# THE DETERMINATION OF BIOACTIVE INGREDIENTS OF GRAPE POMACE (VRANAC VARIETY) FOR POTENTIAL USE IN FOOD AND PHARMACEUTICAL INDUSTRIES

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Wine production generated significant quantities of waste – grape pomace (seeds, skin and stems of grapes). In these vinification by-products a significant amount of bioactive compounds such as phenol compounds can still be found. The phenol composition and radical scavenging activity of grape pomace obtained during winemaking from the red wine grape of Vranac variety (*Vitis vinifera* L.) were investigated to determine their usable values. Reverse-phase high performance liquid chromatography (RP-HPLC) assays were used for determination of the phenolic composition of pomace extracts and the DPPH test was done for their radical scavenging activity. The oil obtained from the pomace was subjected to GC/MS analysis in order to establish its fatty acid composition. As correlation calculations showed ($R^2$=0.9732), phenol compounds are responsible for strong radical scavenging activities of the tested extracts (1.160 ± 0.03 mg DM/ml). Grape pomace oil with a high degree of of unsaturation (over 88%) plays an important role for human health. The phenolic rich extract and grape pomace oil can be used as additives in food and pharmacy industries.

**Keywords:** grape pomace, grape seed, phenolic compounds, radical scavenging activity, fatty acids.

## Introduction

Grape wine (genus Vitis) is considered the world's most abundant fruit crop. Grapes and wine are rich in phenolic compounds which are very important for human health as compounds with antioxidant, anti-cancer, anti-inflammatory and antimicrobial activities [1-3]. There are also studies on the beneficial effects of those compounds on the heart and other chronic diseases [4]. During the processing of grapes into wine, a significant amount of phenolic compounds (soluble in water) passes into wine. But also a certain amounts of these compounds remain in the pomace. On the other hand, grapes and pomace also have a certain amount of vegetable oil which is found in grape seeds, but also in other parts of grapevine as stems [5]. The wine production generated significant quantities of waste (seeds, skins and stems of grapes). This waste was usually used for the production brandy and fertilizer. The phenol composition and antioxidant activity of grape extracts have been well documented [1-4, 6], and there is less data about pomace [7-9]. Also, there were few reports about vegetable oils from grape seeds, and their health benefits, [10] but no reports about the whole grape pomace.

The aim of this study was to determine the extraction yield of phenolics and vegetable oils, their composition and antioxidant activity of the phenolic extract from the grape pomace.

## Materials and Methods

### Samples

The wine pomace of red wine variety Vranac (*Vitis vinifera* L.) was taken from the „Aleksic" winery immediately after a 14-day vinification process. Maceration was performed with crushed grapes without stems. The samples of wine pomace (grape seeds and skins) were washed, dried at 60 °C in the oven for 12 h and crushed in a grinder for 2 min., and then used for extractions.

### Chemicals

Acetonitrile, methanol and formic acid of HPLC-grade were obtained from Merck (Darmstadt, Germany). The standard phenolic compounds and 2,2`-diphenyl-1-picrylhydrazyl free radical (DPPH) and all other chemicals were supplied from Sigma Chemical Co. (St. Louis, MO). The reagents used were of analytical quality.

### Extraction of phenolic compounds

The samples (pomace) were weighed (1 g) and extracted with the 40 ml solvent system of methanol/acetone/water/acetic acid (30/42/27.5/0.5), by stirring continuously at room temperature in the dark for 1 h, and then centrifuged

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for 10 min at 2500 x g. The supernatants were collected and the precipitates were extracted again with the same solvent system. Combined extracts were evaporated to dryness under vacuum rotary evaporator below 50 ºC and diluted in methanol to the concentration of 0.1 g/ml.

Extraction of grape pomace oil

The powder of the grape pomace (10 g) was extracted in a Soxhlet extractor for 3 h with 200 ml of hexane at 60 °C.

Spectrophotometric Determination of Phenolic Compo sition

Total phenolics, hydroxycinnamoyl tartaric acids and flavonols in selected extract samples were determined according to the spectrophotometric method previously described [11]. The results were expressed as milligrams (mg) of gallic acid equivalents (GAE) for total phenols, mg of caffeic acid equivalents (CAE) for total hydroxycinnamoyl tartaric acids and mg of quercetin equivalents (QE) for total flavonols per gram (g) of the extract dry matter (DM).

