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Antioxidant capacity of the algae using a biosensor method

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Abstract

Three different methods, i.e. a biosensor method, a voltammetric method and a spectrophotometric method, have been used to evaluate the total antioxidant capacity of certain types of algae. In the final evaluation of the data also the variation in time of the antioxidant capacity of cultivated algae was considered and some experimental factors, such as the use of different solvent mixtures to extract the antioxidant substances contained in the algae, were discussed.

Keywords: Algae; Superoxide dismutase; Biosensor; Antioxidant capacity

1. Introduction

The control of free radicals is today one of the most intensively investigated medical research topics [1]. Indeed, any imbalance between the production of radicals and the defence system based on the action of antioxidant compounds (radical scavengers) can generate a state of "oxidative stress" that, in some cases, can lead to a series of biochemical alterations even culminating in cellular death [2]. In order to treat and prevent the numerous diseases caused by oxidative stress, efforts have been concentrated on identifying and studying drugs with antioxidant properties [3], or on the boosting of antioxidant enzymes [4,5] already present in the body and lastly on the use of the antioxidants present in plant foods [6]. The latter solution is physiologically more feasible as many antioxidants are present in food and in many cases represent the most natural way of orally administering these substances. However, it is important to evaluate the total "antioxidant capacity" of natural resources, such as fruit and vegetables [7]. This type of research has been carried on by many researchers in recent years [8–11] and also our group has recently done considerable work in this direction [12,13]. Algae constitute a valid alternative to other proteic foods of animal or vegetal origin [14,15]; they actually contain high concentrations of the basic nourishment and are a source of high biological value proteins, minerals, vitamins and polyunsaturated fatty acids, particularly γ-linoleic acid [16,17]. Their benefits also stem from their rapid growth, especially when they are used in large-scale and low-cost systems designed to produce material for use as food or for industrial purposes.

Furthermore, the research performed on the algae confirms their completeness and their regenerative and therapeutic effects for the human organism. Recently more attention has also been paid to their medical properties and their antioxidant and anti-ageing capacities [18]. All their therapeutic and clinical properties linked to their chemical composition have been described in the literature, but so far no formal determination has been made of their total antioxidant capacity or any value defined for it. The present work wants to clear this aspect assessing the total in vitro antioxidant capacity of three types of algae *Spirulina subsalsa* and *Selenastrum capricornutum* (both cultivated) and (powdered) *Spirulina maxima*, using different analytical methods. One devised by us, based on the superoxide dismutase (SOD) biosensor, which has already proved its worth in previous researches [19–21] and two other methods reported in the literature, i.e. a spectrophotometric method [22] based on a cationic radical obtained by oxidizing the *N*,*N*-dimethyl-

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p-phenylenediamine dihydrochloride (DMPD) by means of Fe^{3+} and another one based on cyclic voltammetry involving measurement of the area of the anodic curve which is correlated with the total antioxidant capacity of the sample [23,24].

2. Experimental

2.1. Materials

Xanthine (2,6-dihydroxy purine), sodium salt, ethylenediamine tetracetic acid (EDTA), superoxide dismutase from bovine erythrocytes 4980 U mg^{-1}, dialysis membrane (art. D-9777), *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride, supplied by Sigma (Milan). Acetone RPE, anhydrous dibasic potassium phosphate RPE, anhydrous sodium acetate RPE, analytical grade methanol RPE, supplied by Carlo Erba (Milan). Xanthine oxidase from bovine milk 0.40 U mg^{-1}, cellulose acetate, kappa-carrageenan, supplied by Fluka AG, Buchs (Switzerland). 2-Carboxy-6-hydroxy-2,5,7,8-tetramethylchroman acid (Trolox), supplied by Aldrich (Germany). Potassium chloride supplied by Riedel-de Haen (Seelze, Germany). Ferric chloride, supplied by Merck (Germany).

2.2. Equipment

Perkin-Elmer model Lambda 5 UV–vis Spectrophotometer, equipped with printer; Model 4000-1 electrode supplied by Universal Sensor Inc., New Orleans, LA, USA, coupled to an Amel mod. 551 potentiostat (Milan), connected to an Amel mod. 631 differential electrometer and an Amel mod. 868 analog recorder; thermostable cell connected to a Julabo 58 thermostat supplied by Labospital (Rome); Ultra-Turrax mod. T8 homogenizer, supplied by Ika Labortechnik (Germany); Model F20ST magnetic stirrer, from Falc Instrument (Lurano, Bergamo); Crison gpl 22 pHmeter; Amel mod. 433 polarograph, manufactured by Amel (Milan), equipped with printer and interfaced with a PC (with software: Analyser 7.0); platinum electrode manufactured by Beckman Instruments Inc., Fullerton, CA; 363-S7/120 calomel electrode manufactured by Ingold; Cat.182-02 (pH 0–12) glass electrode manufactured by Crison; Amel glassy carbon electrode, Milan, Italy; CWS 4235 centrifuge; Mettler AE 420 analytical balance; Mettler PM 460 technical balance.

