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A Superoxide Dismutase Biosensor for Measuring the Antioxidant capacity of Blueberry Based Integrators

Luigi Campanella[1], Rosita Gabbianelli[2], Tania Gatta[1], Elisa Mazzone[1] and Mauro Tomassetti[1]*

[1]*Department of Chemistry, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185, Rome, Italy*
[2]*School of Pharmacy, University of Camerino, Via Gentile III da Varano, 62032 Camerino (MC), Italy*

Abstract: The antioxidant capacity of capsules containing blueberry based products which are included among the group of integrators owing to their antioxidant capacity and produced by various drug firms was investigated. The results of the investigation are compared to rank these products in order to their antioxidant capacity. In order to measure antioxidant capacity, our laboratory has recently developed a special electrochemical method based on a superoxide dismutase (SOD) biosensor to determine the superoxide radical. The results obtained by applying the SOD biosensor method to various blueberry based integrators were compared with the results obtained with the spectrophotometric (FRAP) method based on N,N-dimethyl-p-phenylenediamine (DMPD-$FeCl_3$) and with those obtained also using the ORAC fluorimetric (TRAP) method. One of the more interesting aspects of the article is the good agreement it evidences of the results of the three methods for measuring antioxidant capacity. The three methods differ among themselves: an Electron Transfer (ET) method, a Hydrogen Atom Transfer Method (HAT) and an electrochemical based biosensor method of the Monitoring Superoxide Radical (MSR) type. It is also shown how the antioxidant capacity of the fresh vegetable is in any case always greater than that of any food supplement obtained from the same type of vegetable.

Keywords: Antioxidant capacity, SOD biosensor method, TRAP method, FRAP method, Blueberry.

1. INTRODUCTION

Numerous studies and experimental researches have indicated the involvement of reactive oxygen species (ROS) in particular, as well as all types of free radicals in general, in the genesis of numerous pathologies [1]. The antioxidant compounds contained in many foods and beverages are capable of reacting with the radicals [2, 3] and thus play an important role in the prevention and defence against oxidative diseases, so representing a protective factor of fundamental importance for human health. Sometimes, however, in the case of incorrect food habits or physical deficiencies, food intake alone is not sufficient to provide enough antioxidant nutrients. Therefore, in such cases, the use of food integrators is recommended.

This has become a widespread practice although the antioxidant properties of these compounds are often not fully quantified. It is thus of particular and topical interest to be able to come up with new analytical methods to assess the antioxidant capacity of the various 'over the counter' products available in drugstores and that may be purchase without medical prescription.

Blueberry is utilized in several kind of diseases and contain a relatively large amount of acids (citric, malic, etc), sugars, pectins, tannins, mirtillene (glucoside pigment), anthocyanin (which has a beneficial effect on retina capillaries), vitamins A and C and, to a lesser extent, Vitamin B. It is therefore recommended in treating eye disorders (myopia and retinoterapy), in combating eye fatigue, as an antidiarrhoic and against diabetes [4]. It is useful for combating the fragility and excessive permeability of blood vessels by exerting a protective action on capillaries [5]. It is a known fact that the dry extract of blueberry can improve night vision [6]. Therefore, black blueberry leaves and berries are rich in active principle. In addition however, blueberry contain antioxidant compounds like phenols, tannins, flavonoids, anthocyanins, glucosides and anthocyanidins. It is therefore its antiradical action that is of fundamental importance: blueberry anthocyanosides strongly inhibit free radicals as they are able to react with practically all known species of radical [7-10]. Looking at these latter properties, in the present research the experimental results of investigation on several blueberry integrators were compared in order to rank these products in the order of their antioxidant capacity. The aim of the present work was actually to investigate the antioxidant capacity of capsules containing blueberry based products which are included among the group of integrators most widely sold in drugstores owing to this capacity and produced by various drug firms. In order to measure antioxidant capacity, in addition to the various spectrophotometric, voltammetric and fluorimetric methods described in the literature [11-13], our laboratory has recently developed a special electrochemical method based on a superoxide dismutase (SOD) biosensor [14]. The results obtained by applying the SOD biosensor method to various blueberry based integrators were compared with those obtained with the spectrophotometric (FRAP) method based on N,N–dimethyl-p-phenylenediamine (DMPD- $FeCl_3$) [11] and with those obtained using the ORAC fluorimetric (TRAP) method [12],

Address correspondence to this author at the Department of Chemistry, University of Rome "La Sapienza", P.le Aldo Moro 5, 00185, Rome, Italy; Tel/ Fax: +39 06 490631; E-mail: mauro.tomassetti@uniroma1.it

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the most frequently used to determine antioxidant activity in food matrices and adopted as reference method. It was also be compared the antioxidant capacity of these integrators (all sold in drugstores) with that of fresh blueberries.

2. MATERIALS AND METHODS

2.1. Reagents and Apparatus

Xanthine (2,6-dihydroxy purine) sodium salt, N,N-dimethyl-p-phenylenediamine, ethylenediamine tetracetic acid (EDTA), superoxide dismutase 4980 U mg^{-1}, ferric chloride, dialysis membrane (art. D-9777), teflon membrane, and β-phycoerythrin were supplied by Sigma (Milan, Italy).

