A comparison among three different analytical methods to test the scavenging properties of different integrators against radicalic stress

Riccarda Antiochia[1*], Tania Gatta[2], Elisa Mazzone[2], Luisa Mannina[1] and Luigi Campanella[2]
[1]Department of Chemistry and Drug Technologies, Sapienza Università di Roma, Piazzale Aldo Moro 5, Rome, Italy
[2]Department of Chemistry, Sapienza Università di Roma, Piazzale Aldo Moro 5, 00185 Rome, Italy

Abstract: The aim of the present work was to experimentally evaluate the total antioxidant capacity of different commercially available pharmaceutical integrators based on vitamin E, astaxanthin, resveratrol and blueberry using the traditional DMPD-$FeCl_3$ spectrophotometric method and the ORAC spectrofluorimetric method, largely discussed and described in literature and considered as reference methods. The results were compared with those ones obtained with a biosensor based on superoxide dismutase (SOD) enzyme. Precision of antioxidant capacity measures for all integrators was good, generally with a R.S.D.% ≤10% for all methods employed. An evaluation was also performed of the possible influence on the total integrator antioxidant capacity from other compounds and/or excipients present in the formulations which can interfere with the main antioxidant component of the commercial integrator.

Keywords: Antioxidant capacity; pharmaceutical integrators; vitamin E; astaxanthin; resveratrol; blueberry.

INTRODUCTION

The involvement of reactive oxygen species (ROS) and of free radicals in the pathogenesis of many human diseases, including cancer, aging and coronary heart diseases is increasingly being recognized (Moskovitz *et al*., 2002). Superoxide anion radicals ($O_2^{-}\bullet$), hydroxyl radicals (•OH) and peroxyl radicals (ROO•) are reactive oxygen species which are continuously produced in vivo during the aerobic metabolism and more and more often by external sources such as an inadequate diet, environmental pollution and UV radiation. The formation of ROS in excess breaks a balance between oxidants and antioxidants in the body (Ames, 1979). Therefore, much attention has been paid to the use of antioxidants as protecting agents of the body from reactive oxygen species and free radicals (Diplock, 1994). In principle, the consumption of a very well-balanced diet rich in fruit and vegetables can, to some extent, prevent the damages from radicalic oxidative stress. These beneficial effects can be related to the antioxidant properties of speficic compounds such as polyphenols, carotenoids, vitamin E and C, contained in some foods (Prior and Cao, 2000). But the daily intake of these compounds by diet is not always possible and it is not regular. Therefore, in recent years drug industries have marketed a large number of products classified as pharmaceutical integrators, containing one or, more often, several compounds with antioxidant activity, either of natural or synthetic origin (Campanella *et al*., 2004a). Among the most used commercially available pharmaceutical integrators, the ones based on vitamin E, astaxanthin, resveratrol and blueberry have been chosen in this study because of the extraordinary antioxidant properties of their main active principle, particularly efficient to contrast the oxidative stress from noxious environmental conditions.

Vitamin E, a collective name for tocopherols and tocotrienols, is one of the most important lipid-soluble primary defence antioxidants (Rezk *et al*., 2004; Kamal-Eldin and Appelqvist, 1996; Azzi, 2007; Klein *et al*., 2011). Astaxanthin is a red carotenoid (3,3'-dihydroxy-β,β-carotene-4,4'-dione) which recently attracted considerable interest because of its powerful antioxidant activity (Naguib, 2000) reported in literature as ten times stronger than that one of other carotenoids, like lutein, canthaxanthin and β-carotene (Miki, 1991; Nishino, 1998; Iwamoto *et al*., 2000). Resveratrol (3,5,4'-trans-trihydroxystilbene), a kind of polyphenolic trans-stilbene contained in grape skin, is a natural phytoalexin used by plants to protect themselves from fungal and other form of aggressions and has been proved to posses a variety of biological activity including anti-inflammatory, anticarcinogenic and antioxidative activities (Liu *et al*., 2011; Kamiyama *et al*., 2009). The famous "French paradox", the observation of low coronary heart disease death despite high intake of dietary cholesterol and saturated fat, can be explained by a high consumption of red wine by the French population, as reported by many authors (Leger *et al*., 1979). Blueberry is considered one of the fruit with the highest levels of antioxidant activity (Zheng and Wang, 2003; Kay *et al*., 2002) and most of this activity is due to the phenolic compound chlorogenic acid and to antocyanins (Sellappan *et al*., 2002).

The label of the package of pharmaceutical integrators indicate their composition but no data refer to their antioxidant capacity on the market.

**Corresponding author:* e-mail: riccarda.antiochia@uniroma1.it

The aim of the present work was therefore to measure and to compare the antioxidant capacity values of 14 commercially available integrators determined using two conventional analytical methods with those obtained with a cheap, easy to use and "in situ" method based on an amperometric biosensor.

