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A RAPID METHOD OF DETERMINATION OF TRANS-RESVERATROL IN ROMANIAN COMMERCIAL WINES BY REVERSED-PHASE HPLC

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Abstract: *Resveratrol (trans-3,5,4'-trihydroxystilbene) is a phytoalexin produced naturally by 72 different plant species especially grapevines, Japanese knotweed and pines in response to some exogenous factors such as UV radioation, chemical stress and fungal parasites. With the discovery of the „French paradox" resveratrol had entered in the spotlight of researchers. The French paradox is a term generated in 1992 that refers to the cardioprotective effects of resveratrol that have been discovered based on data from French people that has shown an inverse correlation between wine consumption and the incidence of coronary heart disease (CHD) despite the consumption of a diet in hight saturated fat. The aim of the present study is to determine the trans-resveratrol content in 11 Romanian comercial wine samples by direct injection into the HPLC instrument coupled with diode array detector after being filtered through 0.45 µm PTFE membrane filtres.*
The total resveratrol content in alayses samples varied from 0.03 mg/L to 6.64 g/L. Pinot Noir wines had the highest average resveratrol content, while the lowest was found for the Sauvignion Blanc variety. All wines analyzed contained resveratrol in variable quantities. This study could contribute to the analysis made on the variety of Romanian wines for resveratrol content.

Keywords: *trans-resveratrol; phytoalexin; wines; HPLC; „French paradox"*

1. Introduction

People are looking for an anti-aging cure for ages, but they didn't know that this might be closer than expected. The concern for anti-aging substances did the object for many research over the years. It is well known that free radicals are responsible for aging and can contribute to various diseases. The antioxidants can neutralize the effect of those. Among the substances with antioxidant effect are polyphenols, including resveratrol. Resveratrol had entered in the spotlights of researchers once with the observation of „french paradox" [1].

Coronary artery disease is the leading cause of death in Western countries. High consumption of animal fats, especially meat, cheese and various dairy products, results in cardiovascular disease. In addition, the heavy use of tobacco products has an impact on coronary heart disease. The cardioprotective effects of resveratrol were discovered based on research that showed an inverse correlation between red wine consumption and the incidence of cardiovascular disease. Scientists have discovered a phenomenon called the "French paradox", which is based on the hypothesis that a moderate consumption of red wine reduces the occurrence of cardiovascular disease [2,3]. All this is due to the inhibitory effect on cell membrane lipid peroxidation and protection against the oxidation of low-density lipoproteins, such as LDL, which influence the reduction of HDL cholesterol concentration [4].

Resveratrol (3,4 ', 5 - trihydroxystylbene) is a phenolic compound of plant origin, present in a variety of plant species, such as blackberries, grapes, peanuts and currants [33]. Resveratrol is a phytoalexin

and some plants have been found to synthesize it in response to some exogenous stimuli, such as UV radiation, chemical stress and fungal parasites [6]. Grapes and by-products are considered the most important sources of resveratrol [7]. Resveratrol has two isomeric forms, trans-resveratrol and cis-resveratrol. The trans-isomer is present in all component parts of the vine, but in different amounts. The cis-isomer is produced by UV irradiation of the trans isomer. It is generally absent or in small amount in grapes, but both isomers are present in varying amounts in commercial wines [8,9].

The concentration of resveratrol in wine increases during the fermentation process, but the amount present in the wine depends on the wine variety and the oenological conditions. The concentration of this compound is lower in white wines than in red wines due to the fact that the skin of the grapes is removed earlier during the production of white wines. This is confirmed by the fact that the concentration of resveratrol in rose wines is between white and red wines [10].

Viticulture is one of the most important agricultural activities in the world, with approximately 7.4 million ha cultivated in the world in 2018 of which 4.3 million ha are cultivated in Europe. The first country is represented by Spain, with an area of 969,000 ha, followed by China with 875,000 ha and France with 793,000 ha [11].

