Determination of Total Antioxidant Activity of Wines Using a Flow Injection Method with Chemiluminescence Detection

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Total antioxidant capacity (TAC) of several samples of red and white wines was investigated by means of a new analysis method of flow injection with chemiluminescence detection ((FIA-CL). The applied method is based on a chemiluminescence reaction between luminol and hydroxyl radicals generated by the hydrogen peroxide in the presence of Co(II) ions complexed with ethylene diamine tetraacetic acid (EDTA) disodium salt according to a Fenton-type reaction. The FIA-CL assembly is made of three channels through which corresponding liquids are pumped: a carrier flux of 0.05M, pH 9 sodium borate solution where the analyzed sample is injected, a H_2O_2 flux and a flux containing CL reagent, that is a Co(II)/EDTA/luminol solution. This method allows for TAC determination within 2.5 – 300 μmoles L^{-1}domains of gallic and caffeic acid, and with a relative standard deviation (RSD) = 2.41% (n = 10) when gallic acid concentration is 35 μM. The TAC values determined for the analyzed wine samples ranged between 0.19 and 0.55 g of gallic acid equivalents/ L wine and between 0.44 and 1.78 g caffeic acid equivalents/L wine.

Keywords: total antioxidant capacity, *flow injection analysis*, *chemiluminescence analysis*, *luminol*, *polyphenols*

Wines are complex mixtures of water (74 – 87 % (v/v)), ethanol (10 – 14 %), residual saccharides (0.05 – 10%), organic acids (0.05 – 0.7 %), phenolic compounds (0.01 – 0.2%), glycerol etc, and all these components are responsible for the sensorial characteristics of wines [1 – 3]. More and more, wines are considered as important natural sources of polyphenols, the latter being very useful for a good health due to their antioxidant properties (under the caution of a moderate, 1 – 2 glasses of wine as a daily consume) [3].

More than 200 compounds with a phenolic structure as basic wine components have been identified in wines [1]. Total phenols (TP) content, generally between 2 and 3 g/L in red wine is reported in literature [1, 4], which means twice that found in rosé wines and approximately 10 times more than white ones. Wine compositions and respectively their contents in polyphenolic compounds differ as a function of the quality of grapes used for wine making, vinification techniques (different for producing red or white wines), chemical processes occurring during wine processing, maturation and preservation, wine age etc [1, 4 -7].

Some benzoic acids, such as vanillic, gallic and syringic [5, 7 - 9] and hydroxycinnamic acids, such as caffeic acid [5, 8], ferulic acid [8] and its glucoside [7], *m*- and *p*-coumaric acids and its glucosides [5, 7 - 9], caffeoyl tartaric and *p*-coumaroyl tartaric acids [5] etc. have been identified in wines. Shikimic acid, tyrosol and hydroxytyrosol were identified in white wine [8]. An important compound in red wine with particular antioxidant properties is resveratrol (3,5,4'-trihydroxy-stilbene) which can be found free or in glycosylated in one of the two isomer forms, *cis* and *trans* [7 - 13]. It is present in relatively large quantities in the red wines, while in the white ones its concentration is very low [8, 14]. Another very important class of polyphenolic acids in wines is represented by flavonoids, which constitute > 85% (≥ 1g/L) of the TP content in red wines and < 20% (≤ 0.05 g/L) in white wines [1]. Among them, anthocyanidins [1] are considered as having a strong antioxidant activity [15] and procyanidins [5, 16], which are even stronger in as much as wine antioxidant efficiency is considered [16]. Anthocyanidins are found only in red wines [1], free or associated with, for example, tannins [17] or different acids [18]. In wines have been determined flavonols as quercetin [5, 6, 8 - 11], myricetin [5, 7], only in red wines [8], kaempferol [5, 7], in aglycone or glycosidic forms [5, 7], as well as flavan-3-ols (+) catechin [5, 6, 7, 11] and (-) epicatechin [5, 9, 11] etc. Concentrations of tannins and lignans in white wines are much lower than in red ones [1, 19].

