

Short communication

A Comparison of Extraction Methods for Selected Phenolic Compounds from Grape Berry Skins Using Liquid Chromatography and Spectrophotometry

Denis Rusjan,* Zora Korošec-Koruza

University of Ljubljana, Biotechnical Faculty, Jamnikarjeva 101, SI-1001 Ljubljana, Slovenia.
Tel.: +386(0)14231161, Fax: +386(0)14231088, E-mail: denis.rusjan@bf.uni-lj.si.

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Abstract

The content of phenolic compounds in grape skins of the red grape variety 'Merlot' using different extraction solvents and analytical techniques were studied. The 1% solution of 2,6-di-*tert*-butyl-4-methylphenol (BHT) in methanol and ethanol/formic acid/water solution were used as extraction media, and the content of phenolic compounds was determined using HPLC and spectrophotometry. Methanol compared to ethanol/formic acid/water performed better, as statistically significantly higher contents were determined.

The following contents were determined: (+)-catechin 68–74 mg kg⁻¹, (-)-epicatechin 32–35 mg kg⁻¹, oenin 440–450 mg kg⁻¹, rutin 2.9–3.5 mg kg⁻¹, myricetin 0.4–0.5 mg kg⁻¹, kaempferol 2.3 mg kg⁻¹ and quercetin 3.2–3.6 mg kg⁻¹. Using spectrophotometry, an arbitrary procedure was followed for determination of phenolic compounds extracted with ethanol/formic acid/water. Thus, the content of total phenols (791–1127 mg kg⁻¹), tartaric esters (219 mg kg⁻¹), flavonols (163–167 mg kg⁻¹) and total anthocyanins (2179–2219 mg kg⁻¹) was determined. A comparison with literature data is difficult due to different varieties studied, ecological conditions, ripening stage, analytical methods etc., but some similarities can be observed. For our understanding of the process of grape maturation and its quality, it is of importance to develop a reliable and simple method.

Keywords: grape analysis, wine, extraction, antioxidants, chromatography, spectrophotometry

1. Introduction

In the past decades, much research focused on determination of the structure and content of phenolic compounds in grape and wine.^{1–6} The ability to isolate and determine the phenolic compounds in wine enabled researchers to focus on their fate and on their sensory influence. The importance of phenolic compounds for wine colour and flavour is well recognised, while their role in grape, where they are initially produced, is less understood. It is known that while the skin of grape berry contains tannins and pigments, the seeds contain tannins and the pulp contains no pigments.^{1–6}

Tannins, such as flavan-3-ols are the most abundant category of soluble polyphenols in grape berries, found predominantly in the hypodermal layers of the skin and the soft parenchyma of the seeds.⁴ Catechin and epicatechin make up the most important units in grape tannins, with epicatechin usually being the more abundant of the two. Grape tannins are biomolecules usually very diverse in structure, and vary in size from dimers and trimers to oli-

gomers with more than 30 subunits. The flavan-3-ols are represented by catechin and epicatechin, which are isomeric flavan-3-ols: catechin has the 2,3-*trans* configuration and epicatechin has 2,3-*cis* configuration.^{1,4,6,8}

Anthocyanins are the second important group of phenolic compounds, which is co-located with tannins in the thick-walled hypodermal cells of the skin. The main anthocyanidins found in grape of *Vitis vinifera* L. are cyanidin, delphinidin, peonidin, petunidin and malvidin, but their contents differ according to variety, ecological conditions and viticultural practices.^{1,4,5,8}

Flavonols, as the third main group of phenolic compounds (kaempferol, quercetin, myricetin, rutin etc.) are abundant in wine, while in grape they are present in the form of glycosides, galactosides and glucuronides. In most cases, flavonols are less abundant than other phenolic compounds discussed above.^{1,4,7,8}

