Violeta Ivanova-Petropulos[1]
Zaneta Naceva[1]
Viktor Sándor[2]
Lilla Makszin[2]
Laura Deutsch-Nagy[2]
Balázs Berkics[2]
Trajce Stafilov[3]
Ferenc Kilár[2,4]

[1]Faculty of Agriculture, University "Goce Delčev", Krste Misirkov, Republic of Macedonia
[2]Institute of Bioanalysis, Medical School, and Szentágothai Research Center, University of Pécs, Pécs, Hungary
[3]Institute of Chemistry, Faculty of Natural Sciences and Mathematics, "Ss. Cyril and Methodius University", Skopje, Republic of Macedonia
[4]Department of Bioengineering, Faculty of Economics, Socio-Human Sciences and Engineering, Sapientia University, Miercurea Ciuc, Romania

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Research Article

Fast determination of lactic, succinic, malic, tartaric, shikimic, and citric acids in red Vranec wines by CZE-ESI-QTOF-MS

A fast and simple method with CZE coupled to ESI/QTOF-MS was optimized and validated for quantitative determination of organic acids (lactic acid, succinic acid, malic acid, tartaric acid, shikimic acid, and citric acid) in red wines. The BGE was ammonium acetate and the separation of the analytes was performed in a polybrene-coated capillary in the presence of EOF. The sample preparation included dilution and filtration of the wine. The method showed satisfactory performance characteristics: good linearity for each organic acid, with correlation coefficients ranging from $r^2 = 0.9902$ (shikimic acid) to $r^2 = 0.9990$ (tartaric acid). The limit of quantification was between 0.0034 mM (for shikimic acid) and 0.107 mM (for citric acid), and the recovery data fell between 95.8% (malic acid) and 102.7% (lactic acid); the total run time was less than 4 min. The RSD values for the interday repeatability and intraday reproducibility were between 3.44 and 9.50%, and between 1.75 and 8.29%, respectively. Seventeen Macedonian red Vranec wines were studied demonstrating a wide variation in the organic acids' concentration, which should be most probably due to the variation of the climate conditions in the vine areas.

Keywords:
CZE-ESI / QTOF-MS / Organic acids / Vranec wine

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1 Introduction

Wine is a very complex mixture of various compounds such as carbohydrates, organic acids, polyphenols, proteins, alcohols, minerals, etc. Organic acids are important compounds influencing the stability, flavor, aroma and color of grapes and wine and contributing to the pH, and to the chemical and microbiological stability of the wines [1, 2]. Tartaric, malic, and citric acids are the main organic acids in grapes, while lactic, succinic, and acetic acids found in the wine are formed during the alcoholic fermentation [3]. The acid content in grapes ranges from 8 to 13 g/L, depending on the grape variety, as well as climatic conditions during the year, however, the acids in wines occur between 5.5 and 8.5 g/L concentration [4]. Tartaric acid is the main organic acid in grapes and wines, which significantly influence the total acidity of wines. Its concentration—in unripe grapes—may be as high as 15 g/L (0.1 M), whereas in the must it falls between 2 and 6 g/L (13–40 mM), depending on the temperature at which the grapes are exposed. The tartaric acid concentration decreases during fermentation due to precipitation in a form of tartrate crystals. The presence of malic acid causes a "harsh" taste in the wine. Its content decreases during the malolactic fermentation due to its conversion to lactic acid, leading to "wine softening" and releasing fruity flavor. Citric acid slows the yeast growth and participates in biochemical and metabolic processes (e.g., Krebs cycle) [5]. During wine production, this component is allowed to be added into the wine to regulate the acidity, but its level should not exceed 1 g/L (5 mM) concentration. Succinic acid is formed in the wine during the alcoholic fermentation, giving a "bitter" note to the wine. Shikimic acid is present at low concentration in grape must (range: 10–150 mg/L [0.058–0.867 mM]), and it is transferred into the wine during the maceration and fermentation processes. This acid is considered as a factor for the determination of grapes origin [6]. Since organic acids are important for the wine stability, their concentration should be monitored during the whole vinification process, starting from the grapes juices, continuing to the alcoholic fermentation and wine stabilization processes.

Determination of organic acids is usually performed with chromatographic techniques such as HPLC [7–9], GC [10, 11], or ion chromatography [12]. These techniques, however, have limitations, for example, GC can be used only for volatile organic acids analysis, while HPLC or ion chromatography is limited to analyze few organic acids in a single run. Very often, for better chromatographic separation, the time of analysis of

Correspondence: Prof. Ferenc Kilár, Institute of Bioanalysis, Medical School and Szentágothai Research Center, University of Pécs, Pécs 7624, Hungary
Fax: +36-72-536254
E-mail: ferenc.kilar@aok.pte.hu