Romania ranks 10th in the world as an area cultivated with vines, approximately 180,000 hectares. A quarter of the counties where vines are cultivated account for over 65% of the total area, Vrancea being the county with the largest area. Among wine-producing countries, Romania ranks 6th in Europe and 12th in the world [12].

Resveratrol has recently become the subject of intense research due to the fact that it is a powerful antioxidant, anti-cancer, anti-inflammatory, antiviral agent, provides protection against infections, prevents aging, reduces obesity, has neuroprotective and cardioprotective effect and protects against ischemia [12].

Many analytical procedures have been developed for the determination of resveratrol in wine, which is based on the application of gas chromatography (GC), high purity liquid chromatography (HPLC) and capillary electrophoresis [6].

For the determination of resveratrol, the most common technique used is high performance liquid chromatography (HPLC) with various types of detectors, such as photodiode array detector (DAD), electrochemical detectors, fluorimetric detectors and mass spectrometry. Detection based on fluorimetry, electrochemistry and mass spectrometry can provide greater sensitivity and selectivity than the DAD detector [11,13]. In the initial studies, in which the HPLC technique was used as the resolution technique, step extractions were performed to separate the compounds while the detection sensitivity of the HPLC technique was significantly improved using fluorimetric or electrochemical detectors. However, only the trans form of resveratrol was determined. More recently, improved HPLC methods for determining cis, trans, and piceid forms have been reported. The generally used constituents of the mobile phase consist of various mixtures of methanol or acetonitrile with water and electrolytes, such as: acetic acid, formic acid or ammonium acetate. In general, the HPLC method uses a normal phase or reverse phase column [14].

Resveratrol can be easily determined by simply direct injection of the sample into the HPLC system, when the separation is coupled to a DAD detector. In any case, sometimes extraction and / or derivatization procedures are necessary due to the overlapping peaks [6]. In these

cases, the most used procedures are: liquid-liquid extraction, and dansyl chloride is used as a derivatizing agent [15-17]. In some cases the samples can be diluted with 12% alcoholic solutions in 1:3 ratio [10].

In this study, to determine the resveratrol content from comercial Romanian wines was used a simple method by direct injection of the samples into HPLC system coupled with DAD detector. No dilution, derivatization and extraction procedures was needed.

In this study, a simple method of HPLC analysis coupled with DAD detector was performed on samples of comercial Romanian wines to determine the resveratrol content by direct injection of the samples into the HPLC system. This study could contribute to the analysis made on the variety of Romanian wines for resveratrol content. The results can be used in further for the correlation for coronary heart deseases, like the French paradox.

2. Matherials and methods

2.1. Wine samples

Eleven samples of comercial Romanian wine, three white, one rosé and seven red, were purchased from a local store from Suceava, Romania. The selected wines have a controlled designation of origin in order to be able to identify the region where the raw material was grown. The viticultural regions that produced the raw material for the manufacture of these wines, except for one that is located in the Cotnari area - Iasi, is located in the Sub-Carpathian region.

The climate of this unit is temperate-continental transition. In the depression area the predominant is the low hilly floor with temperature values of 8-10 ° C and precipitation values of 600 - 700 mm / year. The predominant sector of climatic influence is continental (of aridity), with a minus of precipitations due to the circulation of the northeastern air masses that produces accentuated cooling in winter [18].

Table 1.

Romanian commercial wine samples

Wine Sample	Grape variety	Vintage	Vineyard region
Red1	Pinot Noir	2018	Odobești, Vrancea
Red 2	Merlot	2018	Cotești, Vrancea
Red 3	Cabernet Sauvignon	2018	Cotești, Vrancea
Red 4	Pinot Noir	2019	Odobești, Vrancea
Red 5	Fetească neagră	2018	Odobești, Vrancea
Red 6	Merlot	2017	Dealu Mare, Domeniile Franco-Române
Red 7	Cabernet Sauvignon	2018	Dealu Mare, Domeniile Dealu Mare
White 1	Sauvignon Blanc	2019	Podgoria Domnească
White 2	Grasa de cotnari	2019	Cotnari, Iași
White 3	Sauvignon Blanc	2018	Cotești, Vrancea
Rosé	Merlot Rosé	2018	Dealu Mare , Domeniile Franco-Române

2.2. Reagent and chemicals

The trans-resveratrol standard (99% GC) was purchased from Sigma-Aldrich Co. Methanol and Acetonitrile (LiChrosolv for HPLC) was obtained from Merk. For preparation of the aquous solutions was used bidistilled and demineralised water.

