POLYPHENOLS ISOLATION AND DETERMINATION IN GRAPE SEEDS BY HPLC/DAD

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Abstract. *Polyphenolic compounds were determined from an extractive solution of grape seeds (Black Magic variety). Separation of the phenolic compounds was done by an adapted USP30 HPLC method. The following phenolic compounds were founded: ellagic acid, gallic acid, E-resveratrol, chlorogenic acid, felluric acid, vanillin and caffeic acid.*

The highest amount of phenolic compounds in grape seeds reaches the value of 2312.737 mg ellagic acid/100g dry seeds of Black Magic grape variety

Keywords: *grape seeds, HPLC/DAD method, phenolic compounds, E-resveratrol.*

1. INTRODUCTION

Grapes (*Vitis vinifera* L. ssp. *sativa*) are one of the most wide-spread fruits crops in the world and they are used for various purposes since antiquity. Pharmaceutical industry is using grapes skin and grapes seeds extracts due to curative properties of polyphenols, resveratrol in particular.

Procyanidins from grape seeds extracts were studied in a keen matter in order to find the potentially curative effects on human health [1-2]. Recent reports indicate a wide range of curative effects such as: antioxidant and radioprotective [1], cataract prevention [2], antihyperglycemic [3], regulator of postprandial lipemia [4], regulator of antioxidant enzyme systems capacity [5], improvement of sensitivity to insulin and prevention of hypertriglyceridemia [6], inhibition and suppression of aromatase activity [7], inhibition of the activity of protein-kinase, decreasing of atherosclerosis and coronary heart disease, anti-inflammatory [8], protective against oxidative damage of brain cells [9]. Marc (byproduct of wine production) capitalization represents an important side of residue management in order to obtain useful products [10-12].

Following all the above issues the paper presents original results obtained during the investigations about the separation and determination of polyphenols in alcoholic extracts of grape seeds.

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2. MATERIALS AND METHODS

2.1. MATERIALS

The grapes (Black Magic variety) were collected in September 2015 from Murfatlar City, Constanta County, Romania.

From 1500 g grapes resulted 8.94 g seeds which were cleaned with water and dried in oven (6 hours at 40°C) until constant weight was archived. Finally resulted 6.71 g dried grape seeds weighted with an accuracy of 0.01 g.

All used reagents were of HPLC reagent grade.

2.2. METHODS

Extraction of phenolic componds from grape seeds

6.71 g of dried grape seeds powder was refluxed 6 h with 67 mL ethanol: distilled water 1:1 (v/v). [13]

The resulted solutions were filtered through Millipore membrane.

HPLC analysis of phenolic compounds

The resulted extractive solution was analized by HPLC.

Adapted USP30 HPLC method [14] was used for separation, identification and quantification of the phenolic compounds.

The identification and quantitative determination of phenolic compounds was performed using HPLC system (Agilent 1200) with quaternary pump, DAD, auto sampler. Separation was carried out on Zorbax Eclipse XDB-C18 column: 250 mm, 4,6 mm; 5 μm (Agilent Technologies). The gradient elution was accomplished using phosphoric acid 0.1% in water (solvent A) and acetonitrile (solvent B) as presented in Table 1.

Table 1. The gradient of elution solvents

No.	Time (min.)	Solvent A, %	Solvent B, %
1.	0-13	90	10
2.	13	78	22
3.	13	78	22
4.	14	60	40
5.	17	60	40
6.	17.5	90	10
7.	22	90	10

The parameters of chromatographic process were:
− the flow rate - 1.5 mL/min;
− the injection volume - 20 μL;
− the analysis time - 22 minutes.

Quantification of phenolic acid was performed using absorbance measurements at 310 nm and 35°C. The retention times and DAD spectra were compared to available authentic standards.