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organic acids with HPLC is longer and/or accompanied with a coelution of the analytes (usually coelution of malic and shikimic acids was observed) [12]. In addition, before HPLC analysis, the matrix effect should be eliminated by the application of some sample preparation protocol, such as SPE [12]. In the last few years, CE has been applied for the determination of organic acids in grapes and wines offering fast analyses and efficient resolution of the analytes [2–4, 13, 14]. In fact, this technique is a valuable tool for analysis of complex samples (as wine is), providing high separation efficiency, good reproducibility, fast analysis, and low consumption of electrolytes and samples [12]. Capillary electrophoresis allows separation and identification of charged and highly polar compounds, which cannot be separated by HPLC methods, and provides simultaneous analysis of analytes with different nature in a single run [12]. The main advantage of CE methods for wine analysis is that in most cases, no previous sample preparation is necessary, for example, dilution and/or filtration. Moreover, CE coupled to MS enables direct identification of the analyte. Thus, this technique was successfully used to study small organic acids in fruit juice [15–17], while CE method coupled with indirect UV detection was developed for organic acid determination in rice wine and beer [18]. Moreover, inorganic and organic acid anions were determined in orange juice and wine samples applying CZE coupled to UV detection. The baseline separation of anions was achieved in less than 14 min with indirect UV detection at 240 nm [19]. In that study, the BGE system contained 1,3,5-benzenetricarboxylic acid, Tris, and tetraethylenepentamine at pH 8.4. A novel method using conductivity detection of small anions in red wines has been published recently [20]. Capillary electrophoresis coupled to MS (CE-MS) was also applied for anionic metabolite profiling of orange juice and wine using a polymeric dynamic coating material [21]. Separation of the anionic metabolites (such as sugars, amino acids, organic acids) was achieved within 12 min, with high separation efficiency and good repeatability. In general, CE methods are useful techniques for analysis of low molecular mass organic acids. As a confirmation of these statements, the review papers on organic acids analysis using CE methods are published by Gomez *et al.* [22] and Klampfl *et al.* [23]. The recent major reviews focusing on the recent advances in the application of capillary electromigration methods for food analysis have summarized some more important aspects in wine analysis [24–27]. These reviews cover CE analysis of a large variety of food-related molecules with different chemical properties (amino acids, biogenic amines, carbohydrates, chiral compounds, contaminants, DNAs, food additives, heterocyclic amines, lipids, peptides, pesticides, phenols, pigments, polyphenols, proteins, residues, toxins, vitamins, small organic and inorganic compounds, as well as other minor compounds).

The use of CE coupled to MS [21], or especially to an accurate-mass QTOF-MS effectively increases sensitivity, providing high mass accuracy and resolution at high acquisition rates. No previous reports have been found in the literature about application of this latter technique on wine analysis. Herein, we report an optimization and application of a CZE coupled to ESI/QTOF-MS (CZE-ESI/QTOF-MS) technique for fast and simple determination of tartaric, citric, malic, lactic, succinic, and shikimic acids in red Vranec wines, applying a very simple sample preparation (wine dilution and filtration). The proposed method is validated, including the validation parameters, such as LOQ, linearity, recovery, repeatability, and reproducibility data.

2 Materials and methods

2.1 Chemicals

Lactic, succinic, malic, tartaric, shikimic and citric acid standards, polybrene (hexadimethrine bromide, PB), acetic acid, formic acid, ammonium hydroxide, and sodium hydroxide were supplied from Sigma-Aldrich (Steinheim, Germany). Ultra-pure deionized water (LC-MS Chromasolv®) was obtained from Fluka (Buchs, Switzerland). Stock solutions of the six organic acids (lactic, succinic, malic, tartaric, shikimic, and citric acids) were prepared in deionized water (LC-MS grade) and stored at 4°C. The standard solutions used for calibration were prepared by appropriate dilution of the stock solutions with the buffer solution before use. The BGE, ammonium acetate or ammonium formate (concentrations between 10 and 75 mM), was obtained by titrating acetic acid (100 mM) or formic acid (100 mM) to the desired pH with NH_4OH (100 mM).

2.2 Winemaking

Grapes from Vitis vinifera L., *cv*. Vranec were grown at vineyards located at 17 wine locations of different geographical surroundings (Table 1). Grapes were manually harvested (20 kg) at optimal technological maturity (18-24°Brix) in September/October 2014 and transported to the wine cellar of BOVIN Winery, Negotino, R. Macedonia. Grapes from each location were processed separately applying the same technology. Thus, grapes were mechanically pressed using a mechanical inox crusher/destemmer and treated with sulfur dioxide (50 mg/L) prior to the undergoing skin fermentation at 22–24°C. Sulfur dioxide was added in a form of 5% sulfurous acid. After the addition of SO_2, a commercial pectolitic enzyme preparation (Endozym Rouge, AEB, Italy) was applied to obtain higher concentration of coloring compounds, skin tannins, and varietal aromas (1 g/hL). After 2–3 h, wines were inoculated with *Saccharomyces cerevisiae* yeast (Fermol Medterranee, AEB). Yeast was prepared by rehydration (20 g/hL) in must, followed by the addition of nutrients, 10 g/hL (Fermol Plus starter, obtained from AEB, containing 59.8% diammonium phosphate, 39.52% cellulose, and 0.6% thiamine hydrochloride). Grape mash was macerated for 7 days and during that period the cap was mechanically punched down two times a day until it remained submerged. After the maceration period, wine was separated from the

pomace by mechanically pressing and stored in 10-L vessels in at room temperature. After 10 days of conservation, wines were racked and treated again with sulfur dioxide (30 mg/L). The second racking was performed after 2 months of storage, when wines were bottled and stored in a cellar at 8–10°C for about 8 months until analyzed.

