Scientific paper

Simultaneous GC-MS Determination of Free and Bound Phenolic Acids in Slovenian Red Wines and Chemometric Characterization

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Received: 20-04-2016

Abstract

Several phenolic acids (PAs), caffeic, vanillic, syringic, p-coumaric and ferulic acid, found in Slovenian red wines were studied using gas chromatography and mass spectrometry. For isolation of the PAs from wine samples, solid phase extraction using hydrophilic modified styrene – HLB cartridges was used. The bound PAs were extracted after basic hydrolysis and *o*-coumaric acid was used as the internal standard. The developed method was validated and the linear concentration range for all analytes was from 1 to 100 mg L^{-1} with correlation coefficients above 0.999. We show that the method is repeatable (RSD<2%), recoveries were above 96%, and LOD and LOQ values were acceptable.
In all of the wine samples tested, caffeic and *p*-coumaric acid were determined to be the predominant PAs (17–72 mg L^{-1}), while other compounds were found in lower concentrations. Principal Component Analysis and Cluster Analysis were used to study differences between wines related towards varieties and Slovenian wine regions. The results demonstrate that variety has more influence on PAs content than wine regions in Slovenian red wines.

Keywords: Phenolic acids, Slovenian red wines, gas chromatography, mass spectrometry, PCA, CLU

1. Introduction

Antioxidant activity of plant materials and natural products has received a great deal of interest over the past years both in the public and scientific communities.[1–4] Generally, it is believed that consumption of plant phenolics decreases the risk of diseases related to oxidative stress.[5]

Wine, as a complex matrix containing several hundreds of different chemical compounds,[6,7] presents an analytical challenge, especially for identification and quantification of compounds in low concentrations. The chemical composition of red wines includes minerals, vitamins, proteins, sugars and phenolic compounds, among them PAs. Red wines are considered to have more protective function than white or rosé wines, because of their higher content in antioxidant substances released from the grape skin and seeds.[8] The total amount of polyphenols in red wines has been estimated in the range from 2000 to 6000 mg L^{-1}.[9] Polyphenols are usually responsible for wine colour and contribute to the bitter flavour of wine.[10] From the literature it is known that lactic acid bacteria (LAB) are responsible for the occurrence of malolactic fermentation (MLF), a secondary fermentation which is considered to be beneficial in most red wines.[11] The phenolic acids content of grapes and wines can positively or negatively affect the rate of MLF.[12] For example, gallic acid at low concentrations has stimulatory effects on the growth and malolactic activity of LAB.[13] On the other hand, some phenolic acids, especially those from the hydroxycinnamic class, delayed the conclusion of the malolactic fermentation by these bacteria.[14] Hydroxycinnamic acids (particularly *p*-coumaric acid) are also known to inhibit growth of a variety of microorganisms including wine-spoilage strains of *L. collinoides*, *L. brevis* and *L. hilgardii*.[15]

Although Slovenia is a small country, its wine production has a significant role in the economy. Altogether, 22,000 hectares of vineyard area is divided among three

major regions (Drava Valley-Podravje, Lower Sava Valley-Posavje and the Littoral-Primorska) with further division into sub-regions.[16,17] The Slovenian Littoral is Slovenia's most widely known and prominent wine region of both white and red wines. Slovenian vineyards are planted with different vine varieties, including Merlot, Cabernet Sauvignon, Chardonnay, Pinot Noir, Syrah, Barbera, and many others.

PAs are present in their free forms or as glycosylated and esterified derivatives.[18–20] Acidic, basic and enzymatic hydrolysis are the most commonly used methods for the extraction of PAs from natural materials.[21–25] From the scientific literature it is obvious that the most commonly used techniques for the determination of PAs are high-performance liquid chromatography (HPLC) with UV or DAD detection or liquid chromatography coupled with mass spectrometry (LC-MS).[26–29] Because of the longer sample preparation process for analysis, using gas chromatography with mass spectrometry (GC-MS) in analysis of phenolic compounds is relatively rare, but in comparison with the other methods mentioned, GC-MS offers several advantages, including complete and high-resolution separation, sensitive detection, unambiguous identification and quantitation of a wide range of phenolics (including all isomers) in one chromatographic run.[30–32]

The aim of our study was to develop a simple and quantitative extraction method of selected PAs to ensure clean extracts in order to obtain a much more sensitive, selective and accurate GC-MS method for identification and quantitation of both free and bound PAs in red wine samples. For extraction of target compounds from the wine samples, solid-phase extraction (SPE) using hydrophilic modified styrene (HLB) cartridges was used. The bound PAs were determined after basic hydrolysis using NaOH in the presence of L-ascorbic acid and EDTA as stabilizers. The applicability of the developed method was tested on Slovenian red wines. Statistical and chemometric analyses were performed and the wines were classified.

2. Experimental

2. 1. Chemicals

All reagents and solvents used were minimally of analytical purity. Standard compounds, *trans*-caffeic acid (99%), vanillic acid (97%), syringic acid (97%), *trans-p*-coumaric acid (98%), *trans-o*-coumaric acid (98%) and *trans*-ferulic acid (98%) and solvents, tetrahydrofuran-THF (99.5%) and pyridine (99.9%), were supplied by Merck (Germany). Derivatization reagent *N*-Methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), HPLC-grade methanol (MeOH) and sodium hydroxide-NaOH (99%) were purchased from Sigma (USA). GC-grade toluene (99.5%) and hydrochloric acid-HCl (36.5%) were purchased from Carlo Erba (Italy). Dichloromethane-DCM was purchased from JT Baker (Germany), L-ascorbic acid (99.7%) was purchased from Alkaloid (Macedonia) and EDTA was purchased from Kemika (Croatia). The water used was obtained from a Milli-Q water purification system.

