Faculty of Agricultural and Food Sciences
University of Sarajevo
Bosnia and Herzegovina

Faculty of Agriculture
Ege University, Izmir
Turkey

24th International Scientific-Expert Conference of Agriculture and Food Industry – Sarajevo 2013

Sarajevo

September 25 – 28, 2013

Proceedings

Sarajevo, Izmir, 2014

22nd International Scientific-Expert Conference of Agriculture and Food Industry – Sarajevo 2011

organized by

Faculty of Agricultural and Food Sciences, University of Sarajevo, Bosnia and Herzegovina
and
Faculty of Agriculture, Ege University Izmir, Turkey

in cooperation with

Uludağ University, Bursa, Republic of Turkey
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Communications presented at the Conference and positively reviewed are published in the Proceedings. Authors are fully responsible for contents, contact information and correctnes of English.
The Proceedigs has been indexed with CAB Publishing – UK (CAB Abstracts and Nutrition and Food Sciences Database) since 2010.

Publisher:
Faculty of Agriculture and Food Sciences, University of Sarajevo – Poljoprivredno-prehrambeni fakultet Univerziteta u Sarajevu
Editor: Prof. dr Milenko Blesić
Technical design: Prof. dr Milenko Blesić, Belma Dučić
Year of publishing: 2013; Year of printing: 2014
Printed by: Print Delivery and Service d.o.o., Ilidža – Sarajevo

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CIP - Katalogizacija u publikaciji
Nacionalna i univerzitetska biblioteka
Bosne i Hercegovine, Sarajevo

631/635:664(082)

INTERNATIONAL Scientific-Expert Conference of Agriculture and Food Industry (22nd ; 2011 ; Sarajevo)
Proceedings / 22nd International Scientific-Expert Conference of Agriculture and Food Industry - Sarajevo 2011, Sarajevo, September 28-October 1, 2011. - Sarajevo : Faculty of Agriculture and Food Sciences = Poljoprivredno-prehrambeni fakultet ; Izmir : Ege University, Faculty of Agriculture, 2012. - 381 str. : graf. prikazi ; 30 cm

Bibliografija uz svaki rad.

ISBN 978-9958-597-27-5

COBISS.BH-ID 19395590

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DETERMINATION OF MAIN ORGANIC ACIDS IN MACEDONIAN WINES BY RP HPLC

K. Tasev [1,3], V. I. Petropulos [2], M. Stefova [3]

Scientific paper

Summary

A reverse phase high performance liquid chromatography (HPLC) method for quantitative determination of tartaric, malic, shikimic, lactic, citric and succinic acids in wines has been validated after the sample clean up. The method was optimized using Supelco LiChrosorb RP-18 (250x4.6mm,5µm) column showing best performance on selectivity between the malic and shikimic acids. Validation parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), precision and recovery of the method, after clean up of the wine samples through C18 solid phase extraction (SPE) columns, were determined. The validated method was applied for analysis of several white and red wines from Macedonian origin.

Key words: *wine, organic acids, separation, HPLC method, validation*

INTRODUCTION

Monitoring of organic acids in wine is very important for the processes control during alcoholic and malolactic fermentation and for the wine quality. Organic acids have influence on the organoleptic properties and stability of wine. Some of the organic acids originate from the grapes (tartaric, malic, citric and shikimic) and some of them as lactic and succinic acids are products of alcoholic and malolactic fermentation (Ribrereau-Gayon & Glories, 2006). Tartaric acid is most abundant acid in wine, and important parameter for stabilization control of wine, which in high concentration gives unpleasant taste. The present of the malic acid in wine results with "harsh" taste in wine. During malolactic fermentation (MLF), malic acid converts to the lactic acid which results with "softens" of the wine and releases fruity flavor in wine. Shikimic acid is important for origin classification of wine. As an acidifying agent, citric acid can be added to the wine, but according the regulation, the level in wine must be below 1 g/L, since larger quantities of citric acid can result with citrus flavor in wine. Succinic acid is a product of alcoholic fermentation and gives a "bitter" note to the wine causing salivation and accentuating (emphases, promote) the overall flavor of the wine. These compounds also indicate alternation or illness of wine (Mato et al., 2005; Valentao et al., 2007; Niculaua et al., 2009 ; Hakan Aktas et al., 2005; Kordiš-Krpež et al., 2001; Zotu et al., 2004).

Several analytical techniques are developed for determination of organic acids in wine. Spectrophotometric methods are very tedious and time consuming, based on reaction of organic acids with specific substances resulting with mixed compounds measured on certain wavelength. There are several publications of enzymatic methods for determination of organic acids. The main advantage of these methods is the high specificity because the determination of L- and D-isomers of some acids is possible and a main disadvantage is time consuming. This method can be automated using flow injection analysis (FIA). The methods using capillary electrophoresis (CE) and capillary zone electrophoresis (CZE) are very widely used in the last years due to the simplicity of sample treatment and cost of the analysis. Gas chromatographic methods are not suitable for determination of organic acid, due to not volatile characteristic of the same. Derivatization reagents such as trimethylsilyl derivates (TMS) are used for derivatization of the organic acid (Mato et al., 2005).

HPLC is one of the most popular techniques for determination of organic acid in wine, applying C18 columns and isocratic elution for separation for relatively short analysis time. (Mato et al., 2005, de Villies et al., 2003, Valentao et al., 2007, Niculaua et al., 2009, Hakan Aktas et al., 2005, Kordis-Krpez et al., 2001, Zotu et al., 2004).

The aim of this work is to optimize and validate an HPLC method for determination of the main organic acids, including tartaric, malic, shikimic, lactic, citric and succinic acids in Macedonian red and white wines, after performing sample clean-up.

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MATERIALS AND METHODS

Chemicals and samples

Organic acid standards have been purchased from Sigma: L(-)malic acid, citric acid, succinic acid and from Fluka (Buchs, Switzerland). L(+)tartaric acid, L(+)lactic acid and shikimic acid. H_3PO_4 (85 %) has been provided by the Carlo Erba (Milano, Italy). All standards were analytical grade purity. Water and acetonitrile with HPLC grade were from Sigma (St. Louise, MO, USA).

The wine samples were provided by the local wineries and also from the supermarkets. Total 8 samples, 4 red and 4 white wines have been analyzed.

Before injection into the HPLC system, all standards and samples were filtered through 25mm x 0.45um PTFE filters provided by Supelco (St. Louise, MO, USA).

Instrumentation

Varian Pro Star HPLC system (pump model 230, autosampler model 410, PDA detector model 330 and Column valve module with thermostat model 500) (Palo Alto, CA, USA) was used. Different types of C18 columns were used: Supelco LiChrosorb RP-18 250x4.6, 5um; Varian C18 150x4.6, 5um; Agilent Zorbax C18 SB 150x4.6, 5um, Perkin Elmer C18 150x4.6, 5um. For the sample clean up a Supelco SPE Manifold and Supelclean LC-18 SPE 500 mg columns were used. Integration of the chromatograms was made by the Varian Star Chromatography Workstation. pH meter Hanna 301 has been used for preparing of the accurate pH of the mobile phase.

Preparing of standards

Standard solutions of organic acids were prepared in water, in the following concentration range: tartaric acid, 1500 - 5500 mg/L; malic acid 60.0 - 1200 mg/L; shikimic acid 5 - 40 mg/L; lactic acid 150 - 1500 mg/L; citric acid 90 - 600 mg/L and succinic acid 200 - 1200 mg/L.

Wine samples preparation

Appropriate amount of wine sample (about 4 ml) was filtered through 25mm x 0.45um PTFE filters. The C18 SPE 500 mg column was conditioned with two C18 SPE volumes of methanol (2x3 mL), followed with 2x3 mL water, HPLC grade. After that, 500 μL of the filtered wine sample was loaded on the C18 SPE column and directly collected in the HPLC vial. The elution was performed with 2x500 μL of buffered water on pH=2.1, with H_3PO_4 ($5x10^3$mol/L). A volume of 10 μL extract was injected into the HPLC system.

Chromatographic condition and validation of method

The isocratic elution was carried out for separation of the organic acids with flow rate of 1 mL/min. Detection of compounds was performed on 210 nm. Injection volume was 10 μL. The mobile phase was prepared using HPLC grade water with concentration of the H_3PO_4 of $5x10^3$mol/L and very carefully set up to pH 2.1 with 85% of H_3PO_4. 1 % of acetonitrile, as an organic modifier, was added in the mobile phase. Total run time was 20 min.

Linearity, precision, accuracy, limit of detection (LOD) and limit of quantification (LOQ) were investigated as method validation parameters. The compound peaks were identified by their retention times, compared with the standards and the UV-Vis spectra. Quantification was performed with 5 points external calibration curves. Precision was determined as repeatability (6 successive injections) and intermediate precision (three injections on three different days in the same week). Accuracy was determined as a recovery for two different concentration levels. The limit of detection was determined as a LOD=3 x SD/slope and limit of quantification as a LOQ=10 x SD/slope in low concentration calibration level.

RESULTS AND DISCUSSION

Influence of the pH on the mobile phase and clean up

The pH value of the mobile phase is the most critical parameter to be control for determination of organic acids in wine. This is due to the low pKa of the organic acids: tartaric (pKa_1=3.03 pKa_2=4.45), malic (pKa_1=3.40, pKa_2=5.20), shikimic, lactic (pKa_1=3.86), citric (pKa_1= 3.09, pKa_2=4.75, pKa3=5.41 pKa_3=6.39, 6.40) and succinic acid (pKa_1=4.2 pKa_2=5.6) (Hakan Aktas et al., 2005; Ribrereau-Gayon, & Glories, 2006). In fact, low pKa value requests low pH in order to keep the organic acids in their un-ionized form. Low pH value was also necessary in the clean up procedure for elution of the organic acids from the C18 SPE 500 mg column.

HPLC columns for separation

Testing the different HPLC columns for organic acids separation, it was shown that Supelco LiChrosorb RP-18 resulted with best performance for their separation, especially between the malic and shikimic acids (please see the chromatograms in Fig. 1 (a) and (b)).

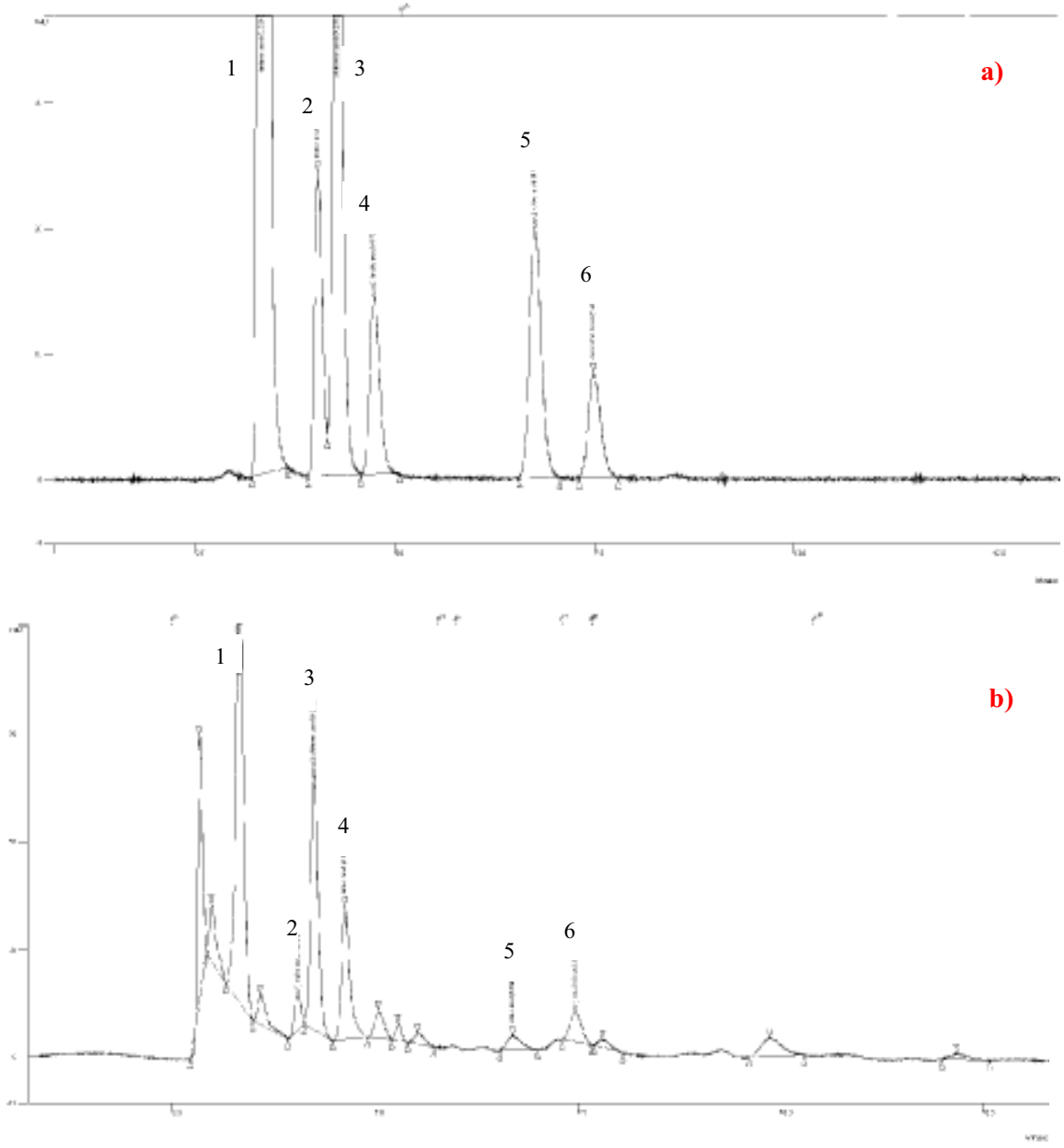

Fig. 1. Chromatograms of standards (a) and wine sample (Vranec) (b)
Elution of the acids was performed on Supelco LiChrosorb RP-18 HPLC column
Labels: 1-Tartaric acid, 2-Malic acid, 3-Shikimic acid, 4-Lactic acid, 5-Citric acid, 6-Succinic acid

Validation parameters

Linearity of the method was tested at five concentration calibration levels for each organic acid. Table 1 contains the range of determination, coefficients of the regression, slope and intercept, and correlation coefficient. As can be seen from Table 1, linearity is satisfactory for all analytes, with correlation coefficient of 0.999.

Tab. 1. Range of determination, coefficients of the regression curves (slope and intercept), correlation coefficient R^2

Organic acids	Range (mg/L)	Slope	Intercept	R^2
Tartaric acid	1500 – 5500	1774.5	-121850	0.9998
Malic acid	60 - 1200	919.56	-22492	0.9994
Shikimic acid	5 - 40	62233	-174620	0.9993
Lactic acid	150 - 1500	740.71	-21249	0.9993
Citric acid	90 – 600	1170.9	-4099.4	0.9995
Succiimc acid	200 - 1200	726.6	-84052	0.9988

Table 2 presents the calculation of the validation characteristics of the method. Relative standard deviation of three replicates within a day (WD) ranged from 1.03 to 2.89 % and between days it was calculated from 2.89 to 3.98 %, showing satisfactory results. The accuracy of the method was checked using standard addition method. One wine sample was spiked with two different concentration levels (50 % and 75 % from the calculated mean value) of standard solutions of tartaric, malic, shikimic, lactic, citric and succinic acids. Calculated recoveries (L1 and L2) ranged between 95 to 105 %, confirming the accuracy of the method.

Limit of detection (LOD) and limit of quantification (LOQ) have been determined in a low concentration calibration level, using the following equations: LOD=3 x SD/slope and LOQ=10 x SD/slope. Results for LOD and LOQ are presented in Table 2.

Tab. 2. Validation parameters

Organic acid	RSD (%)		Recovery (%)		LOD & LOQ	
	WD	BD	L1	L2	LOD (mg/L)	LOQ (mg/L)
Tartaric acid	1.62	3.98	97.6	96.3	2.82	9.39
Malic acid	1.03	3.90	98.7	105	31.8	106
Shikimic acid	1.69	3.71	101	95.0	0.78	2.61
Lactic acid	3.45	4.44	95.9	97.1	45.6	152
Citric acid	2.10	2.89	105	95.5	39.9	133
Succimc acid	2.89	3.28	104	95.9	62.4	208

RSD: relative standard deviation: WD: within a day; BD: between days; L1: concentration level of 50 % of calculate mean value; L2 concentration level of 75 % of calculate mean value; LOD: limit of detection; LOQ: limit of quantification.

Wine analysis

After optimization and validation of the method, several white and red wines from Macedonian origin were analyzed applying the established procedure. The content of the main organic acid in the Macedonian wines are presented in Table 3.

In general, the content of tartaric acid ranged from 2174 to 3388 mg/L in the wines, observing highest amount in Cabernet Sauvignon and Vranec red wines, which is typical for these varieties. Since malic acid is transformed into lactic acid during the malolactic fermentation, white wines contained higher amount of malic acid, compared to red wines, where malolactic fermentation was finished, or almost whole malic acid was transformed into lactic acid. Red wines contained higher amount of shikimc and succininc acids then the white wines and the content of citric acid was similar in both, red and white wines. The obtained results are similar with results from previous authors (Kordiš-Krpež et al., 2001), whereas they have investigated the organic acids in Slovenian wines from different regions. Also Zotu et. al. (2004) found similar content of organic acids in Greek white and red wines.

Tab. 3. Content of organic acids in wines

	Organic acid in white and red wines (mg/L)					
White wines	Tartaric	Malic	Shikimic	Lactic	Citric	Succinic
1 Riesling	2905	1021	11.9	452.9	477.3	459.3
2 Chardonnay	2664	1327	19.8	198.0	373.4	468.5
3 Temjanika	3323	1075	7.3	295.4	501.8	384.2
4 Smederevka	1556	501.8	16.35	911.4	285.3	917.3
Red Wines						
1 Cabernet Sauvignon	3388	181.1	35.4	1461	380.6	1018
2 Vranec	3078	169.4	36.2	1438	370.7	979.5
3 Merlot	2931	168.9	34.8	1364	305.1	1074
4 Cuve	2174	170.4	32.7	1047	298.5	1002

CONCLUSIONS

The established HPLC method presented good separation and appropriate determination of the main organic acids in wine. Simple SPE C18 clean up pretreatment resulted with good recoveries for the sample and prolonged used of the HPLC Supelco LiChrosorb RP-18 column that presented best separation between malic and shikimic acid as can be seen from the chromatograms. The good linearity, sensitivity, precision and accuracy of the method have been obtained confirming the suitability of the method for analysis of organic acids in red and white wines. The optimized and validated method was used for determination of the organic acids in Macedonian wines.

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