Polyphenolic compounds act as reducing agents because they neutralize free radicals or can form complexes with heavy metal ions so that prevent formation of the free radicals [3, 20]. Their antioxidant activity increases with an increase of the number of hydroxyl groups in the structure [21]. A higher consume of phenolic antioxidants (implicit by a moderate consume of wine, especially red wine) is correlated to the decrease of the incidence of some coronary disease [4, 6, 10 - 12] especially due to resveratrol [8] and cancer forms as well [1, 4, 12, 22]. Red wines inhibit platelet aggregation, especially by *trans*-resveratrol [10, 12] and quercetin [10], but also due to their contents in tyrosol and hydroxytyrosol [8]. Catechin and epicatechin [6, 11], tyrosol and hydroxytyrosol [8] can contribute together with resveratrol and quercetin to the cardio- protective effect of the red wines as they inhibit the oxidation of the low-density lipoprotein (LDL) [3, 6] and resveratrol and quercetin distorted eicosanoid synthesis [11, 12]. The flavonoids in wines act as inhibitors of NO [23].

The total antioxidant capacity (TAC) of wines is given mainly by the phenolic content and therefore phenol determination becomes very important. Several methods for polyphenols content determination have been reported

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in literature: (i) spectrophotometric methods, Folin-Ciocalteu method (FCM) for the TP content determination [1, 4, 14], Price and Butler method (PBM) [1] and (ii) chromatographic method, reverse-phase HPLC-DAD [5, 11, 13], HPLC-DA-UV [12], HPLC with colorimetric diode array detector [19], HPLC coupled with a differential refractometer connected in series to a photodiode array detector [2], LC-MS/MS [13], HPLC-DAD-ESI-MS/MS [15], with whom individual polyphenols can be identified. Several other methods developed for total polyphenols and anthocyanins determinations in wines are flow injection analysis (FI), with diode array spectrophotometer [17], based on coupling separation by HPLC with a photometric detection of some anthocyanins [24] and continuous flow methods for on-line determination of polyphenols in wines [25].

The antioxidant capacity (AC) of wines has been determined by: (i) spectrophotometric methods, such as ABTS method for AC determination expressed as Trolox equivalents [1, 5] or vitamin C equivalents [14], DPPH method [1, 6] for testing wine capacity to remove DPPH radical, FRAP method for determining wine antioxidant power to reduce Fe(III) to Fe(II) ion [1] and (ii) methods based on luminescence (fluorimetry and chemi-luminescence). Thus, wine capacity to remove peroxynitrite was determined fluorimetrically by means of a method based on dihydro rhodamine oxidation [23]. AC of the red wines was analyzed by chemiluminescence [3], a method based on the reaction between free radicals, predominantly superoxide anions, resulted by UV irradiation of luminol, in the presence of EDTA. Antioxidant power and polyphenols in red wines were determined in reference [26] by FIA with electrochemical detection.

We present in this contribution a study on the TAC determination in some wine samples by means of a new flow injection analysis (FIA) method. Principles and some applications of FIA methods are given in our previous papers [27, 28]. The proposed method uses a chemiluminescence detection (FIA-CL) and is based on luminol/Co(II)/EDTA/ H_2O_2 system [29].

Experimental part

Reagents and materials

Reagents

Boric acid, cobalt (II) chloride × $6H_2O$ (Reactivul, Bucharest, Romania), 30% hydrogen peroxide (Chimopar, Bucharest), ethylene diamine tetraacetic acid disodium salt (EDTA) (Loba Chemie), luminol, caffeic acid (Sigma), monohydrated gallic acid (Riedel-de Haën), sodium hydroxide (Chemapol, Praha).

Solutions

Co(II)/EDTA/luminol (chemiluminescence reagent) solution, prepared as following: corresponding amounts of cobalt chloride, EDTA-disodic salt and luminol were dissolved successively in 0.05 M, *p*H 9 aqueous buffer solution (prepared in 0.05 M boric acid solution, where the pH was adjusted with 10% NaOH solution). The weighed quantities were so calculated as to correspond to a Co (II): EDTA molar ratio of 0.8, when EDTA concentration was 10^{-3} M. Luminol concentration was 2.3 x 10^{-4} M; 6 x 10^{-4}M H_2O_2 working solution was obtained by dilution the 10^{-1} M H_2O_2 stock solution (prepared in EDTA 2 x10^{-4} M aqueous solution) with the EDTA 2 x 10^{-4} M aqueous solution.

