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STUDY OF THE EXTRACTION OF BIOACTIVE COMPOUNDS FROM THE WINEMAKING WASTE OF THE CABERNET SAUVIGNON VARIETY

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Abstract

The by-products of winemaking have a high impact on the environment, due to their biochemical behaviour. This study aims to extract bioactive compounds from the marc obtained from the winemaking of Cabernet Sauvignon variety from 2019 vintage. Extraction of bioactive compounds was performed using a solvent under various experimental conditions: the traditional method of mechanical stirring, ultrasonic assisted extraction (UAE) and microwave assisted extraction (MAE). In this study, total polyphenols and anthocyanins in the marc result of winemaking of the Cabernet Sauvignon variety were determined by Folin - Ciocalteu method and by ITV method. Resveratrol (cis- and trans-resveratrol) was also identified and quantified by the HPTLC-UV densitometry and confirmed by the ESI-MS method. Trans- and cis-resveratrol were identified in the two extractions and only cis-resveratrol could be quantified at a concentration of 9.75 µg/ml from UAE extraction and 5.94 µg/ml by MAE.

Key words*: bioactive compounds, resveratrol, polyphenols, UAE, MAE.*

INTRODUCTION

Grapes are rich in phenolic compounds, being important for human health having antioxidant, anti-inflammatory, antimicrobial activity (de Lange et al., 2003; Gaziano et al., 1993; Kuulasmaa et al., 2000; Renaud & de Lorgeril, 1992; Stoclet et al., 2004; Tjonneland, Gronbaeck, Stripp, & Overvad, 1999). There are also studies with the beneficial effects of these compounds in the heart and other chronic diseases. At 2019, the vineyards worldwide reached a total area (including areas not yet in production) of 7,400,000 ha, global grape production of 78,000,000 tons and global wine production (excluding juice and musts). 292.000.000 hl. (OIV 2019). Wine production generates significant quantities of waste. These wine wastes have a gross residual biomass (that is residues from winemaking marc and yeast, branches or leaves) (Neamţu, 1983; Neamţu et al., 1983 and 1986; Ruberto et al., 2007; Brist et al., 2014).

From fresh, unfermented pomegranate resulting from the pressing of red grapes, intensely colored, dye substances can be extracted by diffusion, and the solution obtained is concentrated and then used as food coloring (Haslam et al.,1996; Pulido et al., 2000; Moure et al., 2001; Makris et al., 2007; Campos et al., 2008).

Taking into account the percentage of by-products (marc, yeast and tartrate) obtained in the wine making process, which, in some cases, is evaluated at 18 ÷ 20%, and estimates that the rate of grape seeds in marc is 18 ÷ 25% (the rest it has liquid remnants and 55 ÷ 65% skins), this process is cost-effective, only if significant quantities of marc are collected from wine producers. In the world, the processing of grape brands is done entirely, mainly grape seeds - to obtain oil and skins for the food industry and natural dyes (Moţa et al., 2017). From a biochemical point of view, resveratrol is a stilbene (the main stilbene in grapes), belonging to the class of non-flavonoid polyphenols (for example, curcumin or lignans belongs too)

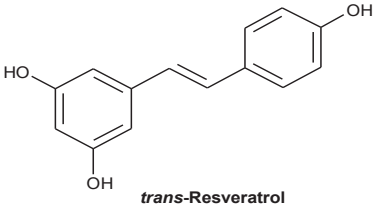

Figure 1. Structure of *trans*-resveratrol

It has been found that some plants synthesize it in response to different stressful circumstances (injury, exposure to high doses of ultraviolet radiation, fungal parasites, etc.). It is found in both stereo-isomeric forms (*cis* - in very small quantities and *trans*-resveratrol most present) and is liposoluble (Geana et al., 2011).

This study aims to extract and bioactive compounds from the mark obtained from the winemaking of the Cabernet Sauvignon variety from the 2019 vintage.

