HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SELECTED PHENOLIC ACIDS IN WINE

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Abstract: The aim of this study was to develop a method for identification and quantification of phenolic acids in different wine samples. The simple reversed-phase HPLC-UV method for simultaneous determination of *p*-coumaric and ferulic acid was developed. The method was validated and working range, linearity, repeatability, accuracy, limit of quantitation *LOQ* and limit of detection *LOD* were determined. The linearity of the method was tested in concentration ranges 0.1-1 mg L^{-1} and 1-10 mg L^{-1}. The correlation coefficients (r^2) were greater than 0.996 and quality coefficients (QC) ≤ 5%. Detection limit for both compounds was 0.01 mg L^{-1}. The method is precise (RSD<10%) and accurate. Two different wine sorts were used for analysis, Slovene red wine Refošk and Slovene white wine Laški Rizling. The wine samples were ultrasonically extracted with ethyl acetate, centrifuged, evaporated, re-dissolved in mobile phase and filtered. The solution was analyzed directly by HPLC-UV. Phenolic compounds were separated with a C18 reversed-phase column by isocratic elution using 2 % acetic acid in water-methanol (85:12, v/v) as the mobile phase at a flow rate of 0.7 mL min^{-1}. Acids were detected and quantitation was performed at wavelength of 320 nm. Extraction procedure was optimized and yields were calculated from internal standard recovery. *O*-coumaric acid was used as internal standard. From the results it is evident that the red wine Refošk contains higher concentrations of investigated compounds then the white wine Laški Rizling. Stability tests were performed with standard compounds of *p*-coumaric and ferulic acid. It was established that both standards occur as *trans*-isomers, but when they were dissolved in methanol and stored exposed to daylight and room-temperature they were gradually transformed to *cis*-isomers. All closely related geometrical *cis*- and *trans*-isomers were successfully separated under the same conditions as described before. The described method is simple and rapid as it involves liquid extraction with no other pre-treatments of samples. It is suitable for simultaneous determination of *cis* and *trans* isomers of *p*-coumaric and ferulic acid in different liquid samples.

Key words: *p*-coumaric acid, ferulic acid, *o*-coumaric acid, HPLC, wine.

1. Introduction

In recent years lots of studies were focused on phenolic acids due to their antioxidant antiviral, antibacterial and anti-inflammatory properties (ZGÓRKA and KAWKA, 2001). A variety of phenolic acids were separated using high-performance liquid chromatography (HPLC) as reported in literature (KALLITHRAKA *et al.*, 2006; ROBBINS and BEAN, 2004; TARNAWSKI *et al.*, 2006; URAKOVA *et al.*, 2008; ZGÓRKA and KAWKA, 2001). In most studies reversed-phase HPLC method was applied using non-polar C18 column. Isocratic elution was applied (TARNAWSKI *et al.*, 2006; URAKOVA *et al.*, 2008; ZGÓRKA and KAWKA, 2001; YI *et al.*, 2009) as well as a gradient method (KALLITHRAKA *et al.*, 2006; ROBBINS and BEAN, 2004; SLADKOVSKÝ *et al.*, 2004). Different mobile phases were prepared, using methanol–aqueous or acetonitrile–aqueous acidified mixtures. It

was determined that pH value is very important due to protonation of the acids. Therefore the best pH was determined to be from 1-3.

GARCIÁ *et al.* (2007) paid a lot of attention to sample pre-treatment. They used liquid-liquid separation with diethyl ether, followed by centrifugation, and vacuum evaporation. A variety of different wines was analysed by KALLITHRAKA *et al.* (2006). Samples were only filtered and no other pre-treatment methods were used. HPLC-DAD method was applied for determination of phenolic acids at different wavelengths (265, 280, 320, and 365 nm, respectively, at 1 mL min^{-1} flow). The concentration of phenolic acids varied a lot depending on weather conditions, geographic sites, the way of wine storage and aging. These discoveries agree also with other studies (ÖZKAN *et al.*, 2006).

The aim of our work was to develop the analytical method to separate and determine two target components in wine samples, namely *p*-coumaric and ferulic acid. Attention was paid to the most appropriate sample pre-treatment technique.

2. Materials and methods

All reagents and solvents used were of analytical grade. Milli-Q water was used. Methanol (MeOH) was purchased from Sigma-Aldrich (Germany), acetic acid (CH_3COOH), ethyl-acetate ($C_4H_8O_2$), 98% *trans* ferulic acid (FA), 98 % *trans p*-coumaric acid (*p*-CA) and 97% *o*- coumaric acid (*o*-CA) were supplied from Merck (Germany).

2.1 Preparation of Calibration Solutions and Calibration Curves

Standard stock solution of *trans* FA, *trans p*-CA and *o*-CA was prepared by dissolving each of them with MeOH in 10 mL volumetric glass flasks to obtain concentration of 1 g L^{-1}. *o*-CA was used as an internal standard (ISTD). This solution was degassed for 20 minutes by sonication. Five working calibration solutions were prepared from standard stock solutions by further dilution with mobile phase consisting of 2% acetic acid in water-methanol mixture (82:18, v/v). Concentration ranges of the final standard solutions used for calibration curves were 0.1-1 mg L^{-1} and 1-10 mg L^{-1}. Calibration solutions were injected into the HPLC-UV system in five replicates. In addition, three replicate analyses of the calibration solutions were performed. Curves were constructed by linear regression of the peak-area ratio (y) of individual acids to the ISTD, versus the concentration (x). All standard solutions were kept at -18 ° C for a maximum one week. Prior to injection the solutions were filtered through 0.45 μm filter.

2.2 Sample preparation

Wine samples were Refošk (red wine) and Laški Risling (white wine) from Slovenia. Wine samples undergo a liquid-liquid extraction prior to their analysis by HPLC. 5 mL of wine sample was transferred into a 50-mL round-bottomed glass-stoppered centrifuge tube, spiked with 50 μL of ISTD (0.5 mg L^{-1}) and extracted with 5 mL of ethyl acetate by sonication (ultrasonic bath model Sonis 4, Iskra, Slovenia) for

20 minutes. The extract was centrifuged (centrifuge model LC 321, Tehnica Železniki, Slovenia) at 1000 rpm for 10 min and the supernatant was accurately transferred into 200-mL conical glass flask using a glass Pasteur pipette. This extraction procedure was repeated three times. Supernatants were combined and concentrated to dryness by rotary evaporation (Büchi Rotavapor R-205, Switzerland). The residue was re-dissolved in 5 ml of mobile phase consisting of 2% acetic acid in water-methanol mixture (82:18, v/v). Finally, the pre-treated sample was filtered through 0.45 μm filter and analysed. The compounds in wine extracts were quantified from the corresponding calibration curves.

2.3 Stability tests

Trans p-CA and *trans* FA standard compounds were dissolved in MeOH separately, to obtain concentrations of 1 mg L^{-1}. The contents of *trans p*-CA and *trans* FA were determined from the corresponding calibration curves and they were used for the control. Then each solution was divided into more equal parts and transferred into graduated glass-stoppered test tubes. Four parts were placed under the daylight and room temperature for varied lengths of time (5 h, 24 h, 48 h and 1 week); another four parts of each solution were stored in the refrigerator in the darkness at -18 °C for varied lengths of time (5 h, 24 h, 48 h and 1 week). After exposure to the mentioned conditions, the content of *trans p*-CA and *trans* FA were determined from the corresponding calibration curves and compared to the control.

Equipment and Chromatographic analyses

A simple and rapid chromatographic separation was carried out using reverse-phase HPLC. Chromatographic analyses were performed on Varian, Prostar (USA) HPLC system, which consisted of Varian Prostar 210 HPLC pump, Varian Prostar injection valve, and UV-VIS detector. Chromatographic system was connected to the PC. Separation was carried out on 250 mm x 3mm i.d., 5 μm non-polar CHROMSPHER 5, C18 column. Elution was performed isocratically with the mobile phase consisting of 2% acetic acid in water-methanol mixture (82:18, v/v). The flow rate was 0.7 mL min^{-1}, the pressure 180×10^5 Pa and the injection volume was 100 μL. Peaks were detected at 320 nm.

3. Results and discussion

Based on linear regression analysis, the responses for investigated compounds were linear over concentration ranges tested. The equations of calibration curves and their correlation coefficients are presented in Table 1. The reproducibility of chromatographic analyses was evaluated by the relative standard deviation (RSD) of five replicate analyses of five calibration solutions. RSD was between 2.91 and 3.81 %.

The extraction procedure was optimized regarding extraction solvent and recovery. Ethyl-acetate was used for extraction of the compounds from wine. The best recoveries (over 89%) were obtained by using three equal volumes of mentioned solvent, while using only two equal volumes gave lower recoveries. The recovery was evaluated using *o*–CA which was added into the wine sample before the extraction.

This compound is structurally related to the investigated compounds and behaves similar on the column as analytes. It does not occur in wine. Therefore it is suitable for quantitative analysis of investigated compounds.

Table 1. Regression equations and correlation coefficients for investigated compounds

Investigated compound	Correlation coefficient (r^2)	Regression equation
trans p–CA	0.9969	y = 0.4088 x + 0.1021
trans FA	0.9968	y = 0.3403 x + 0.0705

Chromatographic separation was optimized with respect to the mobile phase, eluent composition, and flow rate. Several experiments were carried out to resolve the acid mixture using a different mobile phases. To increase the retention behaviour acetic acid was used. Optimal peak resolution was achieved using 2% acetic acid in water-methanol mixture (82:18, v/v). In Fig. 1 chromatograms of investigated compounds, present red wine extract is presented.

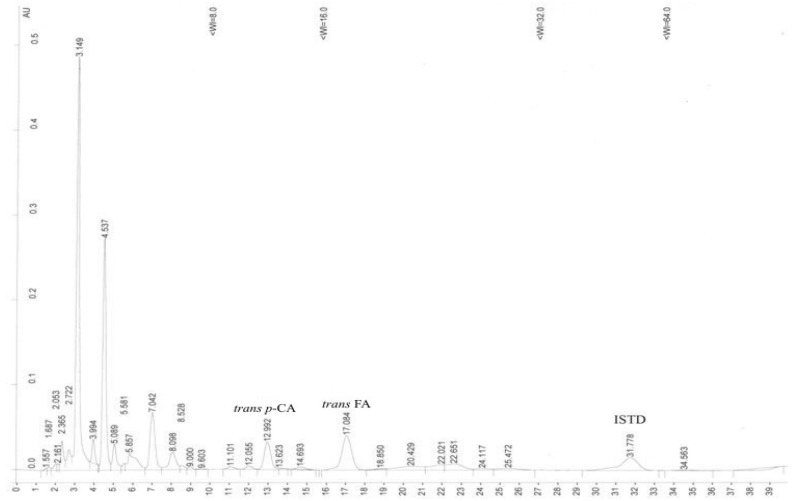

Fig. 1. Chromatogram of investigated compounds present in extract of red wine-*Refošk.*

The separation with good resolution has been achieved for phenolic acids within 32 min. Their average contents in different wine extracts (mg L^{-1}) are presented in Table 2. Concentrations of *trans p*-CA and *trans* FA are higher in red wine. Very high concentration of *trans* FA was determined in Refošk, which was even 100-times higher compared with white wine. Contents of phenolic acids are comparable to those reported in literature (ROBBINS and BEAN, 2004; KALLITHRAKA *et al.*, 2006; GARCIÁ *et al.*, 2007; SLADKOVSKÝ *et al.*, 2004; OZKAN *et al.*, 2006; CASTELLARI *et al.*, 2002). *Trans p*-CA in different brands of red wine were reported in concentrations between 0.9 to 7 mg L^{-1} and *trans* FA from 0.14 to 6 mg L^{-1}L, respectively. Concerning white wine brands lower concentrations of both acids were

found in literature: *trans p*-CA varied from 0.2 to 1.4 mg $L^{-1}$and *trans* FA from 0.1 to 0.7 mg L^{-1}, respectively.

Table 2. Contents of *trans p*–CA and *trans* FA in extracts of red wine-*Refošk* and white wine-*Laški Rizling* (mg L^{-1}). Each content value is the mean of five replications.

Compound	***red wine Refošk*** (n=5)	***white wine Laški Rizling*** (n=5)
trans p–CA	2.95	1.82
trans FA	7.75	0.07

The results of stability tests showed that *trans p*–CA and *trans* FA gradually converted to their *cis* forms when they were dissolved in MeOH and stored in daylight at room temperature. The *trans-cis* isomerization started already after a few hours. The content of *trans p*–CA and *trans* FA has lowered during the time of storing, while the content of *cis p*–CA and *cis* FA has increased. After being stored for 1 week in the refrigerator in darkness at -18 °C, *trans p*–CA and *trans* FA dissolved in MeOH were found to be stable, and no isomer conversion occurred. Although *cis* and *trans* forms of phenolic acids are geometric isomers with very similar structures, they can be well distinguished by order of elution during HPLC (*cis* isomers are retained longer than *trans* isomers) (Fig. 2)

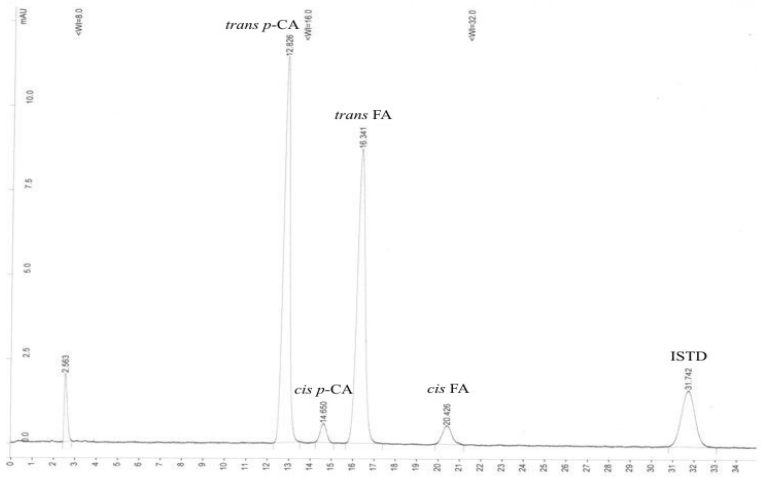

Fig. 2. Chromatogram of compounds present in mixture of *trans p*–CA and *trans* FA in MeOH after being stored in daylight and room temperature for 24 hours.

4. Conclusion

The developed isocratic HPLC method is a rapid and simple one compared with other HPLC methods. The selection of proper mobile phase is crucial for good separation of phenolic compounds. Proper storing conditions are important to ensure

stability of the compounds. The described method can be routinely used for separation and determination of closely related *cis*- and *trans*-isomers of phenolic acids in different wine extracts and other liquid samples with complex natural matrices, even if they are present in the minor contents.

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