Gallic and caffeic acid solutions were obtained by diluting the 10^{-3} M stock solutions (prepared in 2 x 10^{-4} M EDTA aqueous solution) with EDTA 2 x 10^{-4} M solution to concentrations between 2.5 and 300 μM.

All solutions, with the exception of those mentioned otherwise, were prepared in boiled and then cooled double distilled water. The EDTA was employed to complex heavy metal ions possible present in the prepared solutions. Hydrogen peroxide working solutions were prepared on a daily basis. The other solutions were kept for maximum three days since preparation in refrigerator (+4°C) and brought to room temperature just before measurements.

FIA-CL assembly

The FIA-CL experimental set up consists of: a four-channel Gilson peristaltic pump; a Rheodyne type, model 5051 injection valve with six channels; a specially designed for FIA chemiluminescence detection coupled to a computer whose soft was devised to register the analytical signal at different sample concentrations and at variable time intervals; Tygon tubing for pump; Teflon tubing for FIA system (i.d. = 0.8 mm).

The CL detector is made of: photomultiplier tube (PMT), amplifying system, display screen and a flow cell placed in front of PMT. Power voltage for PMT is 1000 V. The flow cell was manufactured by spiraling a Teflon tube (i.d. = 0.8 mm and length = 50 cm) in the same plane so that an eight winding helix was obtained. The cell is placed on a reflecting aluminum thin foil which guides luminous radiation towards the PMT window. The PMT, flow cell and reflecting thin foil assembly is placed in a sealed precinct so that to prevent its exposure to the exterior light.

A FIA-CL set up with three channels for antioxidant capacity determination is show in figure 1. It contains: (a) 0.05M, *p*H 9 sodium borate carrier flux where the analyzed sample is injected; (b) hydrogen peroxide flux and (c) luminol/Co(II)/EDTA flux, which is the chemiluminescence reagent (CL reagent). H_2O_2 flux (b) is mixed with CL reagent flux (c) within the mixing coil (marked with „L" in fig. 1). The resulting oxidant-luminol/catalyst/complexing agent flux meets the one that carriers 0.05M, *p*H 9 sodium borate (a) before entering the flow cell (marked with „F" in fig. 1), where the CL occurs.