2.3. Standard solutions

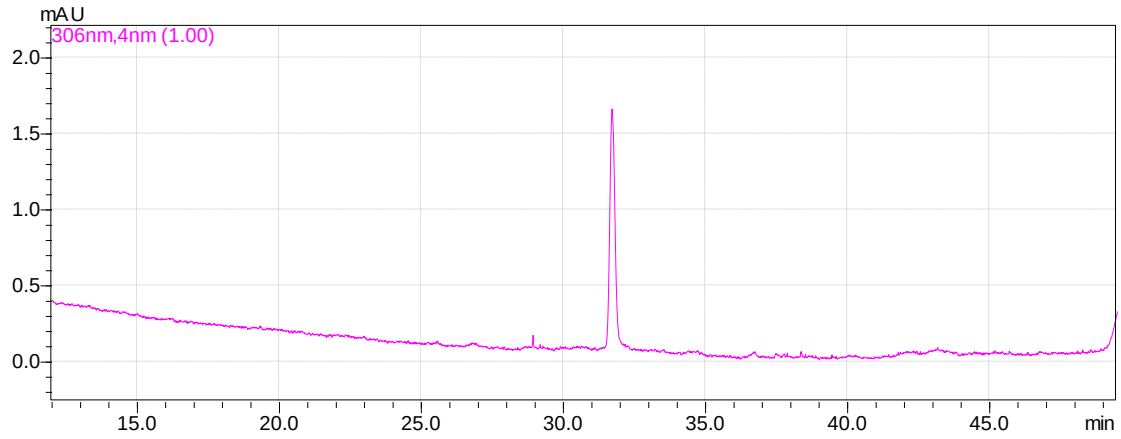
First a stock solution with a concentration of 1g /l was prepared. The resveratrol standard was initially dissolved in a minimal volume of methanol to guarantee a complete dissolution. Before analysis, a set of standards with 0.025, 0.05, 0.075, 0.1, 0.5, 1, 2.5, 5, 10, 20, and 25 mg /L were prepared from the stock solution, diluted in methanol 99%. Special care was taken in connection with the degradation of standard solutions, keeping them protected from exposure to air and light. Standards were stored in brown glass containers at -20^0C.

