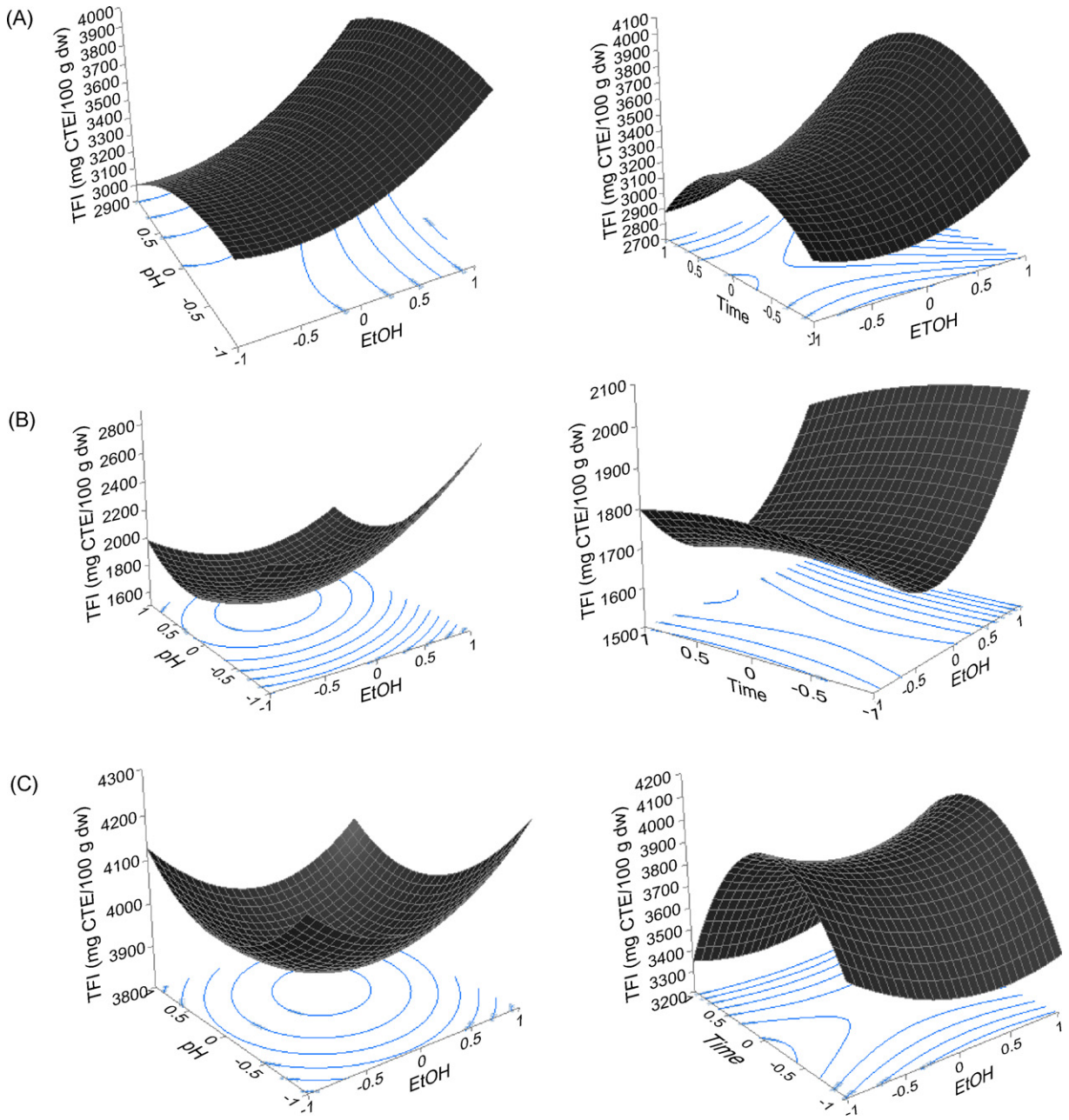

Fig. 2. Response surface plot showing the effect of EtOH/pH (left) and EtOH/time (right) co-variance on the total flavanol yield. (A) Moschofilero; (B) Savatiano; (C) Agiorgitiko.

ues for all responses (TP, TFl, TFn and PC) are analytically given in Tables 2–5.