A mixture of standard solutions in 70% methanol having the next concentrations it was used:

E – resveratrol = 37 mg/mL, Z – resveratrol = 0.22 mg/L, caffeic acid = 0.36 mg/mL, chlorogenic acid = 0.37 mg/mL, cinnamic acid = 0.58 mg/mL, vanillin = 0.42 mg/mL, gallic acid = 0.39 mg/mL, ferulic acid = 0.48 mg/mL, 3-methylgalic acid = 0.34 mg/mL, ellagic acid = 0.43 mg/mL, *p*-coumaric acid = 0.51 mg/mL.

Previously, the retention times of standard solutions have been determined (table 2). Standard deviations of retentions time were obtained after statistical processing of the 6 injections (soft SPSS 10). The retention times were between 0.990±0.025 minutes for gallic acid and 15.867±0.007 minutes for cinamic acid.

Table 2. The retention time of standards.

No.	Standards	Retention time ± SD
1.	galic acid	0.990 ± 0.025
2.	3-*o*-methylgalic acid	2.606 ± 0.008
3.	chlorogenic acid	3.501 ± 0.015
4.	caffeic acid	4.598 ± 0.036
5.	vanillin	6.919 ± 0.051
6.	*p*-coumaric acid	7.187 ± 0.019
7.	feluric acid	8.565 ± 0.058
8.	*E* - resveratrol	14.467 ± 0.017
9.	ellagic acid	15.303 ± 0.027
10.	*Z* - resveratrol	15.751 ± 0.058
11.	cinnamic acid	15.867 ± 0.007

(*standard deviation for six injections)

Z – resveratrol was obtained from E – resveratrol exposed 12 hours at UV 254 nm radiation, and the Fig. 1. presents the chromatogram.
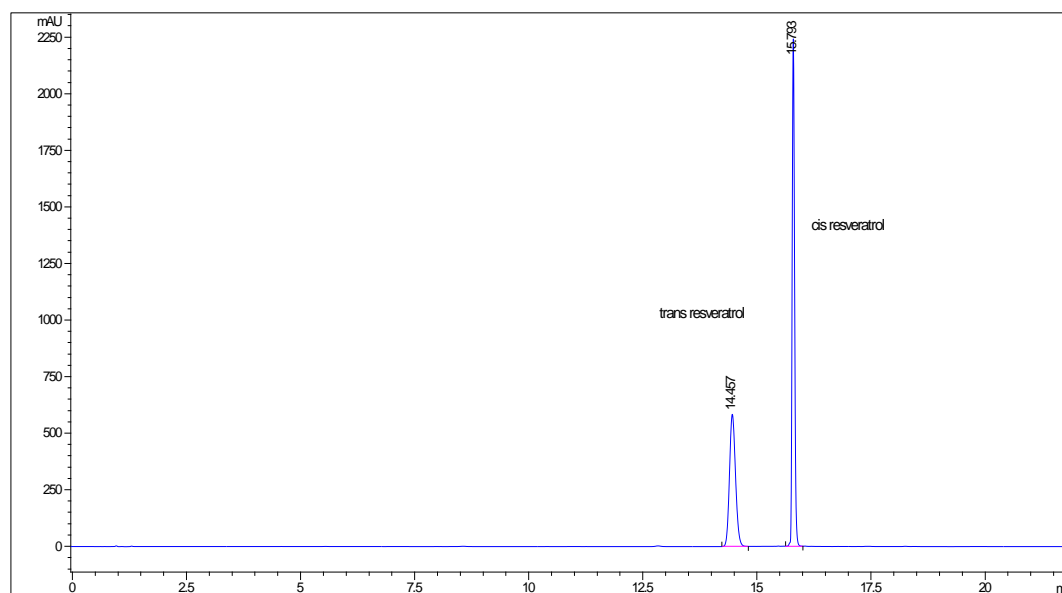
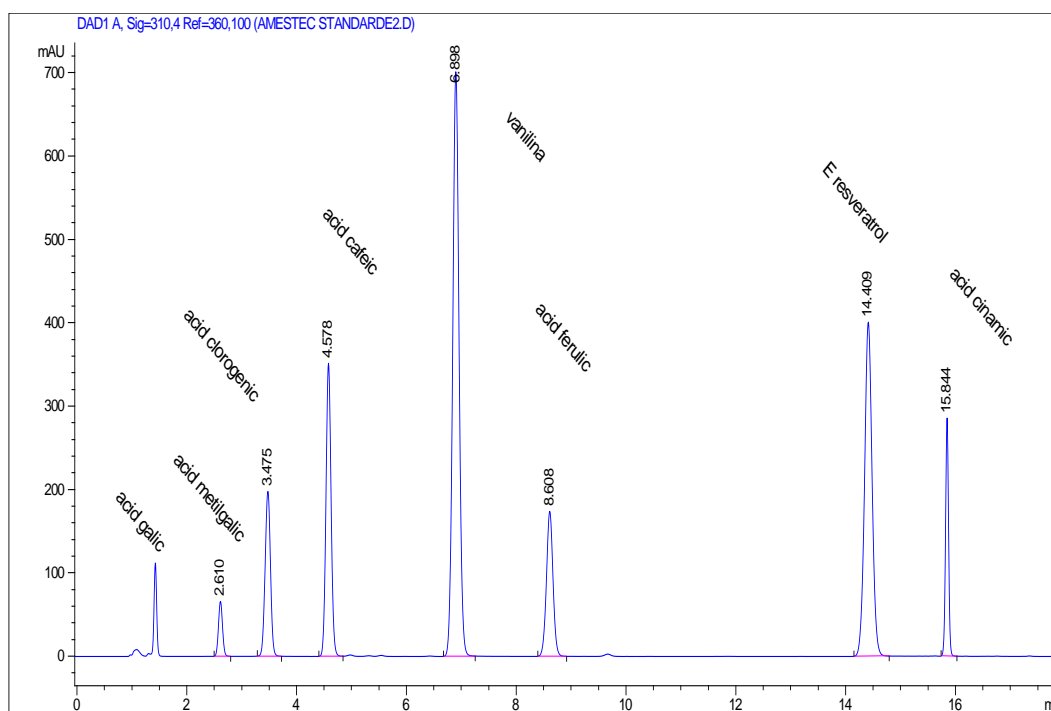**Figure 1. HPLC chromatogram of resveratrol after exposure UV 254 nm radiation.**