2.3. Samples analysed

In the present work, several samples of live algae placed in special culture flasks and one sample of powdered algae were analysed; the total antioxidant capacity of each sample was evaluated, specifically:

- *Spirulina subsalsa* alga, supplied by Culture Collection of Algae and Protozoa (CCAP, SAMS Research Service Ltd.; Dunstaffnage Marine Laboratory, Dunbeg Oban, Scotland).
- *Selenastrum capricornutum* alga, supplied by the University of Naples, used as a water pollution indicator.
- *Spirulina maxima* alga, micronized in capsules, purchased from a drugstore where it is sold as a diet integrator.

The cultivated algae tested (*S. capricornutum* and *S. subsalsa*) are marine algae. Selenastrum is a green eukaryote alga, with a very simple degree of functional organization. The Spirulina algae analysed, both cultivated and powdered, consisted of both blue-green prokaryote algae that form colonies composed of pluricellular organisms. The powdered alga *S. maxima* (micronized in capsules) is a lacustrine alga obtained from intensive farms by a drying process that does not destroy either the cells or the cell membrane and does not alter the proteins and enzymes. The cultivated algae, dispatched to our laboratory from their place of origin, were incubated in a suitable growth medium supplied by CCAP (UK) denoted as ASW: blue-green (BG) medium. This medium consists of two separate solutions that are mixed after sterilization in an autoclave. The first contains a medium extract, sea water and the following salts ($NaNO_3$, Na_2HPO_4, K_2HPO_4); the second contains the salts $NaNO_3$, $K_2HPO_4{\cdot}3H_2O$, $MgSO_4{\cdot}7H_2O$, $CaCl_2{\cdot}2H_2O$, as well as citric acid, EDTA, Na_2CO_3, iron ammonium citrate in distilled water. The pH of the two solutions must be adjusted to 7.4. After the samples were allowed to adapt to this medium for 1 day, analysis of the live algae began and their antioxidant capacity measured. The powdered algae, before being used, were instead hydrated with a 0.05 mol/L solution of phosphate buffer pH 7.5, for 10 min.

3. Method

3.1. Sample treatment

Amperometric analyses using the enzymatic biosensor were carried out on whole, homogenized and centrifuged algae. In practice, in the latter two cases, 0.5 g of algae were placed in 3.0 mL of phosphate buffer 0.05 mol L^{-1}, pH 7.5 and homogenized at 10,000 rpm for 5 min; 0.5 mL of homogenate were placed in the analysis cell containing 20.0 mL of phosphate buffer 0.05 mol L^{-1} pH 7.5. Alternatively, the homogenate was centrifuged (at 3500 rpm for 5 min) and 0.5 mL of the supernatant was analysed in the same way in the same measuring cell. In the case of analysis of whole algae, 0.01 g of whole algae (instead of the 0.5 g used in the case of powdered algae) were weighed out, placed in 0.5 mL of phosphate buffer, and added directly to the measuring cell.

For voltammetric analysis, 0.5 g of whole algae were added to 20 mL of phosphate buffer. After homogenization, 0.5 g of algae were placed in 3.0 mL of phosphate buffer pH 7.5 and added to 17 mL of phosphate buffer in the measuring cell. Furthermore, after centrifuging, 0.5 mL of supernatant were taken and placed in the measuring cell containing 20.0 mL of phosphate buffer.

In both cases, before applying these methods, three drops of glacial acetic acid must be added to 0.5 mL of aqueous solution containing the alga *S. capricornutum* in order to break down the cell wall. The latter is so thick as to otherwise prevent detectable antioxidant values being obtained.

For the spectrophotometric analyses 50 μL of the supernatant of a solution obtained by mixing 0.5 g of algae in 3.0 mL of acetate buffer, 0.1 mol L^{-1}, pH 5.25, was added directly to the measuring cell, the sample being homogenized and centrifuged at 3500 rpm for 3 min.