2. 2. Preparation of Standard Solutions and Calibration Curves

Standard stock solutions of caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid and ferulic acid, as well as of *o*-coumaric acid (ISTD) were prepared by accurately weighing 10 mg of each into a 10 ml volumetric flask, and then dissolving in THF. Five calibration standard solutions were prepared by combining various volumes of PAs stock solutions with 50 µl of ISTD in a 50 mL conical glass flask. Each solution was derivatized by treating it with 100 µL of MSTFA and 50 µL of pyridine for 1 h at 80 °C in a sand bath. After derivatization was finished, TMS derivatives were quantitatively transferred to 1 mL flasks and filled up to the mark with toluene. Five calibration standard solutions in concentration range from 1 to 100 mg L^{-1} were injected in triplicates. The calibration curves were constructed by linear regression of the peak-area ratio of individual PA standard to the ISTD (y), versus the concentration (mg L^{-1}) (x).

2. 3. GC–MS Instrumentation and Working Conditions

TMS derivatives of PAs were analyzed with a Varian 3900 gas chromatograph (GC), coupled to MS/MS Saturn 2100 ion trap mass spectrometer. GC separation was performed using a Varian capillary column VF-5ms CP8944 (30 m × 0.25 mm, with the stationary phase 0.25 µm). 1 µL of the sample was injected in split mode (split ratio 1:10). Carrier gas was He (6.0 UHP) at a flow rate of 1.0 mL min^{-1}. The initial oven temperature was 40 °C, held for 1 min, and then the temperature was raised to 320 °C at a rate of 10 °C min^{-1}, and finally, held for 3 min. The total run time was 32 min. The injection-port and transfer-line were set to 250 °C and 170 °C, respectively. Mass spectra were recorded in SCAN or SIM mode in a range from 50 to 650 *m/z* using electron ionization energy at 70 eV. Peak identification was done by comparing retention times (t_R) and spectral properties with those of standard compounds or by library matching from NIST MS library containing the mass spectra of TMS derivatives of PAs.

2. 4. Validation Parameters for the GC-MS Method

The method was validated for linearity, precision as repeatability, limit of detection (LOD) and limit of quantitation (LOQ). For linearity determination, all calibration curves were constructed using the internal standard method. The curves were fitted to linear least-squares regression. The precision was evaluated through the within-day

(WD) and between-days (BD) repeatability, and expressed as relative standard deviation (RSD). The limit of detection (LOD) was calculated using the equation $(3.3 + s_y)/b_1$ and the limit of quantitation (LOQ) was calculated from the equation $(10 + s_y)/b_1$ (where s_y is standard deviation of linear regression and b_1 is slope of the calibration line).[33]

2. 5. Wine Samples

The developed method was tested using Slovenian red wine samples. Twelve red wines from different Slovenian wineries and different varieties were purchased from local supermarkets. All the tested wine samples orginated from four vintages (2011–2015). **Table 1** shows the varieties, wineries, year of production and percentage of alcohol. Wines were stored in a refrigerator at the temperature of +4 °C until analyzed.

2. 6. Preparation of the Wine Samples and Optimization of the Extraction Procedure

2. 6. 1. Extraction of Free PAs

A standard solution of PAs mixture (in a concentration of 1000 mg L^{-1}) was prepared in MeOH. Solutions of PAs mixture in synthetic wine (hydroalcoholic solution of 5 g L^{-1} tartaric acid, 12% of ethanol, and pH 3.2),[34] were prepared by pipetting 30 and 100 µL of standard solution, respectively, in a 10 mL volumetric flask, and diluted with synthetic wine up to the mark. 1 mL of each solution was transferred into a 50 mL conical flask, spiked with 50 µL of ISTD (1000 mg L^{-1}), diluted with 1 mL of ultra-pure water and acidified with 6 M HCl to a pH value of 2. Prepared samples were added to pre-conditioned HLB Supelco® SPE cartridges (3 mL, 60 mg stationary phase made from hydrophilic modified styrene). A schematic procedure of the sample extraction is shown in **Table 2**.

The free PAs fraction was eluted with 2 × 2 mL of THF. The eluate was collected and dried in a rotary evaporator (at 40 °C) to absolute dryness. Then the sample was derivatized by adding 100 µl of MSTFA and 50 µl piridine, heated at 80 °C for 1 h, diluted with toluene, and analyzed by GC-MS. The analyses were carried out in triplicate. The accuracy of the extraction process was determined through the recovery value in % of the PAs.

Table 2. Sample extraction by SPE (using HLB Supelco® cartridges).

Sample extraction by SPE	
1.	pre-washing of cartridge with 2 × 2 mL DCM
2.	column conditioning: 2 × 2 mL of MeOH and 2 × 2 mL acidified water (pH = 1–2)
3.	sample application: 2 mL of the acidified sample
4.	column washing: 2 × 2 ml ultra-pure water
5.	elution: 2 × 2 ml THF

For the determination of free PAs in selected red wines, the samples were prepared according to the same procedure. 1 mL of homogenized wine sample was spiked with 50 µL of ISTD, diluted with 1 mL of ultra-pure water and acidified with 6 M HCl to a pH value of 2, followed by the previously described steps.

2. 6. 2. Alkaline Hydrolysis of PAs

The stability of the compounds and their recovery percentage after alkaline hydrolysis was first determined

Table 1. Characteristics of the analyzed wine samples.

Sample code	Variety	Variety code	Winery	Wine region	Year of production	% alcohol*
SW1	Cabernet Sauvignon	1	„Vina Koper“	Primorska	2014	13
SW2	Modra Frankinja	2	„Stari Hram“	Posavje	2014	10.5
SW3	Cabernet-Sauvignon	1	„Vipava“	Primorska	2014	11
SW4	Modri Pinot (Pinot noir)	2	Štajerska Slovenia-Ptuj	Podravje	2011	12.5
SW5	Cabernet Sauvignon	1	Goriška Brda	Primorska	2013	12.5
SW6	Refošk	3	Srednje Škofije	Primorska	2014	11
SW7	Refošk	3	„Vina Koper“	Primorska	2014	12.5
SW8	Modra Frankinja	2	Štajerska Slovenia-Ptujska Klet	Podravje	2011	11.5
SW9	Modri Pinot (Pinot Noir)	2	„Vipava“	Primorska	2013	12
SW10	Portugalka	3	Bela Krajina	Posavje	2015	11
SW11	Cabernet Merlot	1	Jeruzalem-Ormož	Podravje	2013	12.5
SW12	Metliška Črnina	2	Bela Krajina	Posavje	2012	11.5