Total anthocyanins were also determined spectrophotometrically [12]. Malvidin-3-glucoside was employed as a calibration standard and the results were expressed as mg malvidin-3-glucoside equivalents (ME) per g of the extract DM.

Reverse-phase High-Performance Liquid Chromatography Analyses

The phenol composition of the selected extracts was analyzed by reverse-phase high performance liquid chromatography (RP-HPLC) of the extracts. The analysis was carried out with an Agilent Technologies 1200 chromatographic system equipped with DAD - photodiode array and fluorescence detectors. The column (Agilent-Eclipse XDB C-18 4.6 × 150 mm) was thermostated at 30 °C. The solvents A: formic acid/water (5:95 v/v) and B: acetonitrile/formic acid/water (80:5:15 v/v) were used and the elution gradient were previously described [12]. The injection volume was 5 µL and the flow rate was 0.9 mL/min. The detection wavelengths were 280, 320, 360 and 520 nm for UV, and 275/322 nm (λEx/λEm) for fluorescence-detection. The phenolic compounds were identified by comparing their spectral characteristics and retention times with the data of the original reference standard compounds and with the data given in literature [13, 14]. The calibration curves of standard phenolic compounds (five data points, n=2) were linear with $R^2$ = 0.99. The results were expressed as mg/g extract DM.

Determination of the Radical Scavenging Activity

The antioxidant activity of all investigated extracts was estimated by determining the radical scavenging activity (RSA, %) of the extracts by the DPPH test previously described [12]. The antioxidant activities of the investigated extracts were expressed as median efficient concentrations (EC50). This is the concentration of the extract (mg/ml) needed for a decrease in absorbance of the DPPH solution to 50%.

Analysis of fatty acids

The fatty acid composition of the oil extract was determined by the gas chromatography mass spectrometry (GC/MS) analysis. GC/MS analysis was performed in a Hewlett-Packard 6890N gas chromatograph, equipped with a fused silica capillary column DB-5MS (5% phenyl methyl siloksan, 30 m × 0.25 mm, film thickness 0.25 µm, Agilent Technologies, USA) and connected with a 5975B MSD mass selective detector (Agilent Technologies, USA). The injector and interface were adjusted for the operation temperature at 250 °C and 300 °C, respectively. It was operated under the following conditions: oven temperature program, 120 °C for 1 min; raised to 240 °C at the rate of 6 °C/min and then kept at 240 °C for 15 min; carrier gas, helium at flow rate of 15 cm/s; split ratio, 1/20 ml/min. Fatty acids were identified on the basis of their retention times (comparison with standards) and spectra using the searchable EI-MS spectra library.

Statistical Analysis

All experiments were performed in triplicate. The values are shown as means ± standard deviation. Computations were realized using Origin software package version 8.0.

## Results and Discussion

The results of the spectrophotometric analysis of the pomace extract are shown in Table 1. The applied spectrophotometric analysis provides fast information of the total phenol content in the tested extracts.

The results showed a significant phenol content in the tested extracts. Total anthocyanins were the most abundant, followed by hydroxycinnamoyl acids and flavonols. Anthocyanins originate from the colored samples i.e. grape skin which is part of the pomace. For both varieties, Merlot and Vranac, Katalinic et al. [7] found approximately the same total anthocyanins content in the skin extracts, around 739 mg/g FW. Iacopini et al. [15] reported that the total anthocyanins content of the skin extracts for 10 studied varieties ranged from 5.94 to 39.29 mg/g DW. Hydroxycinnamoyl acids and flavonols are the ingredients of grape skins and steams which are also parts of grape pomace.

In order to determine the phenol content and composition of the investigated extracts more precisely, the HPLC method was used. The results from Table 2 are in accordance with those obtained by spectrophotometric determination of phenolics (Table 1).