3.2. Superoxide dismutase electrochemical bionsensor

The antioxidant activity of the algae was measured above all by using the superoxide dismutase (SOD) electrochemical biosensor, which is able to determine the superoxide radical. The biosensor used was obtained by coupling a transducer (an amperometric hydrogen peroxide electrode) with the superoxide dismutase enzyme immobilized in kappa-carrageenan gel. For the measurement of antioxidant capacity, the superoxide radical ($O_2^{\bullet -}$) is produced in aqueous solution by xanthine/xanthine oxidase enzyme system [25]. The disproportion reaction of the $O_2^{\bullet -}$ radical in the presence of the superoxide dismutase enzyme immobilized on the electrode produces oxygen and hydrogen peroxide. The hydrogen peroxide is oxidized at the anode, generating an amperometric signal that, when operating under diffusion conditions, is proportional to the concentration of the superoxide radical produced in solution. The addition of a sample having antioxidant capacity causes a decrease in the signal since the antioxidant species present in the sample react with the superoxide radical and reduce its concentration in solution; there is a consequent decrease in both the H_2O_2 produced and the intensity of the amperometric signal. This decrease is proportional to the antioxidant capacity of the sample added [19,20,26].

In practice, the electrochemical biosensor is placed in a glass cell thermostatted at 25 °C containing 20.0 mL of phosphate buffer 0.05 mol L^{-1} at pH 7.5 and allowed to stabilize under constant stirring. After the addition of a fixed quantity of xanthine oxidase enzyme (1.2 mg), a series of further additions of 200 μL of the xanthine solution 0.01 mol L^{-1} is carried out. The current variations are thus recorded as a function of the increasing concentrations of added xanthine, thus obtaining a calibration straight line, the slope of which is measured. The same type of measurement is then repeated, but this time also adding the solution containing the sample to be analysed for its antioxidant capacity to the cell containing the phosphate buffer, and proceeding as described above. A second calibration straight line is thus obtained with a lower slope value than in the previous case whenever the sample has antioxidant properties. From a comparison of the values of the two slopes, it is possible to obtain an index of total antioxidant capacity for our sample, which is computed using the following algorithm:

$$\mathrm{RAC} = 1 - \frac{m_c}{m_x}$$

where RAC is the relative antioxidant capacity, m_x the slope of the straight line obtained from successive additions of xanthine and m_c is the slope of the straight line obtained from successive additions of xanthine but in the presence of sample having antioxidant properties.

3.3. Cyclic voltammetry

Clearly the reducing power of a substance represents a valid expression of its antioxidant capacity. Since the anodic area of a voltammogram may be correlated with the measure of the total reducing power of the test sample, this area may thus be correlated directly with the above mentioned total antioxidant capacity (Fig. 1). Measures are performed by connecting the polarograph to an external cell to which 10 mL of phosphate buffer 0.05 mol L^{-1}, pH 7.5 are placed together with 10 mL of sample. The measuring cell entails the use of three electrodes: a glassy carbon electrode as working electrode, a platinum electrode, as counter-electrode and a calomel electrode as reference; this cell is connected to a bubbler through which a stream of nitrogen is passed. Reduction of working electrode sensitivity is avoiding by cleaning it after each cycle by smoothing the working surface on alumina previously moistened with a minimal quantity of distilled water.

The electrode is further washed and a fresh analysis performed. The cyclic voltammogram is recorded over the range 1300 to −300 mV at a scan rate of 400 mV s^{-1}, with a lower cut off of 40 μA. The results may be expressed as Vitamin C equivalents (mg) using a simple proportion between the anodic area of the standard and that of the sample [23,24]. Ascorbic acid was selected as reference in computing the antioxidant capacity owing to the reproducibility of the anodic

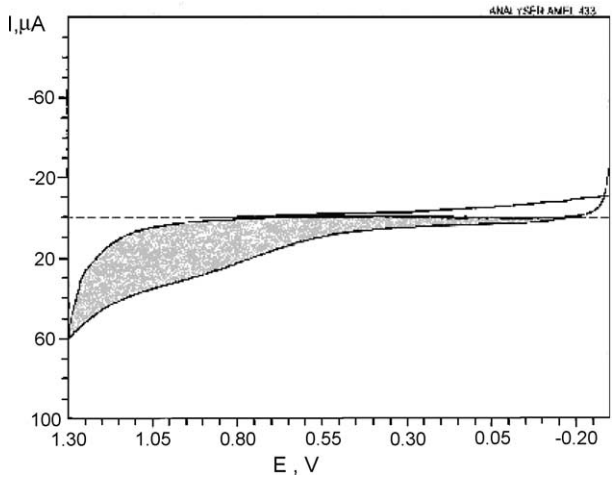

Fig. 1. Cyclic voltammogram in phosphate buffer, containing 5% of methanol, of *Spirulina subsalsa*, homogenized in phosphate buffer.

curve of its voltammogram and also because it is an antioxidant commonly found in nature and in several foodstuffs.

