Chemiluminescence Determination of the Total Antioxidant Capacity of Rosemary Extract

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Total antioxidant capacity (TAC) of some Rosmarinus officinalis L. (rosemary) extracts was investigated by an "in batch" analytical method based on chemiluminescence (CL). *This method is based on a CL 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 method allows for TAC determination in 1 x 10^{-5} – 2.5 10^{-3} moles L^{-1}domain of gallic acid concentration and with a relative standard deviation (RSD) = 2.53% (n = 10) for 8 x 10^{-4} M gallic acid concentration. The TAC values determined for the analyzed rosemary extracts ranged between 1.6 and 4.58 grams of gallic acid equivalents/100 g dw plant. The employed analytical method was verified by applying the standard addition method and a good precision was established.*

Keywords: total antioxidant capacity, chemiluminescence analysis, rosemary extract, gallic acid

Studies of the free radicals and antioxidant capacities of different materials of vegetable nature are widely presented in literature [1-7].

The extracts of medicinal plants and condiments such as rosemary, sage, tea (green or black), of vegetables (red or purple onion, red cabbage, red beetroot, pepper) and of fruit (grapes, sea buckthorn, pomegranate) are constituted of complex matrices with rich polyphenols contents which contribute significantly to their total antioxidant capacity [8-16].

Among medicinal plants and condiments, *Rosmarinus officinalis* L. (rosemary, *Lamiaceae*) is to be mentioned because its leaves are used in digestive disorders, treatment of vertigo and headaches, rheumatism and gout, to control senescent process, as well as condiments, while its floral somities (to prepare volatile oil, condiments and preservatives for meat products and parts of the composition of inhaling substances, soaps and room deodorants) [17]. It has been shown in literature that leaves of this plant contain polyphenols compounds with antioxidant capacity such as polyphenols carboxylic acids (rosmarinic, caffeic, gentisic, vanillic, syringic, gallic), flavonosides (diosmetin, derivates of luteolin and apigenin), tannins, bitter diterpene principles (carnosol, carnosic acid, rosmanol, rosemary diphenol), triterpene (betulin, ursolic and oleanoic acids and their hydroxylated derivates) etc. [17]. Rosemary extracts was obtained with good efficiency in dry state and it is characterized by a high antioxidant capacity. As it is a dry extract, can be weighed, analyzed and used quite easily for different purposes.

In literature is described a HPLC method for the analysis of principal phenolic antioxidants in fresh rosemary [18] and several methods for determination of phenolic content and antioxidant activity of rosemary extracts [19-21].

A variety of methods have been purposed for determining the antioxidant capacity of different products including here vegetable extracts. We will mention in the following some of the reviews [22-29] concerned with these methods of determination. Chemiluminometric analytical procedures can be employed with very good results for determining the antioxidant capacity of several plant extracts [15, 30], wines [31]. When chemiluminescence detection is completed with a flow injection analysis (FIA) methodology [31-34] increased measurement performances are achieved. Good results are obtained also when FIA methodology is coupled with other detection methods [32, 35-37].

The aims of this work were: a) obtaining of rosemary extracts by several methods; b) drawing of calibration curves for establishing an etalon substance (gallic acid) by an "in batch" analytical method based on chemiluminescence [38]; c) determination of the total antioxidant capacity of some rosemary extracts.

Experimental part

Reagents and materials

Reagents. Boric acid, ethylene diamine tetraacetic acid disodium salt (EDTA) (Aldrich), cobalt (II) chloride x $6H_2O$ (Reactivul, Bucharest, Romania), 30% m/v hydrogen peroxide (Chimopar, Bucharest), 5-amino-2,3-dihydrophtalazine-1,4-dione (luminol), gallic acid (Sigma), sodium hydroxide (Chemapol, Praha), ethanol (S.C. PAM Corporation SRL, Bucharest).