**Table 1.** Phenolic content determined by spectrometric methods and radical scavenging activity (EC50) of Vranac wine pomace

| Applied spectrophotometric analysis | Content |
|---|---|
| Total phenolic mg/g DM | 67.40 ± 0.38 |
| Hydroxycinnamoyl acids (mg/g DM) | 2.67 ± 0.05 |
| Flavonols mg/g DM | 1.89 ± 0.02 |
| Total anthocyanins (mg/g DM) | 17.90 ± 0.04 |
| Antioxidant activity - $EC_{50}$ (mg DM/ml) | 1.160 ± 0.03 |

Gallic acid was the most abundant acid present in the tested extracts. Other identified phenolic acids were trans-coutaric and caffeic acid, mainly present in skin and skins of grape pomace extracts. Quercetin-glucoside and rutin were the most abundant flavonols in these extracts, but luteolin-glucoside, kaempherol-glucoside and quercetin were also present.

**Table 2.** Phenolic composition of the pomace from Vranac, red grape variety, determined by HPLC analysis

| Phenolic compound (mg/g DM) | Content |
|---|---|
| Gallic acid | 3.33 ± 0.07 |
| *trans*-Coutaric acid | 0.41 ± 0.02 |
| Caffeic acid | 0.50 ± 0.04 |
| Quercetin glucoside | 0.11 ± 0.01 |
| Rutin | 0.15 ± 0.01 |
| Luteolin glucoside | 0.06 ± 0.02 |
| Kaempherol glucoside | 0.08 ± 0.01 |
| Quercetin | 0.05 ± 0.01 |
| (+)-Catechin | 3.84 ± 0.12 |
| Procyanidins | 10.46 ± 0.21 |
| (-)-Epicatechin | 1.22 ± 0.05 |
| Delphinidin-3-glucoside | 1.03 ± 0.02 |
| Cyanidin-3-glucoside | 0.27 ± 0.01 |
| Petunidin-3-glucoside | 1.24 ± 0.02 |
| Peonidin-3-glucoside | 1.11 ± 0.01 |
| Malvidin-3-glucoside | 4.36 ± 0.02 |
| Delphinidin-3-acetyl glucoside | 2.16 ± 0.02 |
| Delphinidin-3-p-coumoroyl glucoside | 2.01 ± 0.02 |
| Malvidin-3-ac. glucoside | 0.46 ± 0.01 |
| Cyanidin-3-p-coumoroyl glucoside | 0.82 ± 0.02 |
| Petunidin-3-p-coumoroyl glucoside | 0.61 ± 0.01 |
| Peonidin-3-p-coumoroyl glucoside | 0.46 ± 0.02 |
| Malvidin-3-p-coumoroyl glucoside | 7.15 ± 0.07 |
| ∑ Phenolic acids | 4.24 ± 0.09 |
| ∑ Flavan-3-ols | 15.52 ± 0.11 |
| ∑ Flavonols | 0.45 ± 0.04 |
| ∑ Anthocyanins | 21.68 ± 0.13 |
| ∑ Total phenolic compounds | 41.89 ± 1.43 |

Two flavan-3-ol monomers, (+)-catechin and (-)-epicatechin were detected in the pomace extract. We also detected a number of flavan-3-ol oligomers compounds, in a significant quantity. They showed similar UV absorbance spectra to these presented by (+)-catechin, (-)-epicatechin and (-)-epicatechin gallate and may be assigned to nonacylated procyanidins derived from (+)-catechin and (-)-epicatechin, and esterified procyanidins with gallic acid derived from (-)-epicatechin gallate [13-14]. Due to the lack of standards for these compounds we were not able to identify them individually. Their content in the extracts was expressed as procyanidins B2 equivalents. Flavan-3-ol monomers and procyanidins were mainly present in pomace seeds but also in pomace skins and steams in lower quantity.

Anthocyanin compounds had the participation of 51.75 % of the total phenolic content (Table 2). Malvidin-3-p-coumoroyl-glucoside and malvidin-3-glucoside were the main anthocyanidins found in pomace extracts followed by 3-glucosides, acetyl-glucoside and 3-p-coumoroyl-glucoside derivates of peonidin, delphinidin, cyanidin and petunidin (Figure 1).

