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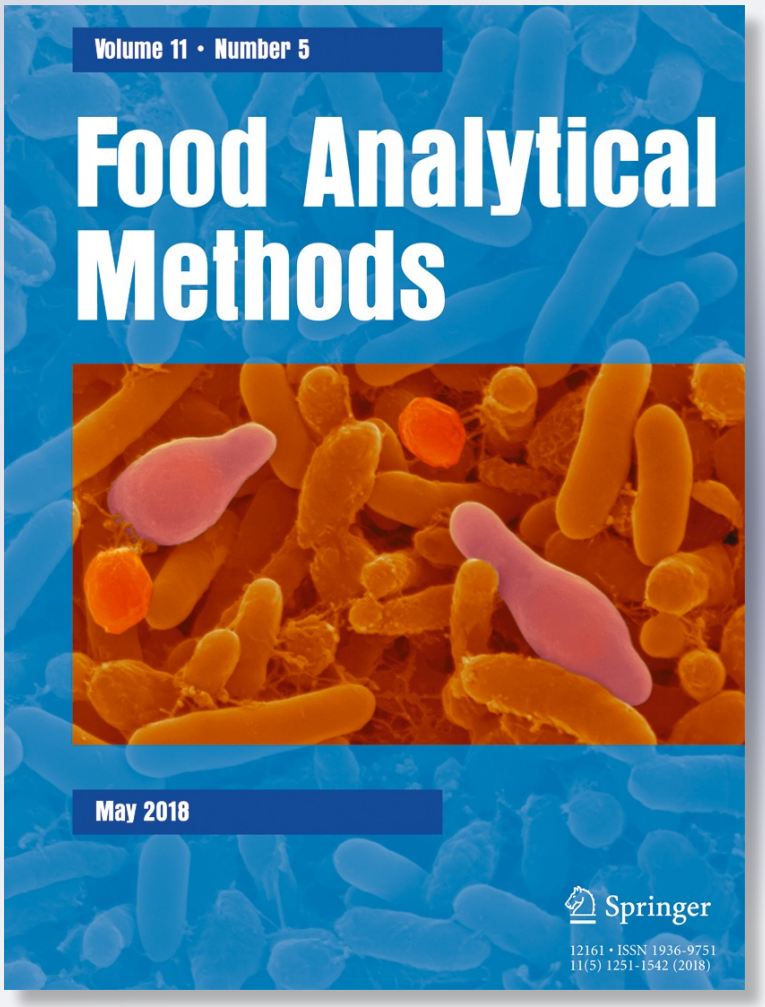

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Optimization and Validation of a New Capillary Electrophoresis Method with Conductivity Detection for Determination of Small Anions in Red Wines

Zorica Lelova[1,2] · Violeta Ivanova-Petropulos[1] · Marián Masár[3] · Klemen Lisjak[4] · Róbert Bodor[3]

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Abstract
A capillary electrophoresis (CE) method has been developed and validated for determination of organic acids (oxalate, tartrate, malate, malonate, pyruvate, succinate, acetate, citrate, and lactate) and inorganic anions (sulfate and phosphate) in red wines. The separations were carried out in an automated separation system equipped with wide-bore (300 μm i.d.) fluoroplastic capillary and contact conductivity detector used for monitoring the separation and quantification of the analytes. The fast method (analysis time less than 5 min.) provided a good linearity of calibration curves ($R^2 > 0.9920$) for the studied acids, as well as a good reproducibility of migration times ($RSD < 1.5\%$). In total, 17 red wines were analyzed with the proposed method, including Vranec, Cabernet Sauvignon, and Merlot wines from various geographic areas (Demir Kapija, Kavadarci, Negotino, and Veles) in Macedonia. The used fully automated separation system (sample dilution not included) predetermined the developed CE method for routine analysis.

Keywords Wine · Organic acids · Inorganic anions · Validation · Capillary electrophoresis

Introduction

Organic acids are important components in grape and wine that determine their acidity and affect the sensory perception, such as flavor, aroma, and color. Organic acids also influence the pH, as well as the microbiological and biochemical stability of wines, particularly in white wine (Castiñeira et al. 2002; Esteves et al. 2004). Most bacteria do not grow at lower pH values in the wine, which means that wine is more stable and has a greater potential for storage and aging (Tašev et al. 2016). During the wine aging, acids are involved into reactions of esterification which influence the development of the desired wine bouquet. Therefore, the content of organic acids should be monitored during the vinification process, starting from the grapes juices and maceration, continuing to the alcoholic fermentation and wine stabilization processes.

The main organic acids in grape juices are tartaric, malic, and citric acids, while lactic, succinic, and acetic acids are formed during the alcoholic fermentation (Mato et al. 2007). The content of acids in grapes ranges from 8 to 13 g/L, while in wines, acids' content is between 5.5 and 8.5 g/L, depending on the variety and climatic conditions during the year (Peres et al. 2009). Tartaric acid is the dominant organic acid in grapes and wines which plays significant role in maintaining the chemical stability of the wine, its color, and influence the taste of the finished wine. The content of tartaric acid decreases during the fermentation as a result of precipitation in a form of tartaric crystals. Usually, the total acidity is expressed as tartaric acid equivalents. During the malolactic fermentation undertaken by the lactic acid bacteria, the content of malic acid decreases due to its conversion to lactic acid, which concentration increases. Citric acid also influences the acidity of wines, and it is an important component in biochemical and metabolic processes (e.g., Krebs cycle), which slow the yeast growth, but do not block it. Succinic acid is as a byproduct of the metabolization of nitrogen by yeast cells during fermentation. About 1 g/L is produced during the

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primary fermentation. This compound is undesirable at high levels because of its bitter and salty taste. Acetic acid is the volatile compound produced in wine during or after the fermentation period and responsible for the sour taste of vinegar. An excessive amount of acetic acid is considered as a wine fault.