Xanthine oxidase 0.39 U mg^{-1} and cellulose acetate were supplied by Fluka AG, Buchs (Switzerland). 2,2-azobis(2-amidinopropan)dihydrochloride (ABAP) was supplied by Waco Chem (Richmond, VA, USA). Potassium dihydrogenphosphate, sodium acetate and sodium hydrogenphosphate were supplied by Carlo Erba (Milan, Italy). Polyvinylacetate, acid-2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman (Trolox) and cellulose triacetate were supplied by Aldrich (Germany).

Several instruments were used to carry out the present research: UV-VIS spectrophotometer Perkin Elmer mod. Lambda 5, provided with printer; Crison pH meter mod. GLP 22; spectrofluorimeter Perkin–Elmer, mod. LS-5, provided with Perkin–Elmer recorder, mod. 561; electrode mod. 4000-1 by Universal Sensor Inc. New Orleans, LA, USA, coupled with an Amel potentiostat mod. 551, connected to an Amel differential electrometer, mod. 631 and to an Amel analog recorder, mod. 868; Ultra-Turrax homogenizer mod. T8 by Ika Labortechnik; mulino, A 10 Yellow line IKA Works Inc.

2.2. Samples

Four different commercial blueberry-based food integrators (in capsules), all sold in drugstores, were analyzed together with a sample of fresh blueberries (analyzed with or without skin). The composition (as reported by the manufacturer) of the four commercial samples, indicated as B1, B2, B3, B4, is shown in Table **1**.

2.3. Treatment of Samples

Five capsules of each product were opened and their content collected and gently homogenized. 1.0 g of each sample was dissolved or dispersed in 6 cm^3 of 0.05 M phosphate buffer (pH=7.5). In the case of blueberry fruit samples, 1.0 g of sample taken from half the entire fruit (pulp + skin, after eliminating all the internal seeds) as well as of 1.0 g of the other half of the fruit without the skin were respectively dispersed in 6 cm^3 of phosphate buffer (0.05 M, pH=7.5), then in treating homogenized and centrifuged for 10 min at 4000 rpm, at room temperature and the supernatant analyzed.

For the analyses carried out using the N,N-diethyl-paraphenylenediamine + Fe^{+3} method the samples were treated in 6 cm^3 of acetate buffer (pH=5.25) before the analysis was performed.

All the samples solutions were diluted 1:10 for biosensor (SOD) and ORAC method application while they were diluted 1:1000 for DMPD + Fe^{+3} method.

2.4. Methods

2.4.1. Superoxide Dismutase (SOD) Biosensor Method

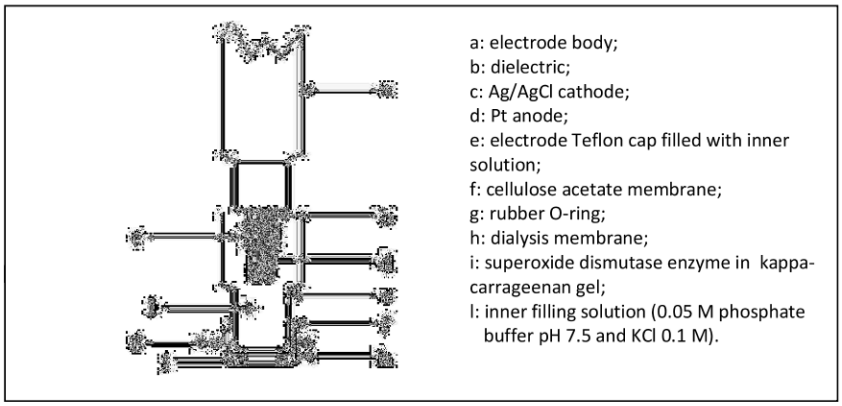
The total antioxidant capacity was measured by SOD biosensor operating as follows: the superoxide radical is determined using a biosensor obtained by coupling a transducer (an amperometric electrode for hydrogen peroxide) with the superoxide dismutase (SOD) enzyme immobilized in kappa-carrageenan gel [15] (see Fig. (**1a**)). The gel containing the enzyme is sandwiched between a cellulose acetate membrane and a dialysis membrane. The whole assembly is secured to the electrode with an O-ring.

A constant potential of +650 mV with respect to an Ag/AgCl/Cl^- cathode is applied to the platinum anode. The dialysis membrane is used to support the gel and to prevent attack to the enzyme. The superoxide radical is produced by the oxidation of 3,7-dihydro-1H-purine-2,6-dione in aqueous solution to 7,9-dihydro-1H-purine-2,6,8(3H)-trione in the presence of the xanthine oxidase enzyme, which is free in solution. The disproportion reaction of the superoxide radical, catalyzed by the superoxide dismutase immobilized on the electrode, produces oxygen and hydrogen peroxide.