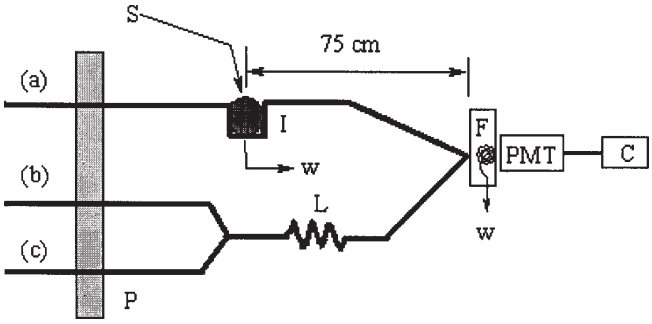


Fig 1. FIA-CL assembly

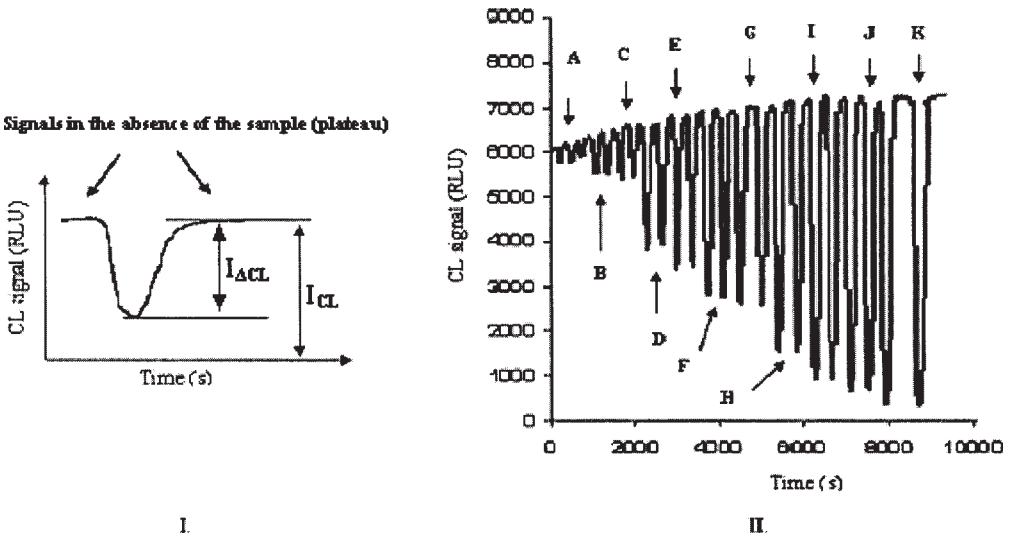
(a) - 0.05 M, pH 9 sodium borate carrier flux; (b) - 6 . 10^{-4} M H_2O_2 flux; (c) - Co(II)/EDTA/luminol (0.80x10^{-3} M / 1x10^{-3} M / 2.3x10^{-4} M) flux;

P - peristaltic pump; I - injection valve; L – mixing coil (L = 150 cm, i. d. = 0.8 cm); F –flow cell; PMT - chemiluminescence detector (photomultiplier tube); C – computer; S – sample; w –waste

Determination of total antioxidant capacity using the FIA-CL method

Principle of method

The proposed FIA-CL method is based on the reaction between luminol and hydroxyl radicals generated by a Fenton-type reaction of H_2O_2 with Co (II) ions (released from Co (EDTA) complex). As Co(II) ions are freed from the complex in a very low concentration, the amount of $HO^{\cdot}$ radicals generated by the reaction with H_2O_2 is accordingly very low. The resulted hydroxyl radicals react with luminol and generate a chemiluminescence radiation for a period of time, of the order of tens of seconds. In the absence of an antioxidant, a relatively constant CL signal



I. II.

Fig. 2. Shape of FIA-CL signals: I. Typical shape of a chemiluminescence signal registered when sample is injected into the flux; II. Signals registered when both etalon solutions and analyzed samples are injected A –doubly distilled water, B -M gallic acid solutions with different concentrations: B- 4 μM, C -6 μM, D -10 μM, E -20 μM, F - 35 μM, G -50 μM, H-75 μM, I -100 μM, J -200 μM, K -300 μM, RLU = relative units; I_{CL} = the background CL signal. Working procedure was as above

of certain intensity is registered as a plateau when carrier and the reagents are pumped by using the FIA-CL assembly (fig. 1).

In the presence of an antioxidant injected in the carrier flux (a) during the constant light emission (plateau), a decrease of the CL signal is registered which is proportional to antioxidant type, activity and concentration. The shape of the signals registered when a sample containing an antioxidant is injected in the carrier is depicted in figure 2.

Working procedure

The total flow rate of fluxes through FIA assembly was 0.5 mL/min and it was adjusted at the beginning of each series of measurements so that to be the same through all three channels. Volume of the injection coil (fig. 1) was 70 μL. Temperature was maintained at 20-25 °C.

Antioxidant solutions (standards/real samples) prepared in 2 x 10^{-4} M EDTA were injected in the 0.05M *p*H 9 borate buffer carrier flux (channel (a) in fig.1). Each sample was injected at least twice and an average was calculated.

Shapes of the signals registered when the antioxidant solutions were injected under the mentioned working conditions are given in figure 2.II.

Computation procedure

The shape of a FIA-CL signal is presented in figure 2.I. I_{CL} value (fig. 2.I) corresponds to the background CL signal and is given by a CL reaction between the active oxygen species formed by H_2O_2 (free radicals) with luminol. $I_{\Delta CL}$ value corresponds to the I_{CL} decrease due to neutralization of a part of the free radicals during their reaction with the antioxidant agent. The $I_{\Delta CL}$ value for CL signal was measured for both standards and analyzed sample solutions. In all cases, the $I_{\Delta CL}$ value for a comparison sample (which does not contain an antioxidant) was subtracted from $I_{\Delta CL}$ and $I'_{\Delta CL}$ was obtained.