*According to the declaration on the wine bottle.

with the standard compounds and later an optimized procedure was used on the real wine samples. Standard solution of PAs mixture (at a concentration of 1000 mg L^{-1}) was prepared in MeOH. Solutions of PAs mixture in synthetic wine were prepared by pipetting 30 and 100 μL of standard solution, respectively, into the 10 mL volumetric flask, and diluted with the synthetic wine up to the mark. 1 mL of each solution was transferred into a 50 mL conical flask, spiked with 50 μL of ISTD, and exposed to alkaline hydrolysis, according to the previously described method with some modifications.[35] 1 mL of the spiked synthetic wine was treated by adding 9 mL of 2 M NaOH (which contained 1% L-ascorbic acid and 10 mM EDTA as stabilizers) for 2 h at room temperature. Then the sample was acidified to pH 2 using 6 M HCl, and PAs were extracted with SPE HLB cartridges. The whole procedure with alkaline hydrolysis was repeated also without stabilizers.

2. 7. Quantitation of PAs

The contents of free and total PAs were determined from the corresponding calibration curves using the ISTD method, taking into account the recovery of the extraction procedure. PAs from the cinnamic group exist in *trans*- and *cis*-forms, both found in plants. *Trans*-forms of PAs are naturally predominant isomers. Therefore, for quantitative determination, the peak areas of the *trans*- and *cis*-forms of caffeic acid, *p*-coumaric acid and ferulic acid were summed.

2. 8. Statistical Analysis

Chemometrical data analysis was carried out in order to discover any statistically or other significant differences between the samples grouped according to two categorical variables – wine variety and wine region. Microsoft Excel was used for the data preparation and result outputs. Statistical data treatment was performed using SPSS Statistics version 22.

3. Results and Discussion

Our study tested isolation and quantitative determination of five target PAs (caffeic acid, vanillic acid, syringic acid, *p*-coumaric acid and ferulic acid) in red wine samples using the GC-MS method. All GC-MS SCAN parameters for trimethylsilylated standard compounds, together with their retention times (t_R) and characteristic fragment ions, are listed in **Table 3.**

Linear regression analysis proved that the responses for all of the investigated compounds were linear over the tested concentration range (1-100 mg L^{-1}), and correlation coefficients (r^2) were above 0.999. The results of the regression analysis and calibration data are shown in **Table 4**. Table 4

Table 3. Retention times and fragmentation parameters for trimethylsilylated PAs obtained after trimethylsilylation using the ion-trap mass detector.

Compound	t_R	Characteristic fragmentation ions *m/z* (relative intensity %)
cis-*o*-Coumaric acid	16.65	147(100), 293, 308
Vanillic acid	17.70	253, 267, 282, 297(100), 312
cis-*p*-Coumaric acid	17.94	219, 249, 293 (100), 308
trans-*o*-Coumaric acid	18.18	147, 219, 293 (100), 308, 381
Syringic acid	19.11	298, 312, 328, 342 (100)
cis-Ferulic acid	19.32	249, 293, 308, 323, 338(100)
trans-*p*-Coumaric acid	19.49	219, 250, 293 (100), 308, 381
cis-Caffeic acid	19.99	219, 381, 396 (100), 397
trans-Ferulic acid	20.95	249, 293, 323, 338 (100)
trans-Caffeic acid	21.38	73, 219, 381, 396 (100)

Table 4. Validation parameters for investigated PAs.

PA	Linear correlation	r^2	[1]WD-RSD	[2]BD-RSD	LOD*	LOQ*
Vanillic acid	y = 0.0477x + 0.0771	0.9999	0.11	0.72	0.05	0.09
Syringic acid	y = 0.0231x + 0.0921	0.9999	0.95	1.81	0.06	0.12
p-Coumaric acid	y = 0.0398x + 0.0555	0.9996	0.38	1.47	0.06	0.13
Caffeic acid	y = 0.0558x + 0.0986	0.9999	1.36	1.97	0.07	0.15
Ferulic acid	y = 0.0324x + 0.0718	0.9996	1.01	1.81	0.03	0.09

[1] Within-day PA/ISTD peak-area ratio repeatability of individual PAs at the concentration 10 mg L^{-1}, expressed as %RSD. [2] Between-days PA/ISTD peak-area ratio repeatability of individual PAs at the concentration 10 mg L^{-1}, expressed as %RSD. * LOD and LOQ are in mg L^{-1}.

also shows the within-day (WD) and between-days (BD) repeatability expressed as relative standard deviation (RSD), and it gives RSD below 2% in all cases. The determined values of LODs and LOQs for all selected PAs are also shown in Table 4.

From the literature it is well known that anthocyanin-type pigments can cause great interference in the chromatographic separation and identification of non-anthocyanin phenolic compounds.[36] In our study, anthocyanins were successfully removed using HLB cartridges. Another advantage of HLB cartridges over conventional C18 columns in the separation of phenolic compounds are that more polar interferences (e.g. sugars) can be eliminated with water without losing analytes, higher sensitivity, good repeatability, reproducibility, and high percentages of recovery were reported by Perez-Magarino et al., 2008.[37]

Accuracy of SPE in determining free PAs was evaluated by spiking a synthetic wine with the standard solution at two different concentrations levels (30 and 100 mg L^{-1}). The recovery of free PAs ranged from 93% to 114%