Table 1. Some Information Reported by Producer Firms Concerning Integrators Based on Blueberry Components

Commercial Name	Active Principles	Excipients
B1	Cryogenically crushed blueberry with a titrated tannin content of 0.7% (expressed as pyrogallol).	Hydroxypropyl cellulose.
B2	Sodium anthocyanoside blueberry complex with 36% anthocyanoside content (mirtocyan).	Mannitol, lactose, methyl cellulose, citric acid, silica precipitate, gelatine.
B3	Powdered blueberry (Vaccinium myrtillus) with a titrated content of 0.3% dry blueberry extract with titrated total anthocyanidin expressed as delphinidin chloride 25%.	Gelatine.
B4	Dry black blueberry extract in water-alcohol mixture with 25% of anthocyanidin.	Soya vegetable oil, partially hydrogenated vegetable fats, gelatine, glycerol, red iron oxide, sodium ethylparaben, sodium propylparaben.

a)



b)

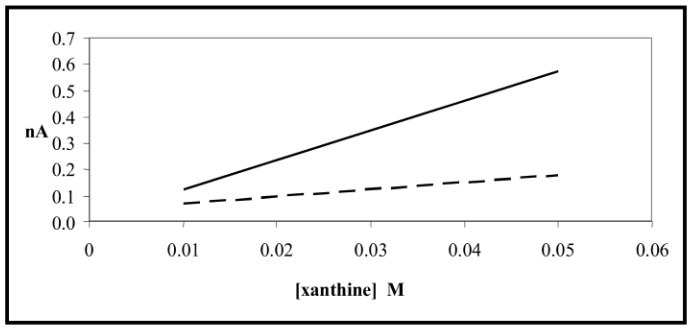


Fig. (1). a): biosensor; **b**): Example of straight line recorded in absence of antioxidant sample (i.e.) "blank" (continuous curve) and in presence of antioxidant sample (dashed curve).

The hydrogen peroxide produced is oxidized at the anode, generating an amperometric signal (in nA) that is proportional to the concentration of the superoxide radical present in solution.

The addition of a sample possessing antioxidant properties produces a decrease in the signal as, by reacting with the superoxide radical, the concentration of these species in solution is lowered. As a consequence, both the released H_2O_2 and the intensity of the amperometric current diminish. In practice, the electrochemical biosensor was placed in a cell thermostated at 25°C containing 15.0 cm^3 of phosphate buffer, 0.05 M, at pH 7.5, and allowed to stabilize under constant stirring.

After addition to the same solution of a fixed quantity of the xanthine oxidase enzyme (1.2 mg), three subsequent additions of 0.2 cm^3 of the solution of 3,7-dihydro-1H-purine-2,6-dione sodium salt 0.01 M were made, waiting for the signal to stabilize between subsequent additions. The values of the recorded current variations were thus plotted versus the 3,7-dihydro-1H-purine-2,6-dione concentration and the slope value calculated (Fig. (**1b**)).

After washing the cell, all the same solutions were renewed in the measuring cell, in which also the sample under test for its antioxidant capacity was dissolved, or dispersed. Lastly, a new straight line was recorded as described above (Fig. (**1b**)).

The value of the antioxidant capacity was expressed in RAC units by the following algorithm:

(RAC) "Relative Antioxidant Capacity" = $1 - (m_c / m_x)$, where:

m_x = slope of the straight line obtained by means of subsequent additions of 3,7-dihydro-1H-purine-2,6-dione;

m_c = slope of the straight line obtained by means of subsequent additions of 3,7-dihydro-1H-purine-2,6-dione, but in the presence of the sample possessing anti-oxidant properties.

The superoxide dismutase assembling and SOD immobilization in kappa–carrageenan were described in detail in previous papers [15, 16].

2.4.2. ORAC Spectrofluorimetric (TRAP) Method

The oxygen radical absorbance capacity (ORAC) spectrofluorimetric method is well known and extensively described in the literature; it is usually chosen as a reference method as highly suitable and reliable, but also is too expensive for routine analysis [17]. In the presence of free radicals or oxidizing species, the protein β-phycoerythrin (β-PE) loses more than 90% of its fluorescence within 30 min. The addition of antioxidant species, which react with the free radicals, inhibits the fluorescence of this protein. This inhibition may be related to the sample's antioxidant capacity. In particular, 2,2-azobis(2-amidinopropane) dihydrochloride (ABAP) was used to generate peroxide radicals ($ROO^{\cdot}$). To perform the measurements, the wavelengths were set at k = 540 nm for excitation and 565 nm for emission. Initially, 0.08 cm^3 of sample was placed in the cuvette together with

0.02 cm^3 of phosphate buffer (0.075 M, pH 7), and 1.46 cm^3 of β-phycoerythrin (0.183 M in phosphate buffer), prepared and allowed to stand at 37°C for 15 min before use.

The cuvette was placed in the spectrofluorimeter and the initial fluorescence (f_0) read off after 30 s. Then a further 0.02 cm^3 of phosphate buffer was added to the solution in the cuvette together with 0.02 cm^3 of ABAP (3.2 10^{-4} M in phosphate buffer). After stirring, the fluorescence was read off after 0.5 s and then every 2 min, for a total time of 70 min. A similar procedure was also carried out using a 20 10^{-6} M solution of acid 2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman instead of sample. The final results are expressed in ''ORAC units'' (micromoles of acid 2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman equivalent per dm^3 of sample) [12]:

$$\text{ORAC Value} : 20k\,(S_{sample} - S_{blank})/\,(S_{Trolox} - S_{blank})$$

where k = dilution factor for the sample; S = integral of the fluorescence curve of the sample, or of the acid 2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman, or of the ''blank".

2.4.3. Spectrophotometric (FRAP) Method: N,N-diethyl-paraphenylenediamine + Fe^{+3}

The cation radical obtained from the *N*,*N*-dimethyl-*p*-phenylendiamine dihydrochloride (DMPD) in the presence of a suitable oxidising solution ($FeCl_3$) displays an absorption peak at 514 nm. The diminution of absorbance at this wavelength, recorded in the presence of the test sample having antioxidant capacity, shows a correlation with the latter's antioxidant capacity.