Chromatographic techniques are the most important techniques for determination of organic acids. Thus, separation and quantification can be performed with high-performance liquid chromatography (HPLC) (Tusseau and Benoit 1987; Schneider et al. 1987), gas chromatography (GC) (Falque-Lopez and Fernández-Gómez 1996; Escobal et al. 1997), or ion chromatography (IC) (Yan et al. 1997; Xiong et al. 2014). Recently, Fourier transform infrared (FT-IR) spectroscopy with partial least squares (PLS) was used for the determination of lactic, succinic, malic, tartaric, citric, and acetic acid in wines, vinegars, and spirits (Regmi et al. 2012). In the last few years, capillary electrophoresis (CE) coupled to UV detection, in direct or indirect modes, has been applied for the determination of organic acids in grapes and wines (Castiñeira et al. 2000; Saavedra and Barbas 2003; Mato et al. 2007; Peres et al. 2009; Liu et al. 2017) offering fast analyses and efficient resolution of the analytes. CE methods combined with conductivity detection (CD) have also been presented. Most frequently, contactless conductivity detectors are used (Kubáň and Hauser 2005). Usually, a reversed direction of electroosmotic flow (EOF) is necessary to separate anionic analytes in a short time with adequate resolution. CE fully adapts to the tendency of miniaturization, and microchip electrophoresis (MCE) represents a great potential in wine analysis (Gomez and Silva 2016). MCE determinations of the small inorganic and organic anions in white and red wines by isotachophoresis (Masár et al. 2001) and zone electrophoresis (Masár et al. 2005) with contact CE have been shown.

Republic of Macedonia has a very long tradition for wine production. There is a need of continuous quality control, such as determination and control of the main organic acids. Until today, only one study on the analysis of organic acids with RP-HPLC (Tašev et al. 2016) has been published, which is not enough for making major conclusions about wine quality. Therefore, further studies are necessary to be performed in order to gain data for the organic acids composition of the Macedonian wines, applying fast and accurate methods. Herein, we report an optimized and validated CE analysis method, hyphenated with CD for determination of organic acids (oxalate, tartrate, malate, malonate, pyruvate, succinate, acetate, citrate, and lactate) and inorganic anions (sulfate and phosphate) in red wines, including Vranec, Cabernet Sauvignon, and Merlot wines from various geographic areas (Demir Kapija, Kavadarci, Negotino, and Veles). Furthermore, to the best of our knowledge, this is the first report on application of the CE-CD technique on determination of organic acids in wines. The quality parameters of method, such as limit of detection (LOD), limit of quantifications (LOQs), linearity, recovery, repeatability, and reproducibility are presented.

Materials and Methods

Chemicals and Reagents

Sodium salts of sulfate, acetate, and hydrogen phosphate, as well as lithium lactate, and oxalic, tartaric, malic, malonic, pyruvic, succinic, and citric acids were purchased from Sigma-Aldrich (Bratislava, Slovakia). Stock solutions of standards were prepared with a concentration of 1 mmol/L, except of acetate (10 mmol/L) and lactate (5 mmol/L). 4-Morpholineethanesulfonic acid (MES), Bis-Tris, Bis-Tris propane used for preparation of electrolyte solutions were BioXtra quality (www.sigmaaldrich.com). Cyclodextrins were obtained from Cyclolab (Budapest, Hungary). Methylhydroxyethylcellulose (MHEC) 30,000 (Serva, Heidelberg, Germany) with viscosity of 30 Pa s (2% (*w*/*V*)) in water at 20 °C, purified on a mixed-bed ion exchanger Amberlite MB-1 (Merck, Darmstadt, Germany), was used as a suppressor of EOF. It was added to the electrolyte solutions. Water demineralized by a Simplicity deionization unit (Millipore, Molsheim, France) was used for the preparation of the electrolyte and sample solutions.

Grapes

Grapes from *V. vinifera* L. varieties Vranec, Cabernet Sauvignon, and Merlot cultivated in the Tikveš wine region (Republic of Macedonia) were harvested in September/October 2015, at optimal technological maturity: 18, 20, and 26° Brix, respectively (levels between 18 and 26° Brix are desirable as objective criteria for estimating optimal grape maturity). Vranec grapes were collected from 16- to 26-year-old vineyards with area of 30 ha, while Merlot and Cabernet Sauvignon grapes were grown at 15 and 26 ha, 17- and 26-year-old vineyards, respectively. The distance between the rows was 1.5 m, and the distance between the vines was 1.0 m. Grapes were manually harvested early in the morning and placed in crates.

Winemaking

In total, 17 red wines were produced and analyzed, including Vranec, Cabernet Sauvignon, and Merlot, originating from four geographic areas: Demir Kapija, Kavadarci, Negotino, and Veles (Republic of Macedonia).

