Thermal Stability Study of the Grape Seeds Extracts in the Aqueous Solutions

Carmen POP, Ançuța M. ROTAR, Liana SALANȚĂ, Sonia SOCACI, Floricuța RANGA, Carmen SOCACIU*

Faculty of Food Science and Technology, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Address - 400372 - Cluj-Napoca, Mănășturi Street, number 3-5, Romania; *Corresponding author e-mail: carmen.socaciu@usamvcluj.ro

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ABSTRACT
This study was carried out to evaluate the effect of parameters the extraction process of grape seeds extracts on the bioactive compounds. The aqueous extracts were screened for total polyphenol content and total flavonoid content which were determined spectrophotometrically using a modified Folin-Ciocalteu method, respectively a chromogenic system of NaNO₂–Al(NO₃)₃–NaOH based method. The antioxidant activity was determined using DPPH method while their polyphenolic composition by means of HPLC-DAD- MS/ ESI(+)-analysis. Total phenols content and flavonoid content varied between 37.835 and 31.830 mg GAE/g, 23.420 and 17.645 mg QE/g respectively in Fetească Neagră seeds; between 24.265 and 27.065 mg GAE/g, 17.970 and 15.205 mg QE/g respectively in Fetească Regală seeds. All extracts showed remarkable DPPH radical-scavenging activity ranging from 94.110 to 95.515%.

The study revealed 14 phenolic compounds belonging to the following groups: flavan-3-ol monomers, proanthocyanidins, hydroxycinnamic acid and hydroxybenzoic acid derivatives. Quantitative differences among the varieties and the level of temperature applied of the extraction process were observed.

The results suggested that the heat treatment of grape seeds liberated phenolic compounds having a significant effect in increasing the amounts of active when a 90°C extraction temperature was used.

Keywords: grape seeds, antioxidant activity, phenolic contents, HPLC.

Abbreviations: GSER - Grape Seed Extract from Fetească Neagră; GSEW - Grape Seed Extract from Fetească Regală; DPPH - 2, 2 diphenyl-1-picrylhydrazyl;

INTRODUCTION
In recent years, special attention has been focused on the isolation of phenolics from different raw materials (medicinal plants, fruits, vegetables, industrial byproducts, and beverages) and on exploration of their potential benefits for human health (Volf et al., 2014).

The annual production of large waste quantities by the food processing industry creates serious environmental problems as a consequence of the absence of efficient policies regarding their disposal. Many processes are being established, targeting the conversion of waste materials into bio-fuels, food ingredients and other added value bio-products. Wine wastes, consisting mainly of skins, seeds and stems and representing 20% of the processed grapes weight, are considered as valuable coproducts due to their important phenolic compounds content (Rajha et al. 2014).

Grapes (Vitis vinifera L.) belong to world’s largest fruit crops. Since about 80% of the total amount is used in wine-making, the rest of 20% (about 10 million tons) of grape pomace arises
within a few weeks from the harvest campaign. The seeds constitute a considerable ratio of the pomace, amounting to 38-52% on a dry matter basis. The results of certain studies have indicated that the polyphenols present in grape seeds in significant concentrations could be classified into two groups: flavonoids and non-flavonoids (Ignat et al., 2011). The phenolic acid, gallic acid, and monomers catechin and epicatechin are the main polyphenolic compounds in grape seeds (Yilmaz and Toledo, 2004).

The most abundant phenolics isolated from grape seeds and skins are flavan-3-ols (catechin and epicatechin) and their oligomers and polymers (proanthocyanidins). The outer seed coat contains the majority of both the monomeric and polymeric flavan-3-ols (2 to 5 times more than the endosperm) (Godevac et al., 2010).

(+)-Catechin shows antioxidant activity in human plasma by delaying the degradation of endogenous α-tocopherol and β-carotene and by inhibiting the oxidation of plasma lipids. (+)-Catechin has hydroxyl, peroxy, superoxide and DPPH radical scavenging activities (Yilmaz and Toledo, 2004). (-)-Epicatechin is able to scavenge hydroxyl radicals, peroxy radicals, superoxide radicals, and DPPH radicals. (+)-Catechin and (-)-epicatechin have a peroxy radical scavenging activity 10 times higher than that of L-ascorbate and β-carotene when tested on bacteria. Gallic acid is a phenolic acid that can scavenge peroxy radicals and DPPH radicals and has also antifungal activity (Yilmaz and Toledo, 2004).