In practice: in a vessel containing 100 cm^3 of acetate buffer (0.1 M at pH = 5.25) 1.0 cm^3 of a solution of *N*,*N*-dimethyl-*p*-phenylendiamine 0.1 M and 0.2 cm^3 of a solution of ferric chloride 0.05 M was added; this produced the purple colored cation radical of *N*,*N*-dimethyl-*p*-phenylenediamine. The final solution was placed in a quartz cuvette and the absorbance at 514 nm read off. To this solution was then added 0.15 cm^3 of a suitably diluted sample or else a solution of acid 2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman 1.0 mg cm^{-3}; the absorbance at 514 nm was then read off after 10 min during which the mixture was maintained under constant stirring at a temperature of 25°C. Only acetate buffer was placed in the reference cuvette. Total antioxidant capacity is evaluated by comparing the diminution of absorbance due to the sample with that due to the 2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman acid (Trolox) used as standard and thus expressed in Trolox Equivalent Antioxidant Capacity (TEAC) units [11].

3. RESULTS AND DISCUSSION

As already stated in the "Introduction" of the present paper, blueberry is well known for its antioxidant properties. Therefore several researches have been published on this topic in recent years [18-27].

In Tables **2**, **3** and **4** are collected the antioxidant capacity values, respectively found after application of three methods (already validated on standard solution in several previous papers [14-16, 28-30]) and expressed each one with proper measurements units. In Figs. (**2**,**3** and **4**) are displayed as histograms the same data. All three methods display the same trend and are therefore in good agreement. This observation perhaps represents the most significant result of the present investigation. Since there are different oxidant sources and free radicals (for instance $O_2^{\cdot-}$, $HO^{\cdot}$, $NO^{\cdot}$, $ROO^{\cdot}$ and so on) and at same time several general sources of antioxidants (that is enzymes such as peroxidase, catalase, superoxide dismutase), proteins like albumin, ferritin, lactoferrin and so on, and other different kinds of molecules (e.g. glutathione, tochopherol, polyphenols, ascorbic acid, carotenoids, melatonin, angiotensin, etc.), it follows that both oxidants and antioxidants may have widely different characteristics and chemical reactivity. Therefore, no single assay can suitably and optimally reflect all radical sources or all antioxidants in a complex real system. On the other hand, a good method for measuring antioxidant capacity needs to satisfy several different requirements: applicability to different radical sources; have good accuracy and reproducibility; to be relatively simple and to require simple instrumentation available in ordinary analytical laboratories. For these reasons it is always advisable simultaneously to apply different methods and that if possible they have a different method of functioning in the measurement of the antioxidant capacity of a complex matrix such as those examined in the present research. For this purpose in the present work three methods were used that were quite different not only from the instrumental point of view: one is of the electrochemical type, one spectrofluorimetric and one spectrophotometric, in which absorbance is measured in the visible range, but also from the standpoint of the functioning mechanism. The ORAC method is actually a classical Hydrogen Atom Transfer (HAT) method, [31], that is, it is used to measure the classical ability of an antioxidant to quench free radicals by hydrogen donation [32-34]. The one we used may actually be considered a variant of the ORAC method and therefore can be included in the group of the socalled TRAP (Total Radical Trapping Antioxidant Parameter) methods. This kind of method monitors the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals, generated for instance by ABAP and a target probe, e. g. R-phycoerythrin [32, 35]. The TRAP assay involves the initiation of lipid peroxidation by generating water – soluble peroxyl radicals and is sensitive to all known chain breaking antioxidants. The other, spectrophotometric method, is instead a classical Electron Transfer (ET) method [36]. The ET – based assay method involves two components in the reaction mixture, antioxidant and oxidant (the probe). The probe is an oxidant that abstracts an electron from the antioxidant, causing the solution to change color. The degree of the color change is proportional to the antioxidant concentration. In particular, the ET – based assay type used by us is one of the socalled ferric reducing antioxidant power (FRAP) methods. [37-41].

Lastly the third method developed in our laboratory and employed in the present research makes use of an electrochemical sensor based on an amperometric H_2O_2 electrode, but may actually be included among the monitoring superoxide radical (MSR) methods. The superoxide anion radical ($O_2^{\cdot-}$), a single-electron reduction product of oxygen, is one of the most abundant radical products in biological and food systems. This species is quite reactive and easily scavenged by enzymes such as superoxide dismutase and antioxidant agents such as ascorbate.

Table 2. Recorded Antioxidant Capacity Values Using Biosensor (SOD) Method

Sample	RAC (%)	RSD %
UB	68.7	4.9
PB	62.2	9.7
B1	61.2	6.9
B2	60.6	9.7
B3	57.2	13.6
B4	51.4	12.3

Table 3. Recorded Antioxidant Capacity Values Using ORAC fluorimetric (TRAP) Method

Sample	ORAC Values	RSD (%)
UB	177.5	2.9
PB	176.9	8.5
B1	150.7	1.7
B2	150.6	8.7
B3	144.3	3.1
B4	122.9	11.0

Table 4. Recorded Antioxidant Capacity Values Using DMPD+Fe^{+3} Spectrophotometric (FRAP) Method

Sample	TEAC Values	RSD (%)
UB	15.09	0.3
PB	15.06	1.1
B1	14.93	0.3
B2	14.92	0.1
B3	14.84	0.9
B4	14.67	0.9