2.3 Wine sample preparation

All wine samples were diluted with deionized water (ratio 1:5), filtered with a 0.22 μm membrane filter (PVDF syringe filter; Nantong FilterBio Membrane Co., Ltd., China) and injected into the CE system.

2.4 Capillary electrophoresis

The separation of the organic acids in wine was performed in a 7100 Capillary Electrophoresis (CE) system (Agilent Technologies, Waldbronn, Germany). The fused-silica, 80 or 120 cm long (50 μm id), capillaries (Polymicro Technologies, Phoenix, USA) were coated with polybrene. The new capillaries were conditioned by flushing with aceton (2 min), water (2 min), 1 M NaOH (20 min), water (5 min), dynamic coating solution (1% solution of polybrene, 15 min), and BGE (5 min). Between the runs, a short preconditioning was performed by flushing with 1% PB solution (2 min), water (2 min), and BGE (4 min). The capillary was purged with deionized water (5 min) after the runs. Samples were injected hydrodinamically at 50 mbar for 2 s. The injection end of the capillary exhibited the negative pole (cathodic side). The observed current was ca. 15 μA at 20 kV (for 80-cm capillary), and the temperature was maintained at 25°C.

Table 1. Assignment of the Vranec wines produced in different wine regions of Macedonia applying the same technological procedure from grapes

Vranec wines	Locality	Wine region
V1	Bistrenci	Tikveš
V2	Barovo	Tikveš
V3	Demir Kapija	Tikveš
V4	Disan	Tikveš
V5	Drenovo	Tikveš
V6	Gradsko	Tikveš
V7	Krivolak	Tikveš
V8	Kurija	Tikveš
V9	Lepovo	Tikveš
V10	Manastirec	Tikveš
V11	Veles	Tikveš
V12	Vilarov	Tikveš
V13	Ridiste	Tikveš
V14	Štip	Tikveš
V15	Bitola	Bitola
V16	Gevgelija	Gevgelija-Valandovo
V17	Radoviš	Strumica-Radoviš

2.5 Mass spectrometry detection

The detection of the organic acids was made with a 6530 Accurate-Mass Quadrupole Time-of-flight Mass Spectrometer (QTOF-MS) (Agilent Technologies, Singapore) equipped with a Jet Stream ES ion source, coupled to the CE instrument. The sheath liquid (1 v/v % solution of formic acid) was delivered at 0.7 μL/min flow using an LC isocratic pump (1260 Infinity series; Agilent Technologies, Waldbronn, Germany). The ESI/QTOF-MS was operated in the negative ionization mode applying electrospray voltage of 4.5 kV. Nitrogen was used as drying gas at 325°C, with a flow rate of 8 L/min; the pressure of the nebulizer gas was set at 35 psi. The sheath gas temperature was 350°C, with flow rate of 11 L/min. The TOF-MS parameters were the following: fragmentator, 100 V, skimmer, 65 V. The scanning mass-to-charge (m/z) range of the TOF analyzer was 50–250 m/z with a maximum accumulation time of 1000 ms/spectrum. The quantitative determination of the organic acids was made by the extracted ion electropherograms for each organic acid [16, 28]. The calculated m/z values of the quasi-molecular $[M–H]^-$ ions (m/z 89.0244 for lactic acid, m/z 117.0193 for succinic acid, m/z 133.0142 for malic acid, m/z 149.0092 for tartaric acid, m/z 173.0455 for shikimic acid, and m/z 191.0197 for citric acid) were extracted from the total ion electropherograms or the base-peak electropherograms, and the peak areas were used for the quantitative evaluation. The data processing was performed with the ChemStation B. 04.03. version and MassHunter B. 04 version softwares.

2.6 Validation parameters

The linearity of the calibration curves for the quantitative determination was controlled for each organic acid by five concentrations of the organic acids using a wine sample matrix. The LOQ was determined by the expression LOQ = 10 × SD/slope. The recovery of the method was determined by the analysis of one wine sample spiked with a standard solution at one concentration for each acid. The intraday repeatability of the method was studied by repeated injections (5×) of the same wine spiked with the acids, whereas the interday reproducibility was tested by triplicate injections of the spiked wine samples in three different days.

2.7 Statistical analysis

Statistical analysis was performed on the data for the organic acids in wine, including calculation of mean, minimum, maximum, SD, and RSD using Excel 2007 (Microsoft, Seattle, WA, USA). In order to ascertain possible significant similarities or differences among the wines, the *Student–Newman–Keuls multiple comparisons* test on the mean values was applied to the results of the concentrations of organic acids using the XLSTAT Software, version 7.5.2, Addinsoft (Paris, France).