Figure 2. HPLC chromatogram of standards mixture.

Identification and quantitative determination of the active constituents from grape seeds extractive solution was done by comparing the chromatogram of standards mixture (Figure 2) with grape seeds extractive solution chromatogram (Figure 3). Reproducibility of the method was appreciated by the correlation coefficient (r^2) of calibration curves for each compound (Table 3).

Table 3. Corelation coefficients of calibration curves.

No	Phenolic compound	Corelation coefficient r^2
1.	*E*-resveratrol	0.99965
2.	*Z*-resveratrol	0.99729
3.	chlorogenic acid	0.99999
4.	caffeic acid	0.99619
5.	cinnamic acid	0.99845
6.	vanillin	0.99691
7.	galic acid	0.99537
8.	feluric acid	0.99863
9.	ellagic acid	0.99885
10.	*p*-coumaric acid	0.99798
11.	3-methylgalic acid	0.99563

3. RESULTS AND DISCUSSION

3.1. RESULTS

HPLC analysis of phenolic compounds

The extractive solution of grape seeds was analized by HPLC.

Identification and quantitative determination of phenolic compound from analized sample was done by comparing the chromatogram of standards with sample chromatogram.

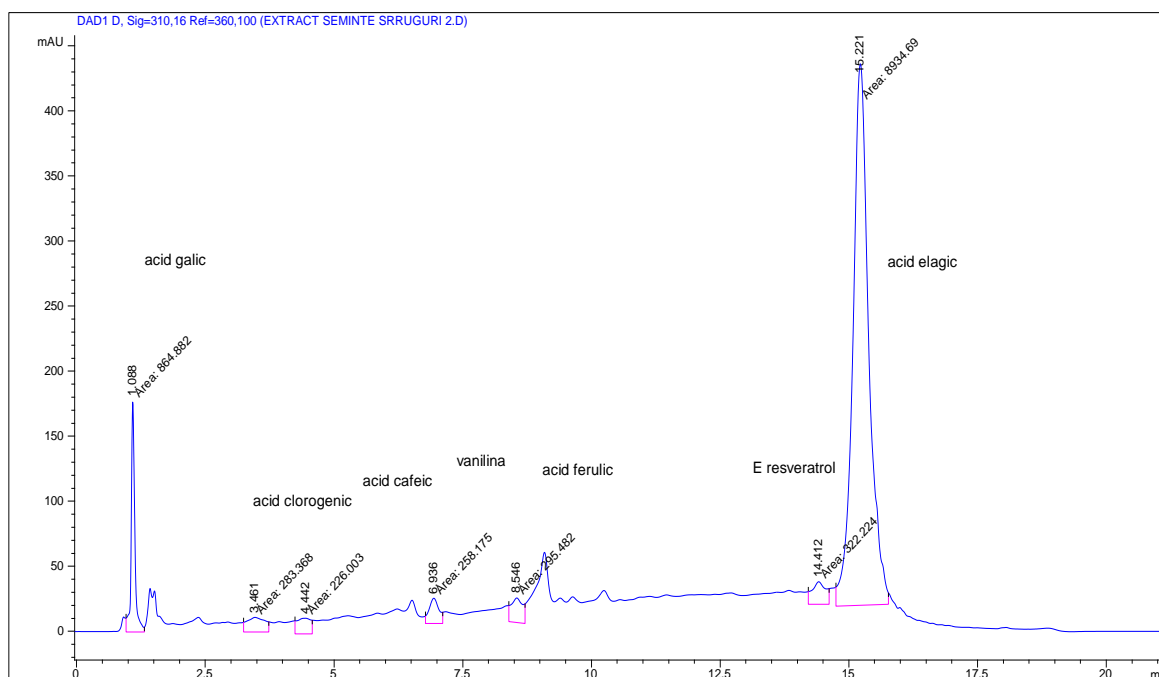The sample chromatograme is presented in Figure 3.

Figure 3. HPLC chromatogram of grape seeds extractive solution.

In Table 4 are presented the concentrations of phenolic compounds identified in the analized grape seeds extractive solution.

Table 4. Phenolic compound concentrations (mg/100 g grape seeds) in grape seeds of Black Magic variety.

No.	Compound	Concentration mg/100 g dry seeds
1.	chlorogenic acid	20.062
2.	caffeic acid	5.868
3.	gallic acid	294.437
4.	vanillin	7.027
5.	feluric acid	16.334
6.	ellagic acid	2312.737
7.	*E*-resveratrol	43.906

3.2. DISCUSSION

The sample chromatograme indicated high concentration of ellagic acid, gallic acid, chlorogenic acid and felluric acid calculated per 100 g grape seeds of Black Magic variety. It's well known also that felluric acid is precursor of caffeic acid and vanillin. In the studied sample was notice also a high content of *E*-resveratrol known for its precious health effects [15-18]

The rich content of grape seeds extractive solution in ellagic acid, gallic acid and *E*-resveratrol indicates to be a truthful source with antiproliferative, antioxidant, anticancer, cardiopreotectoare and tonics of central nervous system effects.

4. CONCLUSIONS

Appling HPLC analysis on the extractive solution of grape seeds (Black Magic variety), many phenolic compounds were separated from which seven were identified.

The seven phenolic compounds are listed as follows taking in consideration the decreasing order of concentration in the extractive solution expressed in mg/100g dry seeds: ellagic acid, gallic acid, *E*-resveratrol, chlorogenic acid, felluric acid, vanillin and caffeic acid.

The data indicates that from all identified phenolic compounds in the studied extract, the highest content is of ellagic acid, gallic acid and *E*-resveratrol while smallest content is on chlorogenic acid, felluric acid, caffeic acid and vanillin.

The presence of these phenolic compounds in grape seeds of Black Magic variety attests their curative properties.

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