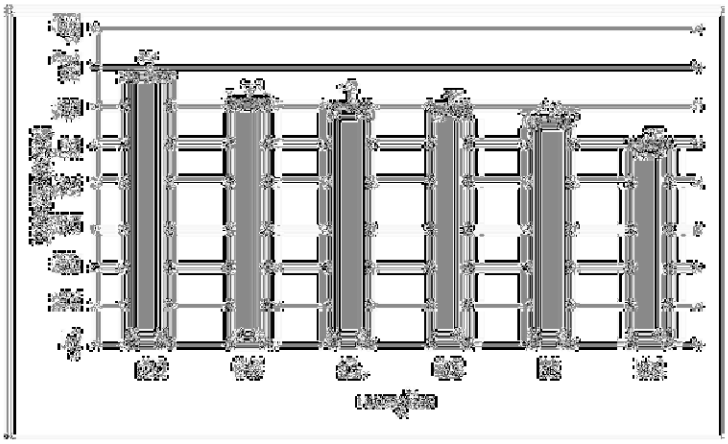


Fig. (2). Antioxidant capacity trend obtained using (SOD) biosensor method; values expressed in RAC units. UB = unpeeled blueberry; PB = peeled blueberry; B_1–B_4 = the four different blueberry integrators (see Table **1**).

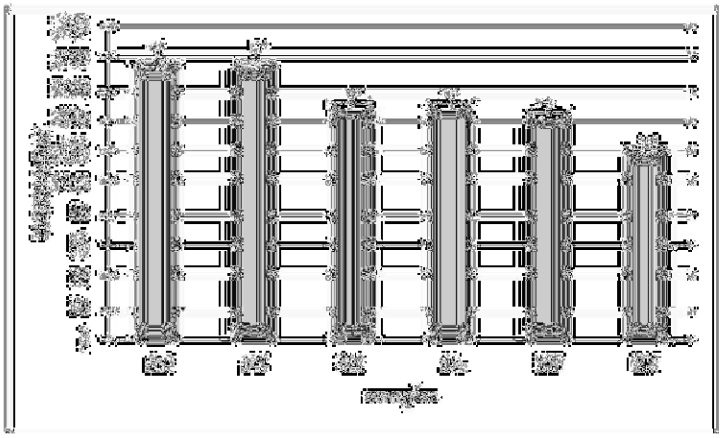


Fig. (3). Antioxidant capacity trend obtained using ORAC fluorimetric (TRAP) method; values expressed in ORAC units. UB = unpeeled blueberry; PB = peeling blueberry; B_1–B_4 = the four different blueberry integrators (see Table **1**).

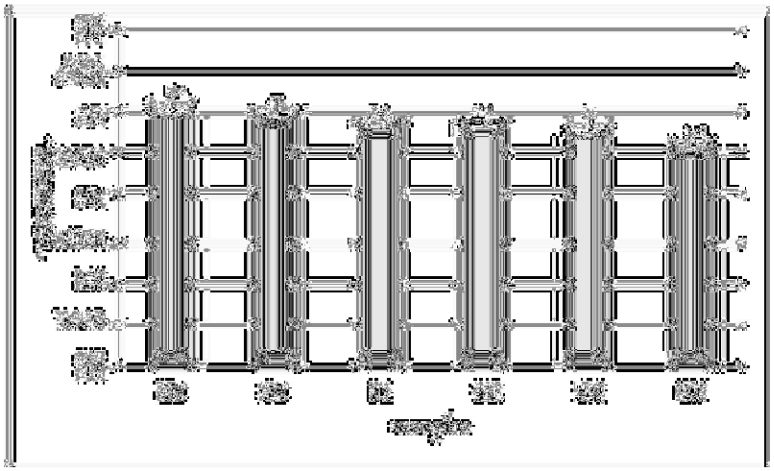


Fig. (4). Antioxidant capacity trend obtained using DMPD + Fe^{+3} spectrophotometric (FRAP) method; values expressed in TEAC (trolox equivalent antioxidant capacity) units. UB = unpeeled blueberry; PB = peeled blueberry; B_1–B_4 = the four different blueberry integrators (see Table **1**).

Electrochemical biosensor methods are particularly suitable for determining the superoxide anion radical in real time and include two main types. In the first type the cytochrome C immobilized on an electrode surface is reduced by the superoxide anion radical enzymatically produced in solution [42]. In the second type (i.e. our method) the superoxide is, also in this case, enzymatically produced in solution, and so the superoxide dismutase immobilized on the electrode surface catalyses the specific dismutation of the superoxide anion radical, producing molecular oxygen (O_2) and hydrogen peroxide (H_2O_2). The electro-oxidation of H_2O_2 at the electrode surface generates the detection signal, as is described in greater detail in the methods section of the present paper.

To improve the selectivity of this sensor a Teflon membrane, impermeable to H_2O_2, but permeable to $O_2^{\circ -}$ radical, was used.

In conclusion, despite the considerable differences between the three methods selected and used for this very reason, the similarity of the antioxidant capacity values obtained is without doubt a result that confirms and supports the information available on the antioxidant capacity obtained for the various samples analysed as well as on the validity of the methods, in particular, the biosensor method developed by us. In this connection Figs (**5**, **6** and **7**) show the relative trends in the correlation straight lines referring to the SOD and the ORAC methods, or to the spectrophotometric method, respectively. The correlations obtained, although not certain perfect, comfort, however, the results provided by the recent biosensor method, which proves to be in good agreement with the two more traditional methods.