3 Results

The main objective of the present work was to develop a rapid and simple method based on the CZE-ESI/QTOF-MS technique for the determination of lactic, succinic, malic, tartaric, shikimic, and citric acid concentration in wine samples. Experiments were performed to optimize the CE parameters for the separation. For the separation of the organic acids the volatile, aqueous ammonium acetate buffer was applied as a BGE, which is compatible with the MS detection. In order to avoid the adsorption of the organic acids on the capillary wall as well as to suppress the EOF, a polybrene coating was applied. Using the respective characteristic ion m/z values for each organic acid, the extracted ion electropherograms for a mixture of the organic acid standards are shown in Fig. 1a, and for a Vranec wine sample (V1) in Fig. 1b.

The main parameters of the proposed CZE-ESI/QTOF-MS method were thoroughly evaluated. The validation parameters, including linearity of concentration curve, LOQ, recovery, repeatability and reproducibility, were determined. Table 2 contains the linearity data in the relevant concentration range for the slope, intercept, and correlation coefficient values. The calibration curves of the standard solutions covered the expected concentration ranges for each acid in the wine samples (after dilution, of course). The calibration, plotting the extracted ion peak area *versus* the concentration of the test organic acids, was linear for all organic acids investigated, with correlation coefficients ranging from $r^2 = 0.9902$ (for shikimic acid) to $r^2 = 0.9990$ (for tartaric acid). The LOQ was determined for each acid, ranging from 0.0034 mM for shikimic acid to 0.107 mM for citric acid. The accuracy of the procedure was estimated using the standard addition method.