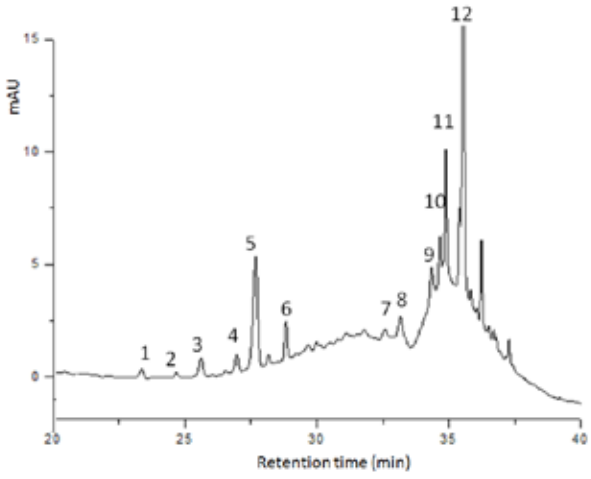


**Figure 1.** HPLC chromatogram of the grape pomace extract recorded at 520 nm: 1 delphinidin-3-glucoside, 2 cyanidin-3-glucoside, 3 petunidin-3-glucoside, 4 peonidin-3-glucoside, 5 malvidin-3-glucoside, 6 delphinidin-3-acetyl glucoside, 7 delphinidin-3-p-coumoroyl glucoside, 8 malvidin-3-acetyl glucoside, 9 cyanidin-3-p-coumoroyl glucoside, 10 petunidin-3-p-coumoroyl glucoside, 11 peonidin-3- p-coumoroyl glucoside and 12 malvidin-3-p-coumoroyl glucoside)

The radical scavenging activity of the investigated extracts was estimated by DPPH test. The results are shown in Table 1 and Figure 2, expressed as $EC_{50}$ value (mg/ml). The grape pomace extract showed a significant radical scavenging activity. The high linear correlation between different concentrations of grape pomace extracts and corresponding radical scavenging activities of these extracts ($R^2$=0.9732) suggests that the phenolic compounds, obviously present in the extract and certainly interact with DPPH radical, are at least partially

responsible for the strong radical scavenging activity of these extracts.

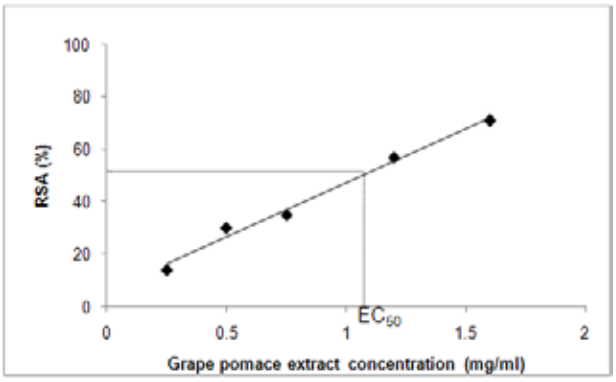


**Figure 2.** Radical scavenging activities (RSA) of the solutions of different grape pomace extract concentration

The analysis of the fatty acid composition of the pomace oil is performed on the GC/MS apparatus under conditions described in the experimental part. The mass fractions (%) of the identified fatty acids from the pomace oil are shown in Table 3.

**Table 3.** Fatty acid composition of the grape pomace

| Fatty acids | Formula | mass fractions (%) |
|---|---|---|
| Palmitic | $C_{16}H_{32}O_2$ | 6,6 |
| Linoleic | $C_{18}H_{32}O_2$ | 72,4 |
| Oleic | $C_{18}H_{34}O_2$ | 16,3 |
| Stearic | $C_{18}H_{36}O_2$ | 4,1 |
| Linolenic | $C_{18}H_{30}O_2$ | <0,1 |
| Palmitolinoleic | $C_{16}H_{30}O_2$ | <0,1 |

The most common fatty acids were linoleic, oleic, palmitic and stearic acid (Figure 3). The linolenic and palmolinoleic acids were present in the concentrations below 0.1%. The major fatty acid in the grape pomace oil was linoleic acid.

