Total Antioxidant Capacity of Some Fruit Seeds Extracts

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The objective of the study has been to determine total antioxidant capacity (TAC) of some fruit seeds extracts by a chemiluminescence (CL) method, based on a luminol/ Co(II)-EDTA/ H_2O_2 system. Extracts of Citrus x limon (lemon), a variety of *Citrus reticulata* (clementine), *Vitis vinifera* (white grapes), and *Citrullus lanatus* (watermelon), were prepared by refluxing *96 % ethylic alcohol. The calibration curve was drawn by using the quercitin over a concentration between 10^{-5} –10^{-3} moles L^{-1}. RSD was 2.65% (n = 10, $c_{quercetin}$ = 3.5 x 10^{-5} M). TAC values determined for the analyzed seeds extracts were as follows: 603 for lemon, 594 for grapes, 437 for watermelon and 279 for Clementine, all in quercetin mg equivalent/100 g dw. The precision of the method was verified by applying a standard addition method.*

Keywords: total antioxidant capacity, chemiluminescence analysis, fruit seeds extract, quercetin

A diversity of secondary plant metabolite with an antioxidant character are present in the vegetal extracts, among which we mention: tocopherols, carotenoids, phenolic acids, flavonoides etc. These compounds intervene in the cellular defense mechanisms against the free radicals and oxidative stress [1-5], as they posses anticancerous and antimutative effect [6-8].

It has been reported in literature [9] that several compounds with a strong antioxidant capacity, such as lycopen, L-ascorbic acid, some polyphenols and their glycosides, contribute to the total antioxidant activity (TAC). Chemical structures of some antioxidants which are usually found in the citric seeds are depicted in figure 1.

Citrus seed extracts have a high antioxidant activity in comparison with those from other plant seeds, but in the most cases TAC is lower than that of the similar extracts obtained from the peel [9, 11]. Flavonoids such as epigalocatechin, rutin, naringin, hesperidin, quercitin and phenolic acids (gallic, caffeic, chlorogenic, vanillic, syringic, ferrulic, rosmarinic, *trans*-2-hydroxycinamic) are common in citric seeds.

Concentrations of these compounds are modified as a function of fruit matureness (and implicitly of the seeds) so that when ripe the fruit seeds contain large amount of antioxidants [10]. The polyphenolic composition of citrus seed compounds differs a great deal from one species to other but 7-O-glicozoflavones prevail in all of them. Different parts of the fruits (seeds, pulp, peel) have different polyphenolic composition. As example, eriocitrin and hesperidin prevail in lemon seeds, while neoeriocitrin, narginin and neohesperidin are mostly present in the peel [12]. Although naginin is found in the peel as well as seeds, it is absent in fruit pulp.

Grape seeds contain approximately 7% polyphenols [13] among which an important part of is represented by proantocianides. The later form a group of bioflavonoids,