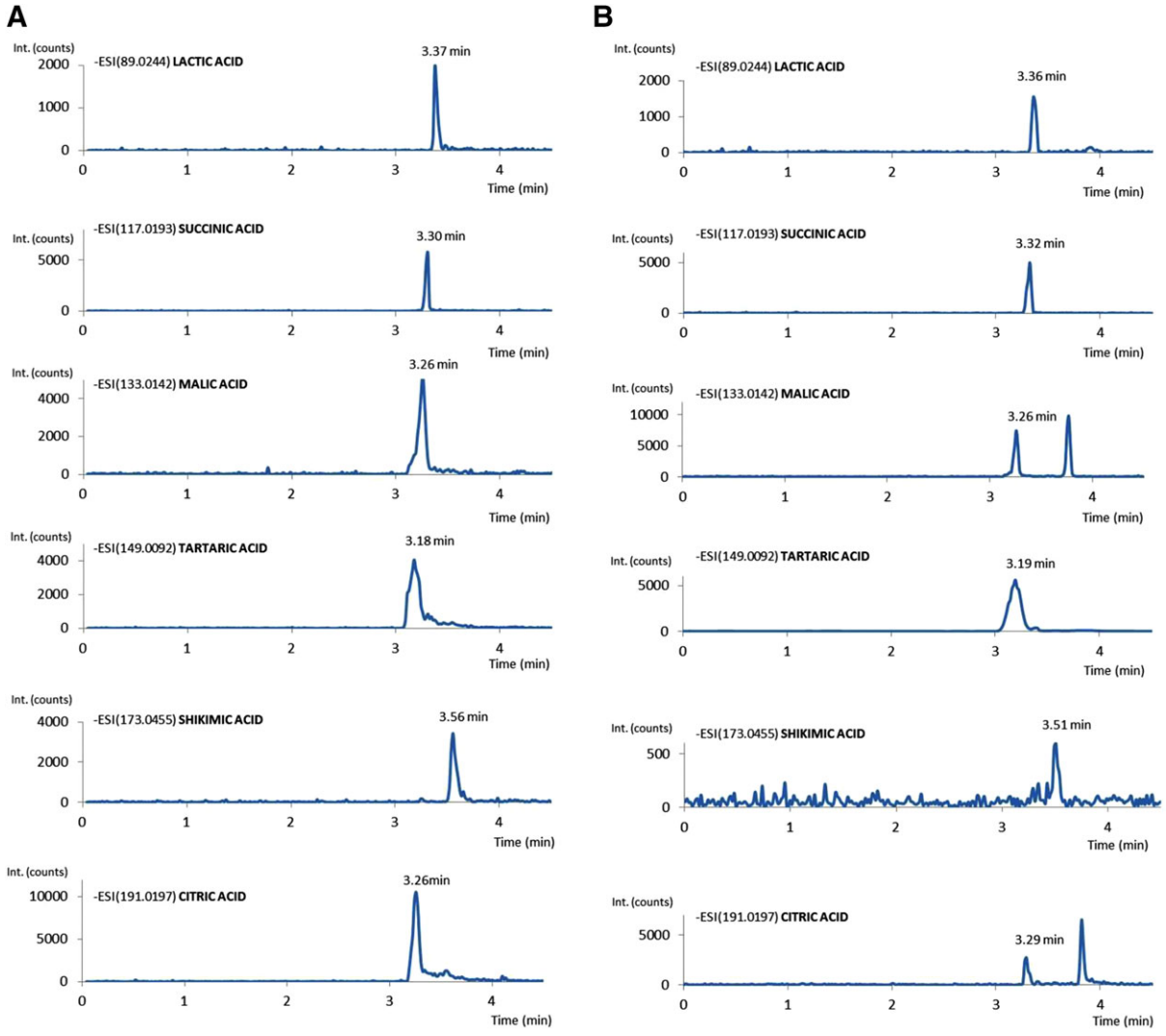

Figure 1. Extracted ion electropherograms of (a) the organic acid standards (100 mg/L each), and (b) the organic acids in the Vranec wine V1. Experimental conditions: capillary: length 80 cm; 50 μm id; BGE, 50 mM ammonium acetate buffer pH 6.0; dynamic coating with polybrene; applied voltage, –20 kV; current: 15 μA; hydrodynamic injection, 50 mbar for 8 s. Mass spectrometry detection: ESI sheath liquid composition, 1% formic acid, flow rate at 0.7 μL/min; sheath gas temperature, 350°C; flow rate, 11 L/min; spray voltage, 4.5 kV; temperature of the drying gas, 325°C; pressure of the nebulizer, 35 psi; accumulation time, 1000 ms/spectrum.

Table 2. Linear regression data

Organic acid	MS (*m/z*) [M-H]$^-$	Migration time (min)	Concentration range (mM)	Intercept	Slope	R^2	LOQ (mM)
Lactic	89	3.5	0.078–1.68	425	512	0.9918	0.081
Succinic	117	3.3	0.034–0.59	2169	2334	0.9912	0.04
Malic	133	3.2	0.003–1.50	1408	2883	0.9970	0.0031
Tartaric	149	3.1	0.033–5.36	−230	3447	0.9990	0.038
Shikimic	173	3.6	0.003–0.35	1328	29721	0.9902	0.0034
Citric	191	3.3	0.104–3.40	779	9684	0.9931	0.107

R^2, correlation coefficient; LOQ: limit of quantification.

Table 3. Standard additions for checking the accuracy of the CE-QTOF-MS method for determination of organic acids ($n = 3$)

Organic acid	Added (mM)	Wine V1 (mM)	Calculated (mM)*	Experimental (mM)*	SD	Recovery (%)
Lactic	5.00	8.43	13.43	13.8	0.26	102.7
Succinic	5.00	7.09	12.09	11.75	0.24	97.19
Malic	5.00	2.18	7.18	6.88	0.21	95.82
Tartaric	5.00	22.35	27.35	27.14	0.15	99.23
Shikimic	5.00	0.18	5.18	4.98	0.14	96.14
Citric	5.00	1.47	6.47	6.25	0.16	96.60

*Values are average of three replicates.

Table 4. Repeatability and intraday reproducibility measurements for the organic acid content in one Vranec wine sample (V13)

Organic acid	Interday repeatability (5 replicates × 1 day)		Intraday reproducibility (3 replicates × 3 days)	
	Mean concentration (mM)*	RSD (%)	Mean concentration (mM)*	RSD (%)
Lactic	3.93	6.91	3.71	5.80
Succinic	4.62	9.50	4.44	6.82
Malic	7.89	3.44	7.89	1.75
Tartaric	31.5	4.20	31.5	5.90
Shikimic	0.31	8.23	0.31	7.74
Citric	1.73	9.45	1.62	8.29

*Values are averages of three replicates.

One Vranec wine sample (V1), diluted five times, was spiked with the mixture of the organic acids (lactic, succinic, malic, tartaric, shikimic, and citric) at appropriate each concentration. Table 3 shows the results for the recovery studies of the organic acids, showing the recovery ranging between 95.82% (for malic acid) and 102.7% (for lactic acid). Additionally, the repeatability and reproducibility of the proposed method were studied with one wine sample (V13), and the results are shown in Table 4. The RSD data for the intraday repeatability ranged from 3.44 to 9.50% for the organic acid contents. The interday reproducibility provided RSD values between 1.75 and 8.29%. Seventeen wine samples were studied, and the organic acid contents are shown in Table 5. As it was expected, the tartaric acid was the dominant organic acid in almost all wines (concentration range: 14.03–33.29 mM), with the exception of the wine V14, in which the dominant organic acid was malic acid (30.3 mM). Succinic acid (concentration range: 1.45–10.17 mM) and citric acid (concentration range: 1.36–4.66 mM) were found in all wine samples at variable concentrations. Malic acid, which is transformed into lactic acid during the malolactic fermentation, ranged between 0.45 and 30.3 mM in the samples, while lactic acid was also found in all wines (concentration range: 1.24–16.4 mM). The content of shikimic acid in the wines was significantly lower compared to the other acids, and its concentration ranged from 0.02 to 0.34 mM. This acid, however, was not detected in the wine samples V10, V11, and V16, since its content was lower than the LOQ.