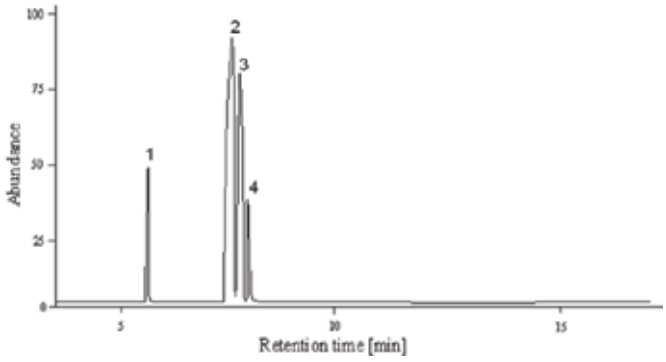


**Figure 3.** GC/MS chromatogram of the grape pomace oil - fatty acid composition: 1 palmitic acid, 2 linoleic acid, 3 oleic acid and 4 stearic acid

The fatty acid composition of the grape pomace are similar to the oils of safflower, sunflower, soybean, maize, cotton seed, popy and tobacco, which belong to the linoleic type [5].The grape pomace oil was rather poor in linolenic acid. Low levels of linolenic acid are desired in edible oils, because high levels of this fatty acid can produce an unfavourable odour and taste in oil. Furthermore, since linolenic acid is oxidised simply due to having three double bonds on its hydrocarbon chain, the stability or shelf-life of the oil rich in linolenic acid will be short [16, 17]. So, the grape pomace oil having low quantities of linolenic acid can be an advantage in terms of the human consumption and the shelf-life of the oil. The second abundant fatty acid in the pomace oil was oleic acid. Oleic acid, a monounsaturated fatty acid, also has great importance in terms of their nutritional implication and the effect on the oxidative stability of oils [18]. The degree of unsaturation in the grape pomace oil was over 88%, coming from unsaturated fatty acids. High levels of unsaturation play an important role in lowering high blood cholesterol and also in the treatment of atherosclerosis [19].

## Conclusion

The grape pomace extract had a high phenolic content. The most abundant phenolic class that was found in the grape pomace extract was anthocyanins followed by flavan-3-ols, phenolic acids (mostly hydroxycinnamoyl acids) and flavonols. As correlation calculations showed, phenol compounds are responsible for strong radical scavenging activities of the tested extracts. The grape pomace oil with a high degree of unsaturation plays an important role in human health. Due to their significant biological activities, the phenolic rich extract and the grape pomace oil can be used as additives in food and medicaments.

## Acknowledgments

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**Izvod**

# ODREĐIVANJE BIOLOŠKI AKTIVNIH SASTOJAKA KOMINE GROŽĐA SORTE VRANAC ZA POTENCIJALNU UPOTREBU U PREHRAMBENOJ I FARMACEUTSKOJ INDUSTRIJI

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(ORIGINALNI NAUČNI RAD)
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Proizvodnja vina generiše značajne količine otpada - komine (uglavnom semenke, pokožice i peteljke grožđa). U komini se i dalje mogu naći značajne količine bio-aktivnih supstanci kao što su fenolna jedinjenja. Fenolni sastav i antioksidativna aktivnost komine, dobijene u procesu vinifikacije crvene vinske sorte grožđa Vranac (*Vitis vinifera* L.), ispitivani su sa ciljem da se ustanove upotrebljivi (vredni) sastojci. Ekstrakti komine analizirani su spektrofotometrijskom i RP-HPLC metodom u cilju određivanja fenolnog sastava, a antioksidativna aktivnost određena je DPPH testom. Ulje dobijeno od komine podvrgnuto je GC/MS analizi kako bi se ustanovio njegov masnokiselinski sastav. Jedinjenja fenola, kako korelacioni proračuni pokazuju ($R^2$=0,9732), odgovorna su za jaku antioksidativnu aktivnost ekstrakata komine (1.160 ± 0,03 mg DM/ml). Ulje komine grožđa sadrži visok stepen nezasićenih masnih kiselina (preko 88%) i igra važnu ulogu za zdravlje čoveka. Ekstrakti bogati fenolnim jedinjenjima i ulje od komine grožđa mogu se upotrebiti kao aditivi u hrani i farmakološkim proizvodima, zbog značajne biološke aktivnosti koju pokazuju.

**Ključne reči:** komina grožđa, semenke grožđa, fenolna jedinjenja, antioksidativna aktivnost, masne kiseline.