The phenylpropanoid biosynthesis of phenols usually starts with the amino acid phenylalanine as the product of the shikimate pathway.^{8,9} The contents of phenylalanine, free cinnamic acids and their CoA esters are reported to be very low in tissues where care is taken to prevent ester

hydrolysis prior to analysis.⁹ Only berry skin anthocyanins appear to behave as typical end-products. Other phenols exhibit patterns of accumulation and subsequent decline during ripening, suggesting their degradation or utilization in biosynthesis. The skin and seeds have features in common with regard to their content of phenols and ripening but also exhibit dissimilar properties; both accumulate tannins as the most abundant phenols at harvest, but anthocyanins only accumulate in the hypodermal cells of the skin. In viticulture and enology the total amount of berry phenols of each category is important at harvest, especially in cases of specific wine production using 'technologically' ripe and over-ripe grape. Carbohydrates, organic acids and phenols are the most used qualitative parameters for grape used in wine production. Their contents differ according to ecological (soil, climate), agro-ampelotechnical practices in vineyard and variety.¹⁻⁹

The importance of phenolic compounds for the wine industry has been demonstrated many times, therefore, the development of appropriate analytical methods became very important. Liquid chromatography is the technique of choice for their separation and quantitative analysis, mainly using diode-array and mass-spectrometric detectors. While a lot of studies have focussed on the identification and quantification of phenols in wine, their quantification in grape skin is less well studied. For determination of phenol compounds in grape berries, their extraction remains the most critical step.¹⁰⁻¹⁵

Therefore, our aim in the present work was to compare a few of the usually used, fast and simple extraction methods for the main phenolic compounds found in grape skins. The subsequent analytical procedure involved liquid chromatographic determination or spectrophotometry.

2. Experimental

2.1. Materials

External standard compounds used in this study were malvidin-3-glucoside (oenin) from Extrasynthese (France), (+)-catechin, (-)-epicatechin, rutin, myricetin, kaempferol from Fluka (Buchs, Switzerland) and BHT (2,6-di-*tert*-butyl-4-methylphenol; 99%), quercetin, caffeic acid and gallic acid from Sigma (Sigma-Aldrich, Germany). The mobile phase consisted of aqueous 0.01 mol L⁻¹ phosphoric acid and methanol, which was HPLC grade (Sigma-Aldrich, Steinheim, Germany). For the preparation of all solutions, additionally purified deionized water was used (MilliQ purification system, Millipore, France).

Grape from the globally-known variety 'Merlot' (*Vitis vinifera* L.) was randomly sampled on the vine and an average sample was made up of all the sampled grape. The contents of selected phenols were determined in berry skins according to different extraction methods.

The phenol contents were expressed as mg kg⁻¹ of fresh weight (FW).

2.2. Instrumentation

The UV/VIS system spectrometer Lambda Bio 20 (Perkin-Elmer) was used to determine the absorbance of sample extracts and the corresponding standards. The absorbance (*A*) at 280 nm was used to estimate the content of total phenols, at 320 nm for tartaric esters, at 360 nm for flavonols and at 520 nm was for anthocyanins.¹²

The HPLC system used was a Thermo Finnigan Surveyor system with a diode array detector set at 280 nm. The absorption spectra of compounds were recorded between 210 and 350 nm. The mobile phase was aqueous 0.01 mol L⁻¹ phosphoric acid (A) and methanol (B). The elution programme was: 5–50% B (in 10 min), 50–70% B (in 5 min), 70–80% B (5 min) and 80–100% B (5 min). The injection volume was 20 µL and the flow rate was 1.0 mL min⁻¹. The column used was a Phenomenex Synergy (4 U MAX-RP 80 A; 250 × 4.60 mm) column, operated at 25 °C.