4 Discussion

Republic of Macedonia has a very long tradition for wine production. Wine is the most important product in the class of alcoholic beverages exported and the second most important agro-product after tobacco, which contributes to high foreign exchange earnings in the country [29]. In order to further increase the export, as well as the competitiveness of the Macedonian wines on the global market, there is a need of continuous quality control, such as determination and control of the main organic acids. Due to the lack of officially published results for these compounds in Macedonian wines, studies are necessary to be performed in order to gain data for the organic acid composition of the wines. Herein, we report a CZE-ESI/QTOF-MS data for the organic acids in red Vranec wines, the most widely spread and most important variety in Macedonia, as well as in the Balkans. To our knowledge, CE directly coupled to ESI accurate-mass QTOF-MS (ESI-ESI/QTOF-MS) has not been used for the determination of organic acids in food samples till now. This

Table 5. Concentration of the organic acids in the Vranec wines from different wine regions of Macedonia (2014)

Wines	Lactic acid (mM)	Succinic acid (mM)	Malic acid (mM)	Tartaric acid (mM)	Shikimic acid (mM)	Citric acid (mM)	Total acid content (g/L)
V1	8.43 ± 1.57	7.09 ± 1.71	2.18 ± 0.38	22.35 ± 3.69	0.18 ± 0.01	1.47 ± 0.10	5.51 ± 0.60
V2	16.40 ± 2.58	9.40 ± 3.59	0.45 ± 0.02	16.85 ± 2.95	0.13 ± 0.01	3.72 ± 0.18	5.87 ± 0.65
V3	6.74 ± 1.80	5.73 ± 0.94	11.43 ± 1.73	28.59 ± 6.44	0.02 ± 0.0001	1.52 ± 0.02	7.34 ± 1.01
V4	4.49 ± 1.12	5.30 ± 0.43	8.35 ± 1.50	19.80 ± 4.03	0.09 ± 0.01	4.24 ± 0.26	5.91 ± 0.64
V5	3.82 ± 0.92	5.38 ± 0.34	13.61 ± 0.54	22.01 ± 2.96	0.04 ± 0.01	2.09 ± 0.02	7.12 ± 0.77
V6	2.70 ± 0.90	6.67 ± 0.43	15.41 ± 2.78	18.79 ± 1.81	0.34 ± 0.05	3.35 ± 0.26	6.57 ± 0.47
V7	1.24 ± 0.45	1.79 ± 0.34	4.59 ± 0.53	14.03 ± 3.02	0.03 ± 0.001	2.67 ± 0.21	3.53 ± 0.46
V8	3.93 ± 1.24	6.07 ± 0.58	12.48 ± 0.54	26.31 ± 3.89	0.09 ± 0.01	1.36 ± 0.05	6.93 ± 0.60
V9	4.83 ± 1.35	1.79 ± 0.43	10.83 ± 0.60	24.56 ± 2.75	0.03 ± 0.01	2.72 ± 0.10	6.67 ± 0.44
V10	4.04 ± 0.57	6.24 ± 0.60	6.39 ± 0.38	33.29 ± 5.50	<LOQ	2.20 ± 0.10	7.32 ± 0.83
V11	2.36 ± 0.56	1.45 ± 0.05	6.24 ± 0.83	15.37 ± 0.74	<LOQ	2.30 ± 0.16	3.92 ± 0.17
V12	2.25 ± 0.90	4.79 ± 0.60	20.23 ± 1.05	24.97 ± 2.42	0.12 ± 0.02	1.88 ± 0.05	7.54 ± 0.40
V13	3.82 ± 0.22	4.27 ± 0.60	7.82 ± 1.05	32.68 ± 5.03	0.32 ± 0.03	1.68 ± 0.05	6.46 ± 0.45
V14	4.16 ± 1.01	5.38 ± 0.68	30.30 ± 4.66	17.52 ± 1.48	0.02 ± 0.01	3.98 ± 0.21	8.50 ± 0.67
V15	2.13 ± 0.90	8.55 ± 0.51	18.42 ± 4.14	21.07 ± 3.49	0.24 ± 0.03	1.52 ± 0.05	7.11 ± 0.76
V16	2.47 ± 0.67	3.68 ± 0.68	10.53 ± 1.20	19.53 ± 3.83	<LOQ	4.66 ± 0.32	5.84 ± 0.60
V17	4.38 ± 1.24	10.17 ± 2.05	17.82 ± 4.96	26.51 ± 4.36	0.08 ± 0.01	2.88 ± 0.21	8.47 ± 0.96
Mean	4.60	5.51	11.59	22.60	0.12	2.60	6.51
Min	1.24	1.45	0.45	14.03	0.02	1.36	3.53
Max	16.40	10.17	30.30	33.29	0.34	4.66	8.50

Values are presented as averages of triplicates. SD (standard deviation) is given. The error for the total acid content is calculated by propagation of the SD values for summation. The abbreviations of wines originating from different wine regions are explained in Table 1. The total organic acid content was calculated by considering the relative ratios of the organic acids.

