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Biosensors for determination of total antioxidant capacity of phytotherapeutic integrators: comparison with other spectrophotometric, fluorimetric and voltammetric methods

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Abstract

Enzymatic electrodes based on superoxide dismutase (SOD) biosensors, working both in aqueous and non-aqueous solutions, recently developed by the present authors, were used to experimentally evaluate the antioxidant capacity of several phytotherapeutic diet integrators. The precision of this method of analysis was found to be reasonable (R.S.D. $\leq$ 10%). The results were also compared with those obtained using a traditional spectrophotometric method as well as a spectrofluorimetric method described in literature. Lastly, the comparison was extended to another method based on cyclic voltammetry currently being trialled by the present authors.

Keywords: Antioxidant capacity; Evaluation; Superoxide dismutase; Biosensors; Phytotherapeutic integrators

1. Introduction

The interest of modern food science to the antioxidant and radical scavenging properties of certain foodstuffs, especially of plant origin, is well known and justified by the number and importance of even serious diseases [1] that can, at least to some extent, be prevented by the regular intake of these foods [2]. Clearly the modern drug and phytotherapeutic industry promptly responded to these acquisitions of medical science.

One of the more immediate consequences was the marketing of a considerable number of products classified as phytotherapeutic integrators [3], generally obtained by different types of process from plant products, the real antioxidant properties of which are in any case not completely clear, nor indicated exactly on the packaging available on the market. One of the main aims of the present research was thus, using a rapid new method, to measure and compare the true antioxidant properties of the better known products of this type available on the market. As we have stated on other occasions, in characterising given matrixes such as foodstuffs or drugs, the determination of free radicals and that of total antioxidant capacity—the latter closely linked to radical scavenger contents—often represent two aspects of the same problem. We actu-

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ally initially developed several electrochemical sensors and biosensors for free radical determination of different kinds: voltammetric or amperometric, based on modified electrodes [4], potentiometric, classical or solid state, or using an FET as transducer [5], based on selective polymeric membranes, and lastly enzymatic sensors, the response of which, being modulated by the presence of free radicals, could be used for the determination of radical species [6]. More recently we also developed biosensors based on the superoxide dismutase enzyme [7,8]. The possibility of obtaining (SOD) biosensors by modifying the response of a classic amperometric electrode for H_2O_2 using a gel-like membrane to immobilise the superoxide dismutase enzyme (SOD) was discussed in previous papers [7]. In subsequent notes [8] we described the optimisation of the operating conditions of a superoxide dismutase biosensor using the H_2O_2 electrode as transducer. This biosensor was then used to evaluate the scavenging properties of several known molecules [8] and was applied also to determine the total antioxidant capacity of numerous fruit varieties, bulbs and vegetables, as well as of some active principles known for their antioxidant properties [9,10]. During this research, however, it was realised that a large number of molecules with interesting scavenging properties are difficult to determine because of their very low solubility in water. This led us to develop a biosensor capable of operating in non-aqueous solvent and to obtain preliminary application results regarding the evaluation of the scavenging properties of known hydrophobic antioxidant molecules using a new SOD biosensor but with a different construction architecture and capable of operating in non-aqueous solvents [11].

In view of the satisfactory results obtained using these biosensor methods it was clear that the more recent developments of this type of research should be focused on the comparison between the biosensor method developed by us to measure total antioxidant capacity and other methods of a different type developed by several authors for the same purpose, since a wide range of different methods of measuring antioxidant capacity have been proposed in recent years by different authors [12–18]. However each of these methods leads to the construction of a different scale of antioxidant capacity, valid solely for the method considered. One of the principal aims of the present work was thus to compare the results obtained using two of the better known of these methods with those obtained using the biosensor recently developed by us. To this end first of all the aim was thus, in view of the foregoing, limited to verifying whether any, even qualitative, experimental correlation existed between the scale of the antioxidant capacity obtained using our biosensor and individual scales based on the better known spectrophotometric or spectrofluorimetric methods described in literature. A spectrophotometric method was chosen for this comparison, namely the *N*,*N*-dimethyl-*p*-phenylenediamine (DMPD)–$FeCl_3$ method [12] as well as the spectrofluorimetric Oxygen Radical Absorbance Capacity (ORAC) method [14], which is without doubt one of the best known and most widely used in recent years to measure the antioxidant capacity of numerous food matrixes. Also quantitative correlation between biosensor and ORAC method values was evidenced.

Lastly, the comparison was extended to include also a method based on cyclic voltammetry [15] which we are currently testing in our laboratory.

2. Materials and methods

2.1. Chemicals

Xanthine (2,6-dihydroxy purine) sodium salt, ethylene diamino tetracetic acid (EDTA), superoxide dismutase 4980 U mg^{-1}, dialysis membrane (D-9777), *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), β-phycoerythrin and potassium superoxide were supplied by Sigma (Milan); acetone RPE, cyclohexanone RPE, monobasic potassium phosphate, anhydrous dibasic potassium phosphate RPE, anhydrous sodium acetate, analytical grade, methanol RPE and dimethylsulfoxide were supplied by Carlo Erba (Milan); xanthine oxidase 0.39 U mg^{-1}, cellulose acetate, kappa-carrageenan, sodium perchlorate monohydrate and glycerine were supplied by Fluka AG, Buchs (Switzerland); polyvinylacetate, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was supplied by Aldrich (Germany); potassium chloride, ferric chloride, diethyl ether and glycine were supplied by Merck (Germany); 2,2′-azobis(2-amidinopropan) dihydrochloride (ABAP) was supplied by Waco Chem. (Richmond, VA, USA). Sodium dibasic phosphate A.C.S., Tween 20, 18-Crown-6 were sup-

plied by Aldrich (Germany). The Teflon membrane, YSI model 5775 was supplied by Yellow Springs (Ohio, USA).

2.2. Apparatus

An Amel mod. 332/P electrode was used to assemble the biosensor; a mod. 551 Amel (Milan) potentiostat was used as potentiostatic power supply and also to convert the current signal into a tension signal, which was recorded using an Amel Model 631 differential electrometer (Milan, Italy), coupled to an Amel Model 868 analog recorder.

The original plastic cap of the electrode could not be used when the probe was used in organic solvents as it was rapidly attacked by them. For this type of measure, therefore, the original cap was replaced with a specially made Teflon cap of the same size. Also the original rubber O-ring of the cap, used to fix the membranes, was replaced in this case by a suitable Teflon ring.

The tests were carried out at 25 °C in a 15 ml thermostatted glass cell equipped with a forced water circulation jacket, coupled to a Model VC 20B Julabo (Germany) thermostat. The solutions used in the tests were kept under constant stirring using a microstirrer supplied by Velp Scientifica (Italy). A Lambda 5 model UV-Vis Perkin–Elmer spectrophotometer, equipped with printer and a model LS-5 Perkin–Elmer spectrofluorimeter, coupled to a model 561 Perkin–Elmer recorder, were respectively used for spectrophotometric and fluorimetric measurements.

An Amel analyser, model 433, manufactured by Amel (Milan), equipped with a printer and interfaced with a PC, was used for voltammetric measurements. An Ultra-Turrax homogenizer, mod. T8, was supplied by Ika Labortechnik (Germania). The vortex was supplied by Continental Equipment.

2.3. Samples

The samples tested consisted of ten phytotherapeutic diet integrators, all purchased at the drugstore and contained in plastic flacons or dark bottles. The majority were purchased in the form of tablets or capsules and one as granules.

Seven of the samples were in the solid state (tablets or powder in capsules), while three were in the oily state, two as capsules and one as granules. For the purpose of analysis, five capsules were opened and their contents carefully ground up, combined and homogenised. Also in the case of tablets and granules five were carefully ground up together. For the purpose of analysis, 500 mg of each sample were carefully weighed out and then treated as described below. Tables 1 and 2 list the phytotherapeutic integrators tested. The same tables also contains an indication of the drug form and the content of each of them; in the same table an indication is given also of the drug form and content of each of them according to the manufacturers.