In this study it will be establish which method of resveratrol extraction is more suitable for Cabernet Sauvignon by-products, resulted from wine production (marc). For that, it will be compared two extraction methods, MAE (Microwave Assisted Extraction) and UAE (Ultrasound Assisted Extraction), using the optimized parameters of each method, established elsewhere (Cho, 2006; Ghafoor, 2009; Wang, 2012; Soural, 2015; Garcia, 2016; Pezzini, 2018).

It will be analysed the efficiency of these methodologies concerning total phenolics (TP) and resveratrol content, followed by antioxidant activity (AA) evaluation.

MATERIALS AND METHODS

The marc was harvested in Sept 24th 2019, brought to the laboratory immediately and frozen at -20° C to avoid oxidation. Samples were oven dried, to a constant moisture of about 4-5%. The dried marc was then separated manually into its components, seeds, skins and stalks, by means of sieves. The skins, that would have been finally extracted, were milled by a mortar to obtain a fine powder with a medium particle size of 0.8 mm (Casazza, 2010).

The extraction of polyphenols and anthocyanins from marc was performed by the traditional method ITV: maceration for one hour of 50 g of marc in 85 ml of 1% HCl solution and 15% ethyl alcohol, after which it was centrifuged at 3000 RPM from the clear solution (Ţârdea, 2007). The determination of the anthocyanin content from Cabernet Sauvignon marc was used by the ITV spectrophotometric method with its reading at 520 nm (Rockenbach, 2011; Rivas-Gonzalo, 1992), and the total content of polyphenolic compounds (CTCF) was determined by the Folin-Ciocalteu method and by the enzyme method with the BS200 analyser. (Fogarasi *et al.*, 2018; Ratola *et al.*, 2004; Singh *et al.*, 2015; Vincenzi *et al.*, 2013; Weiskirchen & Weiskirchen, 2016).

In this study, catechins, monomeric flavonolic units were determined by the reaction method of catechins with vanillin and are based on the reaction of the floroglucinol cycle with vanillin, producing red color, stable in concentrated H_2SO_4 and HCl solutions. Tannins are more or less polymerized flavonol (procyanidine) chains in leaves and strings. The leuco-anthocyanidins method for determining tannins is based on the property of tannins to be transformed into hot and strongly acidic medium (concentrated HCl) in cyanidine, which has a red color. The determination of catechins in wine was done by the method in which vanillin reacts with floroglucinol and produces a red color and is read at 500 nm wavelength. (Ţârdea, 2007; O.I.V, 1990; Ough, 1988; Ribereau-Gayon, 1965)

Antioxidant activity was determined at marc using DPPH-1,1-diphenyl-2-picryl hydrazyl (Sigma) 25 mg/1000 ml methanol (solution A), 1:10 of solution A = solution B, 100 µl of solution B together with 2 ml of marc extract in methanol solution, it was incubated for 30 minutes at t = 25°C. The free radical scavenging activity using the free radical DPPH• reaction was evaluated by measuring the absorbance at 515 nm after 60 min of reaction at 20°C in a spectrophotometer (Specord 205). Antioxidant activity represented by the amount of antioxidant needed to decrease the initial DPPH radical concentration by 50%. This value is called the "effective concentration" or EC50 value (Tarola et al., 2019). It was determined at a spectrophotometer at wavelength 515 nm the initial absorbance and the one after 30 minutes.

$$\% \text{ DPPHrem} = [A_{t0min} - A_{t30min}/A_{t0min}] \times 100$$

A_{t0min} - Absorbance before incubation
A_{t30min} - Absorbance after incubation

Extraction of bioactive compounds - *trans*-resveratrol and *cis*-resveratrol from marc was done by two methods: ultrasound extraction (UAE - Ultrasound - Assisted Extraction

with HIELSCHER UIP1000h DT) and microwave - assisted extraction (MAE - Microwave - Assisted Extraction with MILESTONE NEOS-GR). HPLC and HPTLC were used to determine resveratrol. Sample processing was performed by liquid-liquid extraction.

