ORIGINAL ARTICLE

CHEMICAL PROPERTIES OF SEVERAL RED WINES AVAILABLE ON ROMANIAN AND ALSO ON THE INTERNATIONAL MARKET

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Abstract

The aim of this study was to perform a phytochemical analysis of 28 red wines available on Romanian and also on the international market. The identification of 14 polyphenols and their quantitative analysis were performed by HPLC/ESI-MS (high-performance liquid chromatography-electrospray ionisation tandem mass spectrometry). Resveratrol contents were determined by HPLC/APCI-MS (HPLC coupled online with atmospheric pressure chemical ionization). Total polyphenols and monomeric anthocyanins were assayed by Folin-Ciocâlteu and pH differential methods. The GC-FID (gas chromatography coupled with flame ionization detector) system was used for the volatile congeners determination. The content of 35 toxic metals was investigated using a Q-ICP-MS (Quadruple inductively-coupled-plasma mass spectrometer) analyses. Quality and quantity of polyphenols, volatile congeners and metal content varied greatly among cultivars, and from this point of view, several Romanian wine samples could be considered of high quality when compared with international brands.

Rezumat

Obiectivul studiului a constatat în analiza fitochimică a 28 de vinuri roşii aflate pe piaţa românească şi internaţională. Pentru analiza calitativă şi cantitativă a compuşilor polifenolici (14 polifenoli) s-a folosit tehnica HPLC/ESI-MS. Conţinutul de resveratrol a fost determinat cu un sistem HPLC/APCI-MS. Cuantificarea polifenolilor totali şi a antocianilor monomerici s-a efectuat prin metoda Folin-Ciocâlteu şi, respectiv, prin metoda pH diferenţială. Analiza congenerilor volatili s-a realizat cu un sistem GC-FID, iar determinarea cantitativă a 35 de metale toxice a fost realizată folosind o metodă Q-ICP-MS. Proporţia claselor de polifenoli, conţinutul polifenolic total, congenerii volatili şi metalele variază de la un producător la alta. Pe baza rezultatelor obţinute putem confirma că mai multe mostre de vin roşu din România sunt de înaltă calitate prin comparare cu brandurile internaţionale.

Keywords: red wine, polyphenols, volatile congeners, metals

Introduction

The regular use of moderate amounts of red wines was associated with low incidence of cardiovascular diseases, especially coronary artery disease (CAD), effect that was associated with the content of several compounds with antioxidant ability. There are two classes of such substances: alcohol that (in low doses) can increase the blood antioxidant capacity after it is metabolized to acetic acid, process that releases $NADH^+$; substances that act as spin-traps (polyphenols and anthocyanins), and provide hydrogen atoms to reactive free radicals saving this way some important biomolecules as unsaturated fatty acids, proteins, nucleic acids from oxidative damage, and preventing the oxidation of the low density lipoproteins (LDL) [1].
The most commonly measured polyphenol in red wine is resveratrol; its concentrations range usually within 0.1 mg/L and 14.3 mg/L. The free anthocyanin content varies between 500 and 2000 mg/L [2].
Beside substances with beneficial health effects, harmful compounds as volatile congeners (acetone, methanol, n-propanol, isobutanol, amyl alcohol) and heavy metals can occur. The following amounts of most commonly metal ions from red wines are usually measured: Mn (0.33 - 3.02 mg/L), Zn (0.17 - 1.80 mg/L), Cu (0.02 - 0.63 mg/L), Ba (0.08 - 0.22 mg/L), Ca

(67.39 - 180.22 mg/L), K (915.35 - 1985.33 mg/L), Mg (87.11 - 192.01 mg/L), Mn (0.88 - 3.15 mg/L), Sr (0.44 - 1.75 mg/L), Zn (0.33 - 0.72 mg/L). The sum of 30 different metals (V, Cr, Mn, Ni, Cu, Zn, Rb, Co, Sn, Cs, Pb, Y, Cd, lanthanides, Tl, Th, U) in red wine were 5,620.54 ± 123.86 ppb, higher than in the apple juice (15 metals totalling 1339.87 ± 10.84 ppb) or in the stout (14 metals totalling 464.85 ± 46.74 ppb) [3-5].

The content of both beneficial and harmful substances is greatly influenced by the origin of wine and the climatic conditions in which the grapes were grown. The production of volatile congeners in the red wines is also influenced by the fermenting conditions.