3. Methods

3.1. Pre-treatment of samples for SOD biosensor analysis

500 mg of each phytotherapeutic integrator was taken, weighed and then homogenised (at 10,000 rpm for 5 min) in 3.0 ml of phosphate buffer (50 mM at pH 7.5); a 0.5 ml sample of the homogenate was then used in the analysis.

Alternatively, the homogenate in phosphate buffer was centrifuged (at 3000 rpm for 15 min); a 0.5 ml sample of the supernatant was then taken for analysis both using the biosensor and spectrophotometric, or the fluorimetric methods. However, when the latter method was applied to oily samples, 500 mg of sample were dissolved in 3 ml of acetone. After homogenisation, this solution was diluted, again with acetone (1 + 9)(v + v). At this stage 80 μl of this solution was then used for the analysis.

3.2. SOD biosensors and performance

3.2.1. Preparation of superoxide dismutase (SOD) biosensor working in aqueous solution

The biosensor we used to determine the superoxide radical was obtained by coupling a transducer (an amperometric electrode for hydrogen peroxide, with a platinum anode maintained at a constant potential of +650 mV with respect to an Ag/AgCl/Cl^- cathode) and the superoxide dismutase enzyme immobilised in a gel-like kappa-carrageenan membrane. The gel containing the enzyme was sandwiched between an inter-

Table 1
Integrator-phytotherapeutic products tested and their composition

Product and drug form	Composition	Content per tablet (or capsule) (mg)	Content per tablet (or capsule) (percentage)	Excipients
n.1 Tablets	Dog rose	1143	92.9	Microcrystalline cellulose Sodium carboxymethyl cellulose Antiagglomerants: Magnesium stearate Stearic acid Silica dioxide Lining agent: Hydroxypropylmethyl cellulose
n.2 Tablets	Rutin Fats Proteins Carbohydrates	100 10 Absent Absent	90.9 9.1	Dicalcium phosphate Microcrystalline cellulose Rutin Sodium carboxymethyl- cellulose Antiagglomerants: Magnesium stearate Stearic acid Silica dioxide
n.3 Tablets	Vitamine C Bioflavonoids Betacarotene Fats Carbohydrates (p.d.) Proteins (N × 6.25)	60 60 5 22.3 583 17	8.03 8.03 0.67 2.98 78.0 2.28	Dicalcium phosphate Microcrystalline cellulose Red Orange Complex (10.9%) Vitamine C tit. at 98% Bioflavonoids Betacarotene tit. at 10% Sodium carboxymethyl cellulose Antiagglomerants: Magnesium stearate Stearic acid Silica dioxide
n.4 Tablets	Vitamine C Vitamine E Betacarotene Selenium Fats Carbohydrates (p.d.) Proteins (N × 6.25)	90 15 15 0.055 54 350 57	15.5 2.58 2.58 0.01 9.29 60.2 9.81	Dicalcium phosphate Microcrystalline cellulose Vitamine C tit. at 90% Vitamine E Acetate tit. at 50% Betacarotene tit. at 10% Yeast tit. at 0.2% in Selenium Sodium carboxymethyl cellulose Antiagglomerants: Magnesium stearate, Stearic acid, Silica dioxide
n.5 Tablets	Acerola e.s.	401	36.4	Saccharose Microcrystalline cellulose Raspberry aroma Dog rose e.s. Antiagglomerants: Magnesium stearate Silica dioxide
n.6 Capsules	Ginkgo plv	300	75.7	Alimentary gelatine
n.7 Capsules	Panax Ginseng powdered root	440	82.1	Alimentary gelatine

nal cellulose acetate membrane and a external dialysis membrane (Fig. 1). The whole assembly was fixed to the head of the electrode by means of a rubber O-ring.

The preparation of the Kappa-carrageenan membrane and the immobilisation of the SOD enzyme in the gel membrane were described in detail in a previous paper [7,8].

Table 2
(Oily) integrator-phytotherapeutic products tested and their composition

Product and drug form	Composition	Content per capsule (or granule) (mg)	Content per capsule (or granule) (percentage)	Excipients
n.1′ Capsules	Wheat germ oil	160	37.2	Glycerol Alimentary gelatine
n.2′ Capsules	Wheat germ oil	338	70.5	Glycerol Alimentary gelatine
n.3′ Granules	Garlic oily macerate	150	44.9	
	Hawthorn oil macerate	50	15.0	
	Mistletone	50	15.0	
	Natural vitamine E D-Alpha-Tocopherol 10 UI	84	25.1	

3.2.2. SOD biosensor method working in aqueous solution

The antioxidant capacity using the SOD biosensor is checked as follows: the superoxide radical is produced by the oxidation in aqueous solution of the xanthine to uric acid in the presence of the enzyme xanthine oxidase:

$$\text{xanthine} + H_2O + O_2 \xrightarrow{\text{xanthine oxidase}} \text{uric acid} + 2H^+ + O_2^{\bullet -} \quad (1)$$

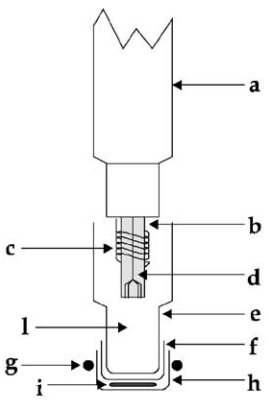
the disproportion reaction of the superoxide radical, catalysed by the superoxide dismutase immobilised on the H_2O_2 electrode, releases oxygen and hydrogen peroxide:

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \xrightarrow{\text{superoxide dismutase}} H_2O_2 + O_2 \quad (2)$$

the H_2O_2 is monitored by the amperometric sensor for hydrogen peroxide.

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

The addition of a sample possessing antioxidant properties produces a decrease in signal strength as the antioxidant species react with the superoxide radical, thus reducing its concentration in solution. There is a consequent decrease in the H_2O_2 released and thus also in the intensity of the amperometric signal and, in the case of several additions, in the value of the slope of the straight line obtained.



a: electrode body; **b:** dielectric; **c:** Ag/AgCl cathode; **d:** Pt anode; **e:** electrode teflon cap filled with inner solution; **f:** cellulose acetate membrane; **g:** rubber O-ring; **h:** dialysis membrane; **i:** superoxide dismutase enzyme in kappa-carrageenan gel; **l:** inner filling solution (0.05 M phosphate buffer pH 7.5 and KCl 0.1 M).

Fig. 1. SOD biosensor (working in aqueous solution) assembly, using an amperometric H_2O_2 electrode as transducer.

3.2.3. Performance of the measurement using the SOD biosensor working in aqueous solution

The electrode is placed in a glass cell thermostatted at 25 °C containing 15.0 ml of phosphate buffer 5×10^{-2} M at pH 7.5 and allowed to stabilise under constant magnetic stirring. After the addition of a fixed amount of the enzyme xanthine oxidase (1.2 mg), a series of further additions of 200 μl of the xanthine solution 1×10^{-2} M is performed, waiting for the signal to stabilise after each addition before proceeding to read off the current. The current values (read after signal stabilisation) are then recorded in the graph as a function of increasing xanthine concentration, thus obtaining a straight-line calibration curve from which the slope may be measured. The same type of mea-

surement is then repeated, but this time adding also the sample to be tested (0.5 ml of extract, or 0.5 ml of centrifugate) to the cell containing the phosphate buffer and then, after stirring, proceeding as described above. If the sample displays antioxidant properties, the observed signal variation will be lower than in the preceding case, in proportion to the decrease in concentration of the superoxide radical in solution; the new calibration curve thus obtained will present a lower slope value than in the preceding case. By comparing the values of the two slopes it is possible to determine the total antioxidant capacity [19] of our sample.