To a 250 ml Erlenmeyer beaker was added a volume of sample to be analysed (Vprob) and a volume of mixture of organic solvents [cyclohexane-n-pentanol 3: 7 (v/v) - Vsolv] (Table 1). The solution was stirred (200 RPM) at room temperature for two hours. The two phases were then decanted from the separating funnels after 24 hours to ensure efficient separation. The organic solvent mixture was removed by vacuum evaporation in a Heidolph rotory evaporator (bath temperature 80°C) to obtain a dry extract. Preliminary studies have shown that pure trans-resveratrol does not degrade at 80°C. The dried organic phases were then taken up in 2 ml of 95% ethanol (Rabesiaka et al., 2011).

Table 1. Sample volume and volume of mixture of organic solvents for liquid-liquid extraction processing

Sample processed	V_{sample} [ml]	V_{solv} [ml]
Cabernet Sauvignon_U_UAE_10:1	8	21
Cabernet Sauvignon_M_MAE_10:1	7.2	14

Vsample - the volume of sample processed; Vsolv - volume of a mixture of organic solvents [cyclohexane-n-pentanol 3: 7 (v/v)].
Cabernet Sauvignon_U_UAE_10:1 - sample of marc extracted by the ultrasonic method, 10:1- ratio between solvent and dry matter.
Cabernet Sauvignon_M_MAE_10:1 - sample of marc extracted by the microwave method, 10:1- ratio between solvent and dry matter.

Resveratrol was separated, identified and quantified by thin layer chromatography high performance (HPTLC) *High-Performance Thin Layer Chromatography* coupled with UV densitometry, taking into account the following experimental conditions (Agatonovic-Kustrin et al., 2015; Babu et al., 2005; Király-Véghely et al., 2013; Lopez et al., 2007; Lotz & Spangenberg, 2016):

- stationary phase: HPTLC silica gel G 60 F254, preformed glass plates 20 × 10 cm (Merck, Darmstadt, Germany);
- stationary phase pre-washing: chloroform – methanol mixture (1: 1, v/v);
- activation of the stationary phase: drying in the oven (110ºC, 30 minutes);
- mobile phase: mixture of toluene-ethyl acetate-formic acid (7: 3: 1, v/v/v), 20 ml in chromatographic graph, with supersaturation for 20 minutes, at 25ºC;
- standard (reference): methanolic solution 200 µg/ml of *trans*-resveratrol;
- the samples to be analysed: two extracts from the Cabernet Sauvignon marc, previously processed by liquid-liquid extraction;
- the migration distance: 62 mm (the sample application line set to 8 mm, and the solvent front set to 70 mm);
- the volumes applied to the starting line: 2, 3, 4, 5 and 6 µl for the standard solution (calibration curve), respectively 2, 4 and 6 µl for the two samples to be analysed;
- the samples to be analyzed were applied by spray, in the form of strips with a length of 8 mm, using the semi-automatic system CAMAG Linomat 5 (CAMAG, Muttenz, Switzerland): gas-air spray, syringe volume - 100 µl, syringe solvent - methanol/ethanol, application rate - 150 nl/s (methanol) and 100 nl/s (ethanol), pre - dose volume - 0.2 µl;
- drying of the chromatographic plate, after development: 5 minutes, at 25°C (with the help of a cold air blower);
- examination (detection): in UV (for photographing the chromatographic plate - λ 254 nm/366 nm (and at λ 320 nm (Figure 2), for obtaining densitocharts), without derivatization (respectively chemical treatment), using the photodensitometer CAMAG TLC Scanner 3: scan speed of chromatographic plate - 20 mm/s, resolution - 100 µm, lamp - deuterium and tungsten, measurement mode - absorption.

For analysis by electrospray ionization (ESI - Electrospray Ionization) coupled with mass spectrometry (MS - Mass Spectrometry), the samples were eluted directly from the chromatographic plate, using the TLC – MS 2 CAMAG interface coupled with the Waters 1525 binary pump. The elution was pure methanol. For the molecular ion detection m/z, the Waters AcquityQDa mass detector in negative mode (ESI–) was used. The source temperature was 120ºC and the capillary was 250ºC. The cone energy was 10 V for *cis*-resveratrol and 15 V for *trans*-resveratrol, and the capillary energy was 0.8 kV. A range of 100–500 M / Z was used (Careri et al., 2004; Chen et al., 2009; Flieger et al., 2017; Mark et al., 2005).