The experimental values of all indices were analysed by multiple regression to fit the second-order polynomial equations shown in Table 6 and the quality of fit was ascertained using the coefficients of determination (R^2). The experimental data obtained showed a good fit with the equations, which were statistically acceptable at 95% significance level ($p < 0.05$), with the exception of TP for Savatiano. This fact indicated a satisfactory agreement between observed and predicted responses and that the equations found can adequately predict the experimental results. The utilisation of the predictive models enabled the theoretical calculation of the optimal sets of conditions, under which maximal values could be attained (Table 7).

The trends revealed in each case were recorded in the form of three-dimensional plots (Figs. 1–4), where on the left is illustrated the effect of simultaneous variation of pH and EtOH, and on the right the effect of simultaneous variation of time and EtOH.

3.1.1. Total polyphenols

The trend seen for TP recovery from Moschofilero stems (Fig. 1A) upon simultaneous variation of pH and EtOH indicated that maximal yield can be achieved at intermediate pH and EtOH values. At higher pH and EtOH levels, TP recovery showed a declining tendency. The same effect was observed for Agiorgitiko (Fig. 1C), but for Savatiano (Fig. 1B) the effect of pH was quite the opposite, as intermediate pH values afforded lower TP yield, which was increased either at lower or higher pH. With regard to the extraction time, maximal yield for Moschofilero were found for short duration, while for Savatiano and Agiorgitiko intermediate and longer durations were proven favourable, respectively (Table 7).