Harvested grapes (6000 kg) of each variety and from each wine area were transported to the Tikveš winery (Kavadarci, R. Macedonia), whereas the grapes were

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processed separately. After processing of grapes with mechanical crusher/destemmer (Selectiv' Process Winery, Pellenc, Pertius, France), each must was collected in a fermentation tank (7 tones). The must was immediately treated with sulfur dioxide (40 mg/L) in a form of 5% sulfurous acid. After the addition of SO_2, a commercial pectolytic enzyme preparation (Vinozym Vintage, FCE, Lamothe Abiet, France) was applied in all tanks (3 g/100 kg) in order to obtain higher color stability, body, mouthfeel, as well as a higher polyphenols and aroma extraction. After 3 h, wines were inoculated with commercial *Saccharomyces cerevisiae* yeast (Lalvin ICV D80, Lallemand, France). Before application, the yeast was previously rehydrated in water (20 g/hL, at 35 °C for 30 min), followed by the addition of nutrients (containing sterols, polyunsaturated fatty acids, vitamins, and minerals) in a dose of 45 g/hL (Go-ferm protect, Lallemand, France) to improve yeast survival, particularly in difficult fermentation conditions. Grape mash from each tank was macerated for 10–12 days, and during that period (alcoholic fermentation), "pumping over" was applied in all lots, two times a day.

After the maceration period, wines were separated from the pomace by mechanical pressing and stabilized in an inox tanks (7000 L) for 24 h. After that period, wines were racked, inoculated with malolactic bacteria (1 g/hL, Christian Hansen) and after finishing the malolactic fermentation, wines were treated with sulfur dioxide again (30–40 mg/L). The second racking was performed after 3 months of storage, bottled, and stored in a cellar at 4–12 °C for 5 months before analysis.

In order to determine the general chemical composition of wines, official methods of analysis of wines (OIV 2016) were used, and following parameters were analyzed: alcohol (OIVMA-AS312-01 A), dry extract (OIV-MA-AS2-03B), specific density (OIV-MA-AS2-01 A), total acidity (OIV-MA-AS313–01), volatile acidity (OIV-MA-AS313–02), total SO_2, and free SO_2 (Ivanova-Petropulos and Mitrev 2014). All wines contained alcohol between 11.02 to 15.29%, dry extract 34.0 to 36.7 g/L, and specific density ranged between 0.9946 and 0.9971. The pH of wines was between 3.4 and 3.7, the total acidity ranged between 4.7 and 6.6 g/L (tartaric acid equivalents), and volatile acidity in wines ranged from 0.4 to 0.6 g/L (acetic acid equivalents). The content of free and total SO_2 was between 15 to 58 mg/L and 60 to 100 mg/L, respectively.

CE-CD Analysis

CE separations were performed using a fully automated Electrophoretic analyzer EA 202A (Villa Labeco, Spišská Nová Ves, Slovakia) equipped with a 300-μm i.d. capillary tube made of fluorinated ethylene-propylene copolymer, polymethylmethacrylate sample injection block with a sample plug length of 3 mm (500 μm i.d.) and Triathlon autosampler (Spark Holland, Emmen, The Netherlands). The AC contact conductivity detector (Villa Labeco) connected to the detection electrodes placed at the end of capillary (90 mm effective length) monitored the CE separations. During the separations, the driving current was stabilized at 120 μA.

The newly developed background electrolyte (BGE) was composed of 35 mmol/L MES, 6 mmol/L Bis-Tris propane, 3.4 mmol/L Bis-Tris, and 0.1% (*w*/*V*) MHEC, pH = 6.0 with addition of different concentrations of α-CD (0 and 20 mmol/L) and β-CD (0 and 10 mmol/L). At the beginning and at the end of the day, the separation and electrolyte units as well as sample loop in autosampler were rinsed by deionized water using built-in peristaltic pumps. Between analyses, a relatively short rinsing procedure (ca. 1 min) with BGE solution was used.

Table 1 Linear regression data: range of determination, coefficients of the regression curves (slope and intercept), coefficient of determination R^2, LOD, and LOQ

Anion	Range (μmol/L)	Slope (mVs.L/μmol)	Intercept	R^2	LOD (μmol/L)	LOQ (μmol/L)
Sulfate	5–60	2.62	− 0.44	0.9979	1.6	4.8
Oxalate	5–50	2.45	0.45	0.9993	1.5	4.5
Tartrate	5–150	2.68	− 0.29	0.9990	1.5	4.5
Malate	5–100	1.64	− 2.69	0.9994	1.6	4.8
Malonate	5–50	1.74	− 3.83	0.9979	1.9	5.7
Pyruvate	10–70	0.68	− 1.16	0.9975	2.5	7.5
Succinate	5–120	1.99	− 2.20	0.9976	1.6	4.8
Acetate	20–300	1.11	− 8.57	0.9989	5.7	17.1
Citrate	10–60	1.76	− 1.62	0.9919	2.1	6.3
Lactate	30–150	1.15	− 0.82	0.9918	3.7	11.1
Phosphate	20–70	1.33	− 0.38	0.9915	4.5	13.5