technique allows obtaining high mass accuracy and resolution, and it is more selective and sensitive compared to the conventional CZE connected to UV detection. For the identification of compounds with MS, standards are not necessary, since determination is made by characteristic ions (*m/z* values). In our study, the LOQ values were mostly lower for the organic acids compared to HPLC-DAD [18] or CE-UV studies [2], but in some cases slightly lower values have also been obtained by CE techniques (see Table 6). It is important to note that no LOQ data for shikimic acid have been determined by CE. The shikimic acid content of Macedonian wines was recently determined using HPLC [30], but the LOQ (0.015 mM) was significantly higher compared to the results in this study (0.0034 mM), confirming that the high sensitivity and accuracy of the QTOF-MS determination is especially valuable for compounds present at low concentrations. Under the optimized and validated experimental conditions, the total run time was less than 4 min. The migration times of the organic acids were not changing over the course of the method development (the RSD was less than 3%), validation, and application to the real wine samples. The identification and the quantification were easy by the characteristic *m/z* values of the organic acids [16, 28]. The organic acids during the ESI ionization formed the negative single charged $[M-H]^-$ precursor ions (see Table 2). The CE conditions provided a separation of the organic acids in the complexes matrix, that is, in the wine samples after a simple sample pretreatment, i.e. dilution and filtration, which is the advantage of this method. The method can be applied for determination of organic acids in red wines to control the quality (this is especially important for citric acid quantitation, because its content should be controlled before wine export and/or wine import). This method will be very useful in scientific purposes, as well as it will be helpful for the wineries for controlling and improving the winemaking fermentation process: an alcoholic fermentation conducted by yeast and malolactic

Table 6. The LOQ values (mM) for organic acids obtained with CE techniques

Reference	Lactic acid	Succinic acid	Malic acid	Tartaric acid	Shikimic acid	Citric acid
[2]	1.37	0.17	0.49	0.44	n.d.	n.d.
[3]	0.007	0.002	0.004	0.009	n.d.	0.003
[4]	0.026	0.024	0.016	0.023	n.d.	0.027
[36]	0.11	0.085	0.075	0.067	n.d.	0.052
[30]	0.505	0.35	0.293	0.062	0.015	0.219
This study	0.081	0.04	0.0031	0.038	0.0034	0.107

n.d.: no data.

fermentation performed by lactic acid bacteria. Malolactic fermentation has an important role in the determination of wine quality, as for the red, but also for the white wines and it is associated with conversion of malic into lactic acid. Therefore, analysis of these two acids can give us a clear view of the stage to which malolactic fermentation has been carried out.

4.1 Testing the experimental conditions

Organic acids are negatively charged under the separation conditions due to deprotonation [16, 28]. To achieve a separation and quantification of the anions, appropriate BGE has to be chosen. Thus, the BGE (1) has to reverse and suppress the EOF sufficiently, (2) has to have mobility matching the analytes (anions) in order to reduce fronting and tailing of the analyte CE peaks, and (3) has to have a high molar absorptivity [16, 28]. When MS is used for detection of analytes, nonvolatile buffers (e.g., phosphate or borate buffers) are not recommended since they can cause dirt in the electrospray chamber and in the MS analyzer, also it can block the ionization of the analyte and result in a decrease in sensitivity. Therefore, volatile buffers should be applied in CE-MS analyses. In our study, the electrolyte composition was optimized for the MS detection, therefore, two volatile buffers were tested, ammonium acetate and ammonium formate. Although no baseline separation of the individual compounds was obtained in either BGEs, which in fact is not necessary, since the extracted ion electropherograms were satisfactorily available, the partial separation is anyway advantageous. Ammonium acetate showed better extracted ion peak shapes, therefore, it was chosen as BGE. Increasing the ammonium acetate concentration from 10 to 75 mM, caused broadening of the peaks, while the lower concentration of BGE provided longer migration time (results not shown). As the optimal condition, a 50 mM ammonium acetate buffer, with pH 6.0 was chosen for the separation of the organic acids. The influence of buffer pH was not tested, but only taken from the literature data [17]. Baseline separation of compounds was not achieved either with the 80 cm long or with the 120 cm long capillary. In fact, tartaric, malic, and succinic acids migrated almost together, followed by lactic acid, shikimic acid, and citric acid. We decided to use the shorter capillary, 80 cm long, achieving run times of 4 min for the experiments.

To prevent analyte adsorption and analyte loss, EOF instabilities, and to provide appropriate conditions for the CZE-ESI/QTOF-MS interfacing, the inner capillary surface should be coated with convenient coating materials. The coating material should provide homogenous coating surface by shielding completely the adsorption sites and should contribute to obtain high plate numbers. In the hyphenated mode, the coating material has to be MS compatible, and it should provide good reproducibility of the EOF, consequently the migration times of the analytes [31]. In our study, hexadimethrine bromide (polybrene) was the coating material chosen to coat the inner surface of the capillary, which fulfilled all the properties described above and presented high coating stability. The positively charged surface resulted in reverse (anodic) EOF allowing repeatable separations, efficient analyte ionization, and detection of the negatively charged analytes. Moreover, the surface coating was compatible with the MS application and provided good compatibility with the sample matrix components. By using polybrene a high EOF was induced due to its high surface charge, and we received reproducible migration times for the organic acids, which were then assigned easily by their characteristic ions in the MS. The increase of the voltage provided worse resolution (broadening of the peaks); therefore, we did not use higher potential difference than 20 kV.

Sheath liquid has significant effects on the sensitivity for CE-ESI/QTOF-MS. Generally, small amount of a volatile acid like formic or acetic acid is added to the mobile phase in MS analysis to facilitate the ionization of analytes. In our study, we used 1% v/v solution of formic acid as a sheath liquid that allowed satisfactory ionization of the organic acids.