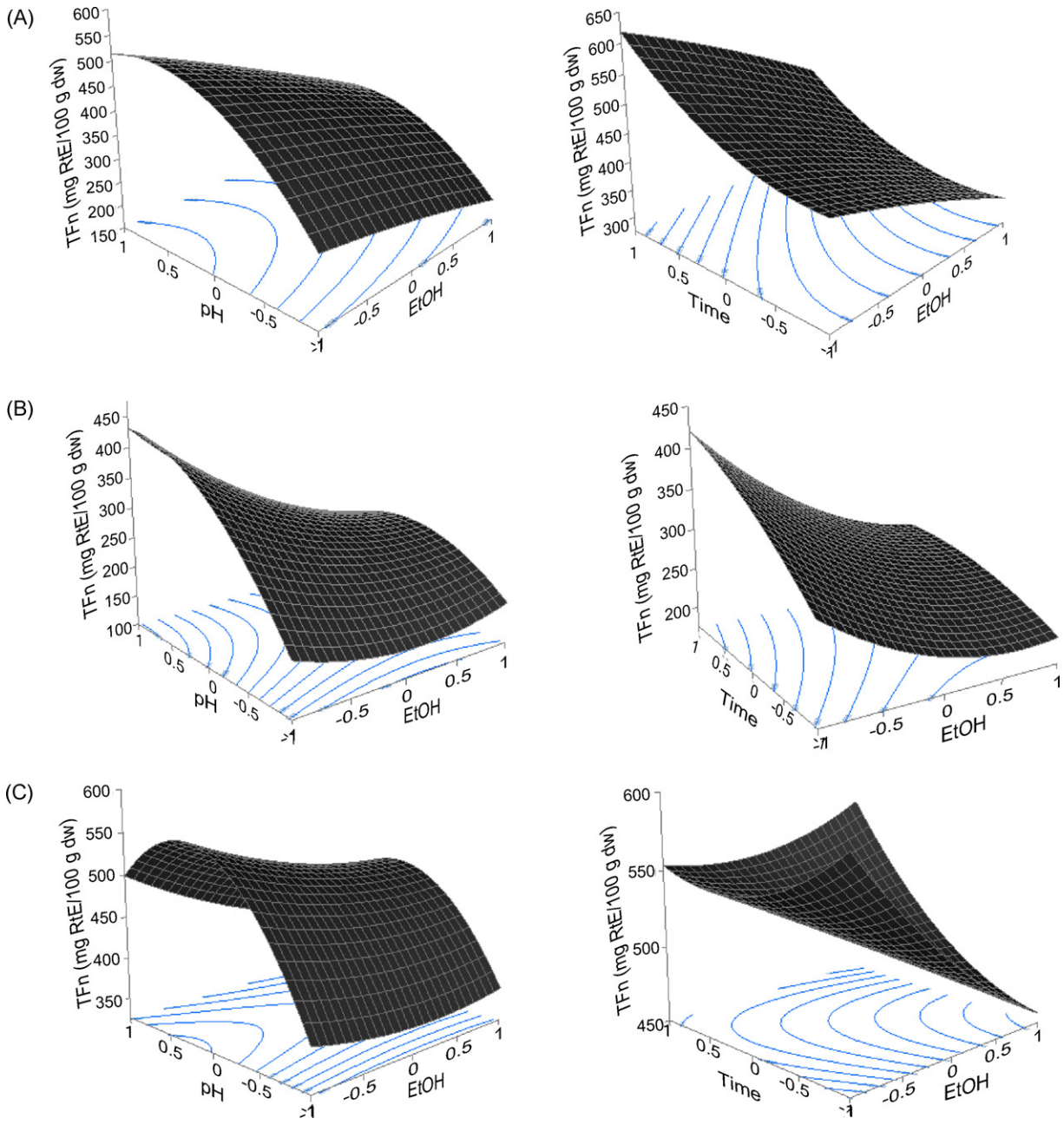

Fig. 3. Response surface plot showing the effect of EtOH/pH (left) and EtOH/time (right) on the total flavone yield. (A) Moschofilero; (B) Savatiano; (C) Agiorgitiko.

3.1.2. Total flavanols

TFl yields were found to obey similar trend for Savatiano and Agiorgitiko, but different for Moschofilero (Fig. 2A–C). In particular, TFl extraction from the stems of the two former varieties was facilitated at pH 2, whereas efficient extraction from Moschofilero required a pH 3. However, there has been a consistency in EtOH concentration and in all cases high EtOH (60%) was demonstrated the most appropriate. Optimal extraction durations varied from 1 to 3.5 h (Table 7).

3.1.3. Total flavones

In all cases it was apparent that low EtOH levels in combination with relatively high pH (4.5–6) might be ideal for flavone recovery (Fig. 3A–C). As opposed to TP and TFl, low pH was unfavourable. Longer extraction time was also shown important, although optimal recovery for Agiorgitiko was found at shorter duration.

3.1.4. Proanthocyanidins

Regarding the effect of pH and time, the findings for all varieties were in accordance, suggesting that low pH and prolonged extraction period are necessary to achieve high yields (Fig. 4A–C). On the other hand, intermediate EtOH levels were very efficient for PC recovery from Savatiano and Agiorgitiko stems, but for Moschofilero lower amounts (Table 7) sufficed to obtain optimal yields.

3.2. Correlation with antioxidant indices

Every extract generated was also assessed with regard to its antioxidant properties, by determining two representative indices, the antiradical activity and the reducing power. The values derived from these tests were correlated with all polyphenolic groups, using simple linear regression analysis. In Table 8 are given the statisti-