The purpose of this work was to measure substances with spin-trapping abilities, volatile congeners and heavy metals in several types of red wines produced in Romania and other countries [6].

Materials and Methods

The polyphenolic profile of the red wines

Reagents: HPLC grade methanol, analytical grade acetic acid and hydrochloric acid were purchased from Merck (Germany). Standards: caftaric acid from Dalton (USA), gentisic acid, ferulic acid, sinapic acid from Roth (Germany), caffeic acid, chlorogenic acid, p-coumaric acid, hyperoside, isoquercitrin (quercetin-3-*O*-glucoside), rutoside (quercetin-3-*O*-rutinoside), myricetin, quercitrin (quercetin-3-*O*-rhamnoside), quercetin and kaempferol were from Sigma (Germany). Wines with years of production ranging between 2009 and 2014 were purchased from local liquor stores. The year of production, the region of origin and the grape varieties are summarized in Figure 1.

Sample treatment: 100 mL sample was lyophilized to constant mass and stored at -20°C. Wines were reconstituted prior analysis with a 13% v/v alcoholic solution, an aliquot of 5 µL was then injected into the chromatographic system. HPLC-UV (detection at 330 and 370 nm) and HPLC-MS (ESI-MS, negative ionization) conditions used for the analysis are described in detail elsewhere [7]. The used chromatographic column was Zorbax SB-C18 100 x 3.0 mm i.d., 3.5 µm particle, and the mobile phase composition was methanol and solution of acetic acid 0.1% (v/v). The elution was started with a linear gradient: methanol 5% to 42 % for 35 minutes, followed by an isocratic elution: methanol 42% for 3 minutes.

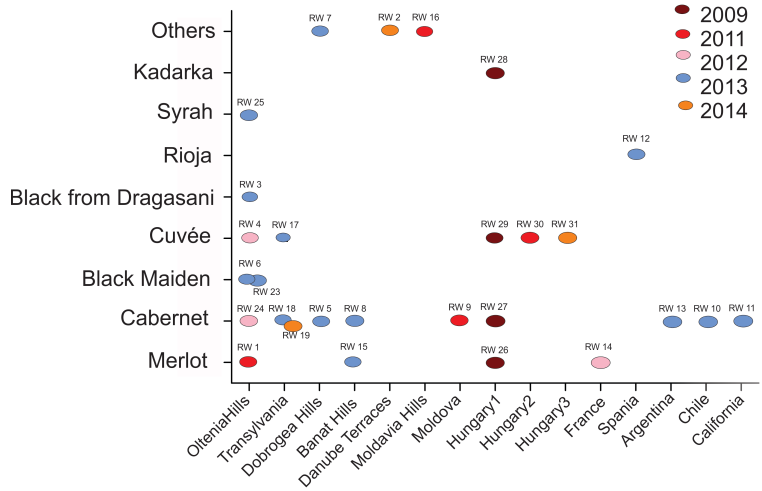

Figure 1.
Year of production, region of origin and the grape varieties of the tested wine samples

Wine resveratrol content

Reagents: methanol and acetonitrile of HPLC gradient grade, ammonium acetate, formic acid, acetic acid of analytical grade were purchased from Merck (Germany). Trans-resveratrol was purchased from Sigma-Aldrich (Germany). Cis-resveratrol was obtained from a standard solution of trans-resveratrol after its irradiation.

Sample treatment: the 28 red wine samples were diluted ten folds with bi-distilled water, then centrifuged and 5 µL was then injected into the chromatographic system. Equipment and chromatographic conditions used for the analysis are described in detail elsewhere [8]. The separation was performed with a Zorbax SB-C18, 100 x 3.0 mm i.d., 3.5 mm particles, reversed phase chromatographic column. An isocratic elution was applied with a mobile phase containing: 1 mM ammonium acetate/acetonitrile (73/27 v/v).