4.2 Characterization of the Vranec wines

The optimized and validated CZE-ESI/QTOF-MS method was applied to obtain the organic acids profiles of Vranec wines produced under same vinification conditions from grapes grown in different wine areas (see Table 5). According to the *Macedonian Wine Law* [32] and the *Regulation of the basic physic-chemical analysis of wine* [33], organic acids have to be determined in all wines before the commercial sale. Moreover, the concentrations of certain organic acids, such as malic and lactic acids, must be controlled during the alcoholic and malolactic fermentation, as well as during the aging process. In this study, the six, most important organic acids were determined, and among them, tartaric acid was the dominant compound in all wines except for one wine (V14), in which the malic acid was the dominant organic acid. The concentration of tartaric acid decreases during fermentation and during the aging process due to the formation of tartrate salts, mainly potassium-hydrogen-tartrates, which precipitates and should be removed from the wine by filtration. All Vranec wines contained relatively high content of tartaric acid, ranging from 14.03 to 33.29 mM, which was slightly higher compared to Brazilian varieties [4]. In fact, high content of tartaric acid is typical for this variety and influence higher chemical stability and color, giving soft freshens of the wines. Moreover, Vranec wines in this study presented higher tartaric acid content compared to commercial Macedonian wines from other varieties, analyzed after 2 years period of storage [4]. During winemaking, some wines undergo spontaneous malolactic fermentation or this fermentation is accomplished by addition of lactic bacteria, whereby malic acid is converted to lactic acid [34]. Malic acid is typically associated with the taste of green apples, while lactic acid is richer and more buttery tasting. In that regard, malolactic fermentation tends to create a rounder, fuller mouthfeel. In Vranec wines, lactic acid bacteria were not added to the wines to start malolactic

fermentation. Almost all wines presented relatively high content of malic acid (range: 0.45–30.3 mM), which means that malolactic fermentation underwent spontaneously in these wines or even it did not start. Exceptions are two wines, V1 and V2, that presented a relatively high concentration of lactic acid (8.43 and 16.4 mM, respectively), which means that malolactic fermentation almost finished in these two wines. In all other wines, lactic acid was formed during the spontaneous malolactic fermentation, ranging between 1.24 and 6.74 mM. Citric acid is usually added to adjust the wine acidity since it does not form insoluble precipitates with calcium and potassium as tartaric acid does, and has a lower cost, compared to tartaric acid [4]. In the wines studied here, it was not necessary to adjust the acidity, and the concentrations of the naturally present citric acid in the samples (ranging from 1.36 to 4.66 mM) were in accordance to the official regulations, that is, not higher than 1 g/L (5.24 mM) [32]. In addition, succinic acid, which is a by-product of the metabolism of yeast during fermentation, with a bitter-salty flavor, was found in concentrations lower than 10 mM except for wine V17, having a slightly higher value (10.17 mM). Compared to results from a study, reported in 2016 for red and white commercial Macedonian wines analyzed with HPLC [13], Vranec wines – produced under controlled winemaking protocols – presented higher content of citric, lactic, malic, and succinic acids. The shikimic acid does not have any important sensory effect in wine. Since this acid is present at low concentration in the grapes, it appears in a significantly lower concentration in the wines. Tessini *et al*. [35] determined the concentration of shikimic acid in different wine varieties (8.69 mg/L, i.e. 45.5 mM for Pinot Noir; 12.69 mg/L, i.e. 66.44 mM for Melbec wines; and 93.57 mg/L, i.e. 489 mM for Cabernet Sauvignon) [35]. Compared to the Melbec and Cabernet Sauvignon wines, the Vranec wines contain significantly lower concentration of shikimic acid. The highest concentrations were 0.34, 0.32, and 0.24 mM for the V6, V13, and V15 wines, respectively. Results for shikimic acid in the wines in this study are similar compared to other red Macedonian wines (Merlot, Vranec, and Cabernet Sauvignon) [30]. In order to know whether the results for shikimic acids are characteristic for the Vranec variety, additional studies have to be performed, including also wine samples from this variety produced by various winemaking protocols, as well as other red and white varieties.

4.3 Concluding remarks

Application of CZE coupled to CZE-ESI/QTOF-MS provides excellent sensitivity and selectivity for fast and simple analysis of lactic, succinic, malic, tartaric, shikimic, and citric acids in red wines after minimal sample pretreatment. The method was optimized and validated, and by this method Vranec wines, from various wine regions in R. Macedonia, were studied, showing a wide variation of organic acid content. A relatively high concentration of tartaric acid was observed in the wines, which is, nevertheless, typical for this variety. Determination of the main organic acids is of crucial importance for the Macedonian wines for their quality control, especially for the wines that are exported to the European countries. Results here will provide a clear view for the organic acids profile in Vranec wines produced from various wine areas. Since this variety has a relatively high acidic content, the results will be useful for winemakers to manage and/or modify the winemaking protocols for this variety to obtain stable and high-quality wines for the global market. Moreover, the proposed method decreases the analysis time compared to the previously reported HPLC and CE methods and allows rapid control of the winemaking processes and the detection of wine alterations and/or illnesses.

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