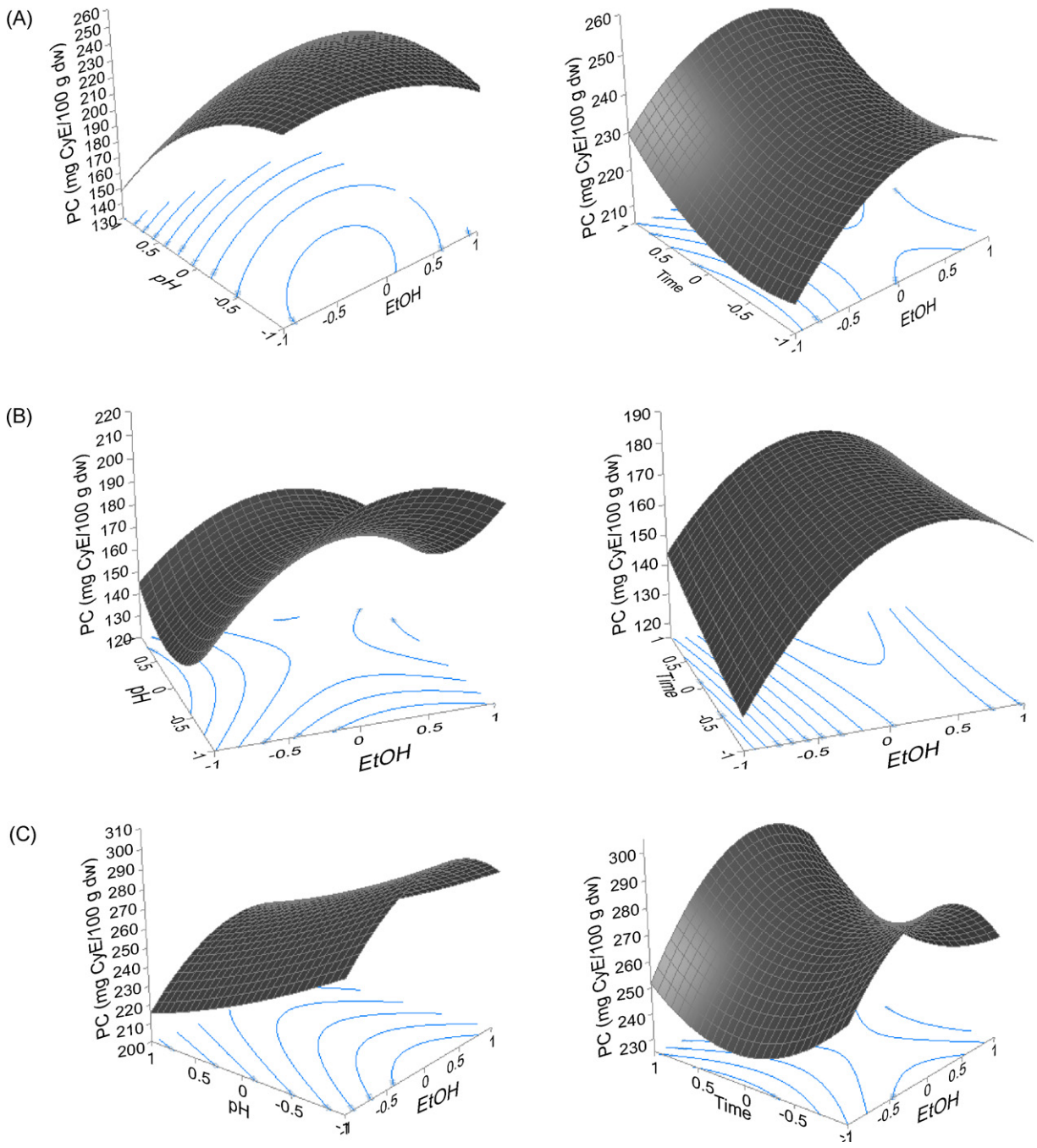

Fig. 4. Response surface plot showing the effect of EtOH/pH (left) and EtOH/time (right) on the proanthocyanidin yield. (A) Moschofilero; (B) Savatiano; (C) Agiorgitiko.

cal parameters calculated for all correlations established. With the exception of Moschofilero extracts, which showed a statistically significant ($p < 0.05$) but low ($R^2 = 0.27$) correlation with TP, in all other cases statistically significant links were found only for PC. More particularly, for Moschofilero and Savatiano, PC correlation was significant with P_R, whereas for Agiorgitiko significant correlation was found between PC and A_{AR} (Table 8).

3.3. Polyphenolic profile of extracts

The HPLC analysis was monitored effectively at 275 nm, where all phenolic classes that occur in grape stems show appreciable absorbance. This being the case, the chromatograms obtained represented the overall polyphenolic profile of the extracts (Figs. 5 and 6). The liquid chromatography–mass spectrometry analysis, performed in positive ion mode, allowed for the tentative identification of almost all major peaks detected, thus permitting a profounder insight into the composition of the optimally obtained extracts. In Fig. 5 can be seen the profile of Moschofilero extract (sample No 1, Table 2), which had the highest TP concentration. The chemical structures of all compounds tentatively identified are given in Fig. 7, numbered in accordance with the peak numbering in Fig. 5.