Wine total phenolic content

Reagents: Folin-Ciocâlteu reagent, Scharlau (Spain); anhydrous sodium carbonate, Merck (Germany); gallic acid (analytical standard), Sigma Aldrich (USA). The total phenolic content of the wines was made using a modified spectrophotometric method official in PhEur 7. The measurement is based on the reduction of the mixture of phosphomolybdic and phosphotungstic acid with the production of molybdenum blue and tungsten blue. Our measurement is based on the method reported by Slinkard K *et al*. [9] with slight

modification made by Waterhouse A. from Department of Viticulture & Enology, University of California, USA. The method is described in detail elsewhere [10]: the reaction mixture contains 40 µL red wine diluted 1:10 with ethanol solution 13% v/v or standard solution, 3160 µL purified water, 200 µL Folin-Ciocâlteu reagents and 600 µL Na_2CO_3 200 g/L solution. The absorbance of the solution was measured at 765 nm. The calibration curve was obtained with gallic acid solution for 5 different concentration.

Measurement of monomeric anthocyanins

Reagents: potassium chloride and anhydrous sodium acetate from Merck KGaA, (Germany); hydrochloric acid from Chemical Company (Romania); cyanidin 3-*O*-glucoside (analytical standard), Sigma Aldrich (USA). Measurement of monomeric anthocyanins content is based on the ability of these compounds to change colour according to the variations of the pH; the coloured form is present at pH = 1, while at pH = 4.5 colourless form is present. A detailed description of the method is given elsewhere [1111].

Measurement of volatile congeners

Reagents: ethanol gradient grade from Sigma Aldrich (USA); ultra-pure water, Millipore Direct-Q^{TM} S (Germany); methanol, isobutanol, amyl alcohol from Merck KgaA (Germany); n-propanol from Sigma Aldrich (USA); acetone from Chemical Company (Romania); GC quality nitrogen, synthetic air and hydrogen, Linde Gaz (Romania).

Equipment and GC method: Dani Master GC coupled with a flame ionization detector (FID), optima wax capillary column (adsorbent thickness: 2 µm, diameter: 0.53 mm, length: 30 m). Temperature program: 60°C for 5 minutes, increase to 90°C with a rate of 5°C/ minute; hold for 5 minutes. FID (flame ionization detector) temperature: 250°C, injector temperature: 250°C, injection volume: 1 µL, mobile phase: nitrogen (10 mL/min debit), combustion gases: hydrogen (40 mL/min debit), synthetic air (220 mL/min debit) and nitrogen (25 mL/min debit). Sample preparation: wine samples were diluted 1:100 with purified water and filtrated through a 0.45 µm nylon filter. Calibration curves were prepared from a mixture of congeners obtained according to the C.E. recommendations (regulation no. 2870/2000) [12-15].

Measurement of the heavy metal content

Reagents: purified water, TKA Microlab purifier (Germany); HNO_3 69% from Sigma Aldrich (Germany); multi-element certificate standard solution (Periodic table Mix 1 for ICP, 10 ppm, Sigma Aldrich, Munich, Germany) was used, containing 33 elements (Al, As, Ba, Be, Bi, B, Cod, Ca, Cs, Cr, Co, Cu, Ga, In, Fe, Pb, Li, Mg, Mn, Ni, P, K, Rb, Se, Si, Ag, Na, Sr, S, Te, Tl, V and Zn); single element standards were used for the construction of the calibration curves for Ti, Ge, Sb, Sn and Mo (10 ppm in 10% HNO_3 Sigma Aldrich, Germany). Optimization of the ICP-MS was made using a ICP-MS Tuning Solution, contains 10 mg/L each of Li, Y, Ce, Tl and Co in a matrix of 2% HNO_3, Agilent Technologies (USA). *Apparatus*: Q-ICP-MS 7500cx, Agilent Technologies (USA) with a MicroMist nebulizer. *Method*: carrier gas debit in the nebulizer: 1 L/min, makeup gas debit: 0.25 L/min, RF power: 1500 W, CeO/Ce = 0.65%, Ce^{++}/Ce = 2.08. Sample preparation: 0.5 mL wine was placed in a 10 mL volumetric flack and 0.5 ml of trace pure HNO_3 and 0.1 ml of internal standard (rhodium) were added. The final volume was adjusted with ultrapure water and homogenized. A detailed description of the method is given elsewhere [16].

Results and Discussion

The polyphenolic profile of the tested samples

A typical chromatogram obtained for a wine sample is shown in Figure 2.

The maximum and minimum concentration of the phenolic compounds in the tested wines is given in Table I.