The percent decrease of CL signal for the sample is calculated with the relation (1):

$$(I'_{\Delta CL}/I_{CL}) \times 100 \quad (1)$$

Calibration curves representing ($I'_{\Delta CL}/I_{CL}$ x 100) as a function of antioxidant concentration (μM) are drawn.

In order to compute the antioxidant capacity value for a sample, the value of the registered analytical signal is interpolated on the calibration curve and sample concentration is determined (in μmoles L^{-1}). Total antioxidant capacity (TAC) of the analyzed samples was expressed in terms of equivalents antioxidant grams (gallic or caffeic acid) in 1000 mL sample.

Results and discussions

Calibration curves ($I'_{\Delta CL}/I_{CL}$ x 100) as a function of gallic acid and respectively, caffeic acid concentration are show in figure 3A. Standard solution concentrations are between 2.5 and 300 μM. Working procedure was as above.

As one can see in figure 3B, the linearity domain for gallic acid lies between 10 and 100 μM, while caffeic acid between 2.5 and 20 μM.

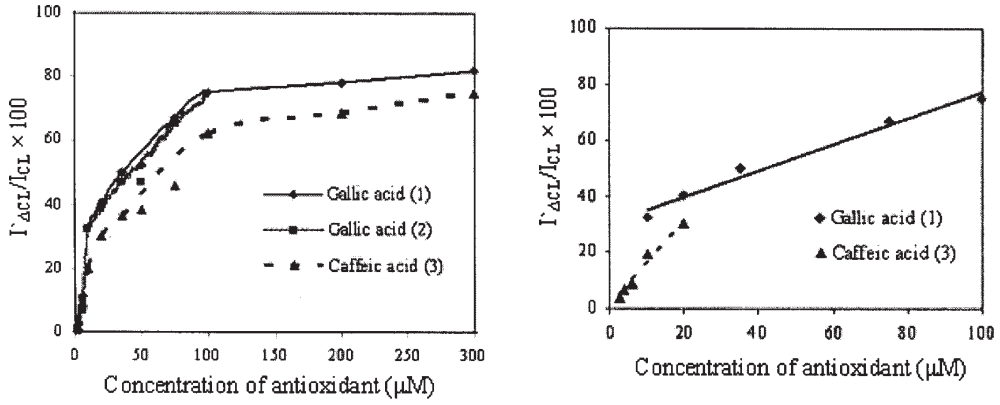


Fig. 3. Calibration curves ($I'_{\Delta CL}/I_{CL}$ × 100) as a function of gallic acid (curves 1 and 2) and respectively caffeic acid (curve 3) concentrations (μM). A. calibration curves; B. linearity domain. Working procedure as above. Calibration curves for gallic acid were drawn in the same conditions but at 24 h time interval

Table 1
ANALYZED WINE SAMPLES AND RESULTS AT THEIR TAC DETERMINATIONS

Nr.	Sample	Sample type/bottling year	TAC	
			g gallic acid equivalents /L wine	g caffeic acid equivalents /L wine
1	P1	„Cabernet" red wine (Recas/ 2009)	0.54	1.77
2	P2	„Bull blood" red wine (Tohani/ 2008)	0.55	1.78
3	P3	„Feteasca" white wine (Jidvei/ 2009)	0.19	0.44
4	P4	„Sauvignon Blanc" white wine (Zoreşti Wine House/ 2008)	0.3	0.73

Two measurements were performed for each sample and the average value is presented