3.3.1. Phenolic acids

Peak 1 exhibited a molecular ion at m/z 313, sodium adduct at m/z 335, and a product of dehydration at m/z 295. This compound was assigned to caffeoyl tartaric (caftaric) acid (Table 9).

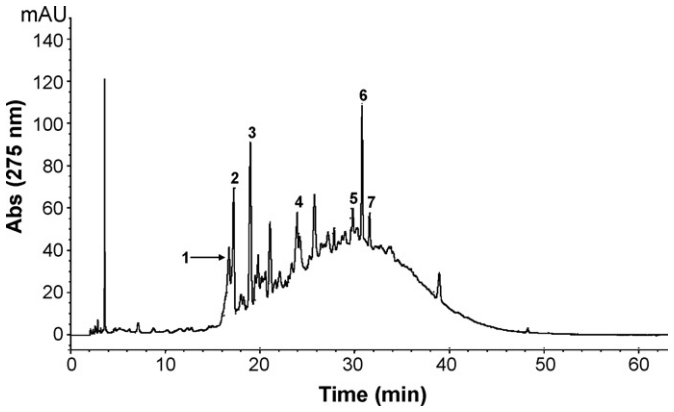

Fig. 5. HPLC trace of the extract with the highest total polyphenol concentration (Moschofilero, No 1, Table 2). Monitoring was performed at 275 nm. Peak assignment: 1, caftaric acid (caffeoyl tartaric acid); 2, a flavanol dimer; 3, catechin; 4, a galloylated flavanol dimer; 5, astilbin (dihydroquercetin rhamnoside); 6, quercetin 3-*O*-glucuronide; 7, rutin (quercetin 3-*O*-rutinoside). For chemical structures see Fig. 7.

3.3.2. Flavanols

Peak 2 gave a molecular ion at *m*/*z* 579, and diagnostic sodium adduct at *m*/*z* 601. This compound was identified as flavanol dimer. Likewise, peak 4 gave a molecular ion at *m*/*z* 731, sodium adduct at *m*/*z* 753 and a characteristic fragment at *m*/*z* 291, which was ascribed to (epi)catechin. These data were consistent with a galloylated flavanol dimer [9]. Peak 3 was identified as catechin, which was further confirmed by comparison of its retention time with original standard.

3.3.3. Dihydroflavonols

For peak 5, a molecular ion was seen at *m*/*z* 451, which upon increased collision energy (70 eV) yielded a daughter ion at *m*/*z* 305.

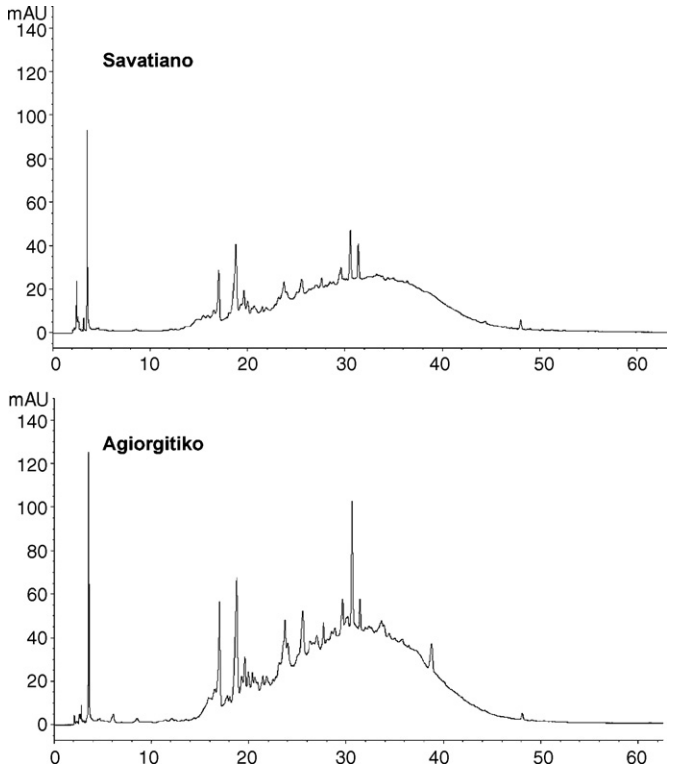

Fig. 6. Chromatograms of Savatiano and Agiorgitiko extract exhibiting the highest polyphenol concentration (No 12 and 14, respectively, Table 2).