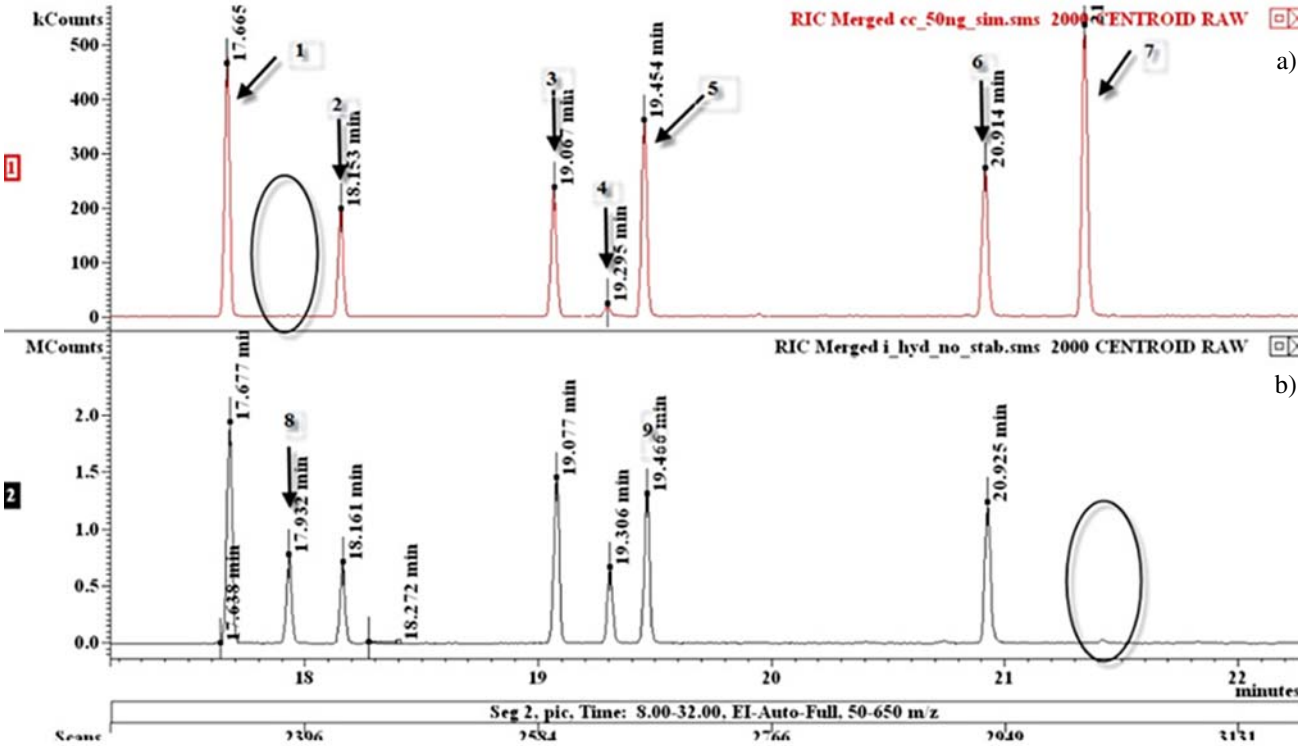

Figure 1. Chromatograms of standard solutions after; **a)** hydrolysis in presence of stabilizer (1. vanillic acid; 2. *trans-o*-coumaric acid; 3. syringic acid; 4. *cis*-ferulic acid; 5. *trans-p*-coumaric acid; 6. *trans*-ferulic acid; 7. *trans*-caffeic acid; 8. *cis-p*-coumaric acid; *cis*-caffeic acid (minimal peak at *t*R 19.99 min); **b)** hydrolysis without stabilizer (1. vanillic acid; 2. *trans-o*-coumaric acid; 3. syringic acid; 4. *cis*-ferulic acid; 5. *trans-p*-coumaric acid; 6. *trans*-ferulic acid; 7. *trans*-caffeic acid (missing peak); 8. *cis-p*-coumaric acid.

Table 5. Determination of the method accuracy expressed as recovery (%).

Recovery of extraction procedure (%)					
Phenolic acid	**Free PAs**		**Bound PAs**		
			In the presence of stabilizer		**Without of a stabilizer**
	Concentration (mg L^{-1})				
	30	**100**	**30**	**100**	**100**
Vanillic acid	105.5	98.9	114.2	105	113.7
Syringic acid	106.8	93.6	107.5	101	105.4
p-Coumaric acid	101.7	106	110.5	103	135.1
Ferulic acid	94.5	96	97.4	104.4	133.7
Caffeic acid	102.7	106	105.9	96.32	NQ[a]

[a] NQ-not quantified. Concentration (mg L^{-1}) <LOQ (for details see Table 4.).