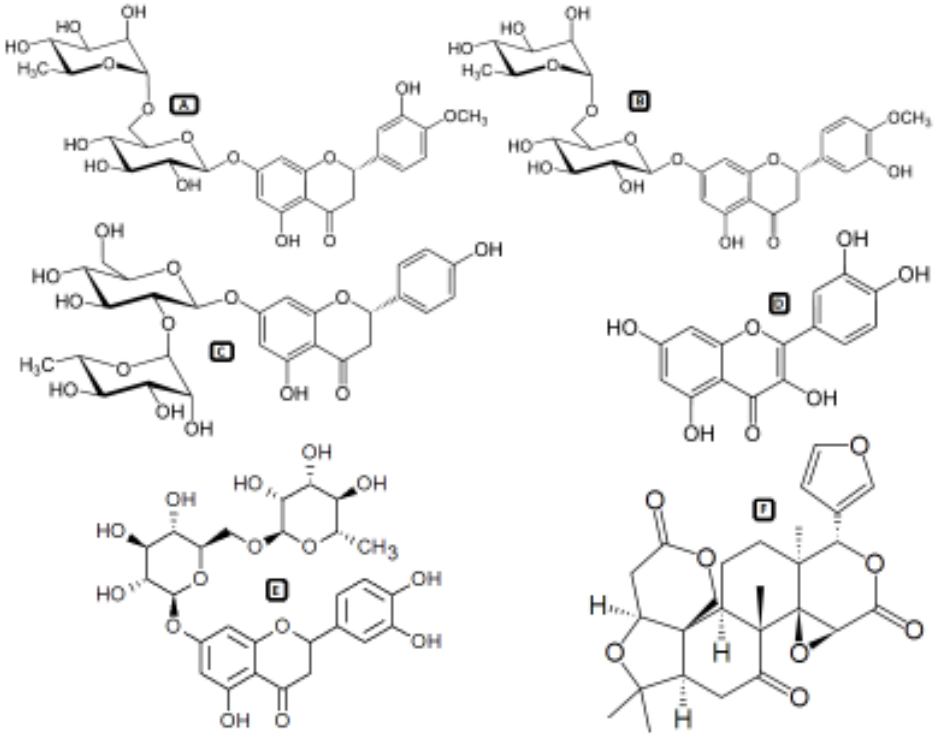

Fig.1. Compounds with antioxidant activity usually formed in citric seeds [9, 10] A-hesperidin, B- neohesperidin, C- naringin, D- quercitin, E- eriocitrin, F- limonin

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which have also a cardioprotective effect [14], as well as a high therapeutic potential for an entire series of disease [13]. The presence of caftaric, cutaric, gallic, caffeic, *p*-cumaric and ferulic acids, catechin, epicatechin, quercitin, epicatechin-gallate and ramnosides was evidenced in grape seeds [15], as some of these compounds have antimutagenic and antiviral characters [16, 17].

The antioxidant activity determination can be performed by several methods as described in the literature: ferric reducing antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity assay (ORAC) [18-20]. Electrochemical methods represent an alternative for antioxidant activity studies, but the matrix complexity makes the result interpretation quite difficult [5, 6, 21, 22].

Several chemiluminescent systems can be employed for antioxidants and TAC determinations. Such systems consists of luminol - Co (II) - H_2O_2 [23, 24] or luminol - Co (II) - perborate [24]. The latter was applied to determining the antioxidant capacity of some grape seed extracts [24]. The selectivity of the above methods is low, making it in fact a determination of total antioxidant capacity, which is expressed by a reference antioxidant[6], such as gallic acid, ascorbic acid, uric acid, caffeic acid as well as quercetin.

In order to indentify the antioxidant compounds and evaluate their corresponding antioxidant activity, the above methods are coupled with separation techniques, such as LC or HPLC, and thus the antioxidant species in the analyzed system can be indentified [11, 25, 26].

The data for antioxidant capacity determination of fruit seed extracts are rather scarce in literature.

In this work total antioxidant capacity of ethanolic extracts obtained from the seeds of some citrus (lemon and clementine), grapes and watermelon was studied by means of an "in batch" analytical method based on chemiluminescence which was described in our previously paper [27]. The results were reported in terms of quercetin equivalents and a comparison between experimental data and several values from literature was performed.

Experimental part

Reagents and materials

Reagents

Boric acid, ethylene diamine tetraacetic acid disodium salt (EDTA) (Aldrich), cobalt (II) chloride . $6H_2O$, 30% (m/v) hydrogen peroxide, 5-amino-2,3-dihydro-phtalazine-1,4-dione (luminol), quercetin (Sigma), sodium hydroxide, ethanol.

Solutions

*S*tock and working solutions were prepared as described in our previously paper, that is in the 0.1 M, *p*H 9 borate buffer solution: 3×10^{-3} M Na_2EDTA solution, 2.4 x 10^{-3} M $CoCl_2 . 6H_2O$ solution with a Co (II)/ EDTA molar ratio of 0.8 and 3.39 x 10^{-4} M luminol solution. The working solution for CL determinations was prepared on a daily basis by mixing 25mL of each of the three solutions mentioned above. 3 x 10^{-4} M H_2O_2 solution was obtained by a corresponding dilution of a 10^{-1} M H_2O_2 stock solution.

Quercetin standard solutions (10^{-5} M - 2 x 10^{-3} M) were obtained by a corresponding dilution of 3 x 10^{-3} M quercetin stock solution. Both stock and working solutions of quercetin were prepared in ethanol: 2 x 10^{-4} M EDTA in water = 80: 20 (v/v). EDTA was added for complexing the metal ions (Fe^{2+}, Fe^{3+}, Cu^{2+}) which can influence measurements as they decompose H_2O_2 by a Fenton-type reaction. This metal ions can also been complexed by quercetol or other flavonoides. With the exception of the mentioned cases, all solutions were prepared in double distilled water.

Apparatus

The CL measurements were carried out by means of a Turner Biosystems 20^n/20 luminometer coupled to a computer whose software allows for recording the light intensity, as described in previous paper [27].

Vegetable materials

Seeds of *Citrus x limon* (lemon, *Rutaceae*), clementine which is a variety of *Citrus reticulata* (mandarin orange) *Vitis vinifera* (white grapes, *Vitaceae*), and *Citrullus lanatus* (watermelon, *Cucurbitaceae*) were used for this study.