An ion at *m*/*z* 473, which was ascribed to Na^+ adduct, was characteristic of its structure that was assigned to astilbin (dehydroquercetin rhamnoside) [9].

3.3.4. Flavonols

Peaks 6 and 7 with corresponding molecular ions at *m*/*z* 479 and 611 were found to yield the same daughter ion (*m*/*z* 303), and corresponding Na^+ adducts at *m*/*z* 501 and 633. Peak 7 gave also a characteristic fragment at *m*/*z* 465, indicating the loss of a rhamnosyl unit (146 Da). These compounds were identified as quercetin 3-*O*-glucuronide and quercetin 3-*O*-rutinoside (rutin), respectively [9].

4. Discussion

4.1. Recovery optimisation and influential parameters

Water/ethanol mixtures have been employed for the extraction of anthocyanins from purple sunflower hulls [17] and black currants [18], phenolics from almond hulls and pine sawdust [19], grapevine shoots [20], olive leaves [11,21], dried sage [22], grape seed meal [23], grape seeds [24], white grape seeds, peels and stems [10], red grape pomace [14], barley [25], *Inga edulis* leaves [26] and lignans from flaxseed [27]. The trends recorded in each case, as well as the discrepancies revealed regarding the optimum EtOH level, suggested that optimisation of polyphenol recovery from tissues with different polyphenolic composition should be based on case experimentation, and that there is not a universal model describing the optimal conditions that should be deployed. In this respect, this study was focused on a particular vinification by-product, derived from different varieties, and the investigation was based on several polyphenolic indices and not only total polyphenols, with the view of performing a multilateral assessment of the extraction efficiency.

This set of examinations highlighted for the first time that the specific groups of polyphenols and their relevant proportion in plant tissues might be crucial in defining the extraction conditions. From the optimisation process it became evident that the extraction of flavanols requires an EtOH level of 60%, as opposed to flavones, which can be efficiently recovered with 40% EtOH (Table 7). For proanthocyanidins, which represent flavanol oligomers and polymers, intermediate EtOH concentrations ranging from 44.2 to 53.1 were the most satisfactory in this regard. Another significant difference was seen in the pH. While flavanol extractions gave higher yields at pH 2–3, flavones were better extracted at pH 4.5–6. The results for PC were absolutely consistent, indicating a pH 2 as the optimal. Such a consistency for PC was also observed for the duration of the extraction, where for all samples the time required for optimal yields was 5 h. On the other hand, optimal extraction times for TFl and TFn were 1–3.5 and 1–5 h, respectively, indicating that in general extraction of higher TFn amounts required extended extraction duration, compared with TFl.

The finding that efficient TFl recovery can be achieved using higher EtOH levels than for TFn cannot be rationalised taking into consideration only their relative polarities. In fact, flavanols are more polar molecules compared even with flavonol glycosides, as also can be seen in the reversed-phase chromatogram (Fig. 5); thus the above-mentioned behaviour of extractability is a paradox. In this case, it would be logical to assume that the final effect seen is an integration of the combined effect of all factors considered.