3.4. DMPD + $FeCl_3$ spectrophotometric method

The cationic radical obtained from the *N*,*N*-dimethyl-*p*-phenylenediamine dihydrochloride, in the presence of a suitable oxidizing solution displays an absorption peak at 514 nm. The decrease in absorbance at this wavelength, recorded in the presence of the sample, is related to the latter's antioxidant capacity. The total antioxidant capacity is expressed by comparing the decrease in absorbance due to the sample with that due to the 2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman acid (Trolox—water soluble analog of Vitamin E), selected as reference standard. The application procedure is the same as that already described in previous article [26].

The samples' antioxidant capacity is expressed in Trolox equivalent antioxidant capacity (TEAC) units, in accordance with the method of Miller et al. [27], using a calibration straight line obtained with different quantities of Trolox, and considering that the inhibition of absorbance at 514 nm due to the Trolox is linear between 0.2 and 11.0 μg of Trolox.

3.5. RT-PCR and semiquantitative analysis

The alga was cultivated in a sterilized flask placed in a Dubnoff bath at a rigorously constant temperature of 17 °C, with a 12-h light/12-h dark cycle. It is important to maintain a constant temperature as any change in it varies the phycobilisome content and consequently the photosynthetic response [28,29]. In the flask containing 160 mL of culture medium as previously described in Section 3, the alga *S. subsalsa* was cultivated in 8 mL of xanthine sodium salt solution (2.78×10^{-3} mol L^{-1}). A catalytic quantity of xanthine oxidase was added to the same solution in order to generate superoxide radicals in situ. Samples of the culture were taken at set intervals so as to monitor the alga's genic response. The alga samples were taken, placed in a 1.5 mL Eppendorf and centrifuged at 2000 rpm for 15 min. After centrifuging, the supernatant was removed and the sample frozen. The total RNA of the various samples was obtained using a ready-to-use commercial solution based on guanidium salts, Trizol (commercial solution supplied by Bio-Rad, Hercules, CA). In extracting total RNA from the individual samples and transforming it into coded DNA (cDNA) the procedure described in the literature [30] was followed. The cDNAs were amplified using polymerase chain reaction (PCR, supplied by Bio-Rad) equipment with specific primers for Fe–SOD and for luciferase, so as to determine the possible variations in expression of this enzyme in the various samples. Luciferase was used as internal control as its expression is not affected by any in vitro cellular modifications. The PCR products were transferred to a nylon membrane (Amersham, Arlington Heights, IL), after electrophoresis on agarose gel and standard hybridization of the radioactive probe of the genes investigated and of the control, obtained using cDNA probes, as described by Teofili et al. [31]. Densitometric analysis for the various samples was made possible by the constant presence of an internal control, which allowed a semiquantitative determination of the expression of the gene examined to be made. A comparison was made of the intensity of patches on the plates (using software supplied by Biorad, Hercules, CA) and the ratio between them established so as to determine the variation in gene expression in the various samples [32,33].

4. Results and discussion

4.1. Comparison of results obtained

All the algae used in the present investigation consisted of microalgae, the chemical composition and the nutrient value of which, according to literature reports [16], displayed wide variations depending on the strain to which they belonged. Fig. 2 shows the results of the antioxidant capacity of the three types of alga considered obtained using the various different methods. A comparison has been made of initial antioxidant values determined for the live algae on the first day of cultivation while, in the case of the powdered alga, of the values obtained as soon as the alga had been rehydrated.

The tests were carried out above all using the electrochemical biosensor (Fig. 2a), which previously gave excellent results in the analysis of different vegetal samples [12,13,34] and that, in this case, proved suitable also for the determination of the antioxidant capacity of the different cultivated and powdered alga samples. Furthermore, the tests were carried out both on whole alga samples and on homogenized alga samples, as well as on centrifuged samples, after homogenization. It is interesting to observe how the results obtained for the powdered alga *S. maxima* are comparable to the antioxidant capacity values found on day one in cultivated *S. subsalsa;* on the other hand, the two algae belong to the same algal strain.