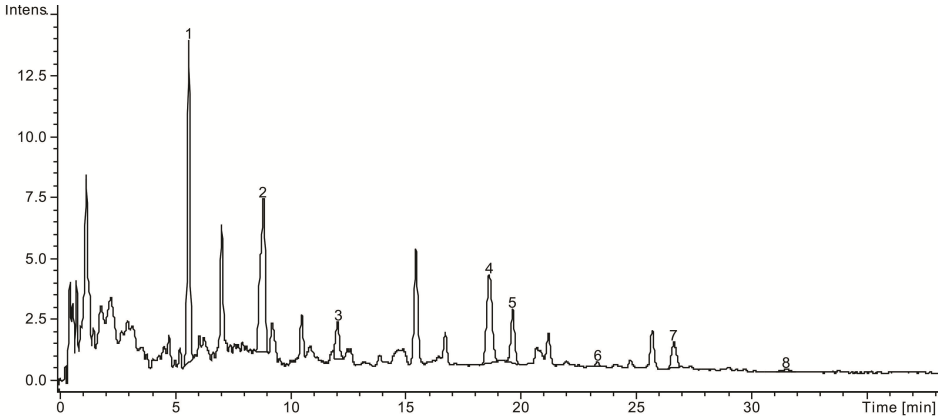

Figure 2.

HPLC-UV chromatogram for sample RW11. Identity of peaks: 1-caffeic acid, 2-p-coumaric acid, 3-ferulic acid, 4-hyperoside, 5-isoquercitrin, 6-quercitrin, 7-quercetin, 8-kaempferol

Table I

Polyphenol and monomeric anthocyanin content of the tested wine samples

Polyphenols	Minimum concentration		Maximum concentration	
(mg/L)	Sample	Value	Sample	Value
Caftaric acid	RW18	BLD[1]	RW1-RW17, RW19-RW31	BLQ[2]
Caffeic acid	RW1, RW23-RW31	BLQ[2]	RW13	32.45
Chlorogenic acid	RW2-RW19, RW25, RW27-RW31	BLD[1]	RW1, RW23, RW24, RW26	BLQ[2]
p-Coumaric acid	RW17, RW18	BLD[1]	RW11	6.03
Ferulic acid	RW4, RW6-RW9, RW12, RW16, RW17	BLD[1]	RW1, RW11	1.06
Gentisic acid	RW1, RW18	BLD[1]	RW2-RW17, RW19-RW31	BLQ[2]
Sinapic acid	RW1-RW31	BLD[1]	-	-
Hyperoside	RW1-RW3, RW5-RW8, RW10, RW13, RW15-RW31	BLD[1]	RW11	6.72
Isoquercitrin	RW1-RW3, RW5-RW10, RW13, RW15-RW31	BLD[1]	RW11	4.20
Kaempferol	RW2-RW7, RW9, RW12, RW15-RW31	BLD[1]	RW1, RW14	0.41
Myricetin	RW17-RW19, RW24, RW25	BLD[1]	RW10	4.56
Rutoside	RW1-RW31	BLD[1]	-	-
Quercetin	RW5, RW17-RW23, RW25, RW27-RW29	BLD[1]	RW1	4.41
Quercitrin	RW1-RW8, RW10, RW12, RW13, RW15-RW31	BLD[1]	RW11	0.36
Resveratrol	RW18	BLQ[2]	RW1	21.28
TPC[3] (g GAE/L)	RW17	0.33	RW29	2.80
MAC[4] (mg Cya-3-O-gluc/L)	RW17	2.97	RW11	187.45

[1]BLD - below limit of detection; [2]BLQ - below limit of quantitation; [3]TPC - total polyphenolic content; [4]MAC - monomeric anthocyanins content

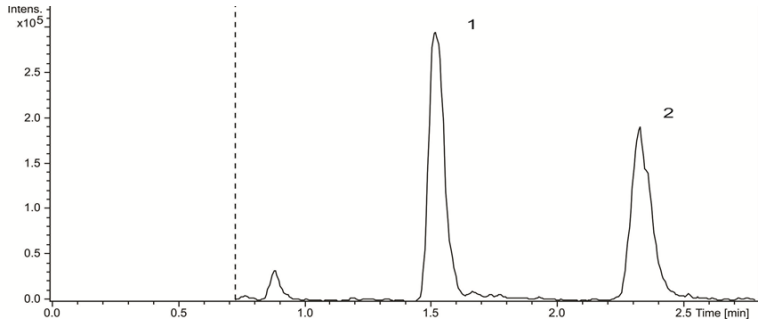

Figure 3.