2.4. Analytical HPLC procedure

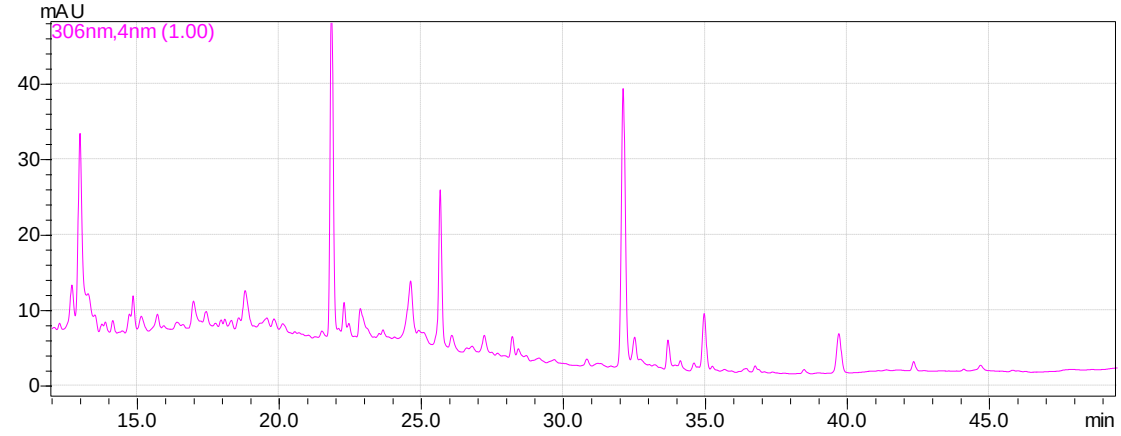
Wine samples immediately after opening and standard solutions were filtered through 0.45 µm PTFE membrane filters and then injected (with a volume of 8 µL) into the HPLC instrument (Schimadzu, Kyoto, Japan) for analysis using an SPD-M-20A diode array detector. The separation was carried out on a Phenomenex Kinetex 2.6 µm Biphenyl 100 Å HPLC Column 150 x 4.6 mm thermostated at 20^0C. Elution was carried out with a solvent system consisting of pure water (solvent A) and acetonitrile (solvent B) as previously described by Marshall et al. [7] with modifications. Binary gradient consisted of solvent A: pure water and solvent B: acetonitrile as follows: linear gradient from 0% to 10% B in 42 min, 10–40% B in 42.6 min, 40–90% B in 46.5 min. Run time was 49.5 min. The analysis were made in triplicate and then the standard deviation was calculated.
The solvent flow rate was of 0.5 mL/min. The determined of trans-resveratrol were carried out at at 306 nm detection wavelength.
The obtained standard calibration curves showed high degrees of linearity (R2 > 0.99). Data collection and subsequent processing were performed using the LC solution software version 1.21 (Shimadzu, Kyoto, Japan). Analyses were perform in duplicate and the quantification was made based on the peak area, using the external standard method.

3. Results and discussion

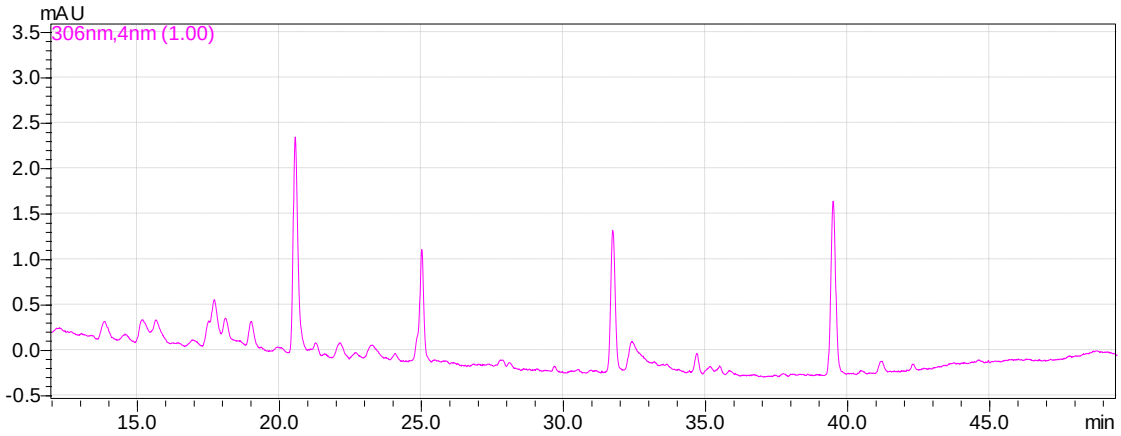
According to the specialized literature, more and more frequently, the methodology of sample analysis is much simplified compared to the data from previous years. Thus, more and more authors recommend analysis for direct injection of samples into HPLC, without pre-concentration, dilution or extraction steps [19,20].
As can be seen from Fig.1, the standard chromatograms are free from interference, with the solvent and trans-resveratrol peaks clearly separated. In wine samples it is observed that the interferences are more significant, especially in the red wine samples.



(a)



(b)



(c)

Fig.1. Chromatogram for :
(a) trans-resveratrol standard solution; (b) red wine sample; (c) white wine sample

The trans-resveratrol standard was used as a standard, and the standard curve equation was used to determine the concentration in the wine samples: y = 117661x, where y is absorbance at 306nm (Figure 2).

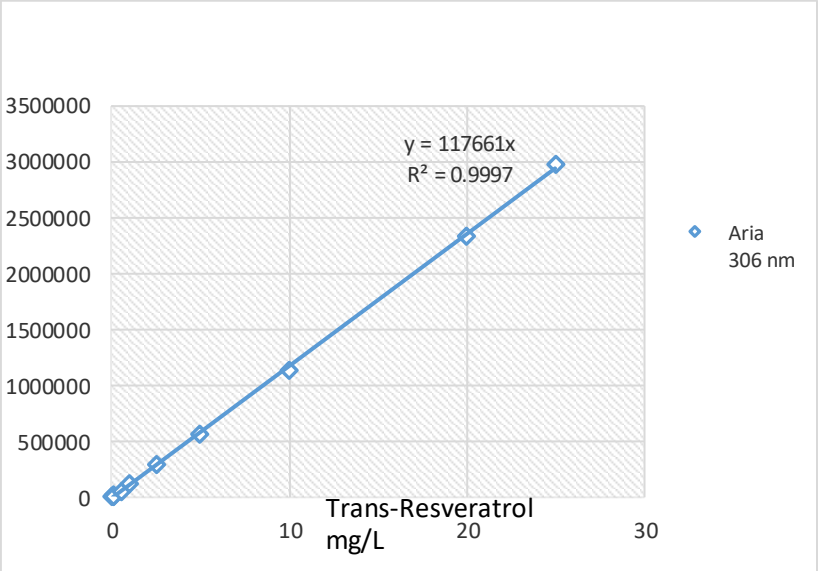


Fig. 2. Standard carve of trans-resveratrol at 306 nm

The concetration of trans-resveratrol ranged from 0.04 to 6,64 mg/L. All results are summarised in Table 2. The concentration of resveratol is much higher in red wines than in white ones, and the content of resveratrol in rose wines is between the two. It can also be seen that the content of resveratrol in wines produced by the same grape variety can vary widely (Pinot Noir 3,14 – 6,64). How the concentration of resveratrol is known to be influenced by various factors (climate, temperature, growing conditions, UV irradiation, exposure to fungal and bacterial infections and production technology) these observation are expected [19]. As can be seen in Table 1, the highest content in trans-resveratrol is in the wine of the Pinot Noir variety – Red 4, and the lowest content is in the wine of the sauvignon blanc variety- White 3. The average trans-resveratrol content in red wines is 4.31 mg/L and in white wines it is 0.07 mg/L. This study is in agreement with a previously reported study made on Romanian wines [21].

Table 2.
Trans-resveratrol content in Romanian commercial wines

Wine Sample	Resveratrol content (mg/L)
Red 1 - Pinot Noir	3.14±020
Red 2 - Merlot	6.17±0.41
Red 3 - Cabernet Sauvignon	2.39±016
Red 4 - Pinot Noir	6.64±0.26
Red 5 - Fetească Neagră	3.91±0.14
Red 6 - Merlot	5.42±0.38
Red 7 - Cabernet Sauvignon	2.50±0.10
White 1 – Sauvignon Blanc	0.04±0.01
White 2 – Grasă de cotnari	0.14±0.02
White 3 – Sauvignon Blanc	0.03±0.01
Rose - Merlot Rose	0.32±0.02

Due to the fact that the concentration of resveratrol in wine is influenced by a multitude of factors (climate, temperature, growing conditions, UV irradiation, exposure to fungal and bacterial infections and production technology) [19], its concentration may differ even if the analysis is performed on the same wine of the same variety, produced under the same conditions but the raw material is harvested in different years [22]. In this sense, Figure 3 shows the average amount of resveratrol in wines produced in various countries in certain years [23-33]. This classification is indicative, because the average results for a given year are taken into account. In order to be able to make a correct comparison it is necessary to make an average of the results from several years. As can be seen from Figure 3., Romanian wines have a high resveratrol content. According to the results obtained, they confirm this once again.

As can be seen from Figure 3., Serbia has the highest concentration of resveratrol in wines. In the work published by Souto et al. In 2001 the concentration of resveratrol in red wines produced in southern region of Brazil varies between 0.82 to 5.75 mg/L.[21] The content of resveratrol in Greek red wines ranged from 0.352 to 1.99 mg/L in the paper realised by Geroginnaki-Christopoulou et al. in 2006 [23]. According to the analyzes performed by Ratola et al. in 2004 in the concentration of resveratrol in red wines from Alentejo Region from Portugal ranged from 0.13 to 2.64 mg/L [10]. In Montenegrin red wines the concentration of resveratrol ranged from 0.4 to 1.1 mg/L according to the work realised by Šćepanović P.S. in 2018 [9].

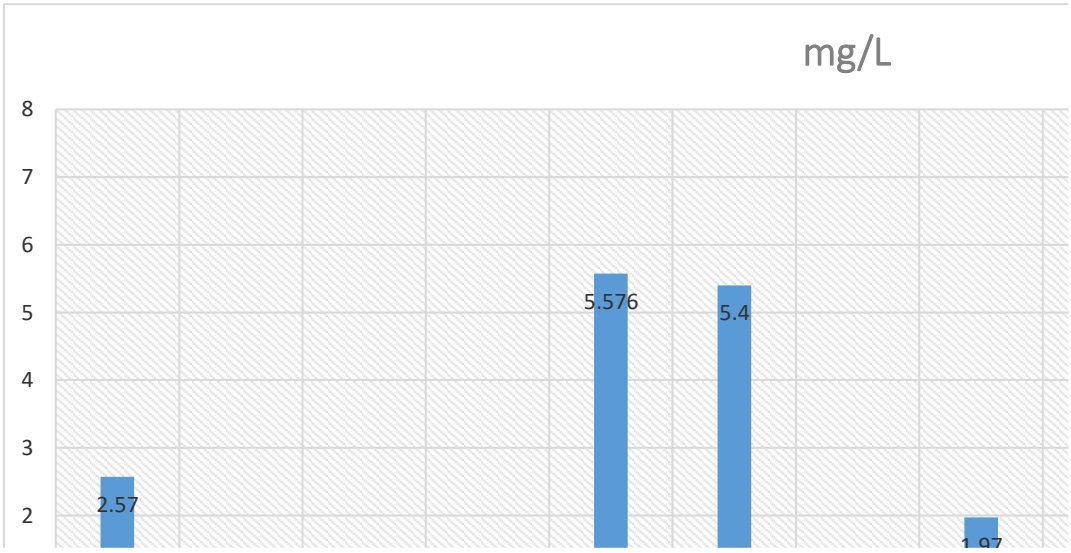


Fig. 3. Concentration of the trans-resveratrol in red wine differentiated according to countries [21-32].

Following the analyzes, this paper shows that the concentration of resveratrol in Romanian red wines varies between 2.50-6.64 mg/L. The results obtained in this study show that commercial Romanian wines are a good source of resveratrol, as they have a higher concentration of resveratrol than wines from other countries. Therefore, several analyzes can be performed for a clearer picture of the resveratrol concentration in Romanian wines.

4. Conclusion

Different types of Romanian wine were

analyzed to determine the trans-resveratrol content. The results of the analyzes are presented in table 1.The concetration of trans-resveratrol ranged from 0.04 to 6,64 mg/L. The concentration of resveratol is much higher in red wines than in white ones, and the content of resveratrol in rose wines is between the two. The content of resveratrol in wines can vary widely even if is produced by the same grape variety(Pinot Noir 3,14 – 6,64).

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The use of the direct injection procedure in HPLC with UV detector allowed to reduce the processing time of the samples.

From the point of view of public health, the values obtained suggest that the physiological effects on Romanian wine consumers may be significant, like the "French paradox". However, trans-resveratrol represents only a small part of all the polyphenols present in wine

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