The value of the relative antioxidant capacity is expressed by the algorithm:

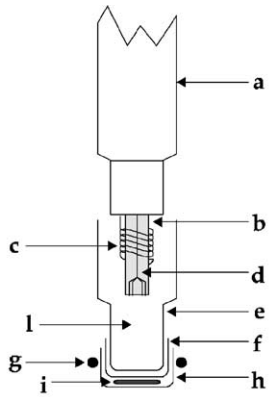
$$\text{Relative antioxidant capacity (RAC)} = 1 - \left(\frac{m_b}{m_a}\right)$$

where m_a is the slope of the straight line obtained by successive xanthine additions, m_b is slope of the straight line obtained by successive xanthine additions, but in the presence of the sample with antioxidant properties. All the samples were analysed using this method.

The choice of a sample volume of 0.5 ml used in the test was the same as that already used and optimised in previous works on other vegetable extracts [8]. It was thus possible also to make a homogeneous comparison with the RAC of different fresh fruit and vegetable foodstuffs [8–10,20].

3.2.4. SOD biosensor assembly able to work in non-aqueous solution

In view of the fact that it is possible [7,21] to generate the superoxide radical also in aprotic organic solvents, especially in the presence of a suitable crown ether, we developed an organic phase enzyme electrode using the assembly shown in Fig. 2: the enzyme was immobilised in kappa-carrageenan gel and sandwiched between two different membranes, an external gas-permeable membrane and an internal cellulose acetate one. Both membranes were secured by means of a Teflon O-ring to the Teflon cap of an amperometric electrode for oxygen (platinum cathode polarised at −650 mV versus an Ag/AgCl/Cl^- anode) containing 0.05 M phosphate buffer (pH 7 and KCl 0.01 M). This configuration of the electrode membrane, completely different from that of classical biosensors of this type



a: electrode body; **b:** dielectric; **c:** Ag/AgCl anode; **d:** Pt catode; **e:** electrode teflon cap filled with inner solution; **f:** cellulose acetate membrane; **g:** teflon O-ring; **h:** gas-permeable membranes; **i:** superoxide dismutase enzyme in kappa-carrageenan gel; **l:** inner filling solution (0.05 M phosphate buffer pH 7.0 and KCl 0.01 M).

Fig. 2. SOD biosensor (working in non-aqueous solution) assembly using an amperometric gas diffusion oxygen electrode as transducer. (Assembly using only one external gas-permeable membrane).

[22,23], was described and optimised by us in previous work [11].

3.2.5. Biosensor method working in non-aqueous solution

In the tests carried out using the SOD/O_2 biosensor operating in non-aqueous solution, the superoxide radical, obtained by means of the following reaction, $KO_2 \rightarrow K^+ + O_2^{\bullet -}$ spreads through the Teflon membrane and is determined by means of the reaction catalysed by the superoxide dismutase enzyme immobilised in the kappa-carrageenan gel layer (supported by the cellulose acetate membrane and placed in front of the amperometric oxygen electrode), in which the superoxide radical produces oxygen and hydrogen peroxide (see reaction (2) in Section 3.2.2.); the variation in dissolved oxygen concentration is detected by the O_2 probe.

3.2.6. Performance of the measurements using the SOD biosensor working in non-aqueous solution

When using the SOD/O_2 biosensor in non-aqueosus solution, in order to obtain the superoxide radical, a

solution of KO_2 0.01 M and 0.02 M of ether18.crown.6 in dimethylsulfoxide (DMSO) is prepared; suitable aliquots (10–100 μl) of this solution are then added to the DMSO solution in the measuring cell and the variations in the signal, are detected by the gas diffusion amperometric oxygen electrode, which is used as the biosensor's indicator electrode. The current variation is recorded (in the order of tens of nA) after each addition; in this way it is possible to construct a suitable calibration curve. The slope value of the latter is compared with that one of a calibration curve obtained under the same experimental conditions, but in the presence of the antioxidant sample to be tested, which has been added to the dimethylsulfoxide solution.

The RAC of the scavenging sample considered is evaluated from the percentage ratio of slope values of two calibration graphs, both in the presence and absence of the antioxidant sample considered, using the same algorithm as the one mentioned in Section 3.2.3.

As shown in the Section 4, after a series of different tests carried out in the present research work, the "definitive" measures of oily products were actually carried out in a more complex solvent mixture consisting of DMSO, glycerine and Tween 20 (10+4+1) (v + v + v), 1% (p/v) in "crown ether".

3.3. Spectroscopic methods to determine antioxidant capacity

3.3.1. DMPD + $FeCl_3$ spectrophotometric method [12]

3.3.1.1. Principle of method. The cation radical obtained from the *N*,*N*-dimethyl-*p*-phenylenediamine 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. 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.

3.3.1.2. Performance of measurements. In a vessel containing 100 ml of acetate buffer (0.1 M at pH 5.25) 1.0 ml of a solution of DMPD 0.1 M and 0.2 ml of a solution of $FeCl_3$ 0.05 M are added; this produces the purple coloured cation radical $DMPD^{\bullet+}$. The final solution is placed in a quartz cuvette and the absorbance at 514 nm read off.

To this solution are then added 150 μl of suitably diluted homogenised and filtered sample in phosphate buffer pH 7.5 or else a solution of Trolox 1.0 mg ml^{-1}; the absorbance at 514 nm is then read off after 10 min, during which the mixture is maintained under constant stirring at a temperature of 25 °C. Only acetate buffer is placed in the reference cuvette.

3.3.1.3. Data processing. the results are reported as the percentage inhibition of the signal I_{514} (%), according to the algorithm:

$$I_{514}(\%) = \left(1 - \frac{A_f}{A_0}\right) \times 100$$

A_0 is absorbance of the cation radical recorded prior to the addition of the sample and A_f is the absorbance recorded 10 min after addition of sample with antioxidant properties.

The antioxidant capacity of the samples is expressed in TEAC (Trolox equivalent antioxidant capacity) units, in accordance with the method of Miller et al. [24], using a calibration curve obtained using different amounts of Trolox and taking account of the fact that absorbance inhibition at 514 nm is linear between 0.2 and 8.0 μg of Trolox.

3.3.2. ORAC spectrofluorimetric method

3.3.2.1. Principle of method [14]. In the presence of free radicals or oxidant species the protein β-phycoerithryn (β-PE) loses over 90% of its fluorescence within 30 min. The addition of antioxidant species, which react with the free radicals, inhibits the diminution of the fluorescence of this protein. The inhibition caused by the action of the antioxidant species is correlated with the sample's antioxidant capacity. The 2,2′-azobis-(2-amidinopropane) dihydro chloride (ABAP) is used to generate peroxide radicals.

3.3.2.2. Performance of measures. The wavelengths are set at 540 nm for excitation and 565 nm for emission. Initially 80 μl of homogenised and filtered sample in phosphate buffer at pH 7 (or else 80 μl of a

solution of oily sample dissolved in acetone, as described in Section 3.1), are placed in the cuvette, together with 15 μl of phosphate buffer (75 mM, pH 7), and 1.46 ml of β-phycoerythrin (18.3 nM in phosphate buffer), prepared and allowed to stand at 37 °C for 15 min before use. The cuvette is placed in the spectrofluorimeter and the initial fluorescence (f_0) read off after 30 s. Then a further 20 μl of phosphate buffer are added to the solution in the cuvette together with 20 μl of ABAP (0.32 M in phosphate buffer). After stirring, the fluorescence is read off after 0.5 s and then every 2 min, for a total time of 70 min. A similar procedure is also carried out using a 20 μM solution of Trolox instead of the sample.

All the products were analysed in this way; only in the case of integrator n. 6 (ginkgo) was a further measurement made since the sample itself gave off a non-negligible fluorescence; the fluorescence of the product alone in phosphate buffer was read off after 0.5 s and then every 2 min, for a total time of 70 min; also this "sample blank" was subtracted from the ORAC value.

3.3.2.3. Data processing. The final results are expressed in "ORAC units" (micromoles of Trolox equivalent per litre of sample).

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

k is the dilution factor for the sample, S is integral of the fluorescence curve of the sample, of the Trolox, or of the "blank".

3.4. Measurement of antioxidant capacity using cyclic voltammetry

3.4.1. Sample treatment

The (non-oily) phytotherapeutic products are treated as follows: 0.5 grams of product are weighed out and placed in a large test tube. The product is then homogenised (5 min in a homogenizer at 10,000 rpm) in 3 ml of distilled water. It is then centrifuged (3500 rpm for 10 min) and lastly the supernatant liquid is subjected to voltammetric analysis after bringing up to volume (20 ml) using a solution of sodium perchlorate monohydrate 0.2 M (supporting electrolyte). However, as described below, the integrators are analysed also using a solution of phosphate buffer 1 M at pH 7.4, instead of perchlorate, and finally also using a (water + acetic acid + acetonitrile)(40 + 30 + 30)(v + v + v) solution.

3.4.2. Performance of measures

The measures are performed by connecting the voltammetric apparatus to a cell thermostatted at 25° centigrade in which the test sample has been introduced in the presence of a supporting electrolyte. The method entails the use of three electrodes: (a) a glassy carbon electrode as indicator electrode, (b) a calomel electrode as reference electrode, (c) a platinum electrode as counter electrode.

In order to avoid diminishing its sensitivity the indicator electrode is cleaned at the end of each cycle by rubbing the surface on alumina that has been previously moistened with a minimum quantity of distilled water. After further washing the electrode is then ready for further tests.

The voltammogram is recorded by scanning the potential over the range (−200/1300) mV at a scanning rate of 400 mV/s. Typical voltammograms obtained for the integrators tested are shown in Fig. 3.

For the purpose of the test the area (in cm^2) subtended by the anodic curve of the voltammogram is measured. The method is actually based on the correlation between the anodic area and the antioxidant capacity of the sample.

4. Results and discussion

As in previous researches [9,10] involving the analysis of vegetal food samples, also in the case of these phytotherapeutic integrators, all obtained from plant and natural products, the interest lies mainly in being able to measure the level of the property for which there is an increasing demand on the market, namely, their total antioxidant capacity, as well as to compare numerical values of the latter for the various integrators considered. To this end, as was done in the previous research, in which the samples consisted of fresh fruit [10], bulbs [9], aromatic herbs [10] and other vegetal products, the total antioxidant capacity per unit weight of product is determined. Indeed the essential information from the medical-pharmaceutical point of view consists in determining the intake level of antioxidant capacity when the same weight of one

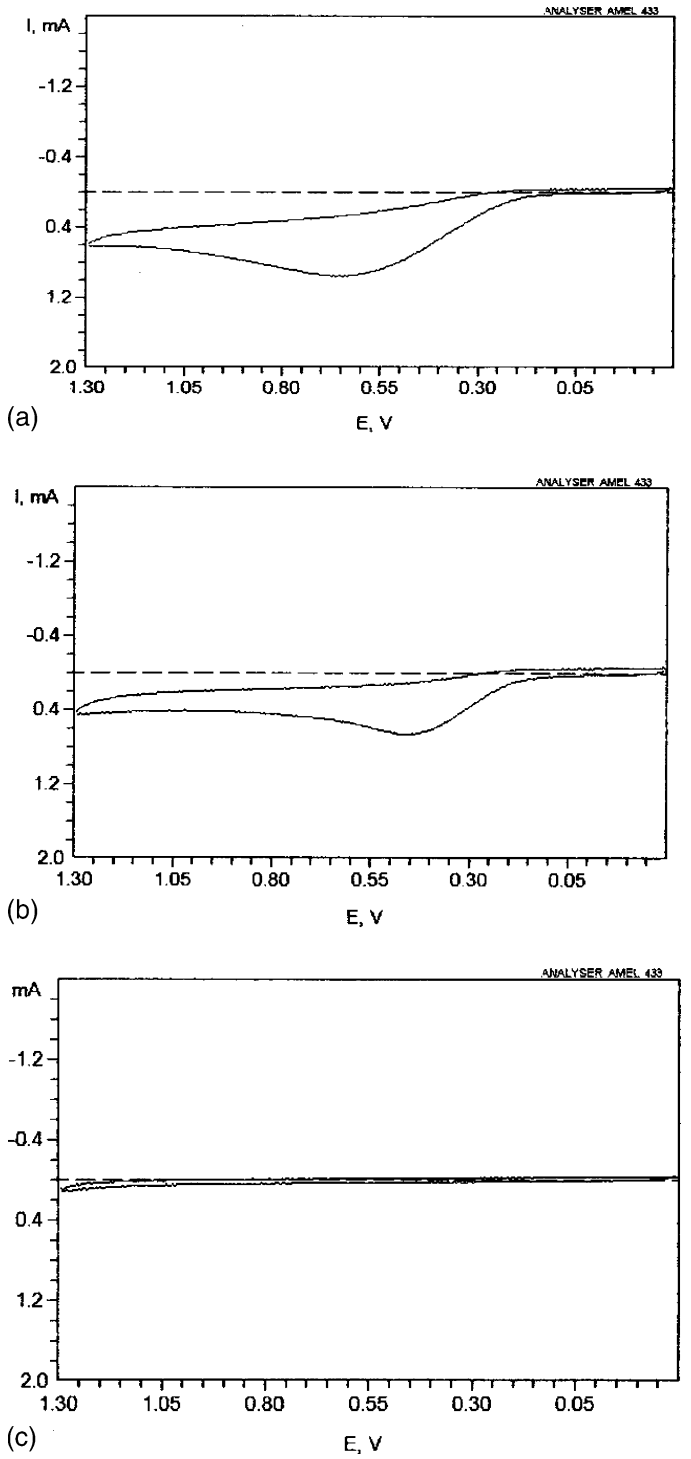


Fig. 3. (a) Voltammogram in $NaClO_4$ 0.1 M of product n.1 (see Table 1). (b) Voltammogram in $NaClO_4$ 0.1 M of product n.4 (see Table 1), (c) Voltammogram in $NaClO_4$ 0.1 M of the supporting electrolyte alone. Scanning rate 400 mV/s.

Table 3
Value of antioxidant capacity of the seven considered integrator-phytotherapeutic products obtained using (a) biosensor method, (b) fluorimetric method, (c) spectrophotometric method and (d) voltammetric method

Sample	(a) Biosensor method (RAC units) ($n \geq 5$)		(b) Fluorimetric method (ORAC units) ($n \geq 5$)	
	Homog.	Centr.	Homog.	Centr.
Sample n.1	0.98*0* ± 0.04*4*	0.87*2* ± 0.06*0*	102*6* ± 65.*3*	46*1* ± 25.*3*
Sample n.2	0.78*2* ± 0.05*0*	0.67*5* ± 0.05*5*	59*2* ± 22.*1*	33*1* ± 23.*9*
Sample n.3	0.61*9* ± 0.03*6*	0.51*4* ± 0.04*3*	59*1* ± 44.*9*	30*2* ± 19.*5*
Sample n.4	0.59*9* ± 0.04*1*	0.51*0* ± 0.02*5*	37*0* ± 36.*2*	16*9* ± 12.*6*
Sample n.5	0.49*5* ± 0.02*5*	0.41*5* ± 0.02*8*	36*5* ± 26.*4*	16*1* ± 11.*4*
Sample n.6	0.35*1* ± 0.03*3*	0.33*4* ± 0.03*2*	29*3* ± 12.*4*	15*8* ± 14.*6*
Sample n.7	0.26*7* ± 0.02*9*	0.21*7* ± 0.02*1*	23*0* ± 10.*2*	13*5* ± 13.*6*
	(c) Spectrophotometric method (TEAC units) ($n \geq 5$)		(d) Voltammetric method (as cm^2) ($n \geq 5$)	
Sample n.1	93.*7* ± 7.*6*		53.3 ± 0.01	
Sample n.2	91.*4* ± 5.*6*		3.08 ± 0.01	
Sample n.3	88.*1* ± 5.*2*		8.11 ± 0.01	
Sample n.4	87.*7* ± 6.*1*		7.38 ± 0.01	
Sample n.5	86.*6* ± 4.*3*		5.89 ± 0.01	
Sample n.6	80.*9* ± 4.*0*		3.34 ± 0.01	
Sample n.7	85.*3* ± 4.*0*		2.72 ± 0.01	

The numbers 1–7 refer to the numbering used for these products in Table 1.

phytotherapeutic product or another is ingested. This is exactly what has been done in the present research using the SOD biosensor optimised for this purpose in previous works [7,8]. The results obtained are shown in Table 3(a). Again, as was done in the case of the fresh vegetal samples, tested in previous researches [8–10], the samples are tested both as homogenates and as centrifuged homogenates obtained using the procedure described in Section 3.1 and already optimised in the previous research [8]. It is immediately apparent from examination of the values shown in the histogram in Fig. 4(a) that homogenates generally tend to have slightly higher antioxidant capacities than centrifugates. In the case of fruit samples it has been demonstrated experimentally [10] that this is due to the fact that when the supernatant of centrifuged samples is taken for analysis, some antioxidant compounds suspended in the homogenised samples remain in the precipitated fibres [10]. Since, as has been seen, these phytotherapeutic products are essentially based on natural vegetal substances, it is quite likely that, also in this case, the same explanation applies. However, the difference in RAC (Relative Antioxidant Capacity) (see Section 3.2.3) found between centrifugates and homogenates is generally lower than that sometimes observed for fresh vegetal products [9,10] and is more or less constant for all the integrators tested. Moreover, in Fig. 4(a), a comparison is made between the RAC of only seven out of the ten integrators tested, namely those showing a comparatively good solubility in aqueous solution (phosphate buffer at pH 7.5), and that it is thus possible to analyse using the SOD biosensor developed by us for use in aqueous solution [7], i.e. a biosensor that has now been fully optimised and has proved to be extremely efficient [8]; on the other hand, three of the integrators, which existed in oily form and were only very slightly soluble in water, had to be analysed using the SOD biosensor capable of operating in non-aqueous solvent [11]. For these products the comparison of RAC values obtained under the conditions in which the biosensor was developed at the time [11] is shown in the histogram in Fig. 5(a). It should be noted in this connection that, in the present research, it was necessary to introduce some methodological enhancements during RAC determination carried out using the biosensor in non-aqueous solvent. As demonstrated in one of our previous articles [11], at the end of some tedious experimental work, it was found that the latter type of SOD biosensor was found to function effectively only under very specific operating conditions. The peculiarities involved refer not only to the unusual

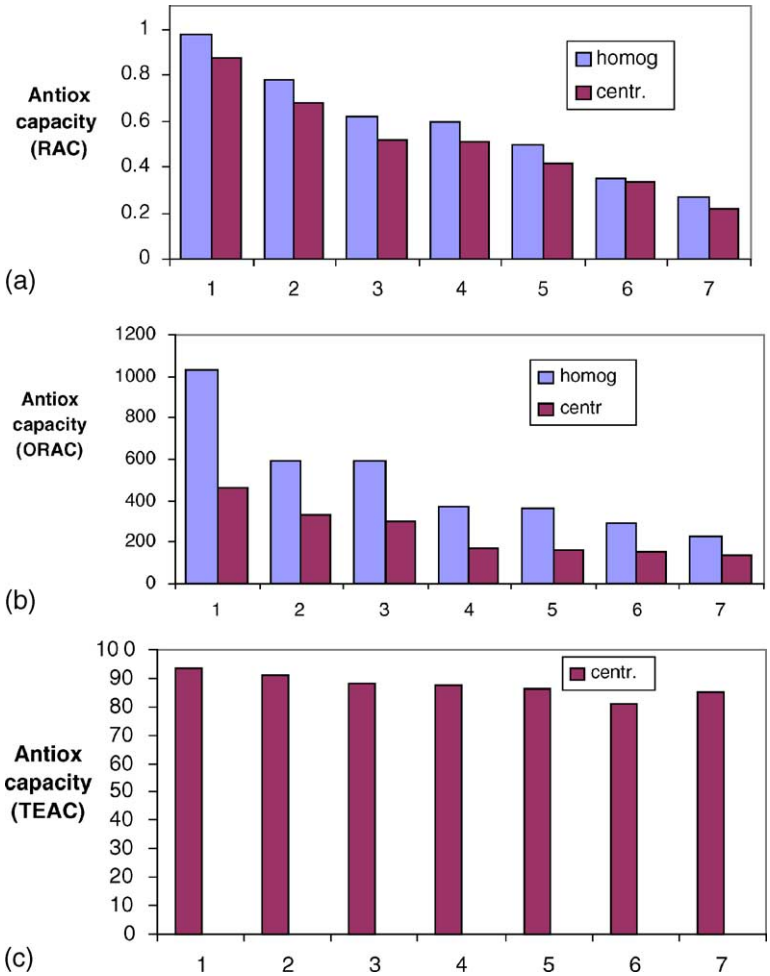


Fig. 4. Comparison of antioxidant capacity trends of seven different phytotherapeutic diet integrators obtained respectively (a) with the biosensor method, (b) with the fluorimetric method and (c) with the spectrophotometric method. The numbers from 1 to 7 refer to the numbering used for these products in Table 1.

configuration [11] of the enzymatic membrane of the biosensor (Fig. 2), but also to the solvent used in the measurements, i.e. dimethylsulfoxide (DMSO) containing crown ether [11]. However, even under these conditions biosensor sensitivity is found to be rather low. Furthermore, also the dimethylsulfoxide is certainly not a solvent that possesses a good capacity for dissolving hydrophobic organic products, particularly when they are oily. Indeed, operating in these conditions during the first few tests carried out, for two out of the three integrators considered, a negative RAC value was actually obtained (Fig. 5a). Taking into account the algorithm contained in Section 3.2.3, this could happen only if the slope of the calibration curve obtained in the presence of the antioxidant was for some reason higher than the slope of the calibration curve obtained in the absence of antioxidant. After careful consideration and examination of the composition of the products as declared by the manufacturer in the case of the two oily samples that produced these rather surprising results, we postulated that the reason was the presence in the two products tested of a certain quantity of glycerine (Table 2). It was hypothesised that when the latter, with the addition of the sample, was mixed to the dimethylsulfoxide a reduction in the surface tension occurred in the latter solvent. This led

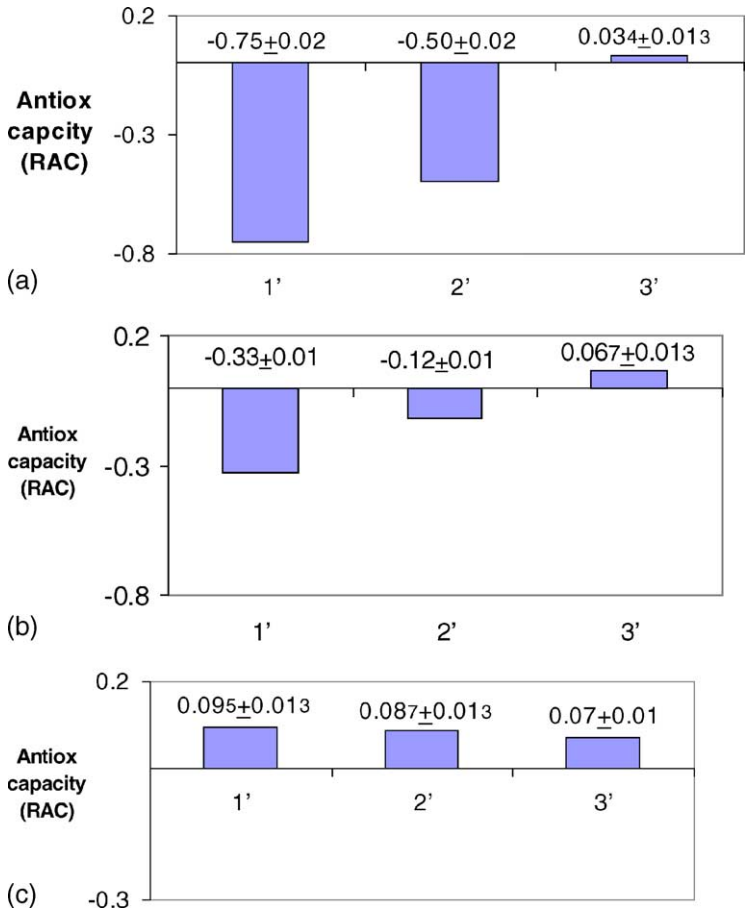


Fig. 5. Antioxidant capacity measurement of three (oily) phytotherapeutic products (a) in DMSO; (b) in DMSO+Glycerine (14.5+0.5)(v+v); (c) in DMSO + Glycerine + Tween 20 (10 + 4 + 1)(v + v + v). All the solution contained also 1% (p/v) of "crown ether". The numbers from 1′–3′, refer to the numbering adopted for these products in Table 2.

to an increase in the permeability of the superoxide radical produced in the solution versus the Teflon membrane of the oxygen sensor. Although detectable [21], this permeability is certainly not high when operating in dimethylsulfoxide alone, as is shown by the low sensitivity of the biosensor when operating in non-aqueous solution [11]. In order to verify this hypothesis the measures on the three oily integrators were repeated, but this time adding a small percentage (3.3% v/v) of glycerine to the dimethylsulfoxide. As may be seen (Fig. 5b) the results indicate a partial reduction in the negative values of the RAC in the two cases in question as well as an enhancement of method sensitivity, also in the third case, in which the RAC was found to be positive also in the preceding test (it should be noted that in the latter case, although oily, the product did not contain any glycerine). Taking into account the observed "improvement", it was decided to repeat the three measures once again, but this time in the presence of a much higher percentage of glycerine in the dimethylsulfoxide (26.6% v/v) so that the excess glycerine added would exert a "buffering" effect, as it were, versus the glycerine contained in the integrator. On the other hand, the addition of a strong excess of glycerine had also a second positive effect, namely to strongly boost the solubility of oily products in dimethylsulfoxide. Lastly, the observation that the decrease of surface tension in the dimethylsulfoxide solution increases the method's sensitivity suggested to us the possibility of introducing a further

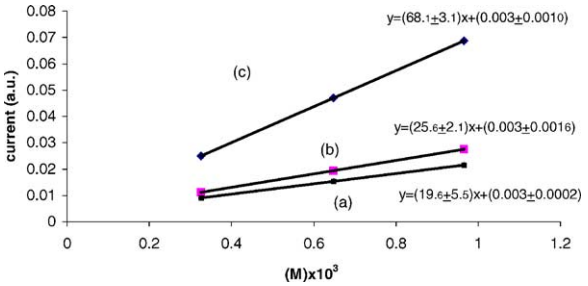


Fig. 6. Calibration curves obtained using the SOD biosensor able to operate in non-aqueous solvents, working in different solvent mixtures: (a) straight line obtained operating in DMSO containing 1% (p/v) "crown ether" (b) straight line obtained operating in DMSO containing 3.3% (v/v) glycerine (i.e. DMSO + Glycerine (15.5 + 0.5)(v + v) and 1% (p/v) "crown ether", (c) straight line obtained operating in DMSO containing (26.6% v/v) glycerine, (6.6% v/v), Tween 20 (i.e. DMSO + Glycerine + Tween 20 (10 + 4 + 1)(v + v + v)) and 1% (p/v) "crown ether".

improvement in this direction, achieved by adding to the dimethylsulfoxide solution a fixed percentage (6.6% v/v) of a non-ionic surfactant (Tween 20). The experimental demonstration of increased biosensor sensitivity thus observed was achieved by constructing different calibration curves using the biosensor, in the first case in dimethylsulfoxide alone, in the second case in dimethylsulfoxide containing 3.3% glycerine, in the third case in dimethylsulfoxide containing 26.6% glycerine and 6.6% non-ionic surfactant. The three calibration curves obtained, shown in Fig. 6, clearly show an increase in the slope in cases two and three, compared to case one. This increase is particularly evident in case three. On the strength of these results the RAC measures for the three oily integrators were repeated in dimethylsulfoxide containing both glycerine and non-ionic surfactant in the percentages stated above. As may be seen, (Fig. 5c) and (Table 4a)

Table 4
Values of antioxidant capacity of the three oily integrators, obtained using (a) the biosensor method and (b) the fluorimetric method

Sample	(a) Biosensor method (RAC units) ($n \geq 4$)	(b) Fluorimetric method (ORAC units) ($n \geq 4$)
1′	0.095 ± 0.009	325 ± 30. 2
2′	0.087 ± 0.008	266 ± 21. 3
3′	0.070 ± 0.007	190 ± 20. 5

The numbers from 1′–3′ refer to the numbering adopted for these products in Table 2.

in this case, as expected, not only were the RAC values found to be positive in all three cases, but it was also possible to obtain a scale of antioxidant capacities for the three different phytotherapeutic products, which represents the ultimate aim of the present research.

After thus obtaining scales of antioxidant capacity by means of two different types of SOD biosensor for all the phytotherapeutic integrators tested, the second part of the proposed research was begun. The aim here, in order to validate the results obtained, was to determine the antioxidant capacity of the integrators not only by the SOD biosensor, but also using the more conventional methods (Table 3b and c) extensively described in the literature, one of the spectrophotometric type [12] and one of the spectrofluorimetric type [14]. It is observed (Fig. 4b and c) that the trends of the antioxidant capacity values of the samples tested, using the biosensor working in aqueous solution, obtained using the spectrophotometric and spectrofluorimetric method, respectively, generally has a good correlation (Fig. 7a–c) with that of the RAC values obtained using the biosensor method. Agreement is particularly good using the ORAC method which, although very expensive, is perhaps the best known and most widely used of the classical methods. Also agreement with the spectrophotometric method based on (DMPD + $FeCl_3$) is quite good: only one different result (in the case of integrators $n = 6$ and $n = 7$) emerges from a comparison (Fig. 4) of the trend observed using this method compared with that obtained using the biosensor method. It should be noted however that, as pointed out on previous occasions [9,10], the selectivity of this spectrophotometric method versus antioxidant products with different antioxidant capacity is comparatively low. Consequently, also the numerical differences in antioxidant capacity of the various samples are very small and so random errors may have a strong effect on the trend obtained.

Moreover, the comparison of the trends indicated in the histograms set out in Fig. 8 shows that an excellent correlation also exists between the RAC trends of the oily products obtained with the biosensor operating in non-aqueous solution (using the previously described 'enhancements' of the method) and the antioxidant capacity trends (Table 4b) obtained using the spectrofluorimetric method (ORAC) (see Fig. 9). It must also be pointed out however that the analysis of these particular products, even using these two methods, is not

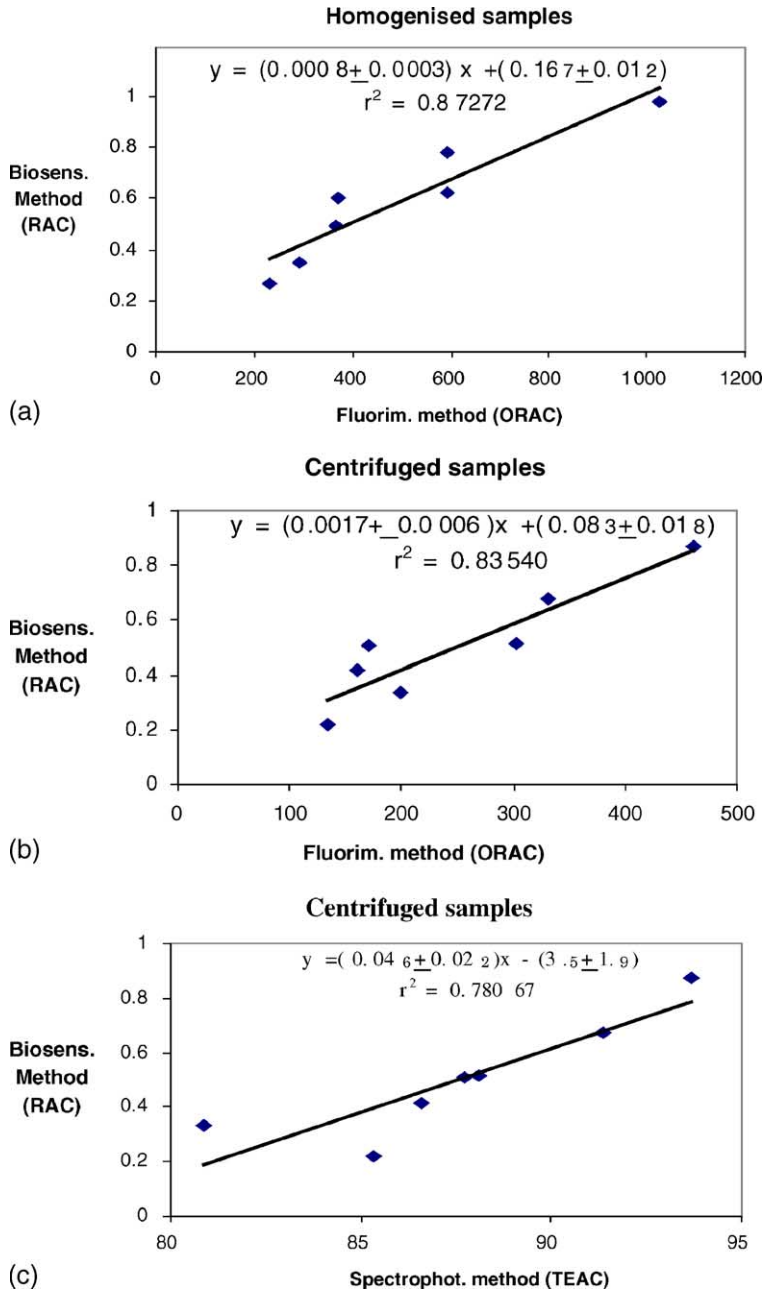


Fig. 7. Correlation curves of antioxidant capacity values obtained for the seven non-oily integrators, using the biosensor method and those found using the fluorimetric and spectrophotometric methods, respectively.

an easy matter. As mentioned earlier, the solvent used for the biosensor method when the biosensor is operating in non-aqueous solution had to be drastically 'improved', but also in the case of the classical ORAC method it was necessary to make some changes. For the test, the sample had to be previously dissolved in acetone and not directly in phosphate buffer, as described in Section 3.1.

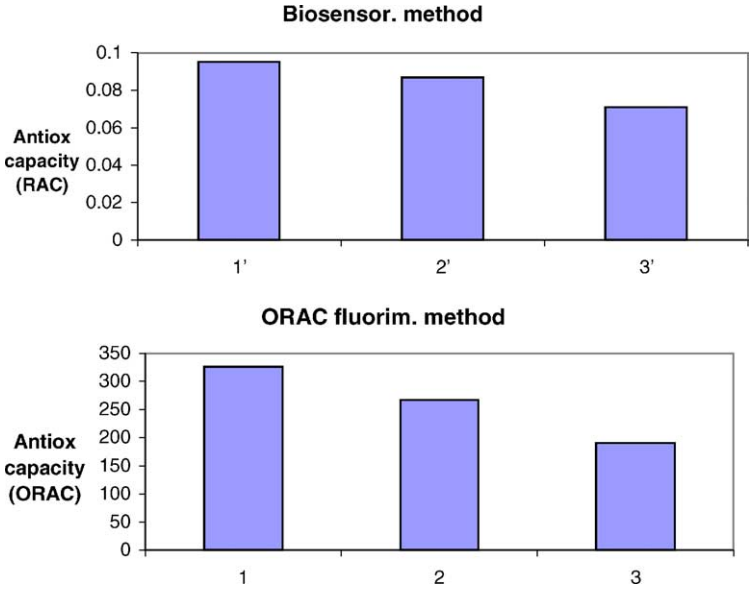


Fig. 8. Comparison of antioxidant capacity trends of the three oily integrators obtained respectively using the SOD biosensor, able to work in non-aqueous solvents (operating in DMSO + Glycerine + Tween 20 (20 + 4 + 1) (v + v + v), containing 1% (p/v) "crown" ether) and with the ORAC spectrofluorimetric method. The numbers from 1′ to 3′ refer to the number used for these products in Table 2.

As regards the method based on cyclic voltammetry [25,26], currently being tested in our laboratory, the trend obtained in the case of the seven non-oily products, shown in Fig. 10(a) and obtained operating in perchlorate 0.1 M, gives variable results. While indicating even too clearly the high antioxidant capacity of product number 1, i.e. the one containing dog rose, which was actually found to be the most highly antioxidant product among those tested, also using the other three methods, although with a difference in antioxidant capacity that was not so exaggeratedly high compared with the other products. Conversely, the method failed when applied to products numbers 2, 6 and 7, indicating, erroneously, practically negligible values for the antioxidant capacity of these products. There is also observed to be a reversal of trend in antioxidant capacity as measured for products numbers 3 and 4 compared with the trends obtained using the other three methods reported in Fig. 4. One of the early hypotheses put forward to account for all this was that in perchlorate solution, at pH 7, the antioxidant capacity of several of the samples is so low as the integrator is not dissolved in sufficiently large quantities during the process of homogenisation-centrifugation performed prior to the analysis. The latter operations were then repeated, this time using a solution of phosphate buffer 1 M at pH 7.4, i.e. in a buffer solution already used previously by other workers [25] and very similar to that used successfully in the preparation of the solutions analysed using the biosensor method [9,10]. However, as pointed out in Fig. 10(b), the results obtained are not very different from those found in the preceding case (Fig. 10a); there is no change in the trend even though the method's sensitivity has been considerably

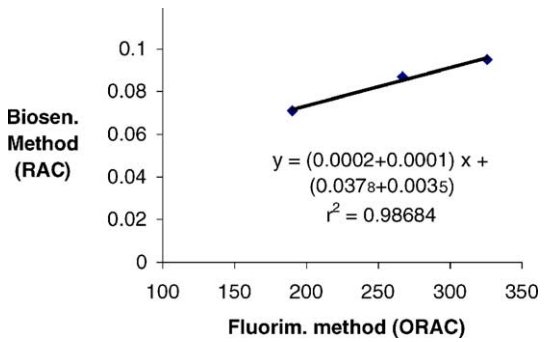


Fig. 9. Correlation curve of antioxidant capacity values obtained for the three oily integrators using the biosensor working in non-aqueous solvent and those found using the ORAC fluorimetric method.

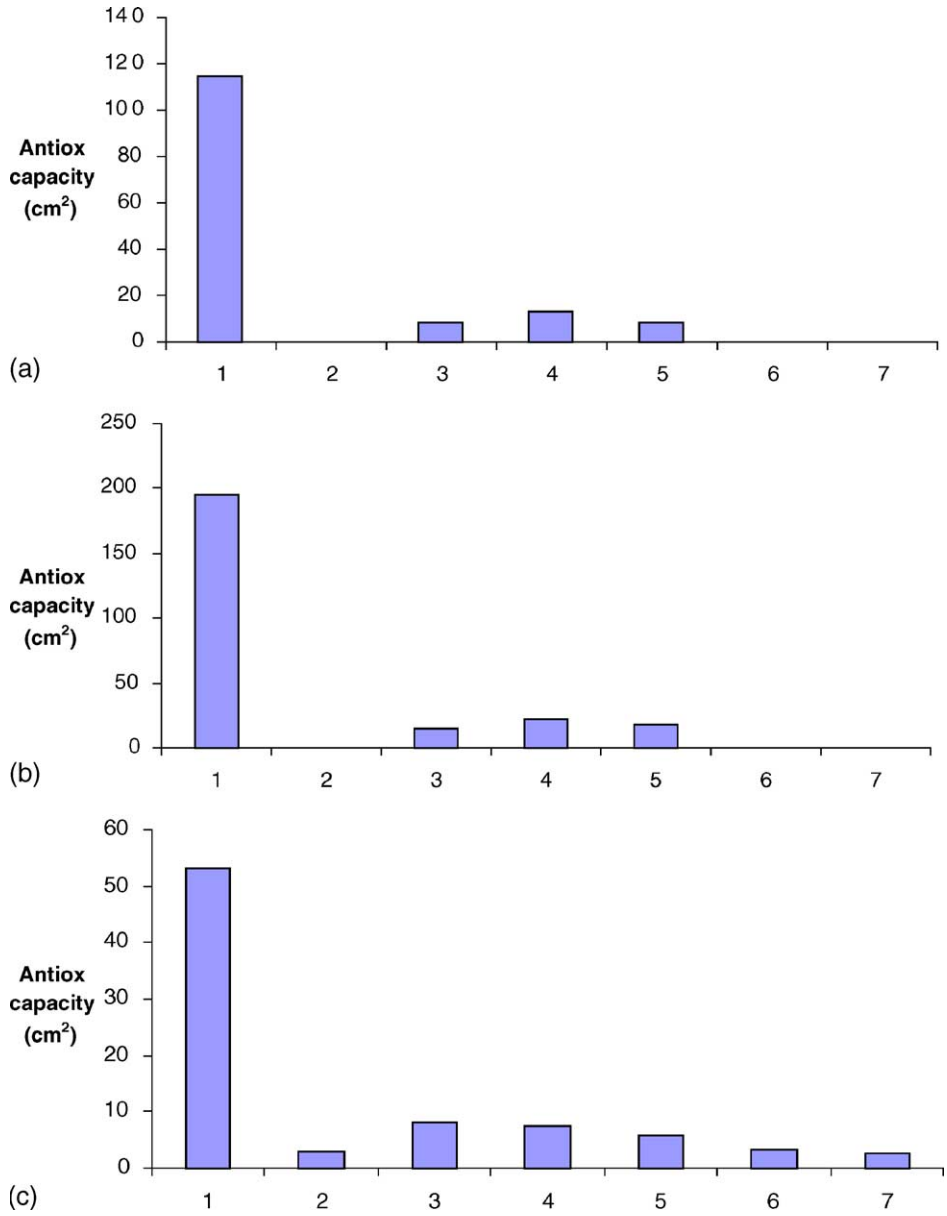


Fig. 10. Antioxidant capacity measurement using cyclic voltammetry of seven (non-oily) phytotherapeutic products (a) in $NaClO_4$ 0.1 M; (b): in phosphate buffer 1 M; (c): in ($H_2O + CH_3COOH + CH_3CN$) (40 + 30 + 30) (v + v + v). The numbers from 1 to 7 refer to the numbering used for these products in Table 1.

enhanced. Lastly, it was decided to pretreat the samples using a completely different solvent mixture (water/acetic acid/acetonitrile) (40 + 30 + 30) (v + v + v) proposed by Chevion et al. [26] when vegetal samples were analysed using voltammetric method. The trend of values in Table 3(d) thus obtained is shown in Fig. 10(c). It should be noted that, when operating in the latter solvent mixture, the trend is actually better than those obtained using the other aqueous solutions, e.g. it is closer to those found using the other meth-

Table 5
Comparison of antioxidant capacity values (all expressed in ORAC units) of the seven integrator-phytotherapeutic products obtained using (a) the biosensor method and by means of the correlation curve equation in Fig. 7(a and b), (b) the fluorimetric method

Sample	(a) ORAC units (calculated)		(b) ORAC units (measured)		Δ%	
	Homog.	Centrif.	Homog.	Centrif.	Homog.	Centrif.
1	101*5*	46*3*	102*6*	46*1*	+1%	−0.4%
2	76*7*	34*8*	59*2*	33*1*	−30%	−5%
3	56*4*	25*3*	59*1*	30*2*	+5%	+16%
4	53*9*	25*1*	37*0*	16*9*	−46%	−48%
5	40*9*	19*5*	36*5*	16*1*	−12%	−21%
6	22*9*	14*7*	29*3*	15*8*	+22%	+7%
7	12*4*	78.*6*	23*0*	13*5*	+46%	+42%

The numbers 1–7 refer to the numbering used for these products in Table 1.

ods set out in Fig. 4. However, a clear-cut 'inversion' always occurs in the case of product number 2 and product number 3. Above all, however, there is still too great a difference (although partly reduced) in the sensitivity displayed by this method towards product number 1 compared with the sensitivity indicated for all the remaining products. At this stage of the research it is still not fully clear what the reasons are for the failure of this method observed in certain cases. What is clear, however, is that it is due only in part to problems linked to the type of solvent used and that it could plausibly be postulated that the kinetics of the oxidative processes taking place at the glassy carbon electrode may be affected, even strongly, by certain substances present in the sample. Indeed, the kinetics of these processes may sometimes be slowed down considerably, but in other cases actually speeded up (for instance, in the case of dog rose), and so the signal obtained for the various different products, when operating in the same conditions, could be severely affected by this fact.

Table 6
Comparison of antioxidant capacity values (all expressed in ORAC units) of the three oily integrators, obtained using (a) the biosensor method and by means of correlation curve equation in Fig. 9, (b) the fluorimetric method

Sample	(a) ORAC units (calculated)	(b) ORAC units (measured)	Δ%
1′	28*6*	32*5*	+12
2′	24*6*	26*6*	+8
3′	16*1*	19*0*	+15

The numbers 1′–3′ refer to the numbering adopted for these products in Table 2.

Lastly, one aspect of this research that we consider important is the possibility of being able first to express and then to compare, for the first time, the antioxidant capacity values obtained using the superoxide dismutase biosensor, also in ORAC units (see Tables 5 and 6). These are the same units of measure as those used by the fluorimetric method which, as already mentioned, is perhaps the one most frequently used. This was made possible by using the equations of the correlation curves shown in Figs. 7 and 9.

5. Conclusions

The biosensor method recently developed by us based on the superoxide dismutase probe proved to be completely valid for the determining total antioxidant capacity not only of vegetal food products, as shown in previous research [8–10], but also of phytotherapeutic diet integrators, as shown in the present research. The repeatability of the method is good (R.S.D. = 5–10%), it is robust [8,9], cheap and rapid to apply, with few operational constraints and does not require the use of any expensive or sophisticated equipment. As concern the validation, the results obtained in the present work indicate an excellent correlation with other chemical methods, and with the ORAC spectrofluorimetric method in particular. Mention should also be made of the improvements made to the biosensor method introduced for the first time in the present article which can be used in the case of certain specific samples, for instance, oily products that are only slightly soluble not only in water but also in dimethylsulfoxide. This, on the contrary, makes the original

method difficult to apply even when the special SOD biosensor capable of operating in non-aqueous solution is used. The method based on cyclic voltammetry undergoing tests in our laboratory has clearly displayed both advantages and drawbacks. Also in this case the former derive from its simplicity, rapidity, repeatability and cheapness. The latter stem from the fact that the method apparently displays a sensitivity that is not uniform for all types of sample. A more thorough investigation will however need to be carried out to determine whether the reasons for this behaviour, as suggested in the present article, actually correspond to experimental truth. It might be possible to make some changes to the method in order to remedy the observed drawback or at least to circumscribe the limits of its applicability more precisely.

Lastly, it may be pointed out that, for the first time in the present research we did not simply make a qualitative comparison of the trends in the values obtained using the different methods, but were able also to make homogeneous quantitative comparisons of the values of antioxidant capacity found by means of the biosensor and fluorimetric methods considered, but all expressed in the same (ORAC) units of measure.

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