Typical chromatogram for the determination of trans- and cis-resveratrol

(RW1; peak 1 = trans-resveratrol; peak 2 = cis-resveratrol)

Wine total polyphenolic content

Total polyphenolic content was measured using gallic acid as analytical standard and based on the calibration curve obtained for the concentration range of 50 - 500 μg/ml (slope 1.078, intercept 0.033). Results are expressed as g gallic acid equivalent/L (g GAE/L) and are shown in Table I. Highest concentration of total phenolic compounds was measured in Hungarian wines (RW26, RW29 and RW31), followed by a Romanian wine (RW4). Very low total phenolic content was measured in all 3 samples of wine originated from the centre of Romania.

Content of monomeric anthocyanins

Quantification of anthocyanins was performed using the following equation:

$$\text{Monomeric anthocyanins (mg cyanidin-3-glucoside equivalents/L)} = \frac{A \times MW \times DF \times 10^3}{\varepsilon},$$

where: $A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$, MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside, DF = dilution factor, ε = 26900 molar extinction coefficient, in L x mol^{-1} x cm^{-1}, for cyd-3-glu, 10^3 = conversion factor from g to mg

Results are expressed as mg cyanidin 3-*O*-glucoside equivalents/L (mg Cya-3-*O*-gluc/L) and are shown in Table I. Major modification (almost 2 orders of magnitude) in the concentrations of monomeric anthocyanins were recorded for the analysed samples. In the same samples the variation in the resveratrol content, the most studied phenolic compound, never exceeded 1 order of magnitude. This can be explained by the influence of the product's age (not only the region of origin and the climatic conditions) on the monomeric anthocyanins content. The aging of the wine tends to decrease by polymerization the monomeric anthocyanins content as can be seen in samples RW29, RW30 and RW31 (same origin and grape variety; different age). The reduction of the monomeric anthocyanins content due to the wine's ageing has been described also by others [17].

Volatile congeners content of the wine samples

The minimum and maximum concentrations of volatile congeners are presented in Table II. The methanol content in the analysed samples ranged between 17.47 - 547.22 mg/L. All samples corresponded to

the established legal limits by OIV (International Organization of Vine and Wine in 2004 established the methanol concentration limit of 250 mL methanol/L for white wines and 400 mL methanol/L for red wines [18]), applied by different countries [19].

The variation in the volatile congeners content of the wines is far less than the variations recorded for the concentrations of substances with spin-trapping ability. The content of amyl alcohol is usually used to differentiate the original wines from the ones obtained from technical quality alcohol.

Metal content of the wine samples

The concentration of the following metals was measured: Li, Be, B, Na, Mg, Al, P, K, Ca, Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn, Ga, Ge, As, Se, Rb, Sr, Mo, Pd, Ag, Cd, Sn, Sb, Cs, Ba, Tl, Pb, Bi.

One can observe that all wine samples meet Romanian governmental regulations regarding their metal content (regulated metal content for: As (0.20 mg/L), Cd (0.01 mg/L), Cu (1.00 mg/L), Pb (0.20 mg/L), Na (60 mg/L), Zn (5 mg/L), B (80 mg/L)) [19, 20]. The concentration of the metals with the highest toxicological interest was compared with the international regulations regarding their accepted daily/weekly intake (Be TDI 2 μg/kg/day [2121], V 1.8 mg/person/day [22], Ni 1 mg/ person/day, Ge 1.5 mg/person/day [23], As 0.3 mg/ person/day and 7.5 μg/kg/day [24], Pd 0.05 mg/kg food [25], Cd 60 μg/person/day [26], Pb 3 mg/adult person/week [27].

Table II

Volatile congeners in the tested wine samples

Volatile congeners (mg/L)	Minimum concentration		Maximum concentration		Average
	Sample	Value	Sample	Value	
Methanol	RW11	45.03	RW26	547.22	228.02
Acetone	RW8, RW9, RW11, RW23-RW31	BLQ[1]	RW7	6.86	-[2]
n-Propanol	RW4	11.77	RW27	67.18	38.86
Isobutanol	RW11	1.99	RW14	155.57	70.45
Amylic alcohol	RW18	74.26	RW27	491.26	270.16
Total volatile congeners	RW18	269.82	RW27	1079.39	-

[1] BLQ – below limit of quantitation; [2] – where at least one sample had BLQ concentration, no average concentration was computed

Three samples (RW24, RW27, RW 28) were found to exceed the limit of concern for Pd. Unfortunate for Cr the oxidation state of the metal was not determined, therefore, a toxicological evaluation of its presence cannot been done (it is known that Cr^{6+} is far more toxic than Cr^{3+}).

Target hazard quotients (THQ) were calculated for the estimation of potential health risks associated with long term exposure to chemical pollutants in case of samples where no Romanian governmental regulation or international regulation was available. The THQ is a ratio between the measured concentration and the oral reference dose, weighted by the length and frequency of exposure, amount ingested and body weight. If THQ < 1 then no adverse health effects are expected as a result of exposure; if 1 < THQ < 5 means that the exposed population is in a level of concern interval, then adverse health effects are possible. But, its numerical value should not be regarded as a direct estimate of risk. Individual THQ values were calculated for 10 metal ions for which oral reference doses exist, by the formula established by the Environmental Protection Agency (EPA) [3, 28]. THQ estimation was made for moderate drinking (150 mL red wine (1 drink)/day for women and 300 mL/day for men) and binge drinking habits (600 mL or more for women and 750 mL or more for men; quantity should be consumed in less than 2 hours), and Table III shows the calculated results [29].

Table III

THQ values calculated for 13 metal ions from red wines

Metals	RfD[1]	Sample	THQ[2] values (min-max)			
			Moderate alcohol consumption		Binge drinking	
			Men	Women	Men	Women
Mg	11.0	RW18-RW13	12.95 - 33.99	6.47 - 16.99	25.90 - 67.98	32.37 - 84.97
Ti	4.0	RW12-RW6	0.09 - 0.32	0.04 - 0.16	0.18 - 0.65	0.23 - 0.81
Mn	0.14	RW18-RW1	5.98 - 38.08	2.99 - 19.04	11.96 - 76.16	14.95 - 95.20
Co	0.03	RW27-RW16	0.79 - 1.48	0.39 - 0.74	1.58 - 2.95	1.97 - 3.69
Se	0.005	RWa[4]-RW1	BLQ[3] - 4.03	BLQ[3] - 2.01	BLQ[3] - 8.06	BLQ[3] - 10.08
Sr	0.6	RW18-RW25	6.28 - 81.82	3.14 - 40.91	12.57 - 163.64	15.72 - 204.55
Mo	0.005	RW26-RW11	2.56 - 23.56	1.28 - 11.78	5.12 - 47.12	6.39 - 58.89
Ag	0.005	RW27-RW17	0.15 - 32.42	0.07 - 16.21	0.30 - 64.84	0.38 - 81.06
Sb	0.0004	RWb[5]-RW26	BLQ[3] - 7.03	BLQ[3] - 3.52	BLQ[3] - 14.07	BLQ[3] - 17.59
Ba	0.2	RW13-RW10	0.26 - 3.24	0.13 - 1.62	0.52 - 6.48	0.65 - 8.10

[1]RfD - oral reference doses, mg/kg/day; [2]THQ - target hazard quotients; [3]BLQ - below limit of quantitation; [4]RWa - RW18, RW29; [5]RWb - RW8, RW13, RW27, RW28

Conclusions

Our work shows that large difference in the content of spin-trapping agents can be found in red wines. The region of origin and the grape variety are the main factors that influence the content of antioxidant substances present in the red wines. Lower influence is brought by the year of production. Regarding the monomeric anthocyanins, the age of the wine is inversely proportional with their concentration due to the polymerization reactions. A series of mechanisms might be related with monomeric anthocyanins decline, such as their adsorption by yeast, their degradation, their precipitation and the progressive and irreversible formation of more complex and stable anthocyanin derived pigments.

Volatile congeners were present in all samples of red wine. The concentrations of volatile congeners are influenced by the conditions present during the fermentation process, therefore, lower amounts are expected to be present in high quality wines fermented in controlled environments.

Concentration of metals in red wines is influenced by the region of origin. The Romanian regulations for toxic metal content were met by all wine samples, but Pd concentrations exceeded, in three cases, the limit of toxicological concern in food set by European regulations.

Summarizing the above information, one can conclude that the region of origin, soil composition has the greatest importance in the quality of a wine, if consumed in moderate amounts for its antioxidant/spin-trapping properties. From this point of view, several Romanian wine samples could be considered of high quality when compared with international brands.

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