However, in the case of the spectrophotometric method, the antioxidant capacity displayed by the various samples is found to be quite similar and the discriminant capacity of the method is very low, which at least partly diminishes the validity of the method. It should also be noted that, weight for weight, as pointed out in previous articles, the antioxidant capacity of the fresh fruit and whole (i.e. including the skin) is always higher than the fruit without skin [28] or longtime stored [29].

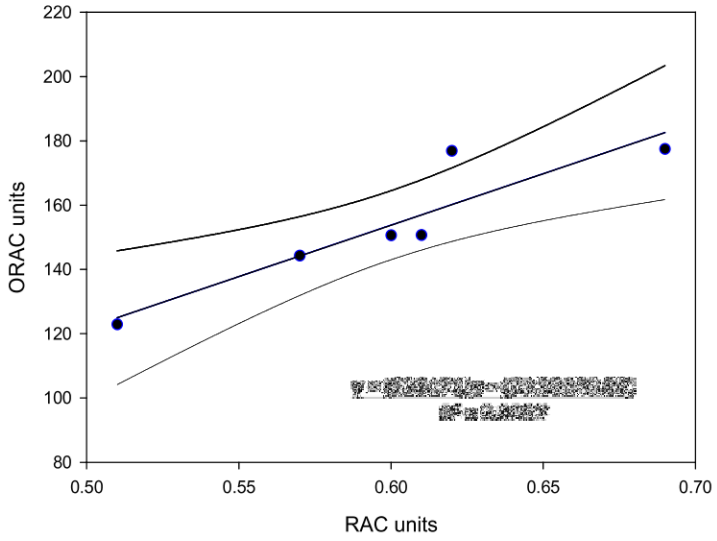


Fig. (5). Correlation curve between biosensor and ORAC methods.

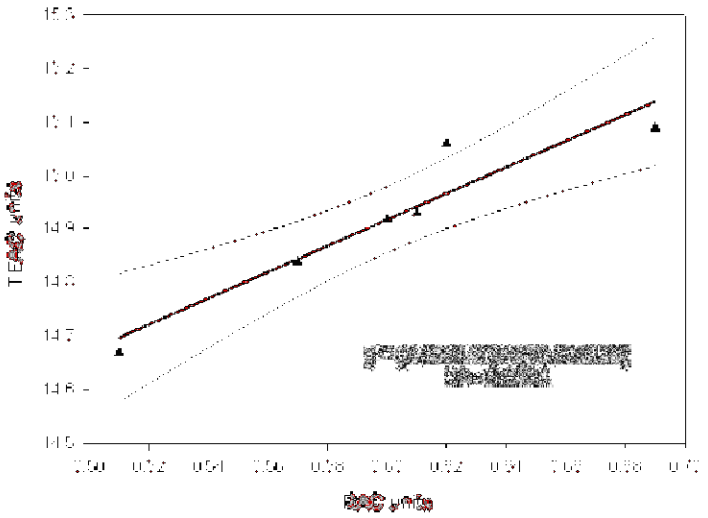


Fig. (6). Correlation curve between biosensor and spectrophotometric methods.

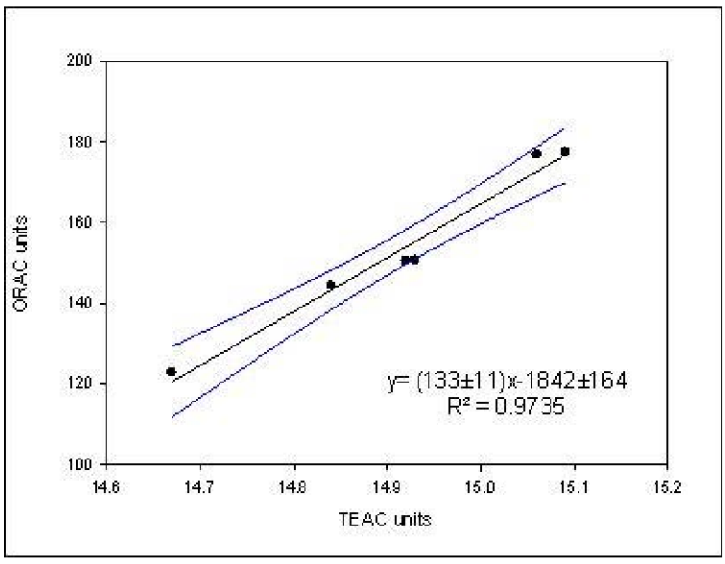


Fig. (7). Correlation curve between ORAC and spectrophotometric methods.

Blueberries (especially the fresh fruit) confirmed the expected antioxidant properties in agreement with the numerous literature reports [7-10]. Also the blueberry-based diet supplements (i.e. food integrators) were found to have a good antioxidant capacity although always lower than that of the fresh product and also this is a confirmation of what was found in previous studies [30] for other types of vegetables.

4. CONCLUSION

Once again the SOD biosensor method confirmed its validity and proved to correlate satisfactorily with the ORAC method. Blueberries, especially the fresh fruit, confirmed the expected antioxidant properties in agreement with the numerous literature reports [7-10]. Lastly, also the blueberry-based diet integrators were found to have a good antioxidant capacity although always lower than that of the fresh product.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

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DISCLOSURE

Take note that a considerable part of the: *2.4. "methods"* section of this paper is practically the same already inserted in our previous paper published on Monatshefte für Chemie - Chemical

Monthly: Campanella, L.; Gatta, T.; Gregori, E.; Tomassetti, M. Determination of antioxidant capacity of papaya fruit and papaya-based food and drug integrators, using a biosensor device and other analytical methods. *Monats. Chem.*, **2009**, *140*, 965-972.