It has been supported that increasing pH values might enhance polyphenol solubility by promoting dissociation of the most acidic phenolic –OH groups, which would render polyphenols higher solubility in a hydroalcoholic medium [11]. Such a hypothesis would explain the tendency recorded for flavones (Fig. 3), and the high yields achieved at pH values varying from 4.5 to 6 (Table 7). Con-

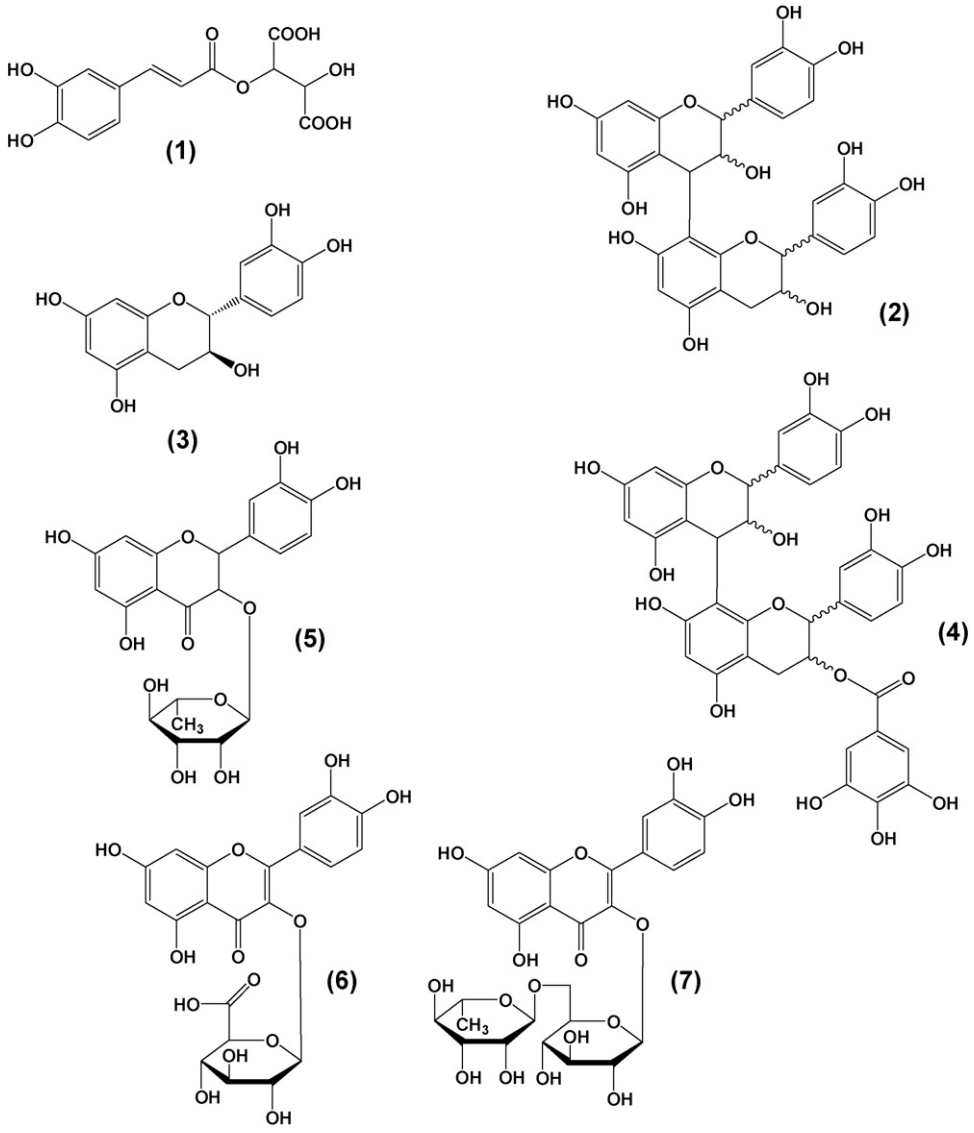

Fig. 7. Structures of the polyphenolic phytochemicals tentatively identified in the optimally obtained Moschofilero extract. For full names see Table 9.

trary to that, the consistent outcome that extraction of TFl and PC is facilitated at pH 2 might suggest that these polyphenols, above a certain pH level, are easily oxidised, a fact that would limit their recovery.

The time necessary to attain optimum levels was, on average, shorter for TFl (2.67 h), somewhat longer for TFn (3.67 h) and even more for PC (5 h). Judging by the relative polarities, it appears that polar monomeric and/or dimeric flavanols, such as those detected in the extracts (Fig. 7), are the first to be released in the extracting medium, followed by the less polar flavones. The liberation of PC from the tissue requires more extended extraction duration, most probably because of their ability to reversibly combine with other macromolecules, such as polysaccharides and proteins, which hinder fast PC solubilisation.