The order of acids is in according to the migration order shown on Fig. 1c

LOD limit of detection, *LOQ* limit of quantification

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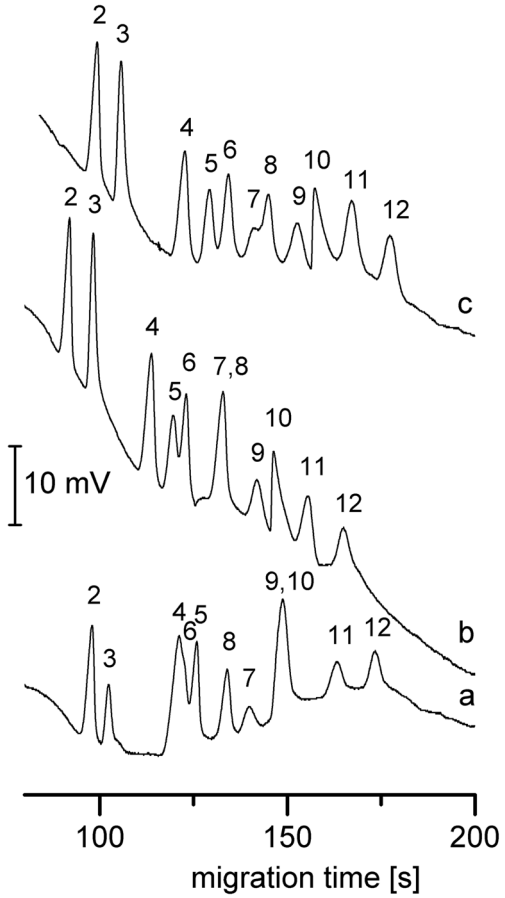

Fig. 1 CE separations of organic and inorganic acids under different separating conditions. The separations were carried out in background electrolytes consisting of **a** 35 mmol/L MES, 6 mmol/L Bis-Tris propane, 3.4 mmol/L Bis-Tris, 0.1% (*w*/*V*) MHEC, pH = 6.0; **b** same as in **a** with addition of 20 mmol/L α-cyclodextrin; **c** same as in **a** with addition of 20 mmol/L α-cyclodextrin and 10 mmol/L β-cyclodextrin. The driving current was stabilized at 120 μA. Peak assignments: 2-sulfate, 3-oxalate, 4-tartrate, 5-malate, 6-malonate, 7-pyruvate, 8-succinate, 9-acetate, 10-citrate, 11-lactate, and 12-phosphate. Chloride (1) is not shown. Concentration of anions in the injected samples were 20 μmol/L, except of acetate and lactate (30 μmol/L)

Calibration and Validation Parameters

For quantification purpose, a six-point calibration curves from the peak areas, assaying the standard solutions of the acids, were constructed for all analytes. The concentration range (μmol/L) for all analyzed compounds is presented in Table 1. Each calibration point was measured three times.

Under the optimized separating conditions, performance of the developed method was validated using linearity, LOD and LOQ, precision, and accuracy.

Statistical Analysis

Statistical treatment, including calculation of mean, minimum, maximum, standard deviation, and relative standard deviation were performed with STATISTICA 6.0 software (Stat Soft Inc., USA). Principal component analysis (PCA) was employed to evaluate the possible grouping of the wines, using XLSTAT Software, Version 7.5.2 (Addinsoft, Paris, France).

Results and Discussion

Optimization of the CE Conditions

In this work, we utilized a sample injection device, firstly shown by Verheggen et al. (1988), for introducing the relatively high volume (590 nL) of the sample in a short plug (3 mm). CE separations of anions were carried out in low conductivity BGE under suppressed EOF. The low conductivity of BGE is necessary when the CE separation is performed in wide bore capillary, and it is beneficial for enhancing the sensitivity of the CD. Short separation path (ca. 10 cm), comprising effective length of column and part of injection device, was reflected in a search for optimum separating conditions. Composition of BGE was chosen based on our previous research (Masár et al. 2005). Several different mechanisms, established by the components of BGE, provided a complete resolution of 11 anions (Fig. 1c). In this context, it should be noted that the BGE contained two counter ions, Bis-Tris and Bis-Tris propane (single and double charged at pH 6), for modification of effective mobilities of mono and divalent acids by ionic strength effect. Host-guest complexations (using α- and β-cyclodextrins as hosts) had the greatest impact on effective mobilities of malonate, succinate, and citrate (Fig. 1b, c).

Validation of the Method

Linearity was tested in 3 days at six concentration levels. The *linearity data*, including slope, intercept, and correlation coefficient (R^2) were calculated, and they are presented in Table 1. As it can be seen from the table, the linearity is satisfactory in all cases with correlation coefficients ($R^2 > 0.992$), ranging from 0.9915 for phosphate to 0.9994 for malate.

LOD was determined as a concentration of the analyte that gives a signal equal to the average background (S_{blank}) plus three times of the standard deviation of the blank (s_{blank}), than LOD = ($S_{blank} + 3 \times s_{blank}$ − intercept)/slope. The calculated intercept was used for estimation of S_{blank}, the blank signal itself. Standard deviation of blank (s_{blank}) was expressed by random errors in the *y*-direction of regression lines ($s_{y/x}$), LOD = (3 $s_{y/x}$)/slope. LOQ was determined as LOQ = 3 × LOD. The obtained values for LOD and LOQ ranged from 1.5–5.7 μmol/L to 4.5–17.1 μmol/L respectively, for all acids.

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Table 2 Precision of the proposed method

Anion	Intra-day precision (RSD of peak area %, $n = 3$)		Inter-day precision (RSD of peak area %, $n = 9$)	
	Low level	High level	Low level	High level
Sulfate	4.8	2.1	5.1	2.8
Oxalate	1.6	2.2	3.6	2.6
Tartrate	0.5	1.1	3.0	1.5
Malate	1.1	0.9	4.3	2.4
Malonate	3.0	1.8	5.8	2.9
Pyruvate	4.0	2.9	5.0	5.2
Succinate	3.5	3.5	4.9	3.9
Acetate	3.9	0.7	4.7	1.3
Citrate	3.4	2.8	5.6	5.1
Lactate	4.4	2.2	5.2	4.1
Phosphate	4.8	0.5	5.5	2.8

The lowest limits of detection were noticed for oxalate and tartrate, 1.5 μmol/L for both analytes (Table 1).

Precision The intra-day and inter-day precision were determined by injection of standard solution with low (10 μmol/L for sulfate, oxalate, tartrate, malate malonate, succinate, 20 μmol/L for pyruvate, citrate, and 30 μmol/L for lactate, phosphate, and acetate) and high concentration (40 μmol/L for sulfate, oxalate, malonate, pyruvate, succinate, citrate, and 70 μmol/L for tartrate, malate, lactate, phosphate, and acetate) of tested analytes. For determination of intra-day precision, freshly prepared solutions were analyzed immediately after preparation, in three repetitions. The RSD values of peak areas for each analyte were lower than 5% for the low concentrations of all acids and lower than 3% for the high concentration of the acids, which confirmed that the proposed method is precise. Inter-day precision was determined during 3 consecutive days with three repeated analyses of daily prepared solutions. The inter-day precision (RSDs of peak areas) was better than 6%. The other results are presented in Table 2.

The accuracy was expressed with the recovery of the determined concentration compared with the true (nominal) value. It was checked using the standard addition method on real wine sample (Vranec-N-1). Wine sample was spiked at two concentration levels with mixed standard solution of acids. The spike recoveries were calculated by following equation: Recovery (%) = (found concentration in spiked sample − original concentration in the sample)/added concentration × 100%. The analysis of these spiked samples led to calculated recoveries ranging between 91.6 and 100.3% (Tables 3 and 4), which confirmed the accuracy of the method and its suitability for determination of selected anions in wine samples.

Repeatability and Reproducibility Repeatability was checked with six repetitions in 1 day, while reproducibility was checked with three repetitions in five consecutive days, both performed on a real red wine sample. Concentrations of the analytes were calculated from their corresponding calibration curves. Values for the relative standard deviation of determined concentrations were low, ranging from 1.1 to 3.5% for repeatability, and 2.9 to 7.5% for reproducibility.

Table 3 Standard additions for checking the accuracy of the CE method for determination of organic and inorganic acids in wine samples ($n = 3$)

Anion	Conc. (μmol/L)	I. conc. level			II. conc. level		
		Added (μmol/L)	Found (μmol/L)	Recovery (%)	Added (μmol/L)	Found (μmol/L)	Recovery (%)
Sulfate	9.3	10	19.2	98.5	20	29.3	99.8
Tartrate	109.0	20	128.3	96.4	40	149.1	100.2
Malate	4.3	10	14.2	98.6	20	24.4	100.3
Succinate	53.0	10	62.7	96.9	20	72.9	99.4
Acetate	104.8	20	123.9	95.7	40	144.0	98.1
Citrate	0	10	9.5	95.1	20	19.5	97.5
Lactate	48.1	10	57.4	93.5	20	67.2	95.7
Phosphate	30.3	10	39.5	91.6	20	50.0	98.3

Wine sample—Vranec-N-1

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Table 4 Repeatability and reproducibility data

Anion	Repeatability (6 replicates)		Reproducibility (3 replicates × 5 days)	
	Mean concentration (μmol/L)	*RSD* (%)	Mean concentration (μmol/L)	*RSD* (%)
Sulfate	9.36	1.1	9.34	2.9
Tartrate	109.3	2.0	109.4	2.0
Malate	3.66	3.3	3.60	7.5
Succinate	53.0	1.8	53.1	2.9
Acetate	104.7	2.0	104.6	3.2
Lactate	47.9	2.7	47.9	4.7
Phosphate	30.6	3.5	30.8	5.3

Wine sample—Vranec-N-1

Identification of analytes based solely on migration times in CE, requires good reproducibilities; therefore, the *migration time precision* is important in assessing the overall performance. RSDs of migration times ranging from 0.6 to 1.6%, expressed in standard deviation, represents 1–2 s. Typically, analytes with smaller effective mobilities (higher migration times) showed a lower RSD. These values, considering the fact, that it represents a data from the separation of model samples at six concentration levels (used also for linearity test), are more than satisfactory. It is also remarkable that the average migration times calculated from the analysis of all wine samples were inside of the interval defined by migration time ± standard deviation, calculated from analysis of model samples. This fact indicates that the used working conditions with eliminated EOF significantly reduced the fluctuation of migration times.

CE-CD Analysis of Red Wines

The optimized and validated CE-CD method was applied for determination of organic and inorganic acids in Macedonian red wines from three varieties, including Vranec, Merlot,

Table 5 Content of organic and inorganic acids (mmol/L) in Vranec, Merlot, and Cabernet Sauvignon wines produces from different wine regions

Wines	Sulfate (mmol/L)	Tartarate (mmol/L)	Malate (mmol/L)	Succinate (mmol/L)	Acetate (mmol/L)	Citrate (mmol/L)	Lactate (mmol/L)	Phosphate (mmol/L)
Vranec-DK-1	1.5 ± 0.1	13.4 ± 1.0	0.7 ± 0.2	6.2 ± 0.3	11.3 ± 0.6	n.d	9.4 ± 0.7	5.5 ± 1.6
Vranec-DK-2	1.8 ± 0.1	13.0 ± 0.3	n.d	5.90 ± 0.1	8.8 ± 0.2	n.d	8.7 ± 0.1	3.1 ± 0.2
Vranec-K-1	1.3 ± 0.1	12.2 ± 0.8	0.6 ± 0.1	5.2 ± 0.2	9.1 ± 0.4	n.d	6.8 ± 0.2	5.3 ± 0.4
Vranec-K-2	1.8 ± 0.1	13.5 ± 0.3	0.4 ± 0.1	5.4 ± 0.2	8.2 ± 0.1	n.d	6.1 ± 0.5	3.5 ± 0.1
Vranec-K-3	1.2 ± 0.1	12.5 ± 0.5	0.8 ± 0.1	5.8 ± 0.3	9.9 ± 0.5	n.d	10.0 ± 0.3	2.1 ± 0.2
Vranec-K-4	1.4 ± 0.1	12.8 ± 0.1	0.7 ± 0.1	6.2 ± 0.2	8.4 ± 0.1	n.d	7.6 ± 0.3	2.2 ± 0.3
Vranec-N-1	1.1 ± 0.1	12.7 ± 0.2	0.5 ± 0.1	6.2 ± 0.1	12.2 ± 0.3	n.d	5.6 ± 0.2	3.5 ± 0.2
Vranec-V-1	1.5 ± 0.1	14.5 ± 0.1	n.d	4.3 ± 0.1	11.0 ± 0.4	n.d	7.0 ± 0.1	4.2 ± 0.3
Merlot-DK-1	2.7 ± 0.1	10.1 ± 0.1	1.0 ± 0.1	7.3 ± 0.3	8.0 ± 0.2	n.d	9.2 ± 0.5	4.3 ± 0.5
Merlot-N-1	1.3 ± 0.1	11.8 ± 0.5	1.2 ± 0.1	6.7 ± 0.5	8.5 ± 0.2	n.d	11.5 ± 0.1	3.8 ± 0.7
Merlot-N-2	4.2 ± 0.1	10.0 ± 0.3	0.6 ± 0.1	5.3 ± 0.1	12.3 ± 0.2	0.4 ± 0.1	6.0 ± 0.6	6.3 ± 0.4
Merlot-V-1	1.4 ± 0.1	11.6 ± 0.4	1.0 ± 0.1	7.0 ± 0.7	8.6 ± 0.3	n.d	9.4 ± 0.2	3.5 ± 0.4
Merlot-V-2	1.2 ± 0.1	11.2 ± 0.3	1.2 ± 0.3	6.9 ± 0.1	8.2 ± 0.4	n.d	11.8 ± 0.1	4.0 ± 0.1
Cab. Sauvig-DK-1	2.2 ± 0.1	13.0 ± 0.3	1.0 ± 0.1	7.6 ± 0.1	6.4 ± 0.1	0.6 ± 0.1	11.0 ± 0.1	7.2 ± 0.8
Cab. Sauvig-N-1	1.8 ± 0.1	13.3 ± 0.3	1.2 ± 0.1	6.7 ± 0.1	7.9 ± 0.1	n.d	12.8 ± 0.4	2.8 ± 0.1
Cab. Sauvig-N-2	2.3 ± 0.1	11.7 ± 0.3	0.8 ± 0.1	5.9 ± 0.2	11.1 ± 0.5	n.d	10.7 ± 0.3	2.6 ± 0.4
Cab. Sauvig-V-1	1.7 ± 0.1	9.1 ± 0.4	0.5 ± 0.1	6.6 ± 0.3	10.8 ± 0.2	n.d	10.0 ± 0.6	4.8 ± 0.4
Min	1.09	9.07	0.43	4.31	6.40	0.00	5.58	2.10
Max	4.17	14.48	1.18	7.56	12.30	0.62	12.85	7.25
Mean	1.77	12.09	0.83	6.13	9.45	0.06	9.00	4.03

n.d. not detected, *Cab. Sauvig* Cabernet Sauvignon

Abbreviations of wine regions: *DK* Demir Kapija, *K* Kavadarci, *N* Negotino, *V* Veles

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and Cabernet Sauvignon wines produces from different wine regions: Demir Kapija (DK), Kavadarci (K), Negotino (N), and Veles (V). The average migration time for all anions in all wine samples was calculated. The differences between the average migration times in model and wine samples were lower than standard deviation in model samples for all anions. Typical electropherograms from the analysis of wine and calibration samples are shown on Fig. 1. The content of the determined acids in the wines is presented in Table 5.

In total, eight acid salts were determined in the wines. Organic acids salts, including the tartrate, malate, succinate, and lactate, were detected in all analyzed wines, since they are naturally present in wine (malate was not detected in two wines (Vranec-V-1 and Cabernet Sauvignon-V-1) and citrate was found in two wines, Merlot (Merlot-N-2) and Cabernet Sauvignon (Cab. Sauvig-DK-1)). Among all organic acids, tartrate was found in highest concentration in Vranec wines, ranging from 12.2 to 14.5 mmol/L. In fact, tartaric acid is synthesized in grapes, and it is extracted into the wine during the maceration. During the fermentation and aging process, its concentration decreases as a result of formation of tartrates, mainly potassium hydrogen tartrates, which precipitate at the bottom of the tanks and afterwards, are removed from the wine by filtration.

The concentration of malic acid is highest at the beginning of the alcoholic fermentation, and afterwards, it is converted into lactic acid, spontaneously or in the presence of malolactic bacteria, during the malolactic fermentation. During this process, the content of malic acid decreases, and the content of lactic acid increases in wine (Davis et al. 1988). In our study, all wines were inoculated with malolactic bacteria, and all of them contained low concentration of malate, ranging from 0.4 to 1.2 mmol/L and relatively high concentration of lactate (range: 5.6–12.8 mmol/L) meaning that malolactic fermentation was completed in the wines.

In addition, succinic acid, which is a by-product of yeast metabolism during fermentation, with a bitter-salty flavor, was found in low concentrations in wines (range: 4.3 to 7.6 mmol/L). Inorganic acids salts, sulfate and phosphate, were determined for the first time in Macedonian wines. The content of both salts, sulfate and phosphate, ranged from 1.1 to 4.2 and 2.1 to 7.2 mmol/L, respectively.

In general, the analyzed wines contained organic acids in amounts that are mostly related not only to the varieties but also to some extent to the applied vinification procedures. The obtained results were in accordance to previously published results for organic acids in Macedonian wines (Tašev et al. 2016) as well as similar to those of previous studies published for Slovenian and Greek white and red wines (Falque-Lopez and Fernández-Gómez 1996; Zotou et al. 2004), as well as for Port wines (Esteves et al. 2004) and Brazilian wines (Peres et al. 2009).

Principal Component Analysis

PCA was applied using the dataset of individual organic and inorganic acids obtained from the CE-CD analysis (excluding the citrate which was detected in only two wines). PCA was used to explore the effect of grape variable vs. geographic wine area based on the acids profile of the analyzed wines. The first two principal components, PC1 and PC2, accounted for 66.17% of the total variance (25.72% for PC1 and 40.46% for PC2), thus explaining a significant information in the dataset. The projection of the wine samples on the first two principal components showed separation mainly into two groups, according to the variety (Fig. 2a): Vranec wines were separated from the Merlot and Cabernet Sauvignon wines, which formed the second group. Vranec wines were mainly

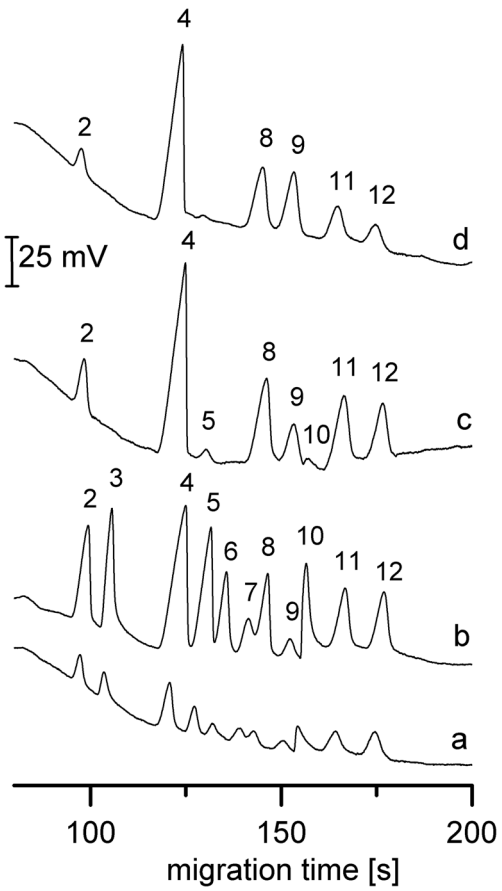

Fig. 2 CE separations of organic and inorganic acids under different separating conditions (**a**, **b**) and 100 times diluted wine samples (**c**, **d**). The separations were carried out in background electrolytes consisting of 35 mmol/L MES, 6 mmol/L Bis-Tris propane, 3.4 mmol/L Bis-Tris, 0.1% (*w*/*V*) MHEC, 20 mmol/L α-cyclodextrin and 10 mmol/L β-cyclodextrin. pH=6.0. The driving current was stabilized at 120 μA. Peak assignments and concentration of the constituents in the injected model samples (μmol/L) 2-sulfate (a-10, b-50) 3-oxalate (a-10, b-50), 4-tartrate (a-20, b-100), 5-malate (a-20, b-100), 6-malonate (a-10, b-50), 7-pyruvate (a-30, b-70), 8-succinate (a-10, b-50), 9-acetate (a-20, b-40), 10-citrate (a-20, b-60), 11-lactate (a-30, b-75), 12-phosphate (a-30, b-70). Wine samples - Cab. Sauvig-DK-1 (**c**), Vranec-N-1 (**d**)

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located in the negative part of PC1 (only three samples, Vranec-DK-1, Vranec-K-3, and Vranec-K4, were located near zero), while Merlot wines and two Cabernet Sauvignon wines were located in the positive part of PC1 (exception were Merlot-N-2 and Cabernet Sauvignon-N-2). Within the group of Vranec wines, clear separation of the wines according to the

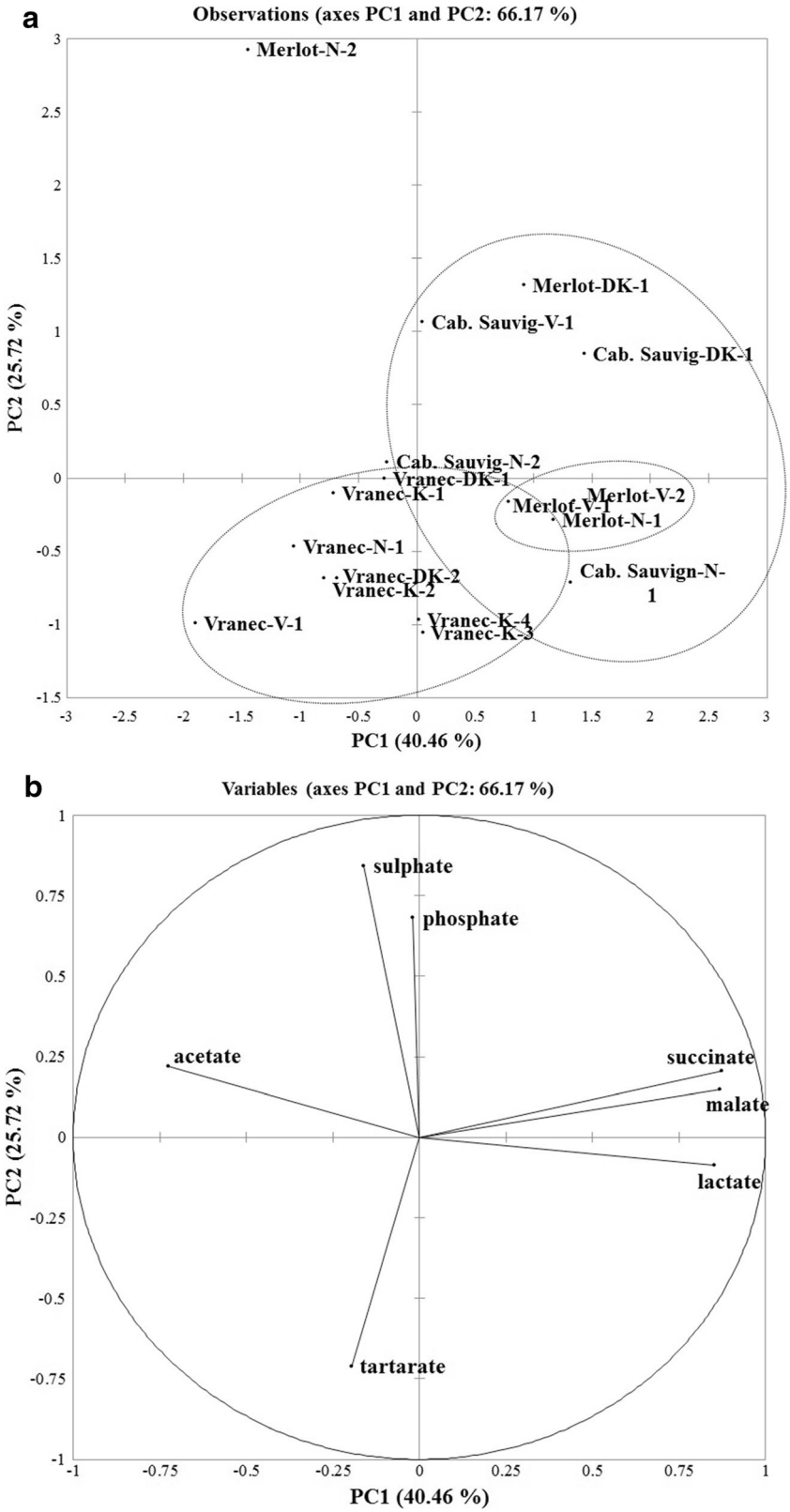

Fig. 3 **a** Eigenvector projection of red wine samples in the space defined for the two first principal components. **b** PCA loadings of organic and inorganic acids in red wine samples

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geographical origin was not observed. Similarly, within the Merlot and Cabernet Sauvignon wines, separation according to the geographical area was not achieved.

The principal components responsible for the differences in the acids composition of the wines produced were determined and presented in the scatter plot in Fig. 2b. The responsible component for the separation of Vranec wines was tartrate salt which prevailed in the negative part of the first principal component, while malate and lactate salts, as well as inorganic salts, sulfate and phosphate, were characteristic for the Cabernet Sauvignon wines. In general, separation of the wines was performed according to the varietal characteristics (Fig. 3).

Conclusion

The proposed CE-CD method is suitable for fast, accurate, and simultaneous determination of the organic acids: acids (oxalate, tartrate, malate, malonate, pyruvate, succinate, acetate, citrate, and lactate) and inorganic anions (sulfate and phosphate) in red wines. The developed method was validated showing satisfactory analytical performance without significant effect of the wine matrix on ionization efficiency. The quality parameters of method, such as LOD, LOQs, linearity, recovery, repeatability, and reproducibility, were determined which confirmed that the method is appropriate for analysis of organic acids in wine. The method was then applied for analysis of real samples, Macedonian red wines from three varieties: Vranec, Merlot, and Cabernet Sauvignon, from various wine regions. All wines contained organic acids in appropriate and recommended concentration levels, protecting the wines from microbiological and chemical oxidation. Vranec wines contained highest concentration of tartaric acid which is the parameter that separates this variety from the other studied. For the first time, inorganic anions, such as sulfate and phosphate, were determined in the local Macedonian varieties.

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Compliance with Ethical Standards

Conflict of Interest Zorica Lelova declares that she has no conflict of interest. Violeta Ivanova-Petropulos declares that she has no conflict of interest. Marián Masár declares that he has no conflict of interest. Klemen Lisjak declares that he has no conflict of interest. Róbert Bodor declares that he has no conflict of interest.

Ethical Approval This article does not contain any studies with animals.

Informed Consent It was obtained from all individual participants included in the study.

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