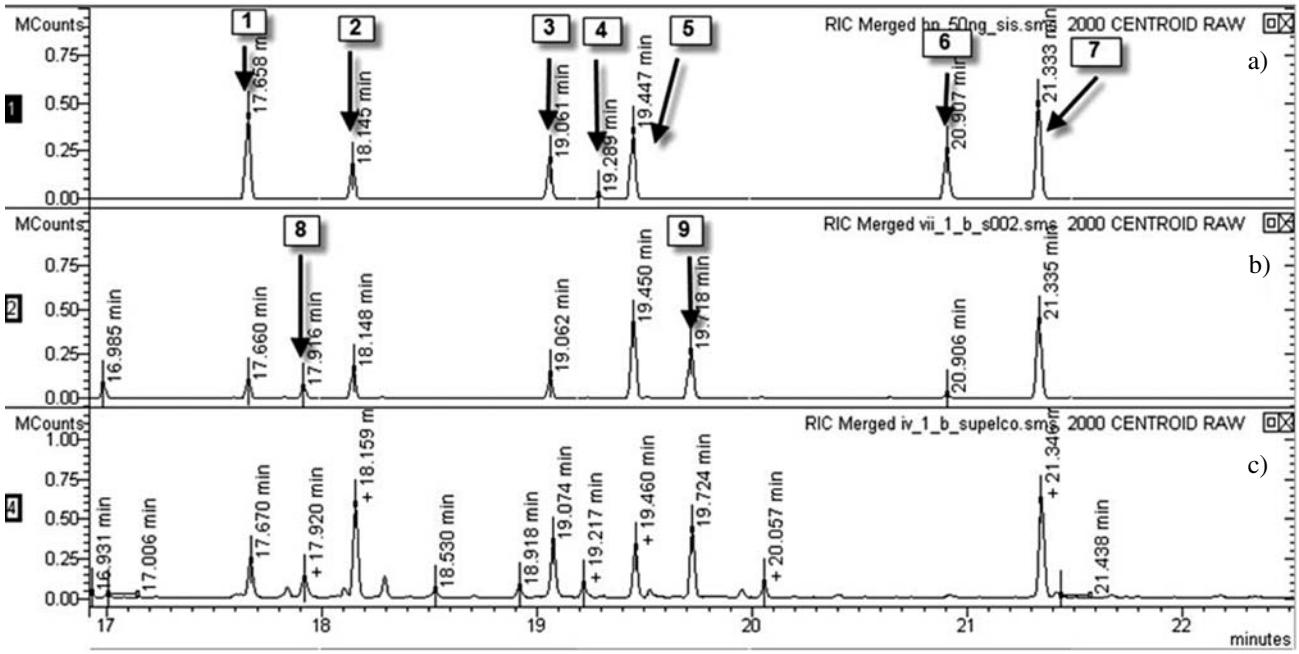

Figure 2. Typical chromatograms of selected PAs recorded in: **a)** SIM mode of standard mixture; **b)** SIM mode of red wine extract and **c)** SCAN mode of red wine extract (1. vanillic acid; 2. *o*-coumaric acid; 3. syringic acid; 4. *cis*-ferulic acid; 5. *trans*-*p*-coumaric acid; 6. *trans*-ferulic acid; 7. *trans*-caffeic acid; 8. *cis*-*p*-coumaric acid; 9. L-ascorbic acid (stabilizer)).

Table 6. Content (mg L^{-1}) of free and total PAs in Slovenian red wines.

Wine code	Form	Phenolic acid[a]				
		Vanillic acid	Syringic acid	*p*-Coumaric acid	Ferulic acid	Caffeic acid
SW1	Free	NQ[b]	3.7 ± 0.5	7.7 ± 0.2	ND[c]	3.2 ± 0.3
	Total	3.8 ± 0.4	19.8 ± 0.4	32.6 ± 0.3	ND	17.1 ± 0.3
SW2	Free	1.0 ± 0.1	3.2 ± 0.3	0.7 ± 0.1	NQ	5.4 ± 0.9
	Total	14.5 ± 2.5	29.8 ± 3.1	28.1 ± 2.2	0.1 ± 0	49.7 ± 3.2
SW3	Free	NQ	NQ	NQ	NQ	NQ
	Total	5.4 ± 1.7	14.3 ± 1.42	37.5 ± 2.1	ND	17.8 ± 0.4
SW4	Free	2.1 ± 0.2	10.7 ± 0.2	1.9 ± 0.1	ND	11.8 ± 1.2
	Total	12.6 ± 1.9	25.9 ± 0.5	31.0 ± 1.1	ND	44.5 ± 2.0
SW5	Free	0.9 ± 0.1	5.1 ± 0.1	1.6 ± 0.1	ND	4.0 ± 0.3
	Total	7.3 ± 0.6	21.9 ± 2.6	48.4 ± 0.9	ND	29.3 ± 0.5
SW6	Free	1.6 ± 0.1	4.7 ± 0.4	0.7 ± 0.1	NQ	4.8 ± 0.1
	Total	9.5 ± 1.0	25.2 ± 2.8	42.8 ± 3.6	2.5 ± 0.8	41.0 ± 0.1
SW7	Free	2.5 ± 0.4	3.4 ± 0.7	1.4 ± 0.1	NQ	3.6 ± 0.4
	Total	10.3 ± 1.4	29.9 ± 3.0	71.9 ± 0.2	4.9 ± 0.1	48.1 ± 1.1
SW8	Free	3.9 ± 0.8	4.4 ± 0.4	2.1 ± 0.2	NQ	3.5 ± 0.4
	Total	14.6 ± 1.0	20.0 ± 0.1	9.5 ± 0.7	NQ	36.9 ± 0.5
SW9	Free	5.1 ± 0.1	5.6 ± 0.2	NQ	ND	0.7 ± 0.0
	Total	10.9 ± 0.6	19.7 ± 0.3	20.9 ± 0.1	NQ	38.4 ± 0.6
SW10	Free	4.9 ± 0.3	7.7 ± 0.3	NQ	NQ	NQ
	Total	7.7 ± 0.8	29.5 ± 2.2	54.2 ± 1.2	0.7 ± 0.1	64.0 ± 0.6
SW11	Free	2.0 ± 0.1	4.6 ± 0.3	2.9 ± 0.1	ND	4.1 ± 0.0
	Total	11.8 ± 0.3	29.7 ± 2.5	63.1 ± 0.9	ND	33.7 ± 0.3
SW12	Free	6.0 ± 0.6	10.3 ± 0.1	NQ	NQ	NQ
	Total	14.6 ± 0.2	27.6 ± 1.1	47.2 ± 0.6	0.2 ± 0.01	64.4 ± 0.6

[a] Each value is the mean (mg L^{-1}) of three independent replicates ± standard deviation.
[b] NQ-not quantified. Concentration (mg L^{-1})<LOQ (for details see Table 4.).
[c] ND-not detected. Concentration (mg L^{-1})<LOD (for details see Table 4.).

(**Table 5.**). These results are in agreement with the results reported by other authors.[38,39] The recovery of the standard compounds after alkaline hydrolysis (without or with stabilizers) extracted by SPE were also determined (**Table 5**). The results prove that hydrolysis without stabilizers (L-ascorbic acid and EDTA) led to a complete loss of caffeic acid[35] and promoted isomerization of *trans-p*-coumaric acid to its *cis*-form (**Figure 1**). For all other investigated compounds, the recoveries were above 96%.

The developed method was then used for the determination of selected PAs in twelve Slovenian red wine samples. **Figure 2c** presents a typical chromatogram of wine extract.