Several methods have been utilized in literature for the measurement of the radical scavenging activity but none of them was reported as the official method to evaluate total antioxidant capacity. The most commonly in recent years to measure the antioxidant capacity of numerous food matrixes are the spectrophotometric method, namely N,N-dimethyl-p-phenylenediamine DMPD)-$FeCl_3$ method (Fogliano *et al*., 1999; Zulueta *et al*., 2009) and the spectrofluorimetric Oxygen Radical Absorbance Capacity (ORAC) method (Zulueta *et al*., 2009; Cao *et al*., 1995), both considered as reference methods. Therefore, in the present paper, the determination of the antioxidant capacity of the different pharmaceutical integrators was firstly performed by using these two methods. Successively, a superoxide dismutase (SOD) biosensor based method (Campanella *et al*., 1999), already applied to the determination of the antioxidant capacity of several herbs (Campanella *et al*., 2001; Campanella *et al*., 2003a), fresh fruits (Campanella *et al*., 2003b), phytoterapeutic integrators (Campanella *et al*., 2004a), drug products (Campanella *et al*., 2004 b), wines (Campanella *et al*., 2004 c) and dry spices (Bonanni *et al*., 2007) was employed to test the antioxidant capacity of the pharmaceutical integrators and the results compared to those ones by the other two methods.

To this end the answer to the aim was limited to qualitative comparisons among the results obtained with the three methods. Successively by homogeneous quantitative comparisons experimental correlations between the scale of measure of the ORAC method and the individual scales of the spectrophotometric and biosensor methods were looked for.

MATERIAL AND METHODS

Chemicals

Xanthine (2,6-dihydroxy purine) sodium salt, superoxide dismutase (SOD) 4980 U mg^{-1}, dialysis membrane (D-9777), N,N-dimethyl-p-phenylenediamine dihydrochloride (DMPD), β- ethylene diamino tetracetic acid (EDTA) and phycoerythrin were obtained by Sigma (St. Louis MO, USA); anhydrous dibasic potassium phosphate RPE, potassium acetate, glacial acetic acid, 37% cloridric acid and anhydrous sodium acetate were obtained by Carlo Erba (Milan, Italy); xanthine oxidase (XOD) 0.39 U mg^{-1} and kappa-carrageenan were obtained by Fluka AG (Buchs, Switwerland); 6-hydroxy-2,5,7,8-tetranethylchroman-2-carboxylic acid (Trolox) was obtained by Aldrich (Milan, Italy); potassium chloride was supplied by Riedel-de-Haen (Seelze, Germany); ferric chloride was supplied by Merck (Germany) and 2,2'-azobis(2-amidinopropan)dihydrochloride (ABAP) was obtained by Waco Chem. (Richmond, VA, USA).

Apparatus

Model 4000-I amperometric electrode, obtained by Universal Sensors Inc. (New Orleans LA, USA), coupled to an Amel mod. 551 potentiostat (Milan, Italy), connected to an Amel mod. 631 differential electrometer and an Amel mod. 868 analog recorder.

The tests were carried out at 25°C in a thermostated glass cell (10 ml) coupled to a mod. VC 20B Julabo thermostat (Germany). The solutions were kept under constant stirring using a microstirrer (Velp Scientifica, Italy).

For spectrophotometric analysis was used a Lamda 16 mod. Perkin-Elmer spectrophotometer, equipped with a printer and for spectrofluorimetric analysis a mod. LS-5 Perkin-Elmer spectrofluorimeter, coupled to a mod. 561 Perkin-Elmer recorder.

Samples

Tests were run on 14 integrators, all in tablet or capsule form. In particular 3 integrators were based on vitamin E (E1, E2, E3) (Vitamina E, Body Spring; Evion, Bracco; Ephynal, Bayer), 3 on astaxanthin (A1, A2, A3) (Massigen Pronto Recupero, Marco Viti; Azyr Mega, Sifi; Astaxantina Complex, Solgar), 4 on resveratrol (R1, R2, R3, R4) (Res Vital, O.T.I.; Resveratrol, Terraternal; Revidox, Paladin Pharma; Vinexpert, Caudalie) and 4 on blueberry (B1, B2, B3, B4) (Mirtillo, Arkopharma; Tegens, Sanofi Aventis; Mirtillo Plus, Aboca; Mirtilene Forte, SIFI). table 1 indicates the composition of the integrators as specified on the respective packages, showing the active principles and the excipients.