The solvents used for HPTLC (LiChrosolv® purity) analysis and the *trans*-resveratrol standard were sourced from Merck - Millipore (Darmstadt, Germany).

RESULTS AND DISCUSSIONS

The phenolic compounds of the grapes are responsible for the color and flavor of the red wines. In 2019, the parameters studied in red grapes were improved greatly due to the climatic conditions, without rains during the ripening period, the grapes besides sugars also accumulated total polyphenols and anthocyanins (Tănase, 2019).
Table 1 presents the polyphenolic potential of grapes at harvest, detecting the total polyphenols, anthocyanins, tannins, catechins content of the resulting marc and the total anthocyanin potential.

Table 1. Phenolic composition of marc obtained from Cabernet Sauvignon grapes at the time of harvest

Harvest date	Variety	The polyphenolic potential of grapes at harvest					
		PFT	PAT	T	C	A	V/La
Sept 24th 2019	Cabernet Sauvignon	191	1291	3.99	0.817	143	0.694

PFT - total polyphenols content mg GAE/l
PAT - total polyphenolic potential mg/kg
C - catechins, mg/l T - tannins, mg/l
A - anthocyanins, mg/l
V/La - degree of tannin polymerization

The results showed that Cabernet Sauvignon marc had an polyphenolic content of 191 mg GAE/l, which represents almost 10% of the total polyphenol content of the wine. Anthocyanins content 143 mg/l also represents almost 1/3 of the anthocyanin concentration of wine (Geana, 2014). The concentration of tannins retained in marc was by 3.99 mg/l.
The total polyphenolic potential registred by the Cabernet Sauvignon variety was 1291 mg/kg marc. The antioxidant activity of Cabernet Sauvignon marc was 8.93%, having a high capacity to eliminate the free hydroxyl radical (Ginjom, 2010).
Data obtained from HPTLC - UV densitometry analysis, with ESI - MS confirmation, about the resveratrol content of the two extracts obtained by ultrasound extraction (UAE – Ultrasound - Assisted Extraction) and microwave assisted extraction (MAE - Microwave - Assisted Extraction).

Resveratrol was separated, identified and quantified by thin layer chromatography high performance (HPTLC) (Figure 1). The examination (detection) was: in UV (for photographing the chromatographic plate - λ 254 nm/366 nm (Figure 1) and at λ 320 nm (Figure 2), for obtaining densitocharts, without derivatization (respectively chemical treatment), using the photodensitometer CAMAG TLC Scanner.

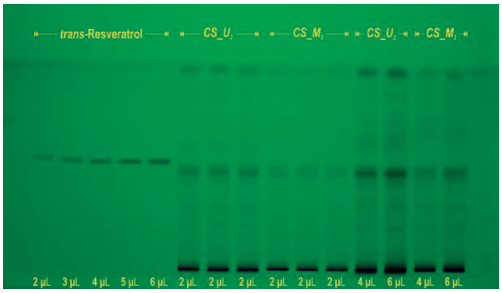

Figure 1. HPTLC chromatochart obtained by analyzing the resveratrol content of the two Cabernet Sauvignon marc extracts. UV photography, λ 254 nm, without derivatization. CS_U: Cabernet Sauvignon Extract_U_2_UAE_10: 1; CS_M: Cabernet Sauvignon Extract_M_2_MAE_10: 1

In Figures 1 and 2 it was observed that *trans*-resveratrol after extraction by both methods (ultrasound and microwave) is detected only qualitatively and not quantitatively.