Extract preparation

Seeds were washed thoroughly with tap water, rinsed with distilled water and dried at room temperature under subdued light. A certain amount of dried seeds was crushed in a grinder and then submitted to a reflux extraction with 96% ethanol for 3 h. The material vegetal: solvent ratio was 1:10 m/v with the exception of watermelon seed extract, when it was 1:3 and mandarin oranges where the same ratio was 1:4. The extracts were filtered on large pore (red band) paper and kept at + 4°C.

Working procedure for the CL determinations has been described in detail in our previous article. Thus, 350 µL of working solution for CL determinations,0.1 M, *p*H 9 borate buffer solution and 3 x 10^{-4}M H_2O_2 solution were homogenized into the reaction vessel(an Eppendorf tube of 1.5mL) which was placed inside the apparatus and then the intensity of chemiluminescence radiation is measured. The intensity value of CL signal registered in the absence of any antioxidant was noted with I_0 [27]. After 600 s (when CL signal values were practically constant and I_0 variation *vs.* time reach a plateau), 25 mL from the analyzed sample (standard/extract) are added to the reaction mixture. After mixture homogenization inside the Eppendorf tube (by means of the pipette tip) a decrease of the CL signal, whose value was assigned I, was registered. Signal shape are similar to those shown in figure 2.

I_0/I ratio values were then computed. The I_0/I value for comparison sample, which was ethanol: EDTA 2x10^{-4} M = 80% (v/v) solution, was subtracted from the I_0/I values of the analyzed samples (standards or seeds extracts).

The calibration curve, I_0/I *vs* quercetin concentration, was drawn as in figure 3. Total antioxidant capacity of seeds extracts was expressed as mg of quercetin equivalents (QE)/ 100g dried seeds (dw).

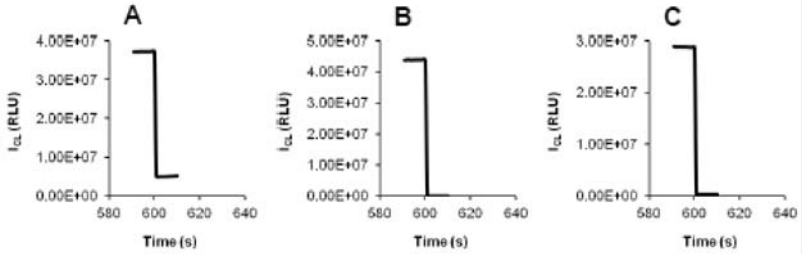

Fig. 2. Decreases of CL intensity signals registered when to the reaction mixture were added: A. 10^{-5} M quercetin solution; B. 10^{-3} M quercetin solution; C. a clementine ethanolic extract. RLU: relative luminescence units. Working procedure was as above

Samples measurements were performed in triplicates.

Results and discussions

Some examples of CL signal decreases registered when a sample (standard or extract) was added to the reaction mixture are given in figure 2.

The CL signal decrease is proportional to antioxidant concentration increase.

Calibration curves

The I_0/I calibration curve is presented in figure 3 as a function of quercetin concentration.

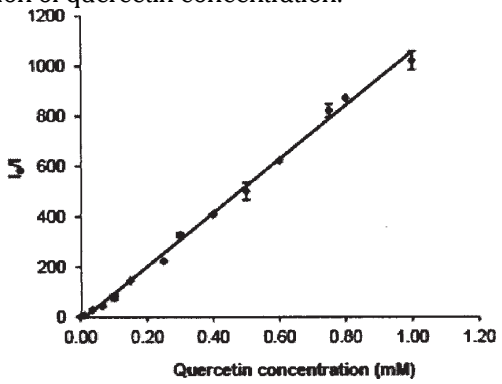

Fig. 3. Calibration curve I_0/I *vs.* quercetin concentration. I_0/I: average I_0/I ratio values for each analyzed solution from which average I_0/I for comparison sample was subtracted. Working procedure as above. Error bars represent ± SD (n = 3)

Calibration curve equation is:

$$y = 1000\,x - 14.2 \quad (1)$$

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

The correlation coefficient is:

$$r^2 = 0.9966\ (n = 14),$$

where n = number of measurements.

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

Relative standard deviation was computed for a concentration of 3.5 x 10^{-5}M quercetol, RSD = 2.65% (n = 10).

Determination of the sample antioxidant capacity

Before analysis, 1mL of each extracts was brought to 10mL with ethanol: 2 x 10^{-4} M EDTA (in water solution) = 80: 20 (v/v). Working procedure for TAC determination was previously presented [27]. The TAC values for the analyzed seeds extracts are given in figure 4.

