Analytical Methods

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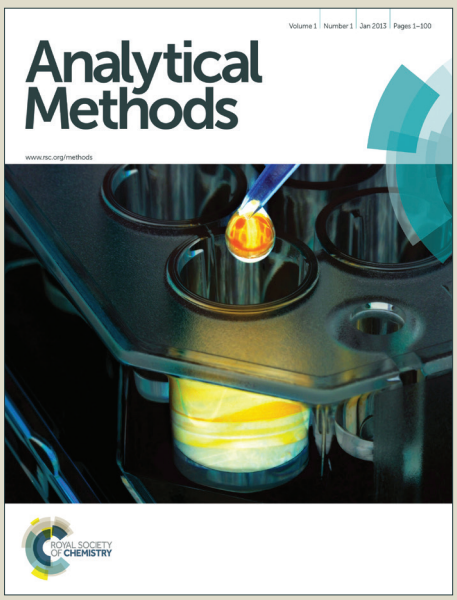

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Title

A reversed phase HPLC-DAD method for the determination of phenolic compounds in plant leaves

Running title

HPLC-DAD method for Phenolic compounds

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Abstract

A simple, fast and efficient HPLC-DAD method was developed for the determination of phenolic compounds in grape leaves. The best separation was achieved in 40 min using gradient elution of methanol, deionized water and acetic acid on Zorbax plus C18 column (4.6 × 100 mm, 3.5 µm) at 25 °C. The phenolic compounds were identified with the help of diode array detector at 280, 320 and 360 nm. The method was precise, accurate, specific and highly reproducible for quantitative and qualitative purposes. The method was used to determine the phenolic composition of grape leaves. A total of 13 phenolic compounds was tentatively identified, namely gallic acid, caffeoylmalic acid, pro-anthocyanin B1, coumaroylhexose-4-O-hexoside, gallic acid derivatives, caffeic acid, quercetin-3-malonylglucoside, quercetin-3-glucoside, kaempferol-3-O-sophorotrioside and kaempferol-3-glucoside. The result demonstrates that this method can be successfully be used for the quality control in the identification of phenolic compounds in grapes and other matrix of plant origin.

Keywords

HPLC-DAD, phenolic compounds, gallic acid, caffeic acid, ellagic acid, grape leaves.

1 Introduction

Phenolic compounds are the main components of plant leaves. These compounds serve as natural antioxidants, and play important role in nutritional, health promoting and commercial properties of plant. [1, 2] Thousands of these plant metabolites have been identified. However, the diversity and importance of the plant kingdom are the main reasons that the phytochemist, biochemist, and plant scientists from academia to industry have focused on the analysis and identification of phenolic compounds. One of the main focus is also to increase the amount of specific phenolic compounds in the plant of interest [3] and subsequently increase the amount and quality of foods. For correct quantification of these compounds, efficient analytical tools are required. Several chromatographic techniques are used to determine the phenolic composition of plant extracts. [4] HPLC with diode array detection (DAD) is one of main technique, widely used for the analysis of phenolic compounds. [5] Phenolic compounds were separated and identified in *Silybum marianum* and *Salvia miltiorrhiza* by HPLC-DAD [6] Brassica species [7] and grape products. [8, 9] The most important product of grape is grape juice. Several anthocyanins from grape juice obtained from a Korean variety of grapes were characterized using HPLC-DAD. [10] Similarly, eight phenolic glycosides were identified in grape juices using HPLC-DAD-MS. The authors stated that a large amount of polyphenolic compounds is lost during preparation of grape juices. Capanoglu, et al. [11]

Grape leaves are one of the important part of the plant in the field to grape production. The grape leaves have been used for stopping bleeding, treatment of inflammatory disorders, pain and hepatitis. The leaf extracts were found to provide protection against the free radical related disease in the liver caused by alcoholic toxicity. [12] The leaves have been found to contain a wide

range of polyphenols such as anthocyanins, flavonoids and other organic acid [13]. Little information is available regarding the determination of phenolic compounds in grape leaves. This study has shown an efficient and accurate HPLC-DAD analytical method for the determination of phenolic compounds in plant leaves.