The antioxidant activity recorded for the whole alga (in which the cells are intact, as they have not been exposed to any mechanical homogenizing action) was found to be slightly higher than that of the powdered alga. It may be postulated that this depends on the defensive action against free radicals possibly deployed by the living alga *S. subsalsa*. Furthermore, the antioxidant capacity value of the homogenized alga is higher than that of the centrifuged product (approximately double). This result can easily be accounted for by observing the findings made on previous occasions [13,21]. Indeed, in drawing off the supernatant the comparatively insoluble antioxidant species present in the homogenized suspension are probably excluded and remain in the precipitate after centrifuging. Lastly, the composition of the cell membrane influenced the antioxidant capacity measures in *S. capricornutum*. The alga actually required preventive lysis treatment of the cell membrane, composed of cellulose and lignin, which give it greater thickness and rigidity. This

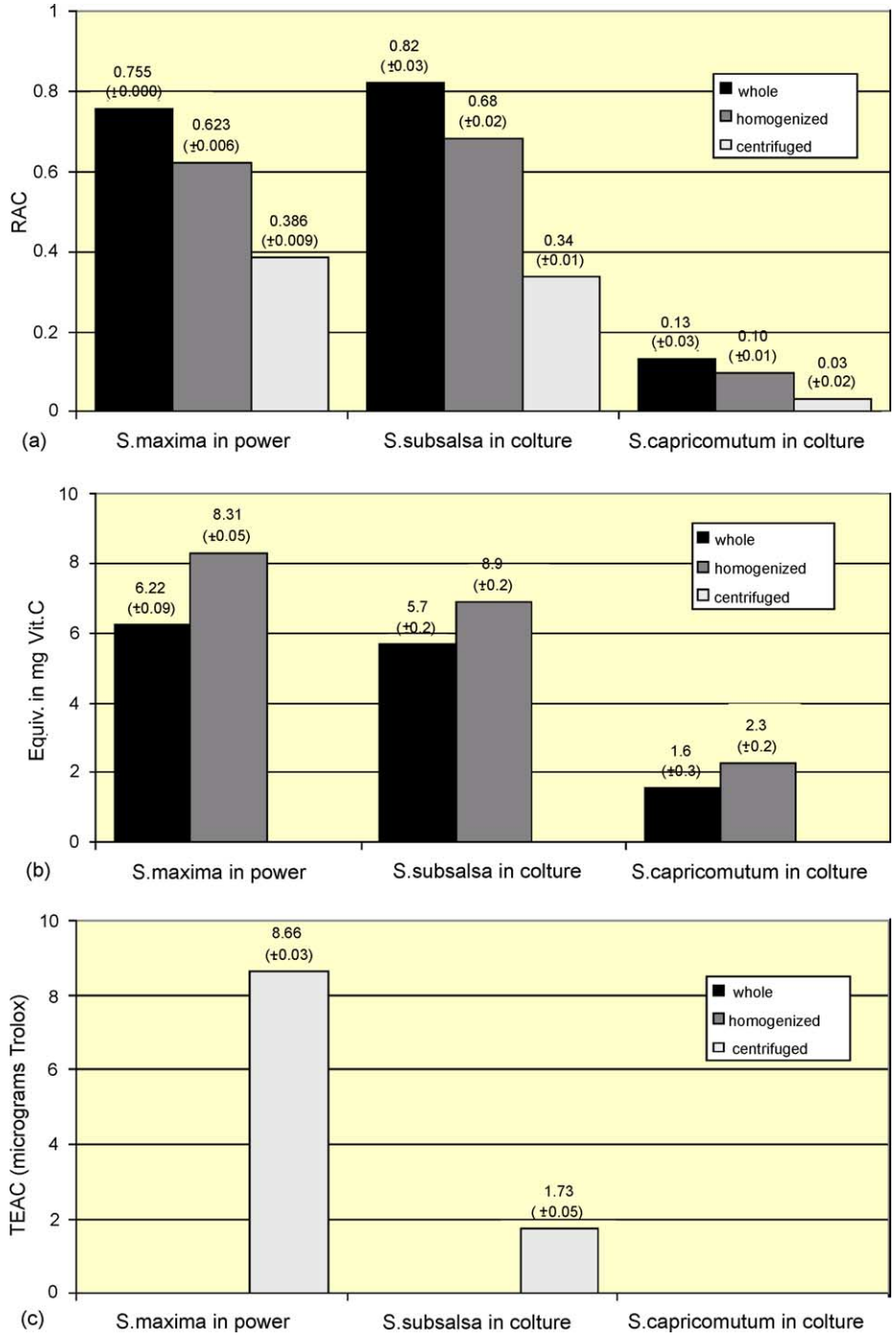

Fig. 2. Comparison of results of measuring the total "antioxidant capacity" obtained (a) using the superoxide dismutase biosensor; (b) the cyclic voltammetry method; (c) the DMPD + $FeCl_3$ spectrophotometric method in the three different alga samples tested.