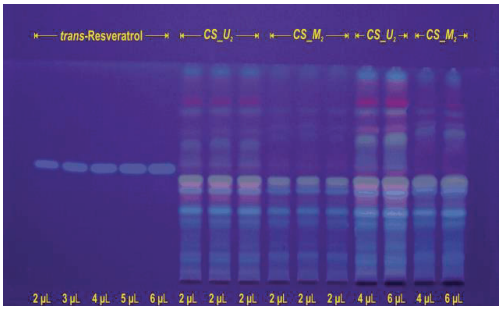

Figure 2. HPTLC chromatogram obtained by analyzing the resveratrol content of two extracts from the Cabernet Sauvignon marc. UV photography, λ 320 nm, without derivatization. CS_U: Cabernet Sauvignon Extract_U_UAE_10: 1; CS_M: Cabernet Sauvignon Extract_M_MAE_10: 1

Figure 3 shows the HPTLC obtained densitochart of the *trans*-resveratrol content of the Cabernet Sauvignon marc extract at 320 nm wavelength without derivatization.

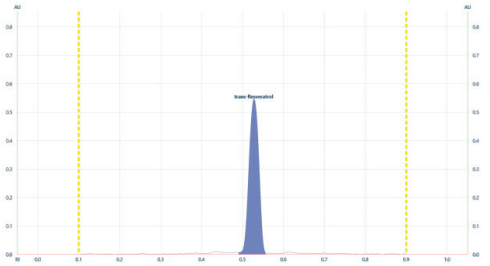

Figure 3. Densitochart obtained by HPTLC analysis of the trans-resveratrol standard (UV λ 320 nm, without derivatization)

Figure 4 shows the densitochart obtained by HPTLC of the cis-resveratrol content of the Cabernet Sauvignon extract of marc obtained by the ultrasonic extraction method, at 320 nm wavelength without derivatization.

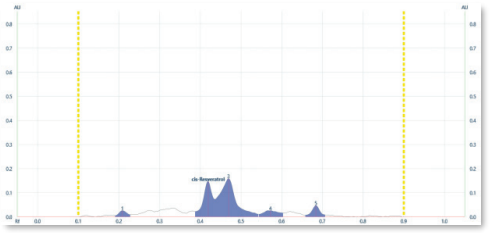

Figure 4. Densitochart obtained by HPTLC analysis of the cis-resveratrol content of Cabernet Sauvignon_U_UAE_10: 1 extract (UV λ 320 nm, without derivatization)

Figure 5 shows the densitochart obtained by HPTLC of the cis-resveratrol content of the Cabernet Sauvignon extract of marc obtained by the microwave extraction method, at 320 nm wavelength without derivatization.

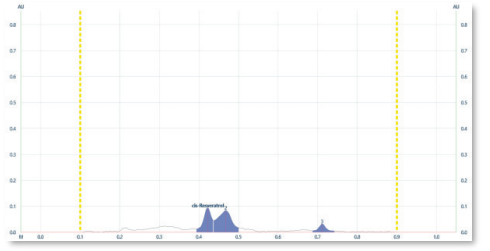

Figure 5. Densitochart obtained by HPTLC analysis of the cis-resveratrol content of Cabernet Sauvignon_M_MAE_10: 1 extract (UV λ 320 nm, without derivatization)

Using the electrospray ionization analysis method (ESI - Electrospray Ionization) coupled with mass spectrometry (MS - Mass Spectrometry) a chromatogram was performed for *trans*-resveratrol in Figure 6. For the electrospray ionization analysis (ESI - Electrospray Ionization) method with mass spectrometry (MS) a chromatogram was performed for *trans*- resveratrol in Figure 6.

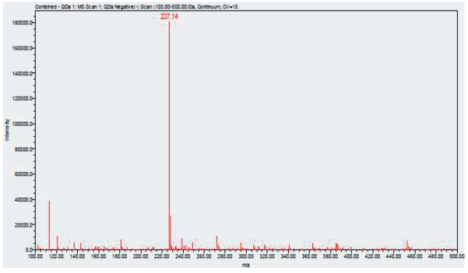

Figure 6. ESI - MS spectrum for the *trans*-resveratrol standard identified in the two extracts from the Cabernet Sauvignon marc (main ion m / z 227)

Figure 7 also shows by electrospray ionization (ESI - Electrospray Ionization) coupled with mass spectrometry (MS - Mass Spectrometry) a chromatogram for *cis* - resveratrol.