2 Materials and Methods

2.1 Chemicals & Reagents

Gallic acid was purchased from BDH (BDH, England), caffeic acid and ellagic acid were from Tokyo Chemical Industries (Tokyo, Japan), methanol was from Sigma-Aldrich (Steinheim, Germany). All other chemicals and reagents were of analytical standard and were purchased from Sigma-Aldrich (Germany). Ultrapure deionized water with less than 5 mΩ and HPLC solvent were sonicated for 30 min before chromatography.

2.2 Preparation of standards and Samples

The fresh grape leaf samples in triplicate were collected from the plant in April. The samples were a shade dried and grinded to fine powder. The powder samples were then stored in refrigerator until analysis. Phenolic compounds were extracted from the powder samples using the modified method reported previously. [14] Briefly 1 g of the powder sample was dissolved in 10 mL methanol-water (60:40) mixture and vigorously shaken for 10 h. The mixture was filtered and centrifuge for 10 min at 4000 rpm. The samples were then filtered and injected in to the HPLC system. Standard solution of gallic acid and caffeic acid were prepared in methanol, while ellagic acid solution were prepared in triethanolamine or acidified ethanol.

2.3 HPLC-DAD Conditions

The HPLC system used was Agilent 1260 Infinity HPLC system consists of quaternary pump, degasser, auto-sampler and diode array detector (DAD). The separation was achieved with the help of Agilent rapid resolution Zorbax Eclipse plus C18 (4.6 × 100 mm, 3.5 µm) column. The column was maintained at 25 °C. The gradient system consists of solvent A (methanol: acetic acid: deionized water, 10: 2: 88) and solvent B (methanol: acetic acid: deionized water, 90: 2: 8). Different gradient programs were evaluated. The most efficient gradient program was started with 100 % A at 0 min, 85 % A at 5 min, 50 % A at 20 min, 30 % A at 25 min, and 100 % B from 30 to 40 min. The chromatograms were obtained using 280, 320 and 360 nm for analysis of phenolic compounds. The spectra were recorded from 190 to 450 nm. The identification was carried out using available standards, retention times, and UV spectra. The quantification of identified compounds was based on the percent peak area.

2.4 Method Validation

The analytical method was validated according to the guidelines of International Conference on Harmonization (ICH), for its linearity, limits of detection (LOD), limits of quantitation (LOQ), precision (inter-day and intra-day precision), repeatability, stability, and accuracy.

3 Results & Discussion

3.1 Optimization of HPLC conditions

HPLC conditions were optimized to obtain a maximum separation with desire purity of each separated peak. Optimum separation was achieved at a flow rate of 1 mL/min. Different mobile

phases consist of methanol, acetic acid and de-ionized water was tested. Because of toxicity and high cost, acetonitrile was not used. Acetic acid was found to contribute to the good shape of the peak as described previously. [15] Because of the separate nature of plant phenolic compounds and the standards, three wavelengths were used, i.e. 280, 320, and 360 nm. Gallic acid was found to give maximum response at the 280 nm than 320 and 360 nm. Similarly caffeic acid and ellagic acid were found to have maximum response at 320 and 360 nm as shown in the **Fig. 1**. The grape leaves samples were also measured at same three wavelengths. This reported method is able to separate phenolic compounds in shorter time (40 min) than the reported method. [16]

3.2 Method Validation

The method was validated for quantitative performance using a calibration curve. Three calibration curves of five points in duplicate were established in the concentration range of 0.333 to 1.666 ng/µL. The linear regressions with a correlation coefficient of 0.9993, 0.9994 and 0.9996 were obtained for caffeic acid, ellagic acid and gallic acid respectively. The LODs and LOQs were evaluated from the slope & residual standard deviations of the respective standard curve. The LODs of 3.08, 1.34 and 6.35 ng/µL were obtained for gallic acid, caffeic acid and ellagic acid respectively. Regression data, LODs, and LOQs for three standard substances are given in **Table 1**. The LOD and LOQ for gallic acid and caffeic acid was comparable with the reported values. Rodriguez-Delgado, et al. [17]

Instrumental precision was determined by replicate (n=7) analysis of standard compounds. The results showed high precision (%RSD) for gallic acid, caffeic acid and ellagic acid with a value of 0.72, 0.92 and 1.2 % respectively. Repeatability (%RSD) was determined using intra-day and inter-day analyses of standard concentration of three standards in replicates (n=5). The intra-day

precision for gallic acid was 1.02 %, for caffeic acid 1.06 % and ellagic acid 0.3 % (%RSD), while inter-day precision was 0.16, 0.37 and 1.2 % of gallic acid, caffeic acid and ellagic acid, respectively as shown in **Table 2**. The precision in terms of retention time was evaluated in the same way as that of the peak area. The %RSD of retention time for gallic acid, caffeic acid and ellagic acid was 1.12, 1.79 and 1.27 % respectively. The overall precision of this method was higher than reported method. [16]

The specificity of the method was assessed using the retention time, and absorption spectra of the available standards in the sample. It was found that absorption spectra was a good tool for determination of specificity of individual compound. A high correlation of the compared spectra was found for the sample and standard. A high precision rate (%RSD) was obtained for the retention time when comparing sample and standard. The stability of the method was evaluated after 0, 2, 4, 6, 8, 10, 12 and 24 h after preparation of the samples. The stability of gallic acid was 1.12, caffeic acid (1.43), and ellagic acid 1.61 (%RSD) as shown in **Table 2**. The stability of the method was higher than already reported methods. [17, 18] The accuracy of the method was assessed by recovery studies. Samples of grape leaves were spiked with three concentrations of standard addition method (1, 3 and 5 mg/g). It was found that gallic acid was recovered lower than other standards with a mean recovery value of 98.01 %. The recovery of caffeic acid and ellagic acid was 100.12 and 99.51 % respectively. The lower recovery of gallic acid may be high reactivity and small size as compared to caffeic acid and ellagic acid.

3.3 Phenolic Composition of Grape Leaves

Fig. 2 shows the separation of phenolic compounds at three wavelengths (280, 320, and 360). Gallic acid eluted at the same retention time as that of the standard. The gallic acid was identified

and quantified using standard calibration curve, retention time and absorption spectra as mentioned in the **Table 3**. The structure of the identified compounds are shown in **Fig. 3**. The present results of gallic acid is comparable to the results obtained previously. Di Lecce, et al. [19] It may be concluded that free gallic are present in grape product below 2.0 mg/ 100 g. Caffeoylmalic acid was the second identified compound eluted at the 3.3 min, was present in highest amount. The compound was identified by comparing the absorption spectra reported recently. Santos, et al. [20] Compound 3 was tentatively identified to be Pro-anthocyanin B1 and compound 4 was p-coumaroylhexose-4-O-hexoside. [21] Compound 5 was gallic acid derivatives, while compound 6 was caffeic acid identified from the standard compound spectrum and retention time. Caffeic acid or its derivatives were previously reported in the grape or its products. [22] Ellagic acid was identified as compound 7 at the retention time of 11.9 min. Compound 8 was found to be present in highest amount was quercetin-3-malonylglucoside, while compound 9 was quercetin-3-glucoside. The later compound was identified previously in the grape leaves. [23] These compounds were previously identified to be present in skin, seed and pulp of grape. [19] Peak 9 & 10 were kaempferol derivatives, namely kaempferol-3-O-sophorotrioside and kaempferol-3-glucoside. The latter was previously to be present in grape skin. [19] Recent studies showed that quercetin and kaempferol were the major phenolic compounds in grape plant. [24] The results of the present study showed the identification of sophorotrioside, which may be formed from the above phenolic compounds. These results showed that grape leaves contains some important phenolic compounds, which can be efficiently identified using the present HPLC-DAD method.

4 Conclusions

In this study, a fast and efficient HPLC-DAD method was developed for the determination of phenolic compounds in grape leaves. The method uses a binary gradient system consists of methanol, deionized water and acetic acid. The separation was achieved in 40 min using a reversed phase C18 column and detection using a diode array detector. The method was precise, accurate, specific and highly reproducible for quantitative and qualitative purposes. The method was used to assess the phenolic composition of grape leaves. A total of 13 phenolic compounds was tentatively identified, namely gallic acid, caffeoylmalic acid, pro-anthocyanin, coumaroylhexose-4-O-hexoside, gallic acid derivatives, caffeic acid, quercetin-3-malonylglucoside, quercetin-3-glucoside, kaempferol-3-O-sophorotrioside and kaempferol-3-glucoside. The method can be successfully be used for the identification of phenolic compounds in other matrix of plant origin.

Acknowledgment

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Figure Legends

Fig. 1. Representative HPLC-DAD chromatograms of the standard phenolic acids. GA, gallic acid; CA, caffeic acid and EA, ellagic acid. Chromatograms were obtained at 280, 320 and 360 nm.

Fig. 2. Representative HPLC-DAD chromatograms of the grape leaves. Chromatograms were obtained at 280, 320 and 360 nm. The identification of the peaks are given at the Table 3.

Fig. 3. Structures of the polyphenolic compounds identified in grape leaves using reversed phase HPLC-DAD method.

Table 1: Linear regression and validation data for the quantitative determination of standard phenolic compounds.

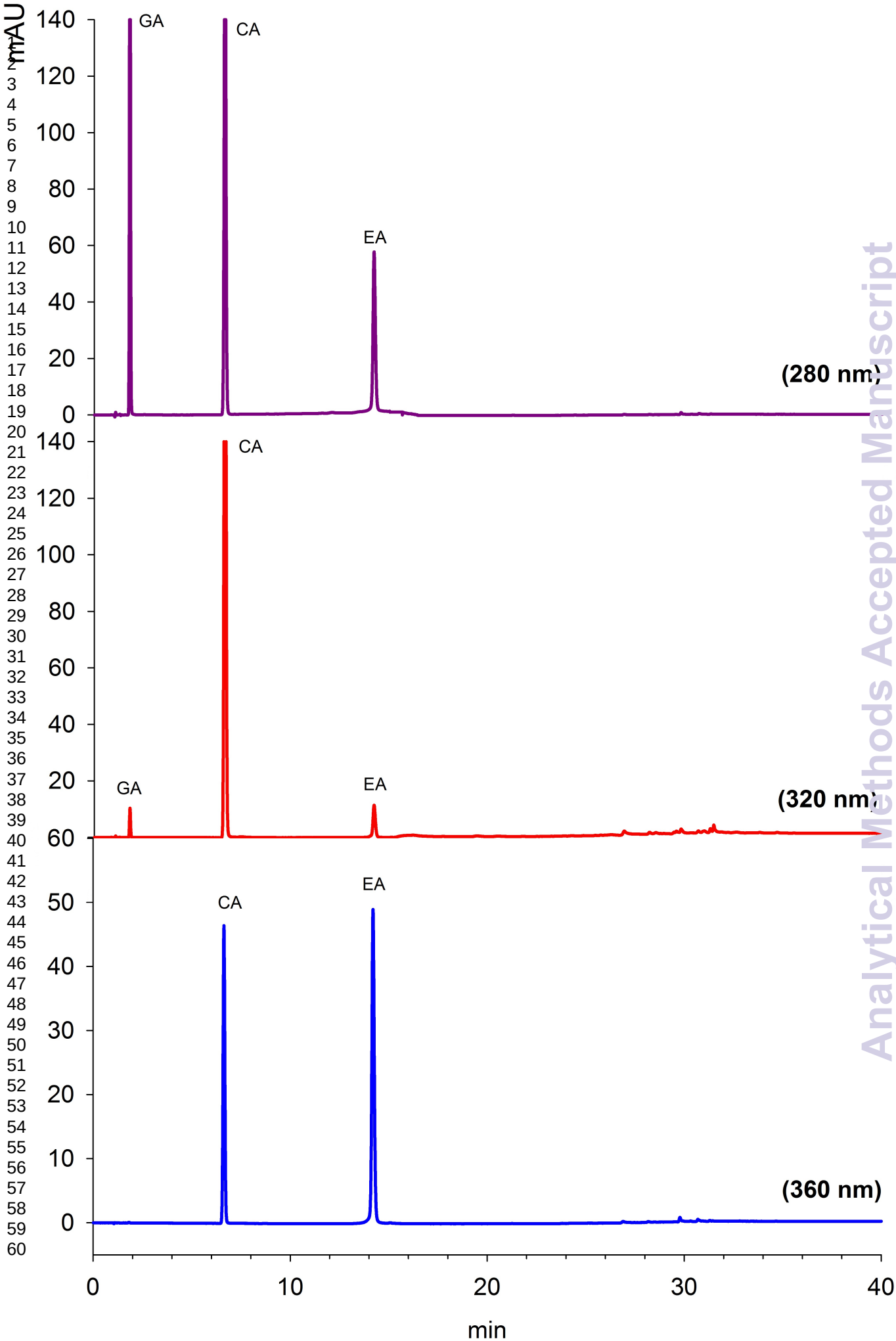
Compound	Calibration Equation (y=ax+b)	Correlation Coefficient	LOQ (ng/µL)	LOD (ng/µL)
Gallic acid	y = 2457.3435x – 48.6606	0.9996	3.08	9.35
Caffeic acid	y = 6621.9955x -88.6467	0.9993	1.34	4.07
Ellagic acid	y = 1297.3362x – 34.4573	0.9994	6.35	19.2

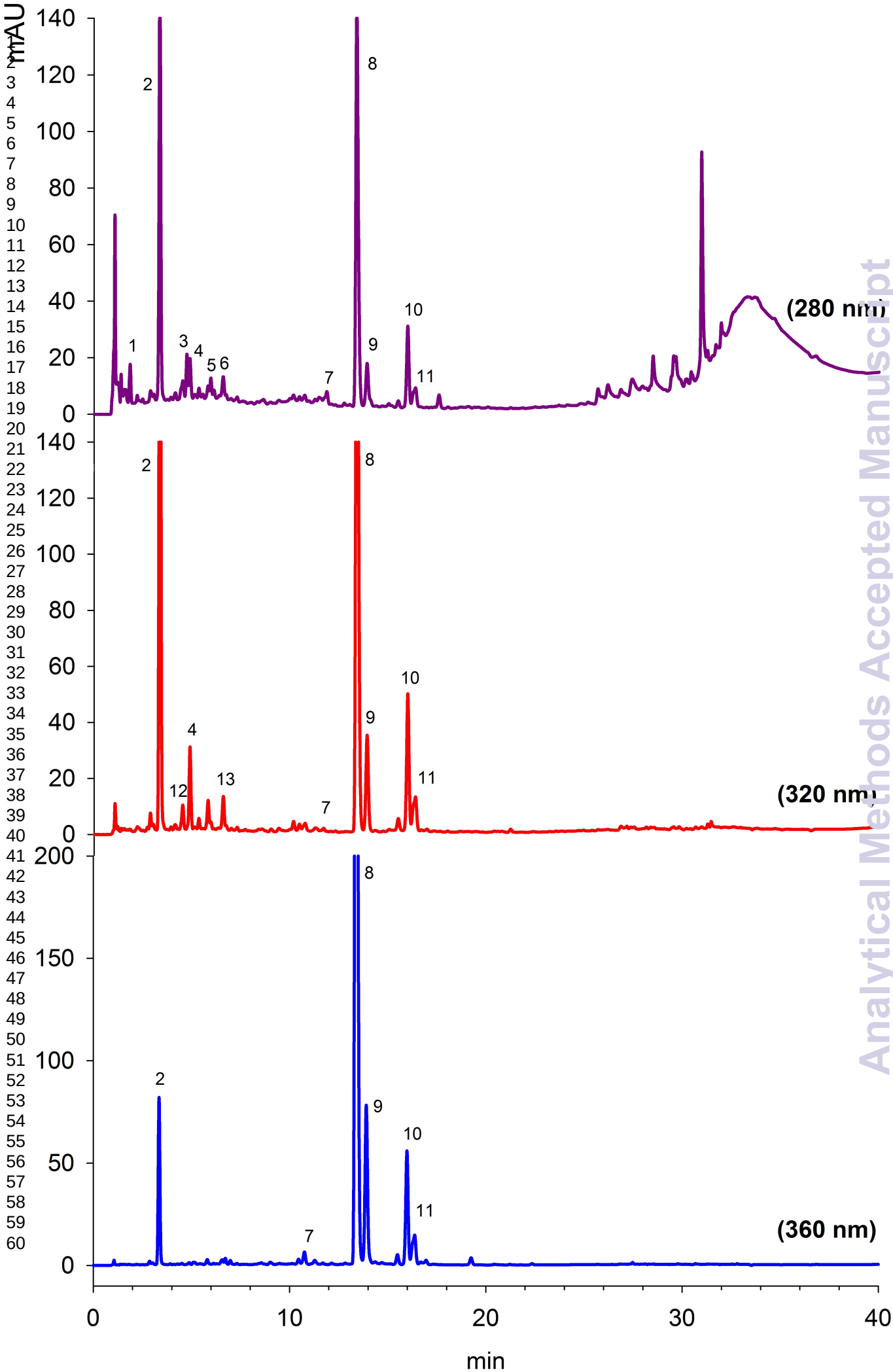
Table 2. Intra-day and inter-day precision of the gallic acid, caffeic acid and ellagic acid in terms of peak area.

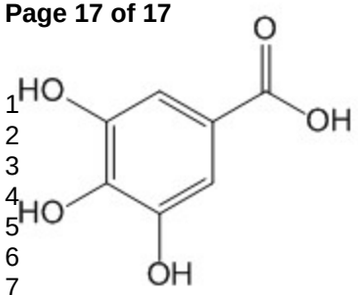
Compounds	Intra-day Precision (n=3)				Inter-day Precision (n =5)				Stability % RSD (n=8)	Recovery (%) n=9	
	Mean Peak Area			RSD (%)	Mean Peak Area			RSD (%)			
	Morning	Noon	Evening		Day 1	Day 2	Day 3			Mean	%RSD
Gallic acid	830.1	817.7	814.1	1.02	828.8	830.1	831.5	0.16	1.122	98.01	2.311
Caffeic acid	2261.8	2227.7	2216.1	1.06	2249.3	2261.8	2265.7	0.37	1.431	100.1	1.455
Ellagic acid	420.4	421.9	419.1	0.3	423.2	420.4	430.3	1.2	1.613	99.51	1.923

Table 3. Identification of phenolic compounds in grape leaves samples. Only compounds with 99.9 % peak purity were selected.

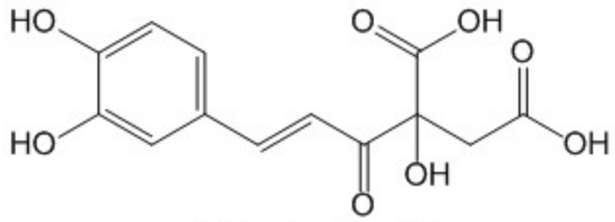
Peak	Retention time (min)	Possible Identity	HPLC-DAD λ_{max} (nm)	Quantity (mg/ 100g)	Identification Reference
1	1.8	Gallic acid	270	1.26	Standard
2	3.3	Caffeoylmalic acid	328, 298sh, 244	31.08	Santos *et al.* [20]
3	4.7	Pro-anthocyanin B1	310, 280	1.68	Santos *et al.* [20]
4	4.9	p-Coumaroylhexose-4-O-hexoside	313, 290	1.53	Aaby *et al.* [21]
5	5.9	Gallic acid derivative	278	0.76	Standard
6	6.5	Caffeic acid	323, 298sh, 238	1.09	Standard
7	11.9	Ellagic acid	253, 305, 368	1.32	Standard
8	13.3	Quercetin-3-malonylglucoside	354,295sh, 256	75.23	Santos *et al.* [20]
9	13.9	Quercetin-3-glucoside	355, 256	8.15	Santos *et al.* [20]
10	15.9	Kaempferol-3-O-sophorotrioside	347, 295sh, 265	5.53	Santos *et al.* [20]
11	16.3	Kaempferol-3-glucoside	348, 264	2.15	Santos *et al.* [20]
12	4.5	Gallic acid derivative	278	0.88	Fischer *et al.* [20]
13	6.7	Caffeic acid derivative	223	1.33	Fischer *et al.* [20]



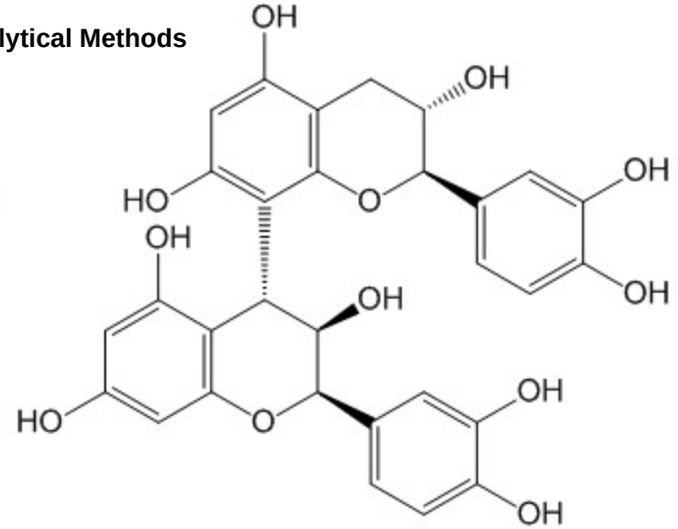




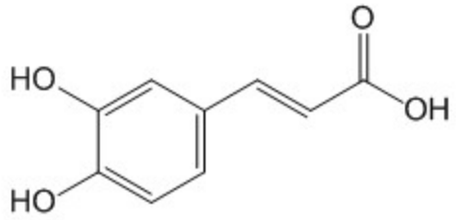
Gallic acid



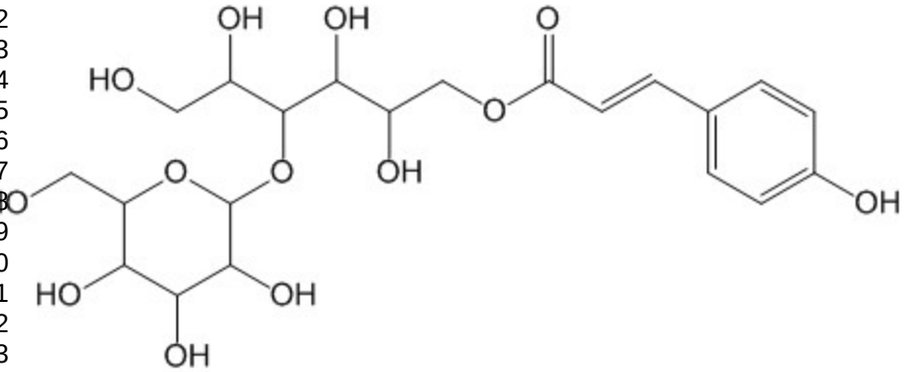
Caffeoylmalic acid



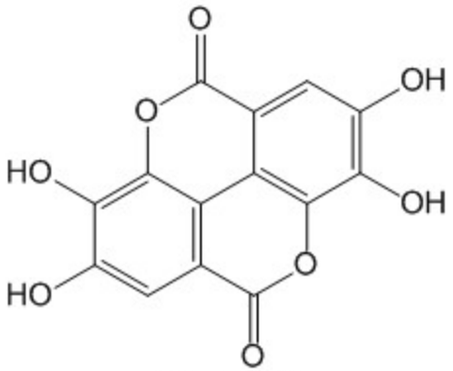
Pro-anthocyanidin B1



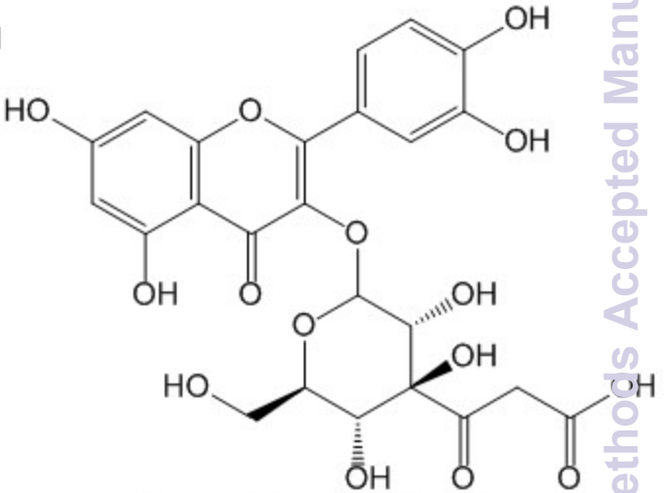
Caffeic acid



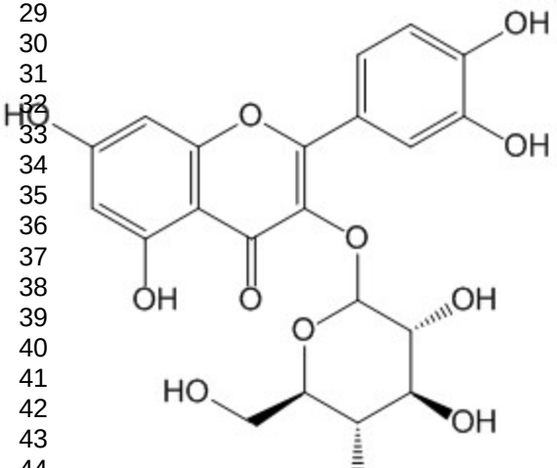
p-Coumaroylhexose-4-O-hexoside



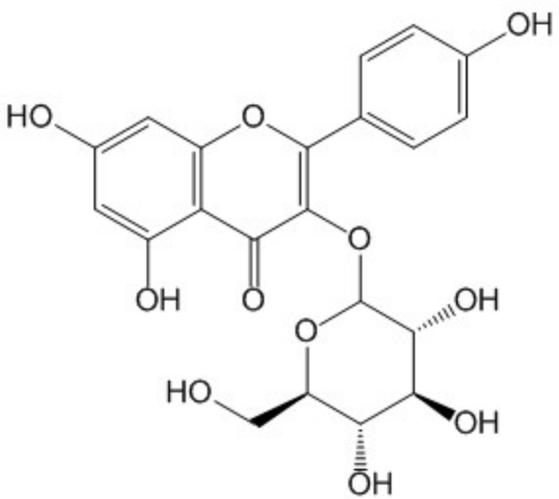
Ellagic acid



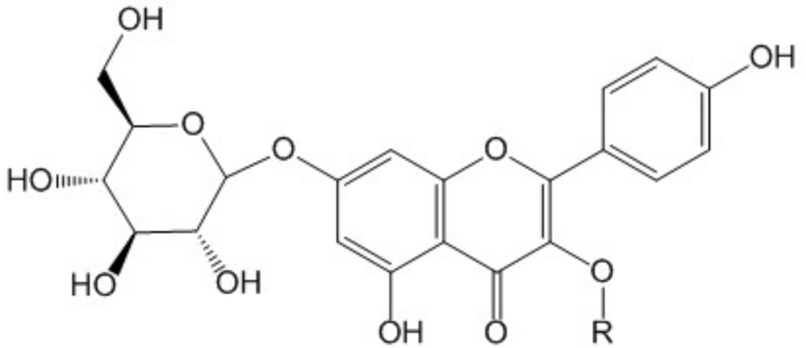
Quercetin-3-malonylglucoside



Quercetin-3-glucoside



Kaempferol-3-glucoside



Kaempferol-3-sophorotrioside