treatment is unnecessary in the case of Spirulinae, in which the cell wall does not contain cellulose, but is made up of muranic acid and diaminopimelic acid. Despite lysis treatment, the antioxidant capacity values of Selenastrum were found to be largely lower than those of Spirulina. This result tends to indicate that the differences found in antioxidant capacity depend also on differences in the composition related to essential fatty acids (especially polyunsaturated) and in the presence of various antioxidant pigments (such as C-phycocyanin contained in *S. subsalsa*) that are not present in green algae (*S. capricornutum*). C-phycocyanin, according to literature reports [35], has a greater radical scavenging capacity than other antioxidant pigments.

Also using the cyclic voltammetric method (Fig. 2b), the values of antioxidant capacity for powdered *S. maxima* and for cultivated *S. subsalsa* were found to be of the same order

of magnitude. However, the whole alga samples, placed directly in phosphate buffer in the measuring cell, gave slightly lower antioxidant capacity values than the corresponding homogenized samples; the centrifugate values were found to be very low, and practically undetectable using this method. Unlike the biosensor based method, using cyclic voltammetry the highest antioxidant capacity values were found for the homogenates; this could be accounted for by the fact that the whole alga can easily cover the surface of the glassy carbon working electrode, thus preventing, or at least slowing down, the oxidation process of the antioxidant species. On the other hand, in view of what has been said above, the concentration of the antioxidant species in the supernatant obtained after centrifuging is so low that it is in practice impossible to detect it using this method. Furthermore, also using the voltammetric method the antioxidant capacity values of *S. capricornutum* were found to be lower, in agreement with the results found using the biosensor method.

Lastly, using the DMPD spectrophotometric method (Fig. 2c), it was possible to analyse only the centrifugates obtained by homogenization of the algae *S. subsalsa* and *S. maxima*, as in the spectrophotometric method the solution must necessarily be perfectly clear in order to measure the absorbance; it was thus not possible to perform measures on solutions of whole cells or on solutions obtained by simple homogenization.

Moreover, in this case, since the possibility of performing the antioxidant measure depends essentially on the solubility of hydrophilous antioxidant substances with strong polarity, such as Vitamin C and several coloured pigments possibly present in the sample, the antioxidant capacity of the alga *S. maxima* was found to be high, while that of *S. subsalsa*, in spite of the mechanical treatment aimed at breaking down the cells, was found to be very low and that of the microscopic unicellular alga *S. capricornutum* non existent. This probably depends on the absence of antioxidant coloured pigments in the supernatant of the live alga *S. subsalsa* and to an even greater extent on that of *S. capricornutum* (completely colourless sample) insofar as the pigments remain attached to the cellular structures. These pigments are instead present in the supernatant of *S. maxima* (blue sample). The blue colour is due to the presence of phycocyanins, water soluble pigments that in the cultivated alga remain attached to the cellular structures of the cytoplasm and the stroma of the chloroplasts.

4.2. Using the polymerase chain reaction to determine the gene expression of iron–superoxide dismutase in S. subsalsa

An explanation of the high value of the antioxidant capacity obtained for the cultivated whole alga *S. subsalsa* using the biosensor method, which as seen above, seems to provide the most reliable values, may be found in the literature [28,29,32]. The living alga apparently uses its own defences against free radicals as its genetic inheritance contains also the superoxide dismutase enzyme. In order to experimentally verify that this is so and thus to evaluate the role of the antioxidant enzymes, in particular Fe–SOD, as regards the response and tolerance of the alga *S. subsalsa* to oxidative stress, we incubated this alga for 6 h with the xanthine/xanthine oxidase (XOD) system which produces in situ a large quantity of superoxide radical. The alga was thus obliged to defend itself by producing more SOD enzyme. The production of this enzyme may be detected by extracting the RNA, which has the task of coding it [33]. The RNA was thus extracted from the samples, and after converting it to coded DNA, the variation in genic expression of the iron–superoxide dismutase enzyme over time was observed and compared with that of a reference enzyme, luciferase. The polymerase chain reaction is known to be a complex process that, through a large number of cycles, leads to the amplification of the cDNAs related to SOD and luciferase. In order to separate the respective bands of the two amplified DNAs, the technique of stratification on 2% agarose gel was used. At this stage, nylon membranes were used on which the bands detected by electrophoresis were adsorbed in order to hybridize the cDNA bands with radioactive probes (that is, with the same primer made radioactive by the addition of phosphorus 32). Subsequently, the membrane was placed in contact with a photographic plate, which on exposure to the P^{32} radiations thus reproduced the intensity of the bands on the nylon membrane. Fig. 3 shows the graph of the ratio of intensity between the two signals obtained as a function of time. The alga's SOD production increases right from the first hour (as shown in Fig. 3) and for at least 6 h this response increases. This trend may be accounted for both by the increased RNA replication encoding the Fe–SOD enzyme (as indicated by the experiments carried out using the PCR technique), and by the increased exposure to the sun which also induces an increased production of antioxidant defences, in particular of endogenous SOD [36,37].