METHODS

Pre-treatment of integrators for spectrophotometric analysis

The tablets were carefully ground in a mortar and 1 g of the resulting powder was dissolved in 6 mL of phosphate buffer (0.05 M, pH=7.5) and homogenized using a vortex. The solution was diluted 1:1000 with phosphate buffer (0.05 M, pH=7.5).

Pre-treatment of integrators for spectrofluorimetric and biosensor analysis

The tablets were carefully ground in a mortar and 1 g of the resulting powder was dissolved in 6 mL of phosphate buffer pH=7.5 and homogenized using a vortex. The capsules containing liquid were opened and 1 g of liquid was added to 6 mL of phosphate buffer pH=7.5 and homogenized using a vortex. The solution was diluted 1:10 with phosphate buffer (0.05 M, pH=7.5).

Table 1: Integrators and their composition

Integrator and drug form	Composition	Content (mg)	Excipients
R1: capsules	Vitis vinifera leaves D.M. (titled in Polyphenols 5%) Resveratrol	200 150	Hydroxypropylmethylcellulose, maltodextrins
R2: capsules	Trans-Resveratrol 99% purity	600	Hydroxy-propyl-methyl-cellulose
R3: capsules	STILVID (Grapes D.M., making Resveratrol 8 mg, Anthocyanosides 0,67 mg, Procyanidins 14,63 mg, Flavonoids 0,40 mg) GRANATA D.M. (making Ellagitannin 8,75 mg, Procyanidins 3,75 mg, Selenium 50 µg)	133 125	Maltodextrin silicium dioxide, magnesium stearate, iron oxide
R4: capsules	Total dry extract of grape (containing grape-seed Polyphenols, Resveratrol and Anthocyanidins) grape seed oil virgin borage oil virgin evening primrose oil Proteid Carbohydrates Lipids	40 120 73 73 4 145 29	Amide, glycerin, glycerol monostearate carragenan, soya lecithin, disodium phosphate, sulphites
M1: capsules	Cranberry cryoground dust titled in Pyrogallol 0,7%	380	Hydroxy-propyl-methyl-cellulose
M2: capsules	Myrtocyan® (Cranberry titled in Anthocyanosides 36%)	160	Mannitol, lactose, methyl-cellulose, citric acid, silica, magnesium stearate, gelatin
M3: capsules	Cranberry Anthocyanidins titled in delphinidin chloride	6,5	Gelatin
M4: capsules	Cranberry D.M. titled in Anthocyanidins 25%	177	Soya oil, vegetable fats, gelatin, glycerol, red and black oxide of iron, sodium-ethil/propyl-paraben
E1: capsules	Triticum vulgare d,l-α-tocoferil acetate	288 10	Edible gelatine (soy lecithin, medium-chain triglycerides), glycerol
E2: tablets	d,l-α-tocoferil acetate	100	Talc, colloidal silica, calcium carbonate, titanium dioxide, cornstarch, milk powred, malt extract, glucose, kaolin, magnesium carbonate e oxide, methilcellulose, glycerol, polimeri di metacrilati, ferro ossido rosso, aroma, cera carnauba, 2,3-butadione, saccarosio
E3: tablets	d,l-α-tocoferolo acetate	100	Glucose, milk powder, sucrose, cocoa powder, cocoa butter, glycerol, lactose, carob seed flour, ethilvanillin, flavouring, rice starch, talc, arabic gum, β-carotene, paraffin

DMPD + $FeCl_3$ spectrophotometric method

According to the method described in literature (Fogliano *et al*., 1999), the cation radical $DMPD^{+}$ was obtained by adding 1/ml of a solution of DMPD 0.1/M and 0.2/ml of a solution of $FeCl_3$ 0.05 M to a vessel containing 100 ml of acetate buffer (0.1 M, pH=5.25). The absorbance was read at 514 nm. Then 150/µl of the properly diluted sample solution or of a solution of Trolox 1.0/mg ml^{-1} was added to the quartz cuvette and the absorbance at 514 nm was read after 10 min, by keeping the mixture under constant stirring. In the reference cuvette was put acetate buffer.

The antioxidant capacity of the sample is expressed in TEAC units (antioxidant capacity in equivalent Trolox), according to the method of Miller *et al*., (Miller *et al*., 1993), reported as the percentage inhibition of the signal I_{514} (%). A calibration curve was constructed using different amounts of Trolox. The absorbance inhibition at

514 nm was linear between 0.2 and 11.0 mg of Trolox with the antioxidant concentration (fig. 1). The calibration curve equation and the correlation coefficient r^2 obtained are the following: $y = (5.26 \pm 0.10)\ x + (3.78 \pm 0.24)$ $r^2 = 0.9923$

where $y = I_{514}$ (%) and x is measured in µg of Trolox.

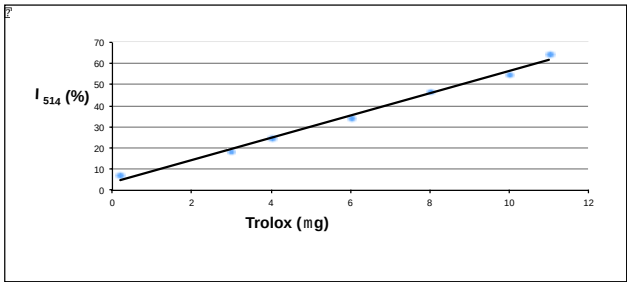

Fig. 1: Trolox calibration curve constructed by the DMPD-$FeCl_3$ spectrophotometric method.

Control samples, containing only the recipients present in each integrator, have been tested with the above reported method showing no antioxidant activity.

ORAC spectrofluorimetric method

The protein b-phycoerithryn (b-PE) loses over 90% of its fluorescence within 30 min in the presence of reactive oxygen species (Cao *et al*., 1995; Huang *et al*., 2005). The decrease of fluorescence of this protein is inhibited by the presence of an antioxidant species. The inhibition is related with the sample's antioxidant capacity.

Peroxide radicals are generated by using 2,2'-azobis-(2-amidinopropane) dihydro chloride (ABAP).

Wavelengths used were 540 nm and 565 nm for excitation and emission, respectively. 40 µl of sample are dissolved in 790 µl of phosphate buffer (75 mM, pH=7.0) and 730 µl of β-phycoerythrin (18.3 nM in phosphate buffer) and placed in a cuvette. The initial fluorescence (f_0) is read after 30s. Successively a further 20 µl of phosphate buffer with 20 µl of ABAP (0.32 M in phosphate buffer) are added to the solution in the cuvette and, after stirring, the fluorescence is read after 0.5 s and then for a total time of 70min every 2/min. The same procedure is carried out using 20/µM solution of Trolox instead of sample.

The antioxidant capacity values are expressed in "ORAC units" (micromoles of Trolox equivalent per litre of sample): ORAC value = $20k\ (S_{sample}-S_{blank}) / (S_{Trolox}-S_{blank})$

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

Control samples, containing only the recipients present in each integrator, have been tested with the above reported method showing no antioxidant activity.

SOD biosensor method

The SOD biosensor was based on a Clark amperometric electrode for hydrogen peroxide formed by a Platinum anode at a constant potential of +650 mV respect to a cathode of Ag/AgCl/Cl^- with the superoxide dismutase enzyme (SOD) immobilised on a membrane of gel-like kappa-carrageenan and put onto the electrode surface sandwiched between a cellulose acetate and a dialysis membrane, respectively. The whole assembly was fixed by a rubber O-ring (fig. 2).

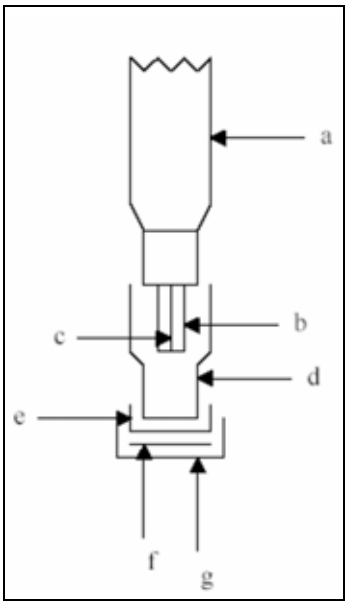

Fig. 2: Superoxide dismutase biosensor: (a) electrode body; (b) Ag/AgCl/Cl^- cathode; (c) Pt anode; (d) electrode plastic cap with buffer solution; (e) cellulose acetate membrane; (f) kappa-carrageenan membrane with SOD enzyme; (g) dialysis membrane.

The kappa-carrageenan membrane and the SOD immobilisation in the gel was carried out according to the method reported in a previous paper (Campanella *et al*., 1999).

The xanthine oxidase (XOD) enzyme catalyzes the production of superoxide radical from an aqueous solution of xanthine and uric: XOD

$$\text{xanthine} + H_2O + O_2 \longrightarrow \text{uric acid} + 2H^+ + O_2^{\cdot-} \quad (1)$$

The superoxide dismutase enzyme, immobilised on the H_2O_2 electrode, catalysed the disproportion reaction of the superoxide radical with the release of oxygen and hydrogen peroxide: SOD

$$O_2^{\cdot-} + O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (2)$$

The H_2O_2 formed by reaction (2) is detected by the amperometric sensor for hydrogen peroxide.

It generates an amperometric signal variation which is proportional to the concentration of superoxide radical in

solution. The presence of a compound with antioxidant properties causes a decrease in the amperometric current as the antioxidant species reduces the superoxide radical concentration in solution. As a consequence, a decrease of the value of the slope of the current vs. xanthine concentration calibration curve is observed.

The electrode is placed in a glass cell thermostated at 25° C containing 1.2 mg of enzyme xanthine oxidase dissolved in 10 ml of phosphate buffer (0.05 M, pH=7.5). Then further additions of 500 µl of 0.01 M xanthine solution are carried out, waiting for the signal to stabilise between each addition and the following one, and the signal current was recorded after each addition. The current values obtained are utilized to construct the calibration curve graphs of the current as a function of increasing xanthine concentration. Same type of measurement is successively performed adding in the cell 1/ml of the sample to be tested and then proceeding as described above. If the sample shows antioxidant properties the new calibration curve will show a lower slope value. By comparing the slope values in the absence and in the presence of the antioxidant compound, it is possible to determine the antioxidant capacity of the sample. The value of the relative antioxidant capacity is expressed by the following equation: relative antioxidant capacity *(*RAC*)* = 1-(m_b/m_a)

where m_a is the slope of the straight line obtained by successive xanthine additions and m_b the slope of the straight line obtained by successive xanthine additions in the presence of the sample with antioxidant properties.

RESULTS

The antioxidant capacity of the integrators was firstly determined with the DMPD-$FeCl_3$ spectrophotometric method and the ORAC spectrofluorimetric method, which were used several times in literature as reference methods to evaluate the antioxidant capacity. The results are shown in fig. 3 and fig. 4, respectively, where the trend of the antioxidant capacity is represented in the form of histograms.

With both the methods, the greater antioxidant capacity is observed for vitamin E and astaxanthin based integrators, although all integrators tested showed good values of antioxidant capacity.

These results agree with those reported in the Antioxidant Food Database, the most comprehensive database used worldwide reporting the antioxidant content of thousands of foods, beverages, herbs and supplements (Carlsen *et al*., 2010), confirming that herbal and traditional plant medicines and dietary supplements represent the highest antioxidant-containing products (Kahkonen *et al*., 1990, Velioglu *et al*., 1998).

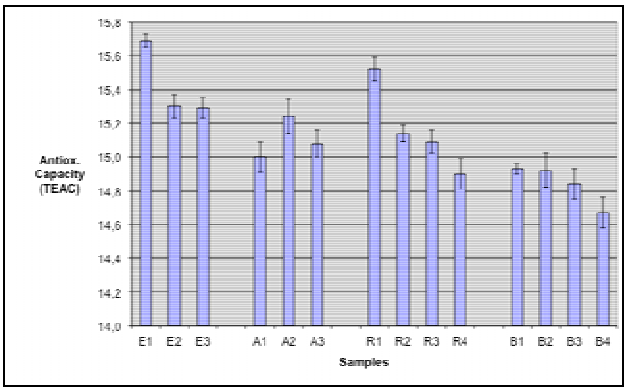

Fig. 3: Antioxidant capacity values (TEAC units) for all integrator samples tested by the DMPD-$FeCl_3$ spectrophotometric method (R.S.D.% values between 3 and 9%).

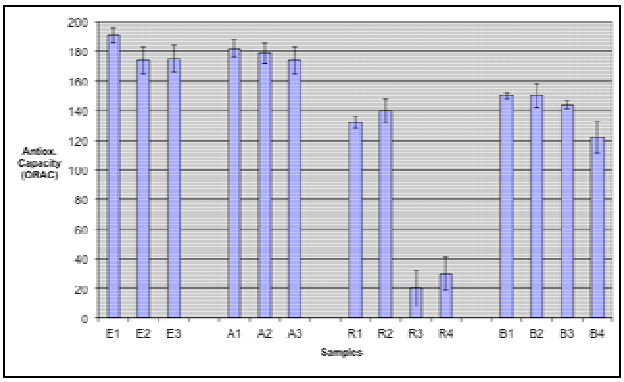

Fig. 4: Antioxidant capacity values (ORAC units) for all integrator samples tested by the ORAC spectrofluorimetric method (R.S.D.% values between 1 and 10%).

In particular, two vitamin E based integrators (E2 and E3 samples) showed almost the same values of antioxidant capacity, lower than E1. This fact was predictable considering the composition of the two samples (table 1). They contain exactly the same amount (100/mg) of the syntetic α-tocopherol mixed with different excipients. As these compounds do not have any antioxidant activity, it is reasonable to believe that the two samples should show the same antioxidant capacity. The first vitamin E based integrator analysed (E1) showed the highest antioxidant capacity value although it contains a ten times lower amount of syntetic α-tocopherol (100/mg) probably because it contains also a compound, wheat germ oil, which is rich in natural vitamin E (with a double biological activity compared to the syntetic one), provitamin A, D and linoleic acid, all substances with antioxidant activity and so exerting a strong synergic effect.

Also for astaxanthin based integrators, the results obtained reflect the prediction. Integrators A1 and A2 showed a greater antioxidant capacity compared to A3

(with the exception of A1 sample in the spectrophotometric method) although they contain the same amount of astaxanthin (4/mg). This can be explained by the fact that in the integrators A1 and A2 astaxanthin is mixed with other substances with known antioxidant activity, as vitamin E, B and C, which are not present in the A3 formulation.

DISCUSSION

It is clear that the antioxidant capacity of a single integrator depends not only on the main active principle but also on other compounds or excipients contained in the integrators themselves which should modify the antioxidant capacity of these formulations with respect to the antioxidant capacity of the principal antioxidant component.

It is therefore possible to compare only the antioxidant capacity values of E2 and E3 samples (vitamin E not mixed with other compounds) with that of A3 sample (astaxantin not mixed): the antioxidant capacity values resulted quite similar with both methods but considering the larger amount of vitamin E (100/mg) compared with that one of astaxanthin (4/mg) contained in the different integrators it is possible to conclude that astaxanthin has a much higher antioxidant activity, as already reported in literature (Naguib, 2000).

As for resveratrol and blueberry based integrators, it is possible to note that R3, R4 and B4 samples showed the lowest values of antioxidant capacity, respectively, probably because they contain a lower amount of active principle. The results obtained for these integrators were not easily predictable as for vitamin E and astaxanthin based integrators, because they are phytocomplexes and their antioxidant capacity is strongly influenced by the extraction method, as well as by the presence of other active principles, eventually co-extracted, neither the former nor the latter known to us.

B1 sample, which is a chrushed powder of blueberry, showed the highest antioxidant activity with both method used, although its concentration is definitely lower than that one contained in the other three integrators. The explanation of this unexpected result is that the cold crushing is a technique which allows to preserve the integrity and completeness of the plant constituents and therefore to ensure higher activity to the final product.

Successively, the antioxidant capacity of the integrators was determined by using an electrochemical method based on a SOD biosensor, recently developed in our laboratory. The results obtained are shown in fig. 5. The method was then validated by comparing the results with those obtained with the spectrophotometric and spectrofluorimetric methods.

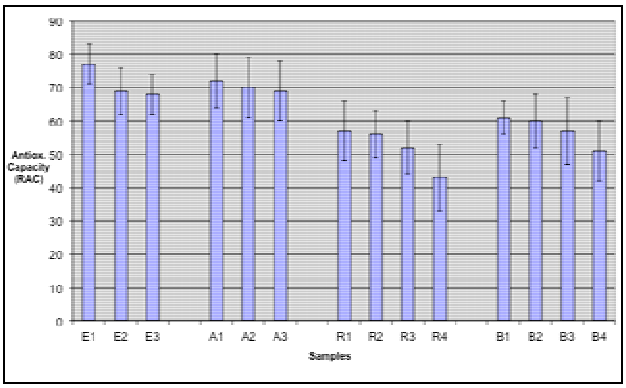

Fig. 5: Antioxidant capacity values (RAC units) for all integrator samples tested by a SOD biosensor (R.S.D. values between 6 and 10%).

It is easy to observe the reasonably good agreement found between the trends in antioxidant capacity values obtained by biosensor method and two previous methods.

In particular, by comparing the trends obtained with the three methods together, it can be noted that the only differences in antioxidant capacity were found for astaxanthin (sample A1) with the spectrophotometric method and for resveratrol (sample R3) with the ORAC method. This can be ascribed to the fact that each method has a different sensitivity towards other active principles than the main one eventually present in the formulations with own antioxidant properties.

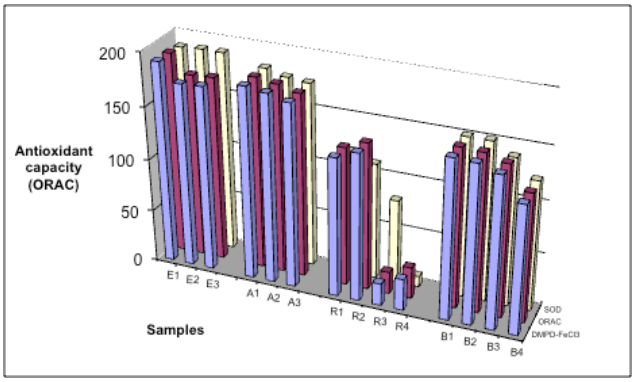

Fig. 6: Antioxidant capacity values (ORAC units) for all integrator samples tested by the three methods.

Lastly, in the present work we did not only perform a qualitative comparison among the antioxidant capacity trends of the integrators but we were able also to homogeneously quantitatively compare the values of antioxidant capacity obtained with the three methods. Using the equation of the correlation curves reported in table 2, it was possible to express the antioxidant capacity values obtained by different methods in ORAC units. The results are reported in form of histograms in fig. 6. By using the same units it was definitely easier to observe the reasonably good agreement between the obtained values.

Table 2: Correlations between methods for the measurement of antioxidant capacity

a) Integrator	Curve equation	Correlation coefficient
Vitamin E	y=(183±9)x+(49±6)	R^2=0.9973
Astaxanthin	y=(250±86)x+(2.5±0.6)	R^2=0.8929
Blueberry	y=(281±37)x-(20±2)	R^2=0.9664
b) Integrator	Curve equation	Correlation coefficient
Vitamin E	y=(4.4±0.1)x+(12.31±0.07)	R^2=0.9995
Resveratrol	y=(3.3±1.7)x-(13.4±0.9)	R^2=0.6673
Blueberry	y=(2.53±0.14)x+(13.38±0.08)	R^2=0.9939
c) Integrator	Curve equation	Correlation coefficient
Vitamin E	y=(41±3)x-(463±47)	R^2=0.9945
Blueberry	y=(111±8)x-(1516±131)	R^2=0.9877

a) ORAC and SOD methods; b) DMPD-$FeCl_3$ spectrophotometric and SOD methods; c) ORAC and DMPD-$FeCl_3$ spectrophotometric methods

CONCLUSIONS

The results obtained in the present work relative to the the antioxidant capacity of 14 commercial pharmaceutical integrators indicate that the highest antioxidant activity is observed for the astaxanthin based integrators, followed by vitamin E, blueberry and resveratrol based ones, confirming the fact that astaxanthin and vitamin E, as reported in literature, are the most powerful antioxidant principles.

A particularly good correlation among two conventional methods, of spectrophotometric and spectrofluorimetric type, respectively, and a new electrochemical method based on a SOD biosensor was observed. By expressing the results obtained with the same units of measure, we made not only qualitative but also quantitative comparisons of all the values obtained. In particular the SOD biosensor method showed a very good correlation for all integrators tested with the exception of astaxanthin and resveratrol based integrators, with the spectrophotometric and ORAC methods, respectively. This can be ascribed to the fact that ORAC and spectrophotometric method show different sensitivity towards other possible antioxidant interfering species present in the formulations, as confirmed by the results obtained for samples A1 and R3 which shows the following antioxidant activity values (ORAC units): 132.93, 182.08 and 190.62 (sample A1) and 139.06, 20.66 and 95.01 (sample R3), determined by the spectrophotometric, ORAC and SOD biosensor method, respectively.

It is important to point out that the antioxidant activity of an integrator is due not only to the main antioxidant principle but also to other constituents and/or excipients contained in the integrators themselves which could raise or even depress the antioxidant capacity of these formulations. For these reasons, the direct comparisons in the antioxidant capacity of the 4 main antioxidant constituents of the integrators studied were possible only for the integrators with no other interfering compound in the formulations.

Lastly, the present research has well highlighted on how the SOD biosensor method resulted to be sufficiently reproducible (R.S.D. ≤10% in all RAC measures) showing a comparable sensitivity to the other methods, also allowing a simple and rapid determination of the antioxidant activity. The biosensors have also the advantages of being easily portable and of operating "in situ". The conventional methods show instead the drawback of being extremely expensive, time consuming and not transportable. For these reasons the SOD biosensor method can represent a valid alternative for evaluation of pharmaceutical integrator antioxidant capacity now supported by the described results.

REFERENCES

Ames BN (1979). Identifying environmental chemicals causing utations and cancer. *Science*, **204**: 587-593.

Azzi A (2007). Molecular mechanism of alpha-tocopherol action. *Free Radic. Biol. Med.*, **43**: 16-21.

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

Campanella L, Favero G and Tomassetti M (1999). Superoxide dismutase biosensor for superoxide radical analysis. *Anal. Lett*., **32**: 2559-2581.

Campanella L, Favero G, Persi L and Tomassetti M (2001). Evaluation of radical scavenging properties of several plants, fresh or from a herbalist's, using a superoxide dismutase biosensor. *J. Pharm. Biomed. Anal.,* **24**: 1055-1064.

Campanella L, Bonanni A and Tomassetti M (2003). Determination of the antioxidant capacity of samples of different types of tea, or of beverages based on tea or other herbal products using a superoxide dismutase biosensor. *J. Pharm. Biomed. Anal.,* **32**: 725-736.

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

Campanella L, Bonanni A, Bellantoni D and Tomassetti M (2004). Biosensors for determination of total antioxidant capacity of phytoterapeutic integrators: Comparison with other spectrophotometric, fluorimetric and voltammetric methods. *J. Pharm. Biom. Analysis*, **35**: 303-320.

Campanella L, Bonanni A, Bellantoni D, Favero G and Tomassetti M. (2004). Comparison of fluorimetric, voltammetric and biosensor methods for the determination of total antioxidant capacity of drug products containing acetylsalicylic acid. *J. Pharm. Biomed. Anal*., **36**: 91-99.

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

Cao G, Verdon G, Wu CP, Wang AHB and Prior RL (1995). Automated assay of oxygen radical absorbance capacity with the COBAS FARA II. *Clin. Chem*., **41**: 1738-1744.

Carlsen MH *et al*., (2010). The total antioxidant content of more than 3100 foods, beverages, spices, herbs and supplements used worldwide. *Nutr. J.,* **9**: 1-11.

Diplock AT (1994). Antioxidant and disease prevention. *Mol. Aspects Med.,* **15**: 293-376.

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

Huang D, Ou B and Prior RL (2005). The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.,* **53**: 1841-1856.

Iwamoto T *et al*., (2000). Inhibition of low-density lipoprotein oxidation by astaxanthin. *J. Atheroscler. Thromb.,* **7**: 216-222.

Kahkonen MP, Hopia AI, Vuorela HJ, Rauha JP, Pihlaya K, Kujala TS and Heinonen M (1990). Antioxidant activity of plants extracts containing phenolic compounds. *J. Agric. Food Chem.,* **47**: 3954-3962.

Kamal-Eldin A and Appelqvist LA (1996). The chemistry and antioxidant properties of tocopherols and tocotreienols. *Lipids,* **31**: 671-701.

Kamiyama M, Kishimoto Y, Tani M, Andoh K, Utsunomiya K and Kondo K (2009). Inhibition of low-density lipoprotein oxidation by Nagano purple grape (Vitis viniferaxVitis labrasca). *J. Nutr. Sci. Vitaminol*., **55**: 471-478.

Kay CD *et al*., (2002). The effect of wild blueberry (Vaccinium angustifolium) consumption on postprandial serum antioxidant status in human subjects. *Br. J. Nutr*., **88**: 389-398.

Klein EA *et al*., (2011). Vitamin E and the risk of prostate cancer: The selenium and vitamin E cancer prevention trial (SELECT). *J. American Medical Assoc*., **14**: 1549-1556.

Leger ASS, Cochrane AL and Moore F (1979). Factors associated with cardiac mortality in developed countries with particular reference to the consumption of wine. *Lancet.,* **1**: 1017-1020.

Liu G, Zhang Z, Yang B and He W (2011). A pilot study for evaluation of antioxidant activity of resveratrol from *Polygonum cuspidatum* in senescence-accelerated mice. *J. Medicinal Plants Research*, **6**: 325-330.

Miki W (1991). Biological functions and activities of animal carotenoids. *Pure Applied Chem.,* **63**: 141-146.

Miller NJ, Rice-Evans CA, Davies MJ, Gopinathan V and Milner A (1993). A novel method for measuring antioxidant status in premature neonates. *Clin. Sci*., **84**: 407-412.

Moskovitz J, Yim MB and Chock PB (2002). Free radicals and disease. *Arch. Biochem. Biophys*., **397**: 354-359.

Naguib YMA (2000). Antioxidant activities of astaxanthin and related carotenoids. *J. Agric. Food Chem.,* **48**: 1150-1154.

Nishino H (1998). Cancer prevention by carotenoids. *Mutat. Res.,* **402**: 159-163.

Prior R L and Cao G (2000). Flavonoids: Diet and health relationship, *Nutr. Clin. Care* **3**: 279-288.

Rezk BM, Haenen GRMM, van der Vijgh WJF and Bast A (2004). The extraordinary antioxidant activity of vitamin E phosphate. *Biochim. Biophys. Acta.,* **1683**: 16-21.

Sellappan S, Akoh CC and Krewer G (2002). Phenolic compounds and antioxidant capacity of Georgia-grown blueberries and blackberries. *J. Agric. Food Chem*., **50**: 2432-2438.

Velioglu YS, Mazza G, Gao L and Oomah BD (1998). Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J. Agric. Food Chem*., **46**: 4113-4117.

Zheng W and Wang SY (2003). Oxygen radical absorbing capacity of phenolics in blueberries, cranberries, chokeberries and lingoberries. *J. Agric. Food Chem*., **51**: 502-509.

Zulueta A, Esteve MJ and Frigola A (2009). ORAC and TEAC assays comparison to measure the antioxidant capacity of food products. *Food Chemistry*, **114**: 310-316.