Solutions. 0.1 M, pH 9 sodium borate buffer solution was prepared from a 0.1M boric acid and 10% NaOH solutions;

- 3 x 10^{-3} M Na_2EDTA solution was obtained by dissolving of 0.114 g EDTA in the borate buffer solution;

- 3 x 0.8 x 10^{-3} M $CoCl_2$ x $6H_2O$ solution was prepared by dissolving 0.0571 g in 100 mL EDTA solution, (Co (II)/ EDTA molar ratio = 0.8) in borate buffer solution (0.1 M, pH 9). The solution brought to a final volume of 250 mL with the respective borate buffer;

- 3.39 x 10^{-4} M luminol solution was prepared by dissolving 0.0060 g luminol in 100 mL 0.1 M, *p*H 9 borate buffer;

- 3 × 10^{-4} M H_2O_2 solution was obtained by a corresponding dilution of a 10^{-1} M H_2O_2 stock solution;

-working solution for chemiluminescence determinations (prepared on a daily basis): 25 mL 3 × 10^{-3} M Na_2EDTA solution is mixed with 25 mL of 3 × 0.8 × 10^{-3} M $CoCl_2$ × $6H_2O$ solution and 25 mL luminol solution, all in borate buffer;

- standard solutions of gallic acid of concentrations between 2 × 10^{-3} and 10^{-5} M gallic acid stock solutions. Stock solution as well as working solutions were prepared in ethanol: 2 × 10^{-4} M EDTA in water = 80: 20 (v/v).

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With the exception of the mentioned cases, all solutions were prepared in double-distilled water.

- solutions for plant extracts were obtained in ethanol: 2 × 10^{-4} M EDTA in water = 80: 20 (v/v).

Plant materials

Vegetable materials: rosemary, dry product from Dacia-Plant (batch 11528).

Plant extracts preparation

Rosemary extract was obtained by three methods:

a) Soxhlet extraction: a know amount of dry and finely ground rosemary was submitted to continuous extraction by means of a Soxhlet extractor, using 96% ethanol as solvent. Extraction time was 10 h;

b) maceration: over a known quantity of dry and finely ground rosemary 100 mL of 96% ethanol were added in an Erlenmeyer flask (200 mL) with neck and ground glass stopper. The vessel content was stirred and then left at room temperature for five days, with occasional stirring.

c) ultrasonically: by means of an ultrasonic batch (Langford Sonomatic, 33 kHz, 100 W power). Extraction time was 90 min (25 – 50 °C). An indirect ultrasonic method was employed when both vegetable product and extraction solvent were placed into an Erlenmeyer flask (100 mL) which was then introduced in the ultrasonic batch closed with an especial rubber stopper.

The obtained extracts were filtered on large pore paper (red band). The solvent was removed by means of a rotary vacuum evaporator and the extracts were taken and dried down to room temperature in Petri boxes.

Apparatus

All CL measurements were carried out with a Turner Biosystems 20^{n}/20 luminometer with two injectors [39] coupled to a computer whose software ("SIS for 2020h") allows for recording the light intensity:

-in a numerical form (relative luminescence units, RLU);

-in a graphical form in MS-EXCEL programmed to record chemiluminescence intensity (RLU) as a function of time (s).

The sample and reagents are mixed together in the reaction vessel, an Eppendorf tube (of approximately 2 mL) which is placed inside the apparatus in order to perform chemiluminescence measurements.

Working procedure

In the reaction vessel, 350 mL of working solution for chemiluminescence determinations, 350 μL of 0.1 M, *p*H 9 of borate buffer solution and 350 μL of 3 x 10^{-4}M H_2O_2 solution are introduced. After the last solution was added, the mixture is homogenized by aspiring liquid several times by means of the pipette and then introducing it back into the reaction mixture. Right away, the intensity of chemiluminescence radiation is measured, whose value is noted with I_0. After 600 min from beginning determinations, 25 mL from the analyzed sample (antioxidant etalon or plant extracts solutions) are added to the reaction mixture. Chemiluminescence measurements are performed after four seconds since the antioxidant solution was introduced, a period of time which includes a reagent homogenisation inside the Eppendorf tube by means of the pipette. Readings were made at intervals of 1 s. When the analyzed sample is introduced in the reaction mixture, a decrease of the chemiluminescence signal is registered, whose value is noted with I.

I_0/I ratio is computed and represented as a function of the gallic acid concentration (used as an etalon) and a calibration curve is thus drawn. The same procedure was used for rosemary extract analysis. The antioxidant capacity of rosemary extracts was expressed as mg equivalents of gallic acid / 100 g dw plant.

Results and discussions

Establishing of the experimental working conditions

The chemiluminescence signal variation as a function of time for the reaction mixture that is working solution for chemiluminescence measurements to which 3 × 10^{-4} M H_2O_2 solution was added is depicted in figure 1.

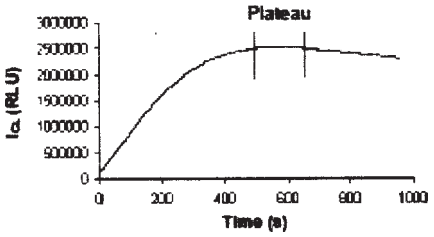

Fig. 1. Shape of CL signal for luminol-H_2O_2–Co(II)EDTA system. I_{CL}: intensity of chemiluminescence signal

One can see from figure 1 that at reaction time values of 600 ± 50 s the chemiluminescence signal values are practically constant. This is why all CL determinations were carried out after 600 s since reagents were introduced in the reaction mixture. A variation of the chemiluminescence signal similar with that presented in figure 1 in a luminol-H_2O_2–Co(II)EDTA medium were reported also in the papers [40, 41].

The CL signal variation when an antioxidant solution is added to the reaction mixture 600 s after 3 × 10^{-4} M H_2O_2 solution was added is shown in figure 2.

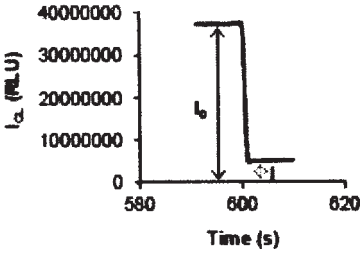

Fig. 2. CL signal variation when an antioxidant solution is added to the reaction mixture I_0: chemiluminescence signal before the analyzed sample was added; I: chemiluminescence signal after the analyzed sample was added

Figure 2 shows that a steep decrease of CL signal is registered when antioxidant solution is added into the reaction mixture whose signal remains then constant for a certain time interval. A detailed study on the experimental parameters of the analysis method in order to establish the optimal working conditions will be given in a next paper [38].

Calibration curves.

The average of the CL signal values were registered for 10 s before (it is obtained I_0) and after (it is obtained I) introducing antioxidant solution (standard or analyzed extract solutions). The I_0/I ratios were computed by using the values for I_0 and I fig. 2). Three recording were done for each analyzed solution, the I_0/I average values were calculated and then I_0/I average (for three measurements) of the comparison sample (ethanol: water = 80%, v/v) was subtracted. The calibration curve was drawn by representing this difference as a function of antioxidant concentration. The values for I_0/I ratios of the different standard gallic acid solutions are listed in table 1.

Gallic acid concentration of samples (M)	I_0/I *
1.0×10^{-5}	6.20
1.0×10^{-4}	11.0
2.5×10^{-4}	24.8
4.0×10^{-4}	34.9
6.0×10^{-4}	83.7
7.5×10^{-4}	98.9
8.0×10^{-4}	103.5
1.0×10^{-3}	130.5
1.5×10^{-3}	118.5
2.0×10^{-3}	181.5

Table 1
AVERAGE VALUES of I_0/I RATIOS for STANDARD GALLIC ACID SOLUTIONS

*I_0/I: I_0/I ratio values for analyzed solutions, from which the I_0/I value for blank was substracted (I_0/I for blank is 2.49). Values are expressed as mean of three measurements. Working procedure according to the one presented above.

The I_0/I calibration curve as a function of gallic acid concentration (mM) was drawn.

Calibration curve equation is:

$$y = 102.48\ x + 4.11 \quad (1)$$

where $y = I_0/I$ ratio and x = concentration of the gallic acid (mM).

The correlation coefficient is:

$$r^2 = 0.9938\ (n = 5),$$

where n = number of measurements.

A good correlation between I_0/I values and gallic acid concentration is noticed.

Relative standard deviation was computed for a concentration of 8×10^{-4} M gallic acid, RSD = 2.53% (n = 10).

Determination of the antioxidant capacity of some rosemary extracts

In table 2 it is presented the yields for obtaining the rosemary plant extracts using extraction methods described in the experimental part.

It can be seen that of the three extraction methods used, the highest extraction efficiency was obtained for continuous extraction. Extraction by maceration and ultrasonic assisted extraction have closely yields.

Sample preparation for analysis.

Some 0.010 g from each extract were ultrasonically dissolved in approx. 5 mL ethanol: 2×10^{-4} M EDTA in water = 80: 20 (v/v). The obtained solution was brought to 10 mL with the same solvent.

Determination of the sample antioxidant capacity

The blank sample was a solution of ethanol: 2×10^{-4} M EDTA in water = 80: 20 (v/v).

The determined I_0/I values and the concentrations (mmoles L^{-1}, respectively mg equivalents of gallic acid/ 100 g dried plant and /g extract) for the analyzed extracts are given in table 3.

As can be seen in table 3, the antioxidant activity of the extracts obtained by different methods ranges between 1.6 and 4.58 g gallic acid equivalents/100 g dw.

The standard addition method was applied in order to verify the accuracy of the obtained results. The same volumes of rosemary extract solution were added in five 10 mL flasks together with known quantities of gallic acid from a 3×10^{-3} M standard solution, so that finally the added gallic acid concentrations in the respective flasks were 0; 0.20; 0.40; 0.60 and 1.00 mmoles L^{-1}. The samples were analyzed subsequently by the method described in the present work. The obtained results are depicted in figure 3.

A straight line is obtained (fig. 3) when representing gallic acid concentrations as determined from the calibration curve equation *vs*. the concentrations of gallic acid added to the rosemary sample (macerated). If the straight line is extended to intersect the abscise (fig. 3) are obtain the gallic acid concentrations in the analyzed rosemary extract. A concentration of 1.01 mM gallic acid equivalents was determined in the analyzed sample, which corresponds to a concentration of 1.04 mM gallic acid equivalents (determined by direct method). The two values differ with less than 3% from each other. The method can be then applied without interference.

A comparison was made between the obtained results and the antioxidant activities of several rosemary extracts reported in literature (table 4) [42-46]. The authors in references [43] and [44] reached the conclusion that rosemary had a very good antioxidant activity.

As can be seen in tables 3 and 4, the results obtained to antioxidant capacity determination of rosemary extracts by method presented in this paper are in a good agreement with the literature data.

Extraction method	Amount of dried plant taken in work (g)	Amount of obtained extract (g)	Yield of dry extract (%)	vegetable material: 96% ethanol ratio (m/v)
Continuous extraction (Soxhlet)	20	4.26	21.3	1:10
Maceration	10	1.10	11	
Ultrasonically assisted extraction	5	0.58	11.6	

Table 2
EXTRACTION YIELDS OF ROSEMARY EXTRACTS OBTAINING BY STUDIED METHODS

Extraction method	I_0/I^*	Antioxidant activity		
		mmol/L (from equation (1))	mg equivalents gallic acid / 100 g dw**	mg equivalents gallic acid / g extract
Continuous extraction (Soxhlet)	134 ± 11.9***	1.27	4580	215
Maceration	97.8 ± 1.92	0.91	1707	156
Ultrasonically assisted extraction	86.9 ± 3.7	0.81	1595	138

*Values are expressed as mean of three measurements.

**dry weight

***standard deviation

Table 3
RESULTS OBTAINED FOR THE ANTIOXIDANT ACTIVITY OF ROSEMARY EXTRACTS BY THE THREE EXTRACTION METHODS

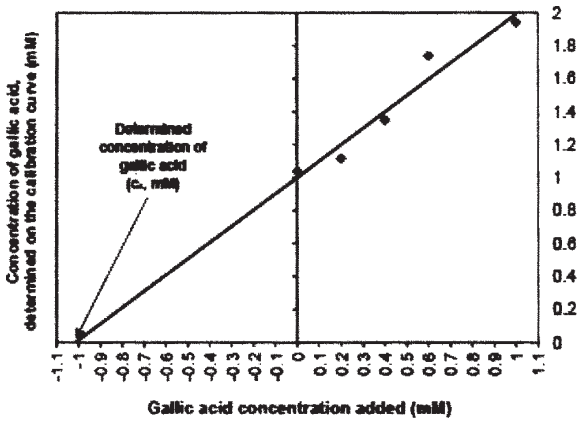

Fig. 3. Experimental results obtained with standard addition method
Results are the average of three determinations

Antioxidant activity (as gallic acid equivalents)	Antioxidant activity (as caffeic acid equivalents/others)	Total phenolic content [a] (as gallic acid equivalents)	Total phenolic content [a] (as caffeic acid equivalents/others)	References
-	-	185 mg equivalents / g dry extract	-	[42]
		22 mg equivalents / g dry extract		[44]
1848 mg equivalents/ 100 g dw[b] (continuous extraction); 1430 mg equivalents/ 100 g dw (UAE [c]) (determined by „d")	3243 mg caffeic acid equivalents/100 g dw (continuous extraction); 1441 mg caffeic acid equivalents /100 g dw (UAE) (determined by „d")	1416 mg equivalents/ 100 g dw (continuous extraction); 565 mg equivalents/ 100 g dw (UAE)	1222 mg caffeic acid equivalents /100 g dw (continuous extraction); 776 mg caffeic acid equivalents /100 g dw (UAE) 1922 mg ferulic acid equivalents/100 g dw (continuous extraction); 879 mg ferulic acid equivalents/100 g dw (UAE)	[45]
38.7 μM Trolox/100 g dw (determined by „e")	513 μM Trolox/100 g dw (determined by „f")	662 μM Trolox/100 g dw (determined by „g")	1.71 mg equivalents/ 100 g dw	406 mg caffeic acid[h]/100 g dw; 36.2 mg ferulic acid[h]/100 g dw	[46]

[a]determined by Folin-Ciocalteu method; [b]dry weight; [c] UAE: ultrasonically assisted extraction; [d]determined by flow injection analysis with chemiluminescence detection (FIA-CL); [e]TEAC: Trolox equivalent antioxidant capacity; [f]DPPH: 2,2-Diphenyl-1-picrylhydrazyl Radical Scavengingg; [g]FRAP: ferric reducing-antioxidant power; [h]phenols content, determined by a HPLC method

Table 4
ANTIOXIDANT ACTIVITY AND TOTAL PHENOLIC CONTENT OF SEVERAL ROSEMARY EXTRACTS REPORTED IN LITERATURE

The rosemary has a significant antioxidant activity being an important source of polyphenols. Rosemary extract is more efficient than ascorbic acid for inhibiting lipide oxidation but no significant differences were found between antimicrobial capacities of the ascorbic and respectively, rosmarinic acids on seafood storage [47]. Rosemary extracts can be used for delaying the fried ground peas to become musty [48].

Conclusions

Rosemary extracts were obtained by three methods: continuous (Soxhlet), maceration and ultrasound assisted extraction. The extraction yields for the three methods were computed. The extraction with Soxhlet extractor has proved to be the most effective.

Determination of the antioxidant capacity of the three obtained rosemary extraction was carried out by means of a chemiluminometric method of analysis based on luminol-Co(II)-H_2O_2 system. Gallic acid was used as an standard substance with antioxidant properties. A calibration curve for gallic acid determination within a 1×10^{-5} – $2.5\ 10^{-3}$ moles L^{-1} domain was drawn. RSD = 2.53% (n = 10) when gallic acid concentration is 8×10^{-4} M. The antioxidant activities of the analyzed rosemary extracts ranged between 1.6 and 4.58 g of gallic acid equivalents/ 100 g dw plant. The obtained results are in a good agreement with literature data. The employed analytical method was verified by applying the standard addition method and a good precision was established.

The experimental results prove that rosemary has a significant antioxidant activity being an important polyphenolic source.

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