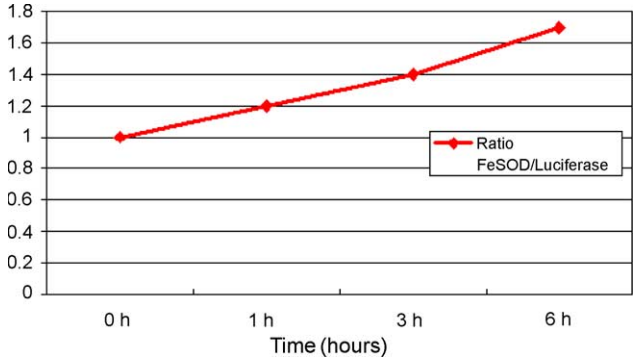

Fig. 3. Graph showing the trend over time of the ratio of the iron–superoxide dismutase (Fe–SOD) produced by the cultivated alga *Spirulina subsalsa* and luciferase, used as reference, obtained using the PCR technique.

4.3. Biosensor method measurement of the variation in time of the antioxidant capacity of cultivated algae

According to several authors [38] the composition of the algae is linked to the 'age' of the culture, the conditions

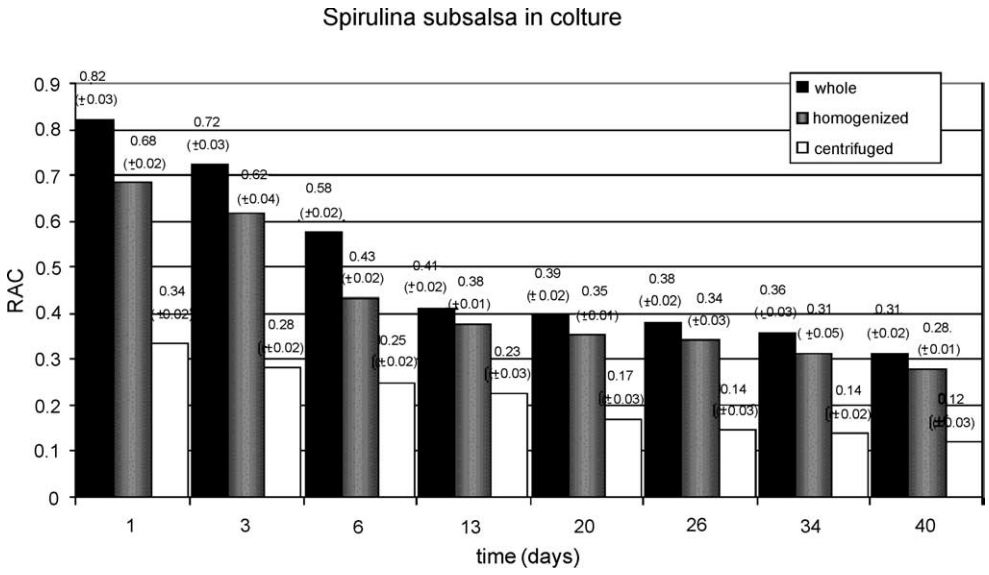

Fig. 4. Histogram obtained using the SOD biosensor and referring to the variation over time of the antioxidant capacity of the cultivated alga *Spirulina subsalsa*.

(temperature, lighting, nutrients supplied) and to the harvesting phase. It was, therefore, investigated whether the variation in antioxidant capacity of the alga *S. subsalsa* and the alga *S. capricornutum* (both whole and homogenized, or also centrifuged) actually depends on these factors by performing continuous measures over time. The measures were carried out for a length of time equal to that required to halve the initial antioxidant capacity value (that is, the value obtained on the first day on which the alga began to be cultivated or was rehydrated). This 'half-life' time was chosen as the most indicative parameter for evaluating and comparing the rate of degradation of the various types of alga. Furthermore, alga sampling was always performed using the same culture flask to show how environmental and physical variations affected the antioxidant capacity trends of one and the same alga population. The results, shown in Figs. 4 and 5, indicate that for the alga *S. subsalsa* it took about 35–40 days to reduce the initial antioxidant capacity by half, while for *S. capricornutum* it took only 20 days.