REFERENCES

[1] Sies, H. Oxidative stress: from basic research to clinical application. *Am J Med,* **1991**, *91*, 31S-8S.

[2] Pedersen, C.B.; Kyle, J.; Jenkinson A.M. Effects of blueberry and cranberry juice consumption on the plasma antioxidant capacity of healthy female volunteers. *Eur J Clin Nutr*., **2000,** *54(5)*, 405-408.

[3] Kay, C.D.; Holub, B.J. The effect of wild blueberry (Vaccinium angustifolium) consumption on postprandial serum antioxidant status in human subjects. *Br J Nutr.*, **2002**, Oct, *88(4),* 389-98.

[4] Lee, I.T.; Chan, Y.C.; Lin, C.W.; Lee, W.J.; Sheu W.H. Effect of cranberry extracts on lipid profiles in subjects with Type 2 diabetes. *Diabet Med.*, **2008**, Dec, *25(12),* 1473-1477.

[5] Mazzanti, G.; Battinelli, L.; Piccinelli, D. Farmaci di origine vegetale ad azione vaso protettrice. *Acta Phytotherapeutica* , **1997**, *2(4)*, 65-76.

[6] Canter, P.H. Anthocyanosides of Vaccinium myrtillus (Bilberry) for Night Vision-A Systematic Review of Placebo-Controlled Trials. *Surv Ophthalmol.* , **2004**, *49(1),* 38-50.

[7] Donnelly, L.E.; Newton, R.; Kennedy, G.E.; Fenwick, P.S.; Leung, R.H.; Ito, K. Anti-inflammatory effects of resveratrol in lung epithelial cells: molecular mechanism. *Am J Physiol Lung Cell Mol Physiol.*, **2004**, *287*, 774–783.

[8] Fang, F.; Kang, Z.; Wong, C. Vitamin E tocotrienols improve insulin sensitivity through activating peroxisome proliferator-activated receptors. *Mol Nutr Food Res.* , **2010**, Mar, *54(3)*, 345-52.

[9] Harikumar, K.B.; Aggarwal, B.B. Resveratrol: a multitargeted agent for age-associated chronic diseases. *Cell Cycle*, **2008,** *7*, 1020–35.

[10] Gao, X.; Xu, Y.X.; Janakiraman, N.; Chapman, R.A.; Gautam, S.C. Immunomodulatory activity of resveratrol: suppression of lymphocyte proliferation, development of cell-mediated cytotoxicity, and cytokine production. *Biochem Pharmacol.*, **2001**, Nov 1, *62(9)*, 1299-308.

[11] Fogliano, V.; Verde, V.; Randazzo, G.; Ritieni, A. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. *J Agric Food Chem.*, **1999**, *47(3)*, 1035–1040.

[12] Cao, G.; Verdon, C.P.; Wu, A.H.B.; Wang, H.; Prior, R.L. Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clin Chem,* **1995**, *41*, 1738-1744.

[13] Hatano, T.; Kagawa, H.; Yasuhara, T.; Okuda, T. Two new flavonoids and other constituents in licorice root; their relative astringency and radical scavenging effects. *Chem. Pharm. Bull.,* **1988**, *36*, 2090–2097.

[14] Bonanni, A.; Campanella, L.; Gatta, T.; Gregori, E.; Tomassetti, M. Evaluation of the antioxidant and prooxidant properties of several commercial dry spices by different analytical methods. *Food Chem.,* **2007,** *102*, 751-758**.**

[15] Campanella, L.; Favero, G.; Tomassetti, M.; Superoxide dismutase biosensor for superoxide radical analysis. *Anal. Lett.*, 1999, *32(13),* 2559–2581.

[16] Campanella, L.; Bonanni, A.; Finotti, E.; Tomassetti, M. Biosensors for determination of total and natural antioxidant capacity of red and white wines: comparison with other spectrophotometric and fluorimetric methods, *Biosens.Bioelectron.*, **2004**, *19*, 641-651.

[17] Gherardi, G.M.; Usberti, M.; Martini, G.; Albertini, A.; Sugherini, L.; Pompella, A. Plasma total antioxidant capacity in hemodialyzed patients and its relationships to other biomarkers of oxidative stress and lipid peroxidation. *Clin. Chem. Lab. Med*., **2002**, Feb, *40(2)*, 104-110.

[18] Muller, D.; Schantz, M., Richling, E. High performance liquid chromatography analysis of anthocyanins in bilberries (Vaccinium myrtillus L.), blueberries (Vaccinium corymbosum L.), and corresponding juices. *J. Food Sci* (United States), **2012**, Apr, *77(4)*, C340-345.

[19] Dastmalchi, K.; Flores, G.; Petrova, V. Edible neotropical blueberries: antioxidant and compositional fingerprint analysis, *J. Agric. Food Chem.*, **2011**, 13 Apr., *59(7)*, 3020-3026.

[20] Johnson, M.H., Lucius, A.; Meyer, T. Cultivar evaluation and effect of fermentation on antioxidant capacity and in vitro inhibition of (+or)-amylase and (+or)-glucosidase by highbush blueberry (Vaccinium corombosum). *J. Agric. Food Chem.*, **2011**, 24 Aug, *59(16)*, 8923-8930.

[21] Kim, S.M.; Shang Y.F.; Um B.H. preparative saparation of chlorogenic acid by centrifugal partition chromatography from highbush blueberry leaves (Vaccinium corymbosum L.). *Phytochem. Anal.*, **2010**, Sep-Oct, *21(5)*, 457-462.

[22] Wei, H.R.; Meng, Y.L. Effects of exogenous nitric axide on highbush blueberry PSII photochemical activity and antioxidant system under high temperature stress. *Ying Yong Sheng Tai Xue Bao*, **2010**, Oct, *21(10)*, 2529-2535.

[23] Rhodes, E.M.; Liburd, O.E.; England, G.K. Effects of southern highbush blueberry cultivar and treatment threshold on flower thrips populations. *J. Econ. Entomol*., **2012**, Apr, *105(2)*, 480-489.

[24] Senevirathne, M.; Kim, S.H.; Jeon Y. protective effect of enzymatic hydrolysates from highbush blueberriy (Vaccinium corymbosum L.) against hydrogen peroxide-induced oxidative damage in Chinese hamster lung fibroblast cell line. *Nutr. Res. Pract*., **2010**, Jun, *4(3)*, 183-190.

[25] Biochemical and molecular changes in response to aluminium-stress in highbush blueberry (Vacciunium corymbosum L.). *Plant. Physiol. Biochem.*, **2011**, Sep, *49(9)*, 1005-1012.

[26] Svobodova, A.; Zdarilova, A.; Vostalova, J. Lonicera caerulea and Vaccnium myrtillus fruit polyphenols protect HaCaT keratinocytes against UVB-induced phototoxic stress and DNA damage. *J. Dermatol. Sci*., **2009**, Dec, *56(3)*, 196-204.

[27] Akerstrom, A.; Jaakola, L.; Bang, U. Effects of latitude-related factors and geographical origin on anthocyanidin concentrations in fruits of Vaccinium myrtillus L. (bilberries). *J. Agric. Food Chem.*, **2010**, *58(22)*, 11939-11945.

[28] Campanella, L.; Bonanni, A.; Favero, G.; Tomassetti, M. Determination of antioxidant properties of aromatic herbs, olives and fresh fruit using an enzymatic sensor. *Anal. Bioanal. Chem.*, **2003**, *375*, 1011-1016.

[29] Campanella, L.; Favero, G.; Persi, L.; Tomassetti, M. Evaluation of radical scavenging properties of several plants, fresh or from a herbalist's, using a superoxide dismutase biosensor, *J. Pharm. Biomed. Anal.*, **2001**, *24*, 1055–1064.

[30] Campanella, L.; Bonanni, A.; Bellantoni, D.; Tomassetti M. Biosensors for determination of total antioxidant capacity of phytotherapeutic integrators: comparison with other spectrophotometric, fluorimetric and voltammetric methods. *J. Pharm. Biomed. Anal.*, **2004**, *35*, 303-320.

[31] Prior, R.L.; Wu, X.; Schaich, K. Standardized methods for the determination of antioxidant capacity and Phenolic in foods and dietary supplements. *J. Agric. Food Chem.*, **2005**, *53*, 4290-4302.

[32] Ghiselli, A.; Serafini, M.; Maiani, G.; Azzini, E.; Ferro-Luzzi, A. A fluorescence-based method for measuring total plasmaantioxidant capability. *Free Radical Biol. Med.*, **1995**, *18*, 29-36.

[33] Glazer, A.N. Phycoerythrin fluorescence-based assay for reactive oxygen species. *Methods Enzimol.*, **1990**, *186*, 161-168.

[34] Cao, G.; Alessio H.M.; Cutler, R.G. Oxygen-radical absorbance capacity assay for antioxidands. *Free Radical Biol. Med.*, **1993**, *14*, 303-311.

[35] DeLange, R.J.; Glazer, A.N. Phycoerythrin fluorescence-based assay for peroxy radicals: a screen for biologically relevant protective agents. *Anal. Biochem.*, **1989**, *28*, 300-306.

[36] Huang, D.; Ou, B.; Prior, R.L. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.*, **2005**, *53*, 1841-1856.

[37] Benzie, I.F.F. An automated, specific, spectrophotometric method for measuring ascorbic acid in plasma (EFTSA). *Clin. Biochem.*, **1996**, 111-116.

[38] Benzie, I.F.F.; Strain, J.J. the ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.*, **1996**, *239*, 70-76.

[39] Benzie, I.F.F.; Szeto, Y.T. Total antioxidant capacity of teas by the ferric reducing/antioxidant power assay. *J. Agric. Food Chem.*, **1999**, *47*, 633-636.

[40] Ou, B.; Huang, D.; Hampsch-Woodill, M.; Flangan, J.; Deemer, E. Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *J. Agric. Food Chem.*, **2002**, *50*, 3122-3128.

[41] Gil, M.I. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. *J. Agric. Food Chem.*, **2000**, *48*, 4581-4589.

[42] Guo, Z.; Chen, J.; Liu, H.; Zhang, W. Electochemical determination of superoxide based on cytochrome c immobilized on DDAB-modified powder microelectrode. *Anal. Lett.*, **2005**, *38(13)*, 2033-2043.

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