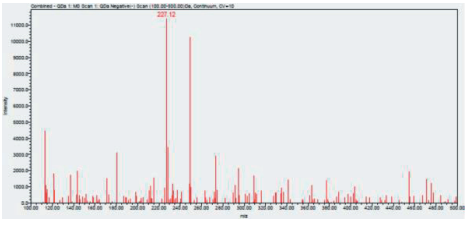

Figure 7. ESI - MS spectrum for *cis*-resveratrol identified in the two extracts from Cabernet Sauvignon marc (main ion m/z 227)

Figure 8 shows the calibration curve for the trans-resveratrol standard at 320 nm wavelength, without derivatization.

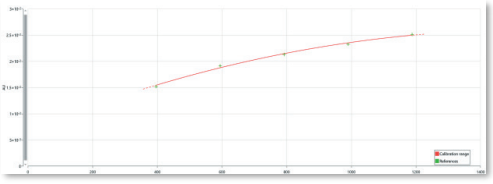

Figure 8. Calibration curve for the *trans*-resveratrol standard (UV λ 320 nm, without derivatization). Polynomial regression, equation: y = -7.998 × 10-15x2 + 2.472 × 10-8x + 6.9 × 10-3, coefficient of variation (CV): 1.21%, correlation coefficient (R): 0.997286

In the two extracts of Cabernet Sauvignon_U_UAE_10:1 and Cabernet Sauvignon_M_MAE_10:1, respectively - it was identified only *cis*-resveratrol from *trans*-resveratrol, by total isomerization, during ultrasound-assisted extraction procedures (UAE - Ultrasound - Assisted Extraction) and, respectively, microwave - assisted extraction (MAE - Microwave - Assisted Extraction).

Table 2. HPTLC - UV densitometry analysis results, confirmed by ESI - MS, for the content of resveratrol in the extracts of Cabernet Sauvignon marc (according to densitochart)

Sample analyzed	Peak No.	ESI–MS $[M–H]^-$ *m/z*	R_f	Concentration [µg / band] (on HPTLC board)	Concentration of *cis*-resveratrol [µg / ml] from the analyzed samples
trans-Resveratrol (standard)	1.	227	0.517	0.396	
	2.			0.594	
	3.			0.792	
	4.			0.99	
	5.			1.188	
*Cabernet Sauvignon*_U_UAE_10:1	2.	227	0.424 *cis*-Resveratrol	0.078	9.75
*Cabernet Sauvignon*_M_MAE_10:1	1.	227	0.424 *cis*-Resveratrol	0.05	5.94

In Table 2 it can be observed that the *trans*-resveratrol by the two methods is detected qualitatively, but not quantitatively. Instead, *cis*-resvatrol is detected in both methods and the highest amount (9.75 µg/ml) is in the method of extracts by ultrasonic processes compared to the microwave extraction method (5.94 µg/ml). From the experiment, it is observed that by extraction, both with ultrasound and microwave, *trans*-resveratrol isomerizes into *cis*-resveratrol.

CONCLUSIONS

Regarding the content of total polyphenols in marc, it is observed that the Cabernet Sauvignon variety has 191 mg GAE/l, almost 10% of the total polyphenols content in the wine and anthocyanins content was 143 mg/l, which represent 1/3 of the anthocyanin content in the wine. It showed, that the Cabernet Sauvignon variety a sufficiently large amount of total polyphenols and anthocyanins remain in marc.

As a result, the antioxidant activity of the marc from the Cabernet Sauvignon variety was high, 8.93%, having a high capacity to eliminate the free hydroxyl radical.

Cis-resveratrol in the composition of the marc can be identified and quantified by HPTLC - UV densitometry, with confirmation by ESI - MS method. The highest concentration of *cis*-resveratrol (9.75 µg/ml) was determined in the method of extracts by ultrasound procedure.

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