Despite the substantial difference between the two types of living alga and the difference in the half-life time values found, it was observed that the rate at which the antioxidant capacity value of the cultivated algae decreased was essentially the same in both cases.

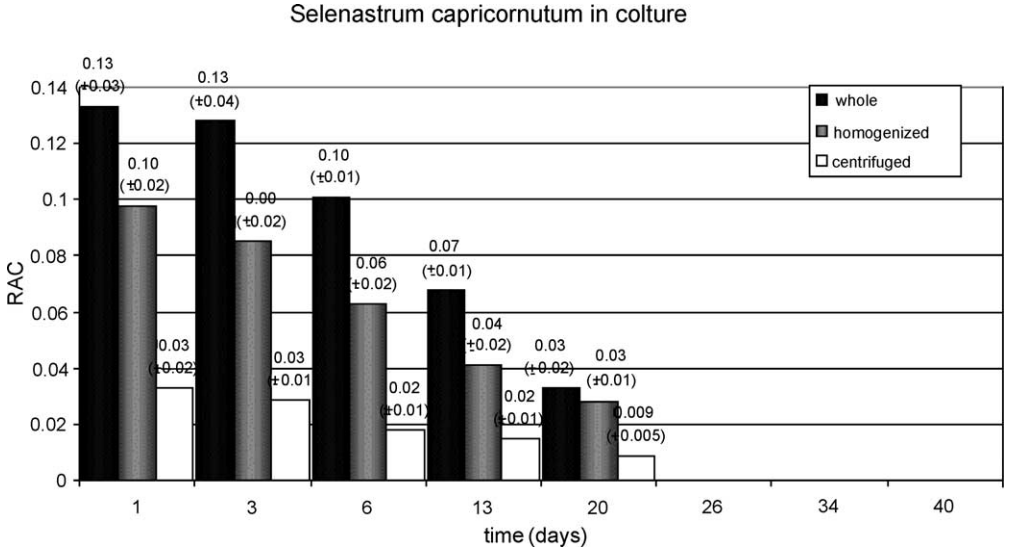

Fig. 5. Histogram obtained using the SOD biosensor and referring to the variation over time of the antioxidant capacity of the cultivated alga *Selenastrum capricornutum*.

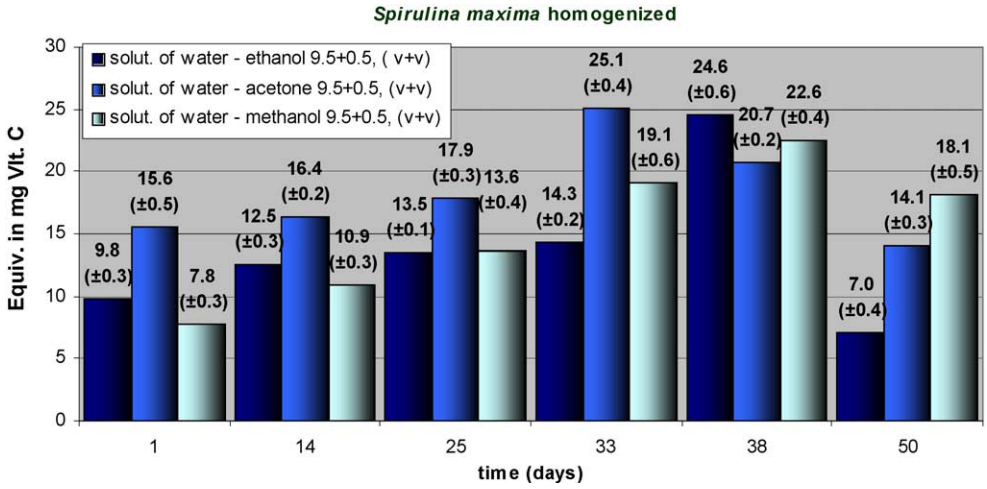

Fig. 6. Histogram, obtained using cyclic voltammetry, referring to the variation over time in the antioxidant capacity of extracts obtained using suitable solvent mixtures from the cultivated alga *Spirulina maxima*.

4.4. Cyclic voltammetry of extracts using different extracts of homogenized Spirulina algae

Higher antioxidant capacity values than those shown in Fig. 2(b) were obtained when the same homogenized alga samples as previously analysed were placed in solution with aqueous mixtures but at a concