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HPLC DETERMINATION OF TWELVE POLYPHENOLS: APPLICATION IN WINE ANALYSIS

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Abstract

Plant polyphenols have longtime attracted scientific attention thanks to their biological activities. Therefore, polyphenols identification is important part of their analysis. In this regard, an HPLC method for simultaneous identification of 12 polyphenols in wine was modified and validated.

The following validation parameters were evaluated: linearity, precision (intraday and interday repeatability), limit of detection (LoD), and limit of quantification (LoQ), recovery. The optimized high performance liquid chromatography (HPLC) method has been applied in the analyses of 12 phenolic compounds in 4 commercials and 2 homemade red wines. Solid state microextraction of wine phenolic compounds was initially carried out in order to partially purify and concentrate the target compounds.

Good linearity for all compounds within the study range was obtained (R^2 greater than 0.9990). Lowest detection and quantification limits were reported for hesperidin - 0.17 and 0.52 μg/mL resp. and highest for gallic acid (1.5 and 4.5 μg/mL) and rutin (1.49 and 4.51 μg/mL). The percentage recovery of all polyphenols ranged from 95.9 for rutin to 100.6% for resveratrol, indicating good accuracy of the method. DL- catechin, caffeic acid and p-coumaric acid predominated in all wine samples. Meanwhile, the homemade wines were relatively poorer in phenolic compounds.

Based on the results, the validated method could be recognized as suitable for wine analysis and recommended for polyphenols identification in plant samples.

***Key words**: HPLC, Method validation, Plant polyphenols, Wine.*

1. Introduction

As a large group of naturally occurring secondary metabolites polyphenols are largely found in plants and beverages. They comprise a wide variety of diverse structures, classified in two main classes: non-flavonoids (particularly phenolic acids, stilbenes, and lignans) and flavonoids, which are all characterized by the presence of basic C_6-C_3-C_6 skeleton. The presence of plant polyphenols is correlated with various beneficial effects for human body. Thus, the increased consumption of polyphenol-rich foods and beverages is associated with a reduction of cardiovascular diseases [1 - 3] also with: anti-obesity, anti-diabetic, anti-hypertensive, anti-hyperlipidemic and anti-inflammatory effects [4]. The risk of some chronic diseases may be lowered at higher dietary flavonoid intake [5]. In addition to their antioxidant activity flavonoids have been reported to provide anti-thrombotic properties [6, 7]. One the most famous polyphenol nowadays is resveratrol, which is a naturally occurring phytoalexin (3, 4′, 5 trihydroxystilbene), commonly found in grapes, berries, and some nuts. It has gained popularity thanks to its powerful antioxidant activity. Many studies have reported the cardioprotective effects of resveratrol found in wine [8], as well as health benefits in prevention of diseases such as cancer, type 2 diabetes, and neurological conditions [9]. However, long-term toxicity data for resveratrol are controversial [10, 11] although clinical trials have shown good tolerability and a pharmacologically safe dose of up to 5 g/day [12].

Wine is a complex matrix. A wide structural variety of molecules in wine are present (e.g.: proteins, amino acids, carbohydrates, phenolic compounds, volatile components, and inorganic compounds), in a wide range of concentrations [13]. Also, during vinification process only limited amount of grape phenolics are extracted

from wine, and it was found that the extraction technique could alter their concentration in limited range [14]. In order to analyze specific polyphenols often an extraction step is needed. Because of the complexity of the matrix multistep extraction process is needed. Solid phase extraction (SPE) is an effective method for the removal of interfering substances and for the enrichment of analytes, since a variety of different extraction sorbents are available [15]. Nonetheless, the use of C18 solid phase extraction (SPE) with red wine has proved effective, but the technique requires alcohol removal and also pH adjustment of the wine prior to loading onto the cartridge [15].

So far, many analytical methods are reported for determination of plant polyphenols. Among them, high performance liquid chromatography (HPLC) techniques are widely used for both separation and quantification of phenolics. Phenolic substances contain a hydroxylated aromatic ring and most of them are water soluble, therefore reversed-phase HPLC with ultraviolet-visible spectroscopy (UV-Vis) detection is the most commonly used technique [16]. However, since polyphenols are structurally similar, their analysis requires high chromatographic selectivity and resolution. Some wine phenolic compounds for example show characteristic absorbance in the UV-Vis region and can therefore be easily detected in HPLC by a photodiode array detector [17].