Contents of five different PAs present in red wine samples are shown in **Table 6** (mean value ± sd). From these results it can be concluded that caffeic acid and *p*-coumaric acid are the most important of total PAs, with contents ranging from 17 to 72 mg L^{-1}. In all of the wines investigated, ferulic acid is present at the lowest concentration level. It was measured only in wines from the Posavje region and in two samples from the Primorska region. It is also worth to mention that red wine sample SW12 represents a mixture of red wine varieties Modra Frankinja, Žametovka, Portugalka and Šentlovrentka and therefore is a very specific red wine sample. This fact was confirmed by the obtained results (Table 6) as the contents of all investigated PAs in the sample SW12 were comparable with contents in the samples SW2 and SW10, both of them belonging to varieties Modra Frankinja and Portugalka, respectively. The largest proportion of PAs was present in bound form.

3. 1. Statistical Analysis

Exploratory data analysis was performed using the SPSS program. In the first step we searched for outliers but no outliers were found in the dataset. Departures from the normal distribution were demonstrated by the Q-Q plots and tested with the Kolmogorov-Smirnov test. Significance value for all tested variables was above 0.05, which indicates normal distribution of data. The Pearson correlation test (0.01 and 0.05 significance levels) was used to determine any inter-relation between two variables. Statistically significant correlations were found only between caffeic acid and syringic acid at the 0.01 level (0.744), and between caffeic acid and vanillic acid at the 0.05 level (0.592).

3. 1. 1. Principal Component Analysis (PCA)

Principal component analysis (PCA) is an unsupervised multidimensional method used for reducing the number of variables along with preserving the information contained in the data table. Projection of the wines on the first two principal components (accounting for 79.8% of the total data variability) demonstrates a clear separation according to the wine variety (**Figure 3.**). The first principal component (PC1) explained 53.7% of the variation between the samples, and the second (PC2) explained 26.1% of the variation. Wines from the Cabernet-Sauvignon variety (group 1 on the biplot) were separated from the other samples, and formed a group in the positive part of PC2, while the Modri Pinot variety (group 2 on the biplot) formed a group in the negative part of PC2.

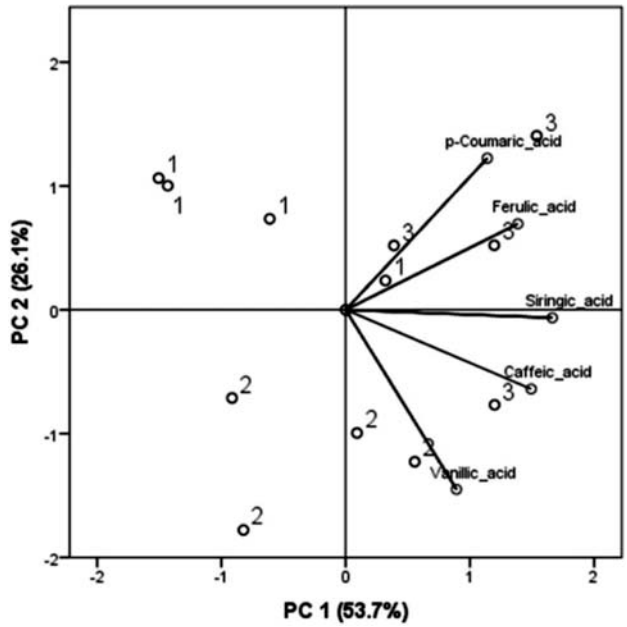

Figure 3. PCA biplot displaying the position of wine samples and descriptors in the plane PC 2 vs. PC 1 for twelve Slovenian red wines. The objects were lebelled by the wine variety.

3. 1. 2. Cluster Analysis (CLU)

Cluster analysis (CLU) is one of the unsupervised multidimensional procedures that involve measuring the distances or similarities between the objects (or variables) to be clustered.[40] In the present work, agglomerative hierarchical cluster (AHC) analysis was performed in order to classify the wines tested according to variety type or wine region. Dissimilarities between the samples were determined based on the squared Euclidean distance, and the objects were clustered using Ward's method. A CLU dendrogram is presented in **Figure 4**. and suggests three groups of clusters. The first cluster group consisted of wine marked as SW2, SW4, SW8 and SW9. All of these wines belong to the Modri Pinot and Modra Frankinja varieties. Samples marked as SW1, SW3, SW5 and SW6 comprised the second group of wines. Three of these wine samples belong to the Cabernet-Sauvignon variety, and sample SW6 belongs to the Refošk variety. All were produced in the Littoral region. Samples marked as SW7, SW10, SW11 and SW12 comprise the third cluster. These results are in accordance with those observed using PCA,

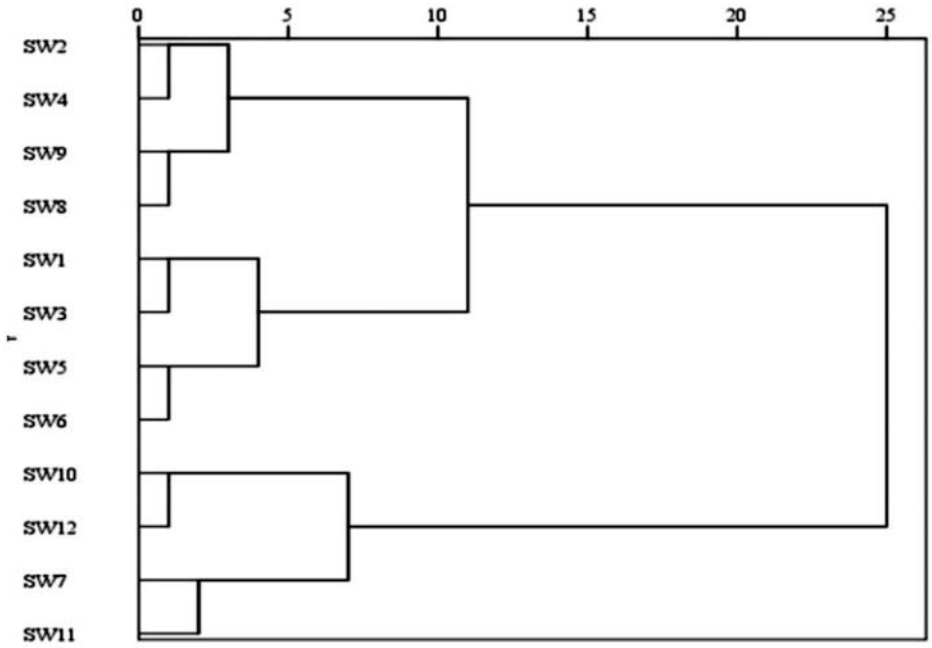

Figure 4. Dendrogram constructed with minimum linkage method for twelve Slovenian red wines.

and confirm that variety has more influence than wine regions on the content of PAs in Slovenian red wines.

4. Conclusions

A simple SPE extraction technique for the isolation of PAs from red wine samples was introduced. The SPE technique allows good separation of target compounds and almost complete removal of matrix influence. A GC-MS method for the identification and quantitative determination of five selected phenolic acids, together with their isomers, was developed. The method was validated with linearity, precision as repeatability, limit of detection and limit of quantitation. The GC-MS method proposed in this study was successfully applied to characterization of Slovenian red wines according to PAs content. From these results it can be concluded that caffeic acid and *p*-coumaric acid are the most important part of total PAs, with contents varying from 17 to 72 mg L^{-1}. The highest contents of all of the PAs investigated were found in wine samples from the Posavje wine region (SW2, SW10 and SW12). Among those wines exist the correlation in the variety. Namely, sample SW12 (Metliška črnina) is a specific wine variety consisting of Modra Frankinja (SW2), Portugalka (SW10), Žametovka and Šentlovrentka varieties. The concentrations of free and bound PAs in Slovenian red wines were between 0.03–11.8 mg L^{-1} and 3.8–71.9 mg L^{-1}, respectively. In accordance with the data obtained using statistical analysis and chemometric methods (PCA and CLU), it can be concluded that variety has more influence on the PA content of Slovenian red wines, but the influence of wine region cannot be completely ignored.

5. References

1. V. Ivanova-Petropulos, A. Ricci, D. Nedelkovski, V. Dimovska, G.P. Parpinello, A. Versari, *Food Chem.* **2015**, *171,* 412-420. http://dx.doi.org/10.1016/j.foodchem.2014.09.014
2. I. Mudnic, D. Modun, V. Rastija, J. Vukovic, I. Brizic, V. Katalinic, B. Kozina, M. Medic-Saric, M. Boban, *Food Chem.* **2010**, *119,* 1205–1210. http://dx.doi.org/10.1016/j.foodchem.2009.08.038
3. E. F. Gris, F. Mattivi, E. A. Ferreira, U. Vrhovsek, D. W. Filho, R. C. Pedrosa, M. T. Bordignon-Luiz*, J. Food Compost. Anal*. **2013**, *31,* 31–40.
4. M. V. S. S. T. Subba Rao, G. Muralikrishna, *Food Chem.* **2002**, *50,* 889–892. http://dx.doi.org/10.1021/jf011210d
5. V. Georgiev, A. Ananga, V. Tsolova, *Nutrients* **2014**, *6,* 391–415. http://dx.doi.org/10.3390/nu6010391
6. D. Serrano-Lourido, J. Saurina, S. Hernández-Cassou, A. Checa, *Food Chem.* **2012**, *135,* 1425–1431. http://dx.doi.org/10.1016/j.foodchem.2012.06.010
7. X. Sun, L. Li, T. Ma, F. Zhao, D. Yu, W. Huang, J. Zhan, *Innov. Food Sci. Emerg.* **2015**, In Press, Corrected Proof.
8. R. Van Leeuw, C. Kevers, J. Pincemail, J.O. Defraigne, J. Dommes, *J. Food Compost. Anal.* **2014**, *36,* 40–50.
9 . I. Garaguso, M. Nardini, *Food Chem.* **2015**, *179,* 336–342. http://dx.doi.org/10.1016/j.foodchem.2015.01.144
10. M. J. Rodríguez Vaquero, M. R. Alberto, M. C. Manca de Nadra, *Food Control* **2007**, *18,* 587–593. http://dx.doi.org/10.1016/j.foodcont.2006.02.005

11. S. Q. Liu, *J App Microbiol* **2002**, *92,* 589–601. http://dx.doi.org/10.1046/j.1365-2672.2002.01589.x
12. F. M. Campos, A. R. Figueiredo, T. A. Hogg, J. A. Couto, *Food Microbilo* **2009**, *26,* 409–414. http://dx.doi.org/10.1016/j.fm.2009.01.006
13. M. R. Alberto, M. E. Farias, M. C. Manca de Nadra, *J Agric Food Chem* **2001**, *49,* 4359–4363. http://dx.doi.org/10.1021/jf0101915
14. A. G. Salih, J. M. Le Quéré, J. F. Drilleau, *Sci Aliment* **2000**, *20,* 537–560. http://dx.doi.org/10.3166/sda.20.537-560
15. F. M. Campos, J. A. Couto, A. R. Figueiredo, I. V. Tóth, A. O. S. S. Rangel, T. A. Hogg, *Int J Food Microbiol* **2009**, *135,* 144–151. http://dx.doi.org/10.1016/j.ijfoodmicro.2009.07.031
16. V. S. Šelih, M. Šala, V. Drgan, *Food Chem.* **2014**, *153,* 414–423. http://dx.doi.org/10.1016/j.foodchem.2013.12.081
17. V. Weingerl, M. Strlič, D. Kočar, *Acta Chim. Slov.* **2009**, *56,* 698–703.
18. M. Ayoub, A. Costa de Camargo, F. Shahidi, *Food Chem.* **2016**, *197,* 221–232. http://dx.doi.org/10.1016/j.foodchem.2015.10.107
19. M. A. Harish Nayaka, U. V. Sathisha, S. M. Dharmesh, *Food Chem.* **2010**, *119,* 1307–1312. http://dx.doi.org/10.1016/j.foodchem.2009.08.044
20. H. Ti, Q. Li, R. Zhang, M. Zhang, Y. Deng, Z. Wei, J. Chi, Y. Zhang, *Food Chem.* **2014**, *159,* 166–174. http://dx.doi.org/10.1016/j.foodchem.2014.03.029
21. N. Ahmad, Y. Zuo, X. Lu, F. Anwar, S. Hameed, *Food Chem.* **2016**, 190, 80–89. http://dx.doi.org/10.1016/j.foodchem.2015.05.077
22. M. P. Germanò, V. D'Angelo, T. Biasini, R. Sanogo, R. De Pasquale, S. Catania, *J. Ethnopharmacol.* **2006**, *105,* 368–373. http://dx.doi.org/10.1016/j.jep.2005.11.029
23. K. H. Kim, R. Tsao, R. Yang, S. W. Cui, *Food Chem.* **2006**, *95,* 466–473. http://dx.doi.org/10.1016/j.foodchem.2005.01.032
24. K. A. Ross, T. Beta, S. D. Arntfield, *Food Chem.* **2009**, *113,* 336–344. http://dx.doi.org/10.1016/j.foodchem.2008.07.064
25. E. Xu, Z. Wu, J. Long, F. Wang, X. Xu, Z. Jin, A. Jiao, *J. Funct. Foods* **2015**, *17,* 214–226. http://dx.doi.org/10.1016/j.jff.2015.05.032
26. Y. Amakura, M. Okada, S. Tsuji, Y. Tonogai, *J. Chromatogr. A* **2000**, 891, 183–188. http://dx.doi.org/10.1016/S0021-9673(00)00625-7
27. M. N. Irakli, V. F. Samanidou, C. G. Biliaderis, I. N. Papadoyannis, *Food Chem.* **2012**, *134,* 1624–1632. http://dx.doi.org/10.1016/j.foodchem.2012.03.046
28. D. Sun, L. Dong, P. Guo, X. Shi, J. Gao, Y. Ren, X. Jiang, W. Li, C. Wang, Q. Wang, *Food Chem.* **2013**, *138,* 139–147. http://dx.doi.org/10.1016/j.foodchem.2012.09.096
29. V. Weingerl, M. Strlič, D. Kočar, *Anal. Lett.* **2011**, *44,* 1310–1322. http://dx.doi.org/10.1080/00032719.2010.512674
30. M. Islamčević Razboršek, *J. Pharm. Biomed. Anal.* **2011**, *55,* 1010–1016. http://dx.doi.org/10.1016/j.jpba.2011.04.003
31. L. Minuti, R. M. Pellegrino, I. Tesei, *J. Chromatogr. A* **2006**, *1114,* 263–268. http://dx.doi.org/10.1016/j.chroma.2006.02.068
32. M. Saitta, S. Lo Curto, F. Salvo, G. Di Bella, G. Dugo, *Anal. Chim. Acta* **2002**, *466,* 335–344. http://dx.doi.org/10.1016/S0003-2670(02)00572-X
33. D. Allen, A. Duyen Bui, N. Cain, G. Rose, M. Downey, *Anal. Bioanal. Chem.* **2013**, *405,* 9869–9877. http://dx.doi.org/10.1007/s00216-013-7405-0
34. M. S. García-Falcón, C. Pérez-Lamela, E. Martínez-Carballo, J. Simal-Gándara, *Food Chem.* **2007**, *105,* 248–259. http://dx.doi.org/10.1016/j.foodchem.2006.11.006
35. M. Nardini, E. Cirillo, F. Natella, D. Mencarelli, A. Comisso, C. Scaccini, *Food Chem.* **2002**, *79,* 119–124. http://dx.doi.org/10.1016/S0308-8146(02)00213-3
36. A. Bimpilas, D. Tsimogiannis, K. Balta-Brouma, T. Lymperopoulou, V. Oreopoulou, *Food Chem.* **2015**, *178,* 164–171. http://dx.doi.org/10.1016/j.foodchem.2015.01.090
37. S. Perez-Magarino, M. Ortega-Heras, E. Cano-Mozo, *Food Chem.* **2008**, *56,* 11560–11570. http://dx.doi.org/10.1021/jf802100j
38. V. M. Burin, S. G. Arcari, L. L. F. Costa, M. T. Bordignon-Luiz, *J. Chromatogr. Sci.* **2011**, *49,* 647–651. http://dx.doi.org/10.1093/chrsci/49.8.647
39. R. Sladkovský, P. Solich, M. Urbánek, *J. Chromatogr. A* **2004**, *1040,* 179–184. http://dx.doi.org/10.1016/j.chroma.2004.04.009
40. A. Bednarova, R. Kranvogl, D. Brodnjak Vončina, T. Jug, E. Beinrohr, *Acta Chim. Slov.* **2013**, *60,* 274–286.

Povzetek

Izbrane fenolne kisline: kavno, vanilinsko, siringinsko, kumarinsko in ferulno smo v različnih rdečih slovenskih vinih analizirali z uporabo GC-MS. Za izolacijo smo uporabljali ekstrakcijski postopek na HLB SPE kolonicah, ki vsebuje hidrofilno modificiran stiren. Pri delu smo vezane fenolne kisline hidrolizirali, kot interni standard pa smo uporabljali *orto*- kumarinsko kislino. Razvito metodo smo validirali: linearno koncentracijsko območje fenolnih kislin je med 1 in 100 mg L^{-1}, korelacijski koeficienti so bili nad 0,999. Potrdili smo dobro ponovljivost, RSD je znašal pod 2%, izkoristke nad 96% in sprejemljive vrednosti LOD ter LOQ. Ugotovili smo, da sta v vzorcih slovenskih vin prevladujoči fenolni spojini kavna in *para*-kumarna kislina (17–72 mg L^{-1}), medtem ko ostale spojine najdemo v nižjem koncentracijskem območju. Metodo glavnih osi (PCA) in analizo klastrov (CLU) smo uporabili za študij podobnosti in razlik med vzorci glede vsebnosti in deležev fenolnih kislin. Potrdili smo korelacijo in večji vpliv sorte grozdja kot vinorodne dežele oziroma regije, iz katere vzorci vin izvirajo.