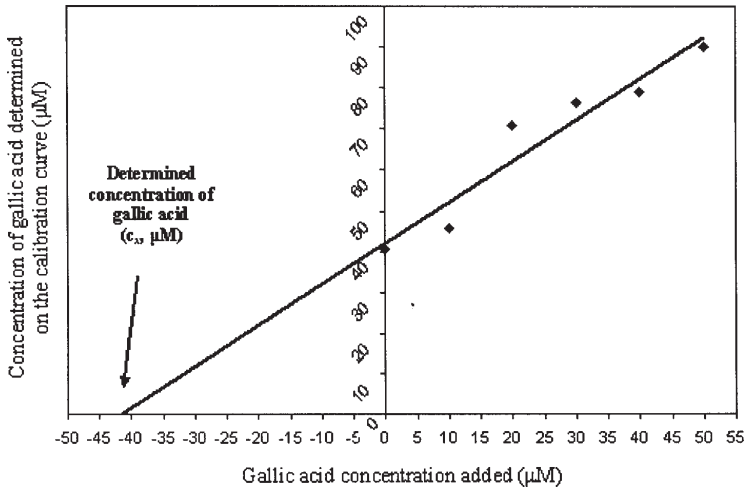


Fig. 4. Experimental results obtained with standard addition method

The curve equation (1) for gallic acid is:

$(I'_{\Delta CL}/I_{CL}$ x 100) = 0.4609 x gallic acid concentration (μM) + 30.925, and correlation coefficient

R^2/n = 0.9819/ 5.

For caffeic acid equation curve (3) is:

$(I'_{\Delta CL}/I_{CL}$ x 100) = 1.5358 x caffeic acid concentration (μM) + 0.9436, and R^2/n = 0.9646/ 5.

Relative standard deviation (RSD) is 2.41% (n = 10) for a 35 μM gallic acid concentration.

Wine samples analysis

The presented FIA-CL method was used for analyses of some Romanian wine samples presented in table 1.

Sample preparation for analysis. Approximately 10 mL of each wine sample were taken in sealed bottles and kept in the refrigerator (+4°C). They were then correspondingly diluted before measurements with a 2 x 10^{-4} M EDTA aqueous solution.

TAC determination

Working procedure was as that given above. The comparison liquid was water.

TAC values for the analyzed wines are listed in table 1.

One can see from table 1 that TAC values lie between 0.19 and 0.55 g gallic acid equivalents / L wine and between 0.44 and 1.78 g caffeic acid equivalents / L wine. In table 1 is seen that red wines exhibit a total antioxidant capacity higher than white ones.

The accuracy of the results was checked with standard addition method. Thus, into six 10 mL volumetric flasks known quantities of gallic acid from a 10^{-3} M standard solution (obtained in EDTA 2 x 10^{-4} M aqueous solution) and volumes between 0 and 500 μL were added. Then, „Cabernet" red wine sample (diluted 1/200 with EDTA 2 x 10^{-4} M aqueous solution) was added to the final volume of 10 mL. Gallic acid added concentrations in samples were 0, 10, 20, 30, 40 and 50 μmoles L^{-1} respectively. The samples were analyzed by the studied method. The obtained results are presented in figure 4.

If one represents gallic acid concentrations obtained by interpolation on the calibration curve (2) in figure 2A as a function of gallic acid concentrations added to the wine sample a straight line is obtained (fig. 4). Concentration of gallic acid in the analyzed sample is obtained from the intercept of the straight line (fig. 4) with abscise. A concentration of 41.5 μM gallic acid equivalents, corresponding to a 40.5 μM gallic acid equivalents was determined thus in the analyzed sample. The two concentration values differ with less than 3% of each other, a fact that proves that the method can be applied without interferences.

Several values of the TP content (TAC of wines is given especially by phenols) and antioxidant activity for wines as reported in literature [1, 4, 14, 30, 31], comparatively with TAC values obtained by our FIA-CL method are show in table 2.

One can see from table 2, our results obtained by FIA-CL method are in agreement with those reported in literature.