In this regard, the aim of this study was to modify and validate a method for plant polyphenols analysis by HPLC with a diode-array detector (DAD). The optimized HPLC method has been applied in the analyses of phenolic compounds in red wines. Solid state micro extraction of wine phenolic compounds was carried out in order to partially purify and concentrate the target compounds.

2. Materials and Methods

2.1 Reagents and samples

Six wine samples were used: 4 commercials (Cabernet Sauvignon - CS; Tempranillo - T; Merlot - M; Syrah - S; and 2 homemade wines (HM1; HM2). The following analytical standards were purchased from Sigma-Aldrich (Steinheim, Germany): gallic acid, DL-catechin, syringic acid, cinnamic acid, hesperidin, chlorogenic acid, caffeic acid, ferulic acid, resveratrol, p-coumaric acid, rutin and quercetin. Stock solutions of all the standards were prepared in methanol.

All chemicals used were of analytical grade. The solvents for the mobile phase were of HPLC grade (Sigma-Aldrich, Darmstadt, Germany).

2.2 Wine samples preparation

Wine samples were first filtered through paper filter. The alcohol fraction was removed by vacuum evaporation at 50°C and the samples were subjected to solid state micro extraction (SPE) of phenolic compounds carried out as follow:

- SPE 1: The solid phase micro extraction was performed in C18 cartridge conditioned with 3 volumes of methanol, 3 volumes of ethyl acetate and 3 volumes of acidified distilled water (pH 2.0). 0.5 mL of wine sample was loaded. The cartridge was washed with 1 volume of acidified distilled water (pH 2.0) and subsequently eluted with 1 volume of ethyl acetate and 4 volumes of methanol. Samples and wash residues were collected and analyzed for total phenolic content to evaluate losses during these steps.
- SPE 2: The cartridge was conditioned with 3 volumes of methanol, and 3 volumes of acidified distilled water (pH 2.0). 0.5mL of wine sample (without pre-treatment) was loaded and then washed with 1 volume of acidified distilled water (pH 2.0) and 4 volumes of methanol.
- SPE 3: The cartridge was conditioned with 3 volumes of methanol, 3 volumes of ethyl acetate and and 3 volumes of acidified distilled water (pH 2.0). 0.5 mL of wine sample (after evaporation of the volatile phase) was loaded and then washed with 1 volume of acidified distilled water (pH 2.0), 1 volume of ethyl acetate and 4 volumes of methanol.
- SPE 4: The cartridge was conditioned with 3 volumes of methanol, and 3 volumes of acidified distilled water (pH 2.0). 0.5 mL of wine sample (after evaporation of the volatile phase) was loaded and then washed with 1 volume of acidified distilled water (pH 2.0) and 4 volumes of methanol.

2.3 Total polyphenol content (TPC) determination

The total phenolic content was determined using the Folin-Ciocalteu reagent [18]. One mL Folin-Ciocalteu reagent was mixed with 0.2 mL sample and 0.8 mL 7.5% Na_2CO_3. The mixture was vortexed and left in dark for 20 min. at room temperature. After incubation, the absorbance was measured at 765 nm against appropriate blank sample. The TPC of the extracts was expressed as milligram gallic acid equivalent (GAE) per gram dw. Gallic acid was used as reference in concentrations 0.02-0.10 mg/mL.

The yield (Y,%) of TPC is determined as follows:

$$Y, \% = \frac{TPCe}{TPCw} x100 \quad (1)$$

Where: TPC_e is the total phenolic content in extract and TPC_w is the total phenolic content in wine.

2.4 HPLC analysis

The HPLC analysis was performed on Elite la chrome (Hitachi), equipped with a gradient solvent pump,

coupled with diode array detector. The data collection and analysis were carried out using the software Elite lachrome (Hitachi). The detection of compounds was performed on Discovery® SHC18 column (25 x 4.6 mm, 5 µm, Supelco), at 278, 306 and 370 nm. The chromatographic separation was performed as described by Özkan and Göktürk, [19], with slight modifications. The temperature of the column was set at 30 ^{0}C and the flow rate was 1.0 mL/min, 20 µL of injection. The following detection wavelengths were used: at 278 nm - gallic acid, DL-catechin, syringic acid, cinnamic acid, hesperidin; at 306 nm - chlorogenic acid, caffeic acid, ferulic acid, resveratrol, p-coumaric acid; at 370 nm - rutin and quercetin. The gradient used for the separation was performed using 2% (v/v) acetic acid (A) and methanol (B) as shown on Table 1.

Table 1. HPLC gradient for phenolic compound analysis

Time, min	A, %	B, %
0	100	0
3	95	5
18	80	20
20	80	20
30	75	25
40	70	30
55	60	40
60	50	50
70	0	100
80	0	100

2.5 Validation procedures

The following performance parameters were evaluated: linearity, precision (intraday and interday repeatability), limit of detection (LoD), and limit of quantification (LoQ), recovery.

To evaluate the linearity, five standard solutions of each polyphenol compound at concentrations varying between 5 - 125 µg/mL, were injected in triplicates. After analyses, a plot was created relating peak area to concentration, and the curve equations and the coefficient of determination (R^2) were determined by linear regression analysis.

The precision of the method was evaluated from independent replicates at three concentration levels of each polyphenol. Intraday and interday precisions were calculated in terms of residual standard deviation (RSD) of the calculated yield of three replicate injections.

The limit of detection and limit of quantification were estimated from the following formulas (2) and (3):

$$LoQ = \frac{3x3xS}{a} \quad (2)$$

$$LoD = \frac{10xS}{a} \quad (3)$$

Where: S is the standard deviation at the lowest concentration level of each polyphenol, and a is the slope of the calibration curve for each polyphenol.

The recovery was determined by adding 0.05 mg/mL of each reference standard to wine sample. The percentage of recovery of each standard was calculated based on the ratio of the calculated standard concentration prior and after HPLC analysis.

2.6 Statistical analysis

Recovery, precision, determination of calibration curve were calculated using Excel 2013 software (Microsoft Corp., Redmond, United States).

3. Results and Discussion

In this study an HPLC method for simultaneous analysis of 12 phenolic compounds was validated. Each phenolic compound was analyzed in 5 concentrations at minimum of three replications. The calculations were performed for each polyphenol standard in a mixture of polyphenols. Three wavelengths of detection were set - 278, 306 and 370 nm. Linearity was evaluated in a wide range of concentrations using the selected wavelengths for each polyphenol as specified in Table 2. The method was linear at least within the range of concentrations of each compound assayed here. The correlation coefficient of the linear regression of the standard curves was greater than 0.9990 for all compounds indicating a good linearity for the study range.

In previous study Burin et al., [20], also validated an HPLC method for 5 wine polyphenols. They determined narrower linear range for p-coumaric and ferulic acids (0.3-30 µg/ mL) than the one stated in this study.

System sensitivity was assessed using LoDs and LoQs, presented on Table 2. Lowest detection and quantification limits were reported for hesperidin - 0.17 and 0.52 µg/mL resp. and highest for gallic acid (1.50 and 4.50 µg/mL) and rutin (1.49 and 4.51 µg/mL).

To assess the precision of the method, repeatability within intra- and interday runs were checked by calculating the RSD of the yield of three replicate injections (on the same day and on three different days). The results are presented in Table 3.

%RSDs for intraday ranged from 0.48 to 1.68% for syringic acid and rutin, respectively. The results for interday runs were similar ranging from 0.40% for gallic acid to 1.77% for rutin.

In order to determinate the recovery wine sample were spiked with 50 µg/mL standard solution of each polyphenol. All samples were measured in triplicate. Spiked sample solutions and unspiked sample solutions were compared for recovery evaluation. The percentage of recovery for each polyphenol was calculated and the results are summarized in Table 4.

Table 2. Retention times, regression equations, linear ranges, LoDs, and LoQs of the standard phenolic compounds

Phenolic compound	R_t, min	Regression equation, R^2	Linear range, µg/mL	LoD, µg/mL	LoQ, µg/mL
Gallic acid	6.61 ± 0.09	Y = 108167x - 38188, 0.9998	25 - 125	1.50	4.50
DL- Catechin	17.24 ± 0.12	Y = 35479x + 56446, 0.9999	5 - 100	0.31	0.94
Chlorogenic acid	19.98 ± 0.15	Y = 237889x - 1426882, 0.9998	25 - 125	0.61	1.83
Caffeic acid	22.59 ± 0.16	Y = 367177x - 393735, 0.9999	25 - 125	0.59	1.79
Syringic acid	25.39 ± 0.19	Y = 297072x + 640492, 0.9999	25 - 125	0.39	1.20
p-Coumaric acid	31.88 ± 0.27	Y = 662904x - 311561, 0.9999	25 - 125	0.75	2.27
Ferulic acid	36.30 ± 0.32	Y = 387572x - 7063, 0.9997	25 - 125	0.42	1.26
Rutin	49.15 ± 0.50	Y = 116056x - 96117, 0.9990	25 - 125	1.49	4.51
Resveratrol	49.74 ± 0.53	Y = 391820x + 506061, 0.9999	5 - 100	0.40	1.30
Hesperidin	52.81 ± 0.27	Y = 118443x - 66803, 0.9999	5 - 100	0.17	0.52
Cinnamic acid	62.96 ± 0.34	Y = 705760x + 499506, 0.9995	5 - 100	0.30	0.91
Quercetin	64.04 ± 0.42	Y = 264497x - 247924, 0.9998	10 - 100	0.38	1.14

Table 3. Intra- and interday repeatability

Phenolic compound	Intraday repeatability RSD, %	Interday repeatability RSD, %
Gallic acid	0.89	0.40
DL- Catechin	1.12	0.50
Chlorogenic acid	0.56	0.90
Caffeic acid	0.49	0.71
Syringic acid	0.48	0.43
p-Coumaric acid	0.67	0.85
Ferulic acid	0.89	0.90
Rutin	1.68	1.77
Resveratrol	1.10	0.68
Hesperidin	0.77	0.50
Cinnamic acid	1.46	1.50
Quercetin	0.74	0.84

Table 4. Recovery of spiked analytes (n = 3)

Polyphenol	Content, µg/mL	Spiked concentration, µg/mL	Observed concentration, µg/mL	RSD, %	Recovery, %
Gallic acid	21.3	50.0	70.7 ± 0.2	0.5	98.7
DL- Catechin	681.5	50.0	731.6 ± 0.5	0.9	100,2
Chlorogenic acid	0.0	50.0	50.0 ± 0.1	0.2	100.0
Caffeic acid	77.4	50.0	126.2 ± 0.2	0.3	97.7
Syringic acid	148.3	50.0	198.1 ± 0.5	1.1	99.6
p-Coumaric acid	42.4	50.0	90.9 ± 0.1	0.1	97.2
Ferulic acid	15.3	50.0	63.8 ± 0.6	1.2	97.1
Rutin	47.6	50.0	95.5 ± 0.4	0.8	95.9
Resveratrol	39.6	50.0	89.9 ± 0.6	1.1	100.6
Hesperidin	0.0	50.0	49.9 ± 0.1	0.2	99.8
Cinnamic acid	0.0	50.0	49.6 ± 0.2	0.4	99.2
Quercetin	19.2	50.0	68.2 ± 0.2	0.3	98.1

The percentage recovery of all polyphenols ranged from 95.9 for rutin to 100.6% for resveratrol, indicating good accuracy of the method.

Wine is one of the most popular beverages in the world. Part of its popularity is due to the presence of phenolic compounds that have attracted much interest due to their antioxidant properties and their potentially beneficial effects for human health [21]. Many research studies focus on the benefits of moderate wine consumption [22]. Although a principle agreement exists among the general public and the scientific community, there is no conclusive evidence on the protective role of red wine against oxidative stress associated diseases (for example - coronary heart disease

development) [23, 24]. More likely, the cardiovascular benefits of wine are due to combined effect of alcohol and other wine components (mainly resveratrol and other polyphenolic compounds) [24, 25, and 26].

Optimization of wine polyphenol extraction procedure was performed in order to partially purify and concentrate the target compounds. Four variants of extraction were set (Figure 5).

Based on the results, presented in Table 5, higher extraction yield (76.0 ± 5.2%) was achieved when alcohol fraction was removed from wine samples and acidified water and methanol were used for elution (SPE 4). The total yield in all fractions was the highest as well. Therefore, this extraction method was used for further analysis of tested wine samples. Expectedly, the methanol fraction was richer in polyphenols regardless the extraction conducted.

Total polyphenol content in tested wines and extracts is shown in Table 6.

The initial phenolic content in tested wines varied between 1.67 - 2.08 g GAE/L. After extraction TPC yield ranged from 74.2 ± 0.4% for Syrah to 97.7 ± 2.1% for Tempranillo wine extract. On average, the established yield is relatively high for all samples - more than 74%.

Radovanovic *et al.*, [27], studied the content of phenolic compounds in Cabernet Sauvignon wines from selected Balkan vineyard region and noted considerable variations depending on the agro climatic factors and oenological practices of the vineyard region. A mean of 1.364 g/L was reported which is less than the obtained in this study for this cultivar.

HPLC analysis of wine samples was performed. The studied polyphenols in the samples were identified from the retention times of chromatographic peaks and the characteristic wavelength of detection for each compound in comparison with those of pure standards. The results are presented on Table 7.

As seen chlorogenic acid, hesperidin and cinnamic acid were absent in the investigated samples. The predominant content was established for DL- Catechin, caffeic acid and p-Coumaric acid. Meanwhile, the homemade wines were relatively poorer in phenolic compounds.

Table 5. Total polyphenol content yield in extracts

Extraction	Yield of polyphenols in extract, %			
	Aqueous fraction	Ethyl acetate fraction	Methanol fraction	Total
SPE 1	0	29 ± 0.51	42 ± 2.71	71
SPE 2	0	-	68 ± 4.5	68
SPE 3	0	23 ± 1.09	49 ± 0.31	72
SPE 4	0	-	76 ± 5.2	76

Table 6. TPC in wine samples and in methanol extracts

Wine	TPC_{wine}, gGAE/L	$TPC_{extract}$, gGAE/L
HW1	1.67 ± 0.11	1.41 ± 0.02
HW2	1.74 ± 0.07	1.52 ± 0.01
M	1.77 ± 0.02	1.64 ± 0.08
S	2.08 ± 0.01	1.55 ± 0.01
T	2.04 ± 0.08	1.99 ± 0.04
CS	1.96 ± 0.05	1.54 ± 0.03

Table 7. Phenolic compounds profile of wine samples, µg/mL

Phenolic compound	HW1	HW2	M	S	T	CS
Gallic acid	77.9	55.9	53.5	90.1	106.7	81.5
DL- Catechin	234.8	-	170.8	216.8	414.8	160.8
Chlorogenic acid	-	-	-	-	-	-
Caffeic acid	174.0	122.3	191.6	284.9	305.4	139.9
Syringic acid	-	397.4	376.7	-	-	404.9
p-Coumaric acid	102.2	94.8	105.1	69.2	128.9	190.8
Ferulic acid	-	36.3	-	-	-	-
Rutin	-	-	-	11.7	-	-
Resveratrol	-	5.9	9.9	-	-	-
Hesperidin	-	-	-	-	-	-
Cinnamic acid	-	-	-	-	-	-
Quercetin	-	-	4.8	4.1	-	7.7
Total detected	588.9	712.6	912.40	676.8	955.8	985.6

The presence of gallic acid in all investigated wine samples is probably due to its abundance in oak wood, which is widely used in winemaking and aging processes [28]. In addition, gallic acid is known for copigmentation promotion reaction during fermentation [29].

4. Conclusions

- In this study an HPLC method for simultaneous identification of 12 polyphenols was modified and validated. The optimized method has been applied in the analyses of phenolic compounds in red wines.

- Good linearity for all compounds within the study range was obtained (R^2 greater than 0.9990). The percentage recovery of all polyphenols ranged from 95.9 for rutin to 100.6% indicating good accuracy of the method.

- Based on the results, the validated method could be recognized as suitable for wine analysis and recommended for polyphenols identification in plant samples.

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