2.3. Preparation of Standard Solutions

Solutions of phenolic compounds, i.e. external standards, were prepared by dissolving the weighted compounds in 1% solution of BHT in methanol or ethanol/formic acid aqueous solution to obtain the concentration of 0.01 g L⁻¹. The standards used for spectrophotometry were gallic acid diluted in 10% ethanol for determination of total phenols, caffeic acid in 10% ethanol for determination of tartaric esters, quercetin in 95% ethanol for determination of flavonols and malvidin-3-glucoside in 10% ethanol for determination of anthocyanins.¹²

2.4. Extraction Procedures

The extraction procedures were carried out according to literature references 11 and 12 with minor modifications in sample weight (1 g instead of 50 g of grape) and in dilution (in 10 mL instead of 50 mL). The following two methods were used:

- Method 1:¹² around 1 g of grape skins were randomly selected and weighted. Using frozen berries, the skins were peeled and separated from the pulp and the skins were later lyophilised. The skin samples were homogenised for 8 min at 20.000 r.p.m. (Ultra turrax T25 basic IKA, Labortechnik) in 10 mL of 50:1.5:48.5 methanol/formic acid/MilliQ water (v/v/v) at 4 °C. The mixture was then centrifuged for 10 min at 9.500 r.p.m. at 4 °C. The supernatant was filtered through a 0.45 µm syringe filter (Chromafil PVDF P-45/25; Macherey-Nagel) and stored at –20 °C prior to injection. For spectrophotometric analysis, the samples were

then diluted 1:10 with 10% ethanol (*v/v*) and 0.25 mL of this sample (or standard) was put in a test tube and added 0.25 mL of 0.1% HCl in 95% ethanol and 4.55 mL of 2% HCl. The solution was mixed and allowed to sit for 15 min before reading the absorbance.¹² The spectrophotometric measurements were performed in the same media as standards.

- Method 2:¹¹ a sample of ~2 g of skins was extracted in 98% methanol containing 1% BHT using an ultrasonic bath (Sonis 4; Iskra Pio). The samples were extracted first with 10 mL for 1 h, then with another 10 mL for 30 min and with another 5 mL for 30 min. The extracts were joined and filtered through a 0.45 μm syringe filter (Chromafil PVDF P-45/25; Macherey-Nagel) and stored at $-20\text{ }^{\circ}\text{C}$ prior to injection. BHT was added as antioxidant and as it does not interact with the extracted phenols or interfere with the subsequent HPLC analyses.¹¹

3. Results and Discussion

3.1. The Analytical Methods

During spectrophotometric analysis, measurements on all standard solutions were done in triplicate. Although the method is arbitrary, absorbance maxima (nm) for gallic acid, caffeic acid, quercetin and oenin were checked. The obtained linear calibration curves were applied for quantification of specific groups of phenolic compounds.

Table 1. Parameters of the spectrophotometric method: analytes, the prescribed standards and measurement wavelengths.¹²

Group of phenolic compounds	Standard used	$\lambda_{\text{measurement}}$ (nm) ¹
Total phenols	Gallic acid in 10% ethanol	280
Tartaric esters	Caffeic acid in 10% ethanol	320
Flavonols	Quercetin in 95% ethanol	360
Anthocyanins	Malvidin-3-glukozid in 10% ethanol	520

For HPLC analysis, optimisation of gradient separation was performed using a suitable mixture of standards. For red grapes, we selected (+)-catechin, (-)-epicatechin, oenin, rutin, myricetin, kaempferol and quercetin.

The uncertainty of determination was estimated by calculating the standard deviation (S.D. in %) for triplicate analyses. Using each extraction procedure, fifteen determinations of phenols were carried out at various stages of berry ripeness. If the difference between the three parallel results was more than 10%, the analyses were repeated. Identification of phenol compounds was done by matching the retention times of peaks in chromatograms

of extracts with those of standards. The order of elution was the following: (+)-catechin, (-)-epicatechin, oenin, rutin, myricetin, kaempferol and quercetin (Figure 1).

The two extraction procedures led to statistically significantly different results (Table 2), commented later (LSD test; $P < 0.05$).

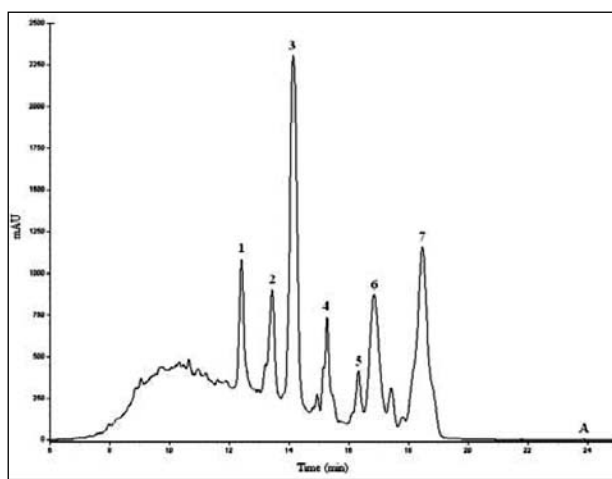
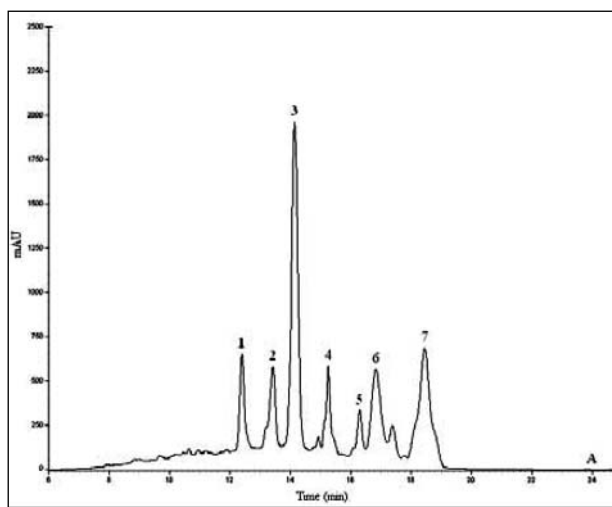


Figure 1. Comparison of chromatograms of two samples obtained by two extraction procedures of 'Merlot' berries at ripe stage: with Method 1 – ethanol/formic acid/water solution (left); with Method 2 – BHT/methanol (right). Peak assignment: 1 – (+)-catechin, 2 – (-)-epicatechin, 3 – oenin (malvidin-3-glucoside), 4 – rutin, 5 – myricetin, 6 – kaempferol, 7 – quercetin.

3.2. Quantification of Phenolic Compounds

Flavan-3-ols: We focused on determination of (+)-catechin and (-)-epicatechin, which are known to be the most abundant flavan-3-ols in grape skin. They are responsible for rapid browning grape juice and for its astringent taste. At ripe stage, the contents of (+)-catechin ranged 60.9–74.3 mg kg^{-1} FW. With maturation, the content decreased to 56.2–68.5 mg kg^{-1} FW. At over-ripeness, the content of (-)-epicatechin in grape skin also decreased to

22.6–32.1 mg kg⁻¹ FW, compared to 25.4–34.5 mg kg⁻¹ FW at ripe stage. In general, these data agreed with findings of other authors^{6,15,19} with some exceptions. Compared to data mentioned by some authors,^{16,17} the determined contents of (+)-catechin and (-)-epicatechin in our study were higher, but lower compared to data in reference 18, which might be explained by different grape variety and ecological conditions. The contents of (-)-epicatechin in all samples were lower than those of (+)-catechin, which confirms other findings.^{6,15,17,19}

Anthocyanins: Malvidin-3-glucoside (oenin) is the main anthocyanin in the red grape variety 'Merlot', constituting 60–70% of all pigments.^{17,18,20} Using extraction Method 1, we determined lower oenin content in berry skins at over-ripe than at ripe stage: average contents were 308 mg kg⁻¹ FW at ripe and of 291 mg kg⁻¹ FW at over-ripe stage, although the difference was not statistically significant. On the other hand, the average content determined using extraction Method 2 was approximately 445 mg kg⁻¹ FW. The determinations according to the two extraction methods were statistically different.

The determined oenin contents are similar to those cited by other authors^{5,20,21} who determined the average contents at ripe stage to be around 360 mg kg⁻¹ FW of grape skin. On the other hand, several times higher contents of oenin in 'Merlot' grape skin (1060 mg kg⁻¹) was determined by Mazza et al.¹² This may be due to the different

method for quantification used. The anthocyanin contents in extracts according to Method 1 and using spectrophotometry is surprisingly high¹² and ranged between 2179 and 2219 mg kg⁻¹ FW (Figure 2). This is higher than the total phenolic content, which is difficult to explain, however, one must bear in mind that the spectrophotometric method is an arbitrary one and inherently inaccurate, as demonstrated here.

Flavonols: The most abundant flavonols in the sampled skins were quercetin followed by rutin, kaempferol and myricetin. The content of quercetin varied from 3.14 mg kg⁻¹ at ripe to 2.88 mg kg⁻¹ FW at over-ripe stage extracted with ethanolic/formic acid/water.¹² The differences to determination using Method 2 were statistically significant: the average contents determined using this method were 3.2 mg kg⁻¹ at ripe to 3.6 mg kg⁻¹ FW at over-ripe stage. These quercetin contents did not differ from values cited in the literature.^{14,15,22} The second most abundant identified flavonol was rutin with the average content of 2.2–3.5 mg kg⁻¹ at ripe and 2.3–2.9 mg kg⁻¹ FW at over-ripe stage. The contents of rutin determined by the two extraction methods differed significantly. The cited rutin contents¹⁹ in red wine were in the interval 0.8–12.4 mg kg⁻¹, and our determinations were in agreement with these data, although they were extracted from berry skins. Extraction with BHT/methanol (Method 2) also led to higher kaempferol determinations compared to extraction with ethanol/formic acid/water (Method 1). Kaempferol contents at ripe stage ranged from 1.8–2.3 mg kg⁻¹ FW, and at over-ripe stage from 1.6–2.2 mg kg⁻¹ FW. The average contents of kaempferol in grape skin were in agreement with values cited in the literature.^{14,15} The content of myricetin ranged from 0.3–0.5 mg kg⁻¹ at ripe and from 0.3–0.4 mg kg⁻¹ FW at over-ripe stage. Again, the two extraction methods led to statistically different values. Wang and Huang¹⁴ cited the average myricetin content to be 1.6 mg L⁻¹, Volikakis et al.²³ between 3.6–6.2 mg L⁻¹, Tsanova-Savova and Ribanova²⁴ between < 1–9.4 mg L⁻¹ and Gambelli and Santaroni²⁵ 1.7–9.7 mg L⁻¹.

The observed differences in flavonol contents as determined using HPLC and spectrophotometry may be expected because the arbitrary method does not include all flavonol components of interest.

The knowledge on flavonol contents in grape berries (skin, pulp and seed) is still poor and more research is needed to better understand the production of phenols during berry ripening process.

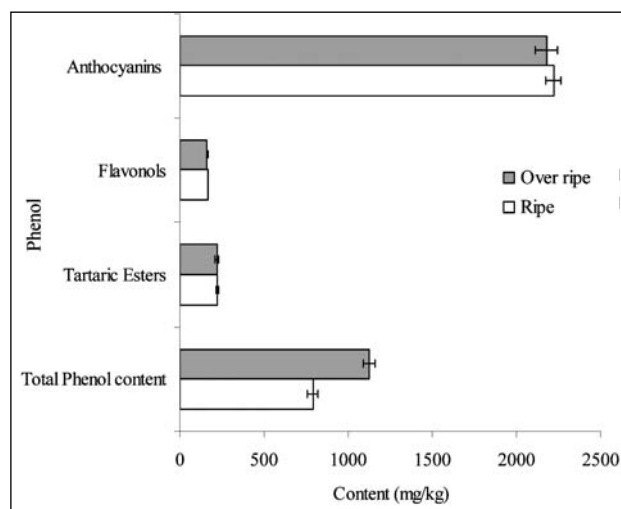


Figure 2. Contents of groups of phenolic compounds in grape skin of red variety 'Merlot' (mg kg⁻¹ FW) according to the spectrophotometric method of quantification.

Table 2. Contents of phenolic compounds (mg kg⁻¹ FW) as determined using the two extraction methods and HPLC.

Extraction	Maturity	(+)-Catechin	(-)-Epicatechin	Oenin	Rutin	Myricetin	Kaempferol	Quercetin
Method 1	Ripe	62 ± 13	25 ± 6	310 ± 30	2.2 ± 0.6	0.34 ± 0.07	1.9 ± 0.2	3.1 ± 0.5
	Over-ripe	56 ± 20	23 ± 8	290 ± 56	2.3 ± 0.6	0.32 ± 0.08	1.6 ± 0.4	2.9 ± 0.6
Method 2	Ripe	74 ± 23	35 ± 9	450 ± 65	3.5 ± 1.1	0.46 ± 0.12	2.3 ± 0.3	3.2 ± 1.2
	Over-ripe	68 ± 20	32 ± 8	440 ± 77	2.9 ± 1.0	0.42 ± 0.07	2.3 ± 0.3	3.6 ± 0.7

4. Conclusions

Differences in determinations of phenols in grape skins of the red variety 'Merlot' using two methods of extraction and two detection techniques were established. Using a methanolic solution of the antioxidant BHT for extraction, higher contents of phenols especially epicatechin, oenin, and kaempferol were established then when using an ethanolic solution of formic acid. In several cases, the differences were statistically significant, and the use of BHT/methanol extraction medium is recommended.

In all cases, the determined values are in agreement with those cited by other authors, while small variations can be explained by differences in variety, grape maturation stage, ecological influences and by different extraction methods, but also detection techniques. No statistically significant differences were observed in contents of individual phenols between ripe and over-ripe berries with same extraction.

These data are not in agreement with the spectrophotometrically determined content of total phenols. In addition a higher content of anthocyanins were determined than the content of total phenolic compounds.

5. Acknowledgement

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Povzetek

Preučevali smo vsebnost fenolnih spojin v kožicah rdečega grozdja kultivarja 'Merlot', pri čemer smo uporabljali različne ekstrakcijske medije in analizne tehnike. Za ekstrakcijo smo uporabljali 1% raztopino 2,6-di-*tert*-butil-4-metilfenola (BHT) v metanolu in mešanico etanol/mravljjična kislina/voda. Slednja raztopina je dala slabše rezultate.

Določili smo naslednje vsebnosti: (+)-katehina 68–74 mg kg⁻¹, (-)-epikatehina 32–35 mg kg⁻¹, oenina 440–450 mg kg⁻¹, rutina 2.9–3.5 mg kg⁻¹, miricetina 0.4–0.5 mg kg⁻¹, kaempferola 2.3 mg kg⁻¹ in kvercetina 3.2–3.6 mg kg⁻¹. Pri spektrofotometričnih določitvah smo sledili dogovorni metodi po ekstrakciji v mešanico etanol/mravljjična kislina/voda. S to metodo smo določili vsebnost vseh fenolov (791–1127 mg kg⁻¹), estrov vinske kisline (219 mg kg⁻¹), flavonolov (163–167 mg kg⁻¹) in vseh antocianinov (2179–2219 mg kg⁻¹). Primerjava z literaturo je težavna zaradi razlik, do katerih pride zaradi kultivarja, ekoloških pogojev, zrelosti, analizne metodologije ipd., čeprav smo opazili podobnosti s podatki drugih raziskovalcev. Za razumevanje procesa zorenja grozdja in njegove kvalitete je pomemben razvoj enostavne in zanesljive metode.