4.2. Polyphenolic composition and antioxidant properties

The polyphenolic profile of grapes stems is rather largely uncharacterised, as opposed to grape skins and seeds, for which considerable effort has been expended on the study of their composition. Stems are known to contain hydroxycinnamate derivatives, such as caftaric and coutaric acid, but also flavonol and dihy-

Table 9
UV–vis and mass spectral characteristics of the main polyphenolic phytochemicals detected in the optimally obtained grape seed extract (*V. vinifera* var Moschofilero).

Peak	λ_{max} (nm)	$[M+H]^+$	Other ions (m/z)	Compound
1	328	313	335 $[M+Na]^+$, 295 $[M-H_2O+H]^+$	Caftaric acid
2	278	579	601 $[M+Na]^+$	(epi)Catechin-(epi)Catechin
3	278	291	–	Catechin
4	278	731	753 $[gDm+Na]^+$, 291 $[(epi)CT+H]^+$	(epi)Catechin–(epi)Catechin gallate
5	290	451	305 (aglycone), 473 $[M+Na]^+$	Dehydroquercetin rhamnoside (astilbin)
6	254, 354	479	501 $[M+Na]^+$, 303	Quercetin glucuronide
7	254, 354	611	303 (aglycone), 465 $[M-146+H]^+$, 633 $[M+Na]^+$	Quercetin rutinoside (rutin)

droflavonol glycosides [28]. Furthermore, stems may have an important stilbene burden, and apart from *trans*-resveratrol and its dimers, such as ε-viniferin, numerous other stilbenic metabolites may be encountered, including resveratrol *C*- and *O*-glycosides, as well as trimers and tetramers [8]. Although in a recent study concerning the polyphenolic composition of stems from Roditis, a Hellenic native *Vitis vinifera* variety, *trans*-resveratrol as well as a viniferin were shown to occur at significant amounts [9], in this study no stilbenes were detected in any of the three varieties tested. On the other hand, the existence of a galloylated flavanol dimer, astilbin, quercetin glucuronide and rutin was confirmed.

Careful interpretation of the data given in Table 8 shows that in Moschofilero and Savatiano extracts, the only statistically significant correlations were those established between PC concentration and P_R, while for Agiorgitiko significant link was found between PC and A_{AR}. For Moschofilero, the correlation of TP was also significant but low. This observation dictates that the PC fraction is likely to govern the antioxidant characteristics of the extracts.

An explanation that might lie behind this finding is that PC molecules, composed of several (usually 2–7) monomer units, have been claimed to express antioxidant effect proportional to the hydroxyl groups they bear [29]. Thus increases in antioxidant potency were associated with increased monomer number, up to 7, but controversial behaviour was evidenced when some monomer units were galloylated [30]. Further, it has been argued that proanthocyanidin quinones, formed from the initial semiquinone radicals after hydrogen abstraction, are capable of producing oligomeric compounds by various pathways. These coupling reactions (or nucleophilic additions) retain the number of hydroxyl groups and the commensurate higher number of radical target sites is primarily responsible for their enhanced antioxidant capacity [31].

Thus in line with other studies that demonstrated the efficacy of PC to exert substantial radical-scavenging in extracts from various plant material [10,32,33], but also their higher potency compared with monomeric flavanols [34], it is likely that PC content in grape stems defines to a large extend their antioxidant properties.

5. Conclusions

The examination presented herein demonstrated for the first time that the set of conditions employed to optimise extraction of phenolics from plant material may vary substantially, even for the same tissue originating from different varieties. Grape stems were shown to contain mainly flavanols and flavonol glycosides, but it appeared that flavanol oligomers and or polymers (proanthocyanidins) define the antioxidant magnitude of the extracts generated. The finding that extraction of different polyphenol classes from grape stems requires different set of conditions might be of value in selective recovery, for the generation of extracts enriched in particular components. On the other hand, these crucial differences in the conditions should be carefully considered when extractions are not directed and recovery of as many phenolics as possible is sought.

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