Table 2
DETERMINED VALUES FOR TOTAL PHENOL CONTENT AND ANTIOXIDANT ACTIVITY OF SOME WINES REPORTED IN LITERATURE AND TAC VALUES OBTAINED BY OUR METHOD

Phenolic compound content (as gallic acid equivalents)					Antioxidant activity (as Trolox equivalents)			References
Determined by FCM[a]		Determined by PBM[b]		Determined by He-Ne Laser OW[c]	Determined by TEAC[d]	Determined by FRAP[e]	Determined by DPPH[f]	
mmol/L	mg/L	mmol/L	mg/L	mg/	mmol/L	mmol/L	mmol/L	
Red wines (n = 29)								[1]
5.14 – 3.30	874 - 2262	1.80 – 4.80	306 - 816	-	13.89 – 34.69	4.92 – 13.94	2.91 – 8.62	
White wines (n = 8)								
0.53 – 0.97	90 -166	0.53 – 1.21	90 – 206	-	4.3 – 8.44	0.86 – 2.14	0.61 – 1.78	
Red wines (n = 3)				Red wines (n =256)	-	-	-	[4]
-	782.79– 1615.80	-	-	786.9 – 1629.9	-	-	-	
Red wine (n = 1)								[14]
-	243*	-	-	-	-	-	-	
Red wines (n = 5)								[30]
	1724 – 1936				732 – 1105**	3.45 – 3.86***	0.56 – 0.71	
White wine (n = 5)								
	282 - 434				344 – 508**	0.44 – 0.67***	0.04 – 0.08	
Rosé wines (n = 1)								
	665				502**	1.37***	0.24	
Red wines (n = 3)								[31]
-	-	-	-	-	8.95 – 12.14	23.90 – 31.53****	11.73 – 16.09[g]	
White wine (n = 3)								
-	-	-	-		1.61 – 1.94	3.72 – 5.04****	1.86 – 2.32[g]	
Rosé wines (n = 3)								
-	-	-	-		1.52 – 2.42	4.66 – 8.33****	1.98 – 3.20[g]	
Red wines (n = 2)								Our results (obtained by FIA-CL)
TAC (as gallic acid equivalents)					TAC (as caffeic acid equivalents)			
mmol/L		mg/L			mmol/L		mg/L	
3.2 -3.22		544.38– 547.79			9.80 – 9.90		1765.57 – 1783.58	
White wines (n = 2)								
1.10 – 1.75		187.13– 297.71			2.45 – 4.05		441.39– 729.65	

*reported in [14] as 340 mg GAE/ red wine serving (140 mL) and 163 mg epicatechin equivalents/ serving; **reported as mg/L gallic acid equivalents; *** mM quercetin equivalents; ****mmol Fe^{2+}/L
[a]FCM = Folin-Ciocâlteu method; [b]PBM = Price-Butler method; [c]He-Ne Laser OW = He-Ne Laser Optothermal Window assay; [d]TEAC = Trolox equivalent antioxidant capacity; [e]FRAP = ferric reducing-antioxidant power; [f]DPPH = 2,2-Diphenyl-1-picrylhydrazyl Radical Scavengingg; [g]TRAP = Total Radical-Trapping Antioxidant Parameter Assay

Conclusions

A FIA-CL method based on Co(II)/EDTA/luminol system is proposed for determining the total antioxidant capacity of some liquid samples. It implies a CL reaction between luminol and hydroxyl radicals generated by the hydrogen peroxide in the presence of Co(II) ions (catalyst for the CL reaction) complexed with EDTA, according to a Fenton-type reaction. The resulted hydroxyl radicals react with luminol and produce a luminol radical. This reacts further with antioxidant species.

We designed a FIA-CL assembly to implement the method. This assembly is made of three channels through which corresponding liquids are pumped: a carrier flux of 0.05 M, *p*H 9 sodium borate solution where the analyzed sample is injected, a H_2O_2 flux and a Co(II)/EDTA/luminol flux (fig. 1).

In the absence of an antioxidant injected in the carrier a CL signal of certain and relatively constant intensity value is registered. When an antioxidant-containing sample is injected into the carrier during the constant light emission, a decrease of CL signal occurs proportionally to antioxidant type, concentration and activity.

The studying method allows for determining the TAC of several liquid samples expressed in gallic and caffeic acid equivalents within a concentration domain between 2.5 and 300 μM, with a linearity range from 10 to 100 μM for gallic acid and from 2.5 to 20 μM for caffeic acid. Our method was employed for determining the TAC of several wines.

A comparison between TAC values for the analyzed wine samples and the data in literature in terms of TP and AC and between TAC values for different wine samples was making. An agreement between TAC values determined by the proposed method and those reported in literature was found.

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