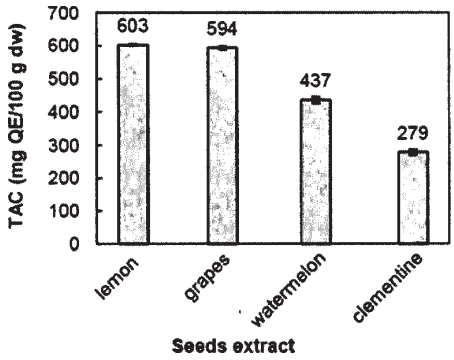

Fig. 4. TAC values for the analyzed seeds extracts
QE: quercetin equivalents. Error bars represent ± SD (n = 3)

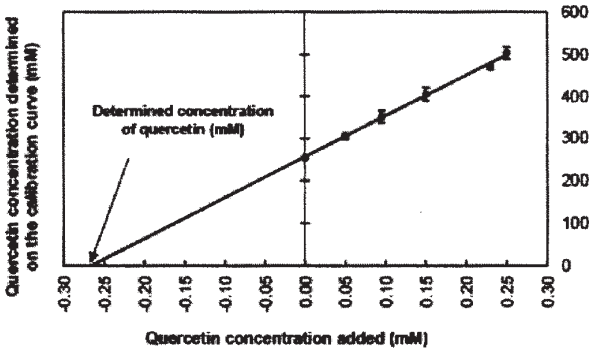

Fig. 5. Experimental results obtained with standard addition method I_0/I: the average (I_0/I) ratio values for each analyzed solution from which average (I_0/I) for comparison sample was subtracted. Working procedure according to the one presented above. Error bars represent ± SD (n = 4)

As can be seen in figure 4, the antioxidant activity of citrus seeds ranges between 279 and 603 mg quercetin equivalents/100 g dw. TAC values of white grapes and lemon extract are comparable with each other.

In order to verify the accuracy of the obtained results, a standard addition method was applied in the same way as described in our previously paper [27]. Thus, the same grapes seeds extract volumes were introduced in six 10mL flasks. Known amounts of quercetin from 3 x 10^{-3} M standard solution (prepared in ethanol: 2×10^{-4}M EDTA in water = 80: 20, v/v) were subsequently added in these flasks, so that concentration of the added standard to be: 0, 0.05, 0.095, 0.21, 0.25 and 1.50mmoles/L, respectively. The resulting samples were analyzed by the method described previously [27] as well as in the present work.

A straight line is obtained when quercetin concentrations determined from the calibration curve equation is represented *vs.* concentration of quercetin added to the extract.

Extract	Antioxidant activity / polyphenolic content		References
	Experimental (mg QE/100g dw)	Literature	
Lemons	603	1800 mg Trolox /100g dw	[28]
Grapes	594	850 mg catechin	[28]
		17.9- 418 mg Trolox/100g dw	[15]
Watermelon	437	1600 mg Trolox/100g dw	[28]
		0.13 - 0.30 mg gallic acid/100g dw	[29]

Table 1
LITERATURE VALUES FOR ANTIOXIDANT ACTIVITY AND POLYPHENOLIC CONTENT OF SEVERAL SEEDS EXTRACTS

If this straight line is extended to intersect the abscissa as in figure 5, quercetin concentration value in the analyzed seeds extract is obtained. A concentration of 267mM quercetin equivalents were determined in the analyzed sample, which correspond to a concentration of 268mM quercetin equivalents determined by direct method. The two values are very close to each other and confirm the experimental results presented in our previously paper [27], and therefore the applied method can be then employed without any interference.

Our results were compared with the values obtained by the different methods reported in literature, which are listed in table 1.

No values expressed in quercetol equivalents were found for the analyzed extracts and therefore a comparison between the results is impossible.

Conclusions

Seed extracts from four fruits (lemons, clementines, grapes and watermelon) were obtained by reflux in ethanol.

The seed extracts total antioxidant capacity was measured by means of a chemiluminometric method based on luminol-Co(II)/EDTA-H_2O_2 system. A calibration curve for quercetin (1×10^{-5} – 1×10^{-3} M) determination was drawn. RSD was 2.65% (n = 10, $c_{quercetin} = 3.5 \times 10^{-5}$ M). The seed extract antioxidant capacity expressed as mg quercetin equivalents/100g dw was 603 for lemon, 594 for grapes; 437 for watermelon and 279 clementines.

The accuracy of the applied method was verified by applying the standard addition method when a difference smaller than 1.5% between concentration obtained by this method and the direct one was found. The proposed method can be used for rapid screening of antioxidant activity and also for providing increased accuracy and high sensitivity compared to other analytical methods, especially spectrophotometric assays.

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