In the literature were described the various conditions (time, solvent, and the manner) for the extraction of polyphenols from grape seeds (Godevac et al., 2010).

Due to the acidic lability of interflavan linkages within proanthocyanidins and the susceptibility of polyphenols to oxidation, a valid extraction method should provide the complete as possible extraction of the polyphenolics while limiting their degradation. Methanol/ water or ethanol/water systems are the common solvents used for extracting polyphenols from grape seeds. In particular, lower molecular weight polyphenols, such as phenolic acids, anthocyanins, and flavanol monomers and oligomers, are well extracted with methanol, while the higher molecular weight flavanols are better extracted with aqueous solutions than with methanol (Godevac et al., 2010).

Recognition of the health benefits of catechins and procyanidins has led to the use of grape seed extract as a dietary antioxidant supplement. The main phenolic antioxidants can also be used to preserve food because of their protective effects against microorganisms. Phenolic antimicrobial compounds are found in grape seeds, skins and stem extracts (Jayaprakasha et al., 2003; Butkhup et al. 2010).

Based on numerous evidence of the strong biological activity of phenolics in grape seeds from Fetească Neagră and Fetească Regală, this study has been focused on determining the optimal parameters of extraction process in water and to investigate the influence of the extraction conditions on phenolic compounds.

**MATERIALS AND METHODS**

**Grape Seeds Material**

Grape seeds samples from two different species Fetească Neagră and Fetească Regală were obtained from Viticulture Center Pietroasa and were produced in the Dealu Mare (“Big Hill”) Wine Region.

The samples of Fetească Neagră are residues left after the wine was separated following fermentation of the crushed grapes for 10 days at 17°C. The residue included pulp, skin, and seeds; the seeds were separated from skin. Fetească Regală residue resulted after the pressing of the grapes prior to fermentation (Coldea et al., 2014); the seeds were also separated from skin. All seed material was dried in air at 28°C for 33 h and was grounded to a fine powder.

Aqueous extraction, was carried out on 15 g of dried material, using 100 mL distilled water at different temperatures (70, 80, 90 and 100°C) for 15 min. The seed sample was extracted three successive times for each temperature and then in cold water (4°C) in dark place at room temperature 25°C. The collected extracts were filtered using filter paper (Whatman No. 1). Filtrate was centrifuged at 6000g for 10 min (Hettich centrifuge, model Micro 22R; Tuttingen, Germany); the obtained Grape Seed Extracts (crude extracts) were kept in a freezer at (-20°C) until use.

**Determination of total phenolic content (TPC)**

The determination of the total phenolics content was performed using the Folin-Ciocalteu
Thermal Stability Study of the Grape Seeds Extracts in the Aqueous Solutions

reagent, according to Socaci et al. (2013); Muresan et al. (2012) and Kodama et al. (2010) with some modifications. A 0.25 mL aqueous extract obtained above were mixed with 0.12 mL of the Folin-Ciocalteu reagent and 1.8 mL of distilled water. After 5 minutes at room temperature, 0.34 mL of a sodium carbonate (Na$_2$CO$_3$) solution 7.5% were added and the mixture placed at room temperature for 2 hours. The absorbance was measured at 750 nm on a Shimadzu UV-1700 PharmaSpec spectrophotometer. A calibration curve was performed using different concentrations of standard gallic acid solutions ($r^2 = 0.9997$) and the concentration of TPC was expressed as mg GAE / g plant material.

Total flavonoid content (TFC)

Total flavonoid content was determined by the aluminum chloride spectrophotometric method according to Zhu et al. (2010). The samples' concentration was determined at 500 nm using a quercetin (0.006–0.800 mg/mL) standard curve ($r^2 = 0.9944$) and expressed as mg QE / g plant material.

Determination of the Free Radical Scavenging Activity by the 1,1-Diphenyl-2-picrylhydrazyl Free-Radical Scavenging Assay

The scavenging activities of the extracts on the stable free radical, DPPH, were assayed using the method adapted after Anesini et al. (2008).

A volume of 0.1 mL of an aqueous extracts were mixed with 0.9 mL distilled water and 3.9 mL methanolic DPPH solution. After 30 minutes incubation in darkness, the absorbance of each sample was measured at 515nm against a blank of methanol.

The percentage of DPPH was calculated by measuring the absorbance of the sample and applying the following equation: % of inhibition = $[1 - (As/A0)] \times 100$, where As is the absorbance of sample, and A0 is the absorbance of the DPPH solution and As is Gallic acid solutions of different concentrations were used as positive controls for antioxidant activity.

HPLC determination

Analysis of grape seeds extract was performed using a HPLC Agilent Technologies 1200 Series chromatograph coupled with DAD detector and MS single quadrupole Agilent 6110 detector. Eclipse XDB C18 column (Agilent), 5 μm, 150 x 4.6 mm was used. The gradient elution was performed with mobile phase A, composed of distilled water: 0.1% acetic acid/ Acetonitrile (99:1 v/v) and mobile phase B, comprising Acetonitrile: 0.1% Acetic acid (99:1 v/v), at a flow rate of 0.5 ml/min. A gradient system was applied as follows: 0-30 min, 5% B; 0-2 min, 5% B; 2-18 min, 40-90% B; 18-20 min, 90% B; 20-24 min, 90%- 5% B; 24-25 min, 5% B; 25-30 min. All samples and solvents were HPLC grade solvents, filtered through a 0.45-μM membrane (Millipore, U.S.A.). The chromatograms were monitored at 280 nm.

Quantification of the compounds was achieved using calibration curves obtained by HPLC of pure (+)-catechin (98%) standards from Sigma-Aldrich. The linearity plot is shown in Figure 1. As shown, the responses for the standard were strictly linear ($r^2=0.9993$) in the concentration range of 10-1500 μg/mL.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) from two replicates for each sample. An analysis of variance (ANOVA) of the data was performed using the SPSS 19.0 statistical analysis system, and a Duncan test with a confidence interval of 95 or 99% was used to compare the means. Differences were considered significant at P < 0.05.

RESULTS AND DISCUSSIONS

Effect of Extraction temperature on TPC, TFC and Antiradical activity

Statistical analysis of the mean reveals that Grape Seed Extract from Fetecasă Neagră at 90°C showed a higher content of total phenols (37.835 mg GAE/g), differing significantly (p< 0.05) from the other extracts. For the samples of

![Calibration curve of Catechin](image-url)
Fetească Regală, the total phenolic content varied between 24.265 and 27.065 mg GAE/g (Tab. 1). The levels of total phenols obtained in this study are higher than those reported in the literature for aqueous extracts, a possible explanation being the optimization of extraction method. Ignat et al. (2011) found about 5,0625 mg GAE/g when applying an extraction at 70°C.

Weidner et al. (2012) reported that the total content of phenolic compounds in acetonic extracts was higher than in methanolic extracts, the level of phenolic compounds was 29.7 mg/g of acetonite extract and 19.7 mg/g of methanolic extract.

Wang et al. (2005) using 70% methanol and 95% ethanol extracts observed that the phenolic compounds varied between 27.27 mg/g and 35.84 mg/g.

Kim et al. (2006) in their study, reported that the heat treatment of grape seeds liberated phenolic compounds, and thus increased the amounts of active compounds in extracts. Astill et al. (2001) and Chen et al. (2001) also reported the influence temperature on the extraction of phenolics and antioxidant activity.

The values of flavonoids in grape seeds extracts varied between 23.38 and 15.27 mg QE/g (Tab. 1.) were significantly different (p< 0.05) when considering all samples. Levels of the flavonoids present in grape seeds identified in literature data vary by species and the processing method of the samples.

Flavonoids extraction was reported to be affected by many parameters such as time, temperature and of the solvent extraction. Thus, for the samples analyzed, the highest concentration of flavonoids was determined for the GSER90 (23.38 mg QE/g), this sample being extracted at 90°C.

Rajha et al. (2014) showed that a 94°C thermal treatment had a positive linear effect on flavonoids. Many authors (Sheng et al. (2013); Rajha et al. (2014)) showed the effect of temperature on flavonoids extraction, they explained this better liberation of bioactive compounds from plant cells and the increase of molecular movement with high temperature. Regarding the time, it had a negative effect, after 76 minutes, the bioactive compounds have registered a decrease, probably due to the decomposition phenomenon observed with relatively extended extraction time.

The radical scavenging activity of samples were not significantly influenced by the heat treatments (P> 0.05) (Tab.1.). The results for the antioxidant activity ranged between 94.110 and 95.515%.

The power of certain antioxidants is associated with their reducing power (Jayaprakasha et al. (2001); Kim et al. (2006)). Duh (1998) reported that reducing properties of antioxidants are generally associated with the presence of reductions.

Identification and quantification of phenolic compounds by HPLC

The high-performance liquid chromatographic technique was developed to identify and quantify the major phenolic compounds present in the grape seed extracts. The chromatographic profiles of the samples are shown in Fig. 2. The qualitative analysis of the chromatograms did show clear differences between some analyzed samples, a variation in the intensities of the same signals could be observed in some samples. This difference

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**Tab. 1. Effect of heat treatments extraction on total phenolic contents and total flavonoid in the extracts**

<table>
<thead>
<tr>
<th>Samples</th>
<th>TPC (mg GAE/g Plant Material)</th>
<th>Flavonoids (mg QE/g Plant Material)</th>
<th>DPPH (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSER70</td>
<td>31.830±0.707c</td>
<td>22.340±0.170b</td>
<td>94.041±0.056a</td>
</tr>
<tr>
<td>GSER80</td>
<td>32.330±0.467c</td>
<td>19.610±0.509c</td>
<td>94.265±0.530a</td>
</tr>
<tr>
<td>GSER90</td>
<td>37.855±1.322a</td>
<td>23.420±0.057a</td>
<td>95.515±0.884a</td>
</tr>
<tr>
<td>GSER100</td>
<td>35.700±0.566b</td>
<td>17.645±0.318d</td>
<td>94.870±0.424a</td>
</tr>
<tr>
<td>GSEW70</td>
<td>26.695±0.233d</td>
<td>16.435±0.177e</td>
<td>94.755±0.191a</td>
</tr>
<tr>
<td>GSEW80</td>
<td>24.585±0.304e</td>
<td>15.440±0.028f</td>
<td>94.130±0.283a</td>
</tr>
<tr>
<td>GSEW90</td>
<td>27.065±0.559d</td>
<td>17.970±0.057d</td>
<td>94.585±0.191a</td>
</tr>
<tr>
<td>GSEW100</td>
<td>24.265±1.082e</td>
<td>15.205±0.092f</td>
<td>94.110±0.042a</td>
</tr>
</tbody>
</table>

Note: Each value represents the mean of two measurements± standard deviation.
Means within each column with same letters are not significantly different (P > 0.05).
Different letters within a column indicate very significant differences among formulation (P < 0.01).
Fig. 2. HPLC profile of grape seed aqueous extract
Identified compounds: 1 - Gallic acid glucoside; 2 - Gallic acid; 3 - Gentisic acid glucoside; 4 - p-Hydroxybenzoic acid; 5 - Resveratrol; 6 - Protocatechuic acid; 7 - Methyl gallate; 8 - Proanthocyanidin dimer; 9 - Proanthocyanidin dimer; 10 - Catechin; 11 - Caffeic acid; 12 - Epicatechin; 13 - Proanthocyanidin trimmer; 14 - Proanthocyanidin dimer monogallate.
concerning the content of phenolic compounds were significantly influenced by different heat treatment in the extraction process (Tab.2).

HPLC analyses were carried out to provide a quantitative measurement of the phenolic profiles (Tab.2.), expressed in mg/ g on a dry weight basis.

The HPLC method was useful to identify the main phenolic markers, specific for plant material and by-products from wine processing. By comparison with standard mixture of phenolics it could determine quantitatively the major phenolics concentration (Coldea et al., 2011).

The identified phenolic compounds in the samples could be classified into the following groups: flavanol monomers (catechin and epicatechin), proanthocyanidins, flavonols, hydroxycinnamic acids, and hydroxybenzoic acid derivatives (Tab. 2, Fig. 2.).

The major compounds identified were gallic acid, catechine, epicatechin, caffeic acid, proanthocyanidin trimmer and proanthocyanidin dimer monogallate. The content of compounds in grape seeds extracts is dependent on the temperature extraction and variety.

A higher content of gallic acid (1.565 mg/g); catechine (1.559 m/100 g); epicatechin (2.866 mg/g); caffeicacid (3.054mg/g); proanthocyanidin trimer (1.878) and proanthocyanidin dimer monogallate (1.694) was identified in sample GSER90, grape seeds from Fetească Neagră, extracted at a temperature of 90°C.

Ignat et al. (2011) reported that the major compounds identified in the aqueous grape seed extract were gallic acid (6.12 mg/100 g) and catechine (44.36 mg/100 g).

Based on numerous literature sources, the most important compounds found in grape seed extracts were gallic acid, catechine and epicatechine, which were significantly different depending on variety and area. For the analyzed varieties (Fetească Neagră and Fetească Regală) were not reported studies in the literature.

Gođevac et al. (2010) reported that the range of free gallic acid varied from 4 to 23 mg per 100

| Peak | t<sub>r</sub> (min) | [M-H]<sup>-</sup> (m/z) | Compound Description | GSER 70 | GSER 80 | GSER 90 | GSER 100 | GSEW 70 | GSEW 80 | GSEW 90 | GSEW 100 |
|------|---------------------|--------------------------|----------------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| 1    | 4.5                 | 333, 171, 155            | Gallic acid glucoside| 0.161   | 0.164   | 0.164   | 0.158   | 0.171   | 0.171   | 0.172   | 0.171   |
| 2    | 5.7                 | 171, 155                 | Gallic acid          | 1.395   | 1.542   | 1.565   | 1.511   | 0.813   | 0.894   | 0.950   | 0.881   |
| 3    | 7.4                 | 317, 156                 | Gentisic acid glucoside | 0.285  | 0.297   | 0.310   | 0.266   | 0.263   | 0.268   | 0.278   | 0.259   |
| 4    | 8.0                 | 139                     | p-Hydroxybenzoic acid | 0.195   | 0.212   | 0.215   | 0.210   | 0.194   | 0.201   | 0.216   | 0.214   |
| 5    | 8.3                 | 229                     | Resveratrol          | 0.197   | 0.204   | 0.210   | 0.180   | 0       | 0       | 0       | 0       |
| 6    | 8.9                 | 155                     | Protocathecuic acid  | 0.263   | 0.278   | 0.282   | 0.247   | 0.194   | 0.204   | 0.207   | 0.200   |
| 7    | 10.1                | 185, 155                | Methyl gallate      | 0.530   | 0.552   | 0.556   | 0.545   | 0.521   | 0.533   | 0.545   | 0.506   |
| 8    | 11.3                | 579                     | Proanthocyanidin dimer | 0.838  | 0.650   | 0.657   | 0.650   | 0.616   | 0.643   | 0.646   | 0.615   |
| 9    | 12.1                | 579                     | Proanthocyanidin dimer | 1.036  | 1.080   | 1.094   | 1.077   | 0.745   | 0.783   | 0.847   | 0.799   |
| 10   | 12.5                | 291                     | Catechin             | 1.421   | 1.520   | 1.559   | 1.420   | 1.226   | 1.345   | 1.356   | 1.225   |
| 11   | 13.1                | 181, 183                | Caffeic acid        | 2.878   | 3.009   | 3.054   | 2.732   | 1.674   | 1.725   | 1.736   | 1.579   |
| 12   | 13.9                | 291                     | Epicatechin         | 2.581   | 2.780   | 2.866   | 2.781   | 2.035   | 2.254   | 2.286   | 2.079   |
| 13   | 14.1                | 867                     | Proanthocyanidin trimer | 1.706  | 1.776   | 1.878   | 1.846   | 1.408   | 1.438   | 1.508   | 1.352   |
| 14   | 14.5                | 731                     | Proanthocyanidin dimer monogallate | 1.538 | 1.632   | 1.694   | 1.670   | 1.264   | 1.291   | 1.354   | 1.185   |
g of grape seeds from some grape cultivars grown in Serbia.

The bioactive compounds of the grape seeds extracts were analyzed for the their applications in matrix of edible film because of their protective effects against microorganisms.

**CONCLUSION**

The results of the present study indicate that the grape seeds represent a valuable natural source of phenolic compounds. The aqueous extract seems to contain higher amounts of gallic acid, catechins, epicatechin, caffeic acid, proanthocyanidin trimer and proanthocyanidin dimer monogallate. These data suggest that the heat treatment of grape seeds liberated phenolic compounds, and thus increased the amounts of active at 90°C, for both varieties analyzed.

After these screening experiments, further work will be performed to describe the antimicrobial activities in more detail and their application to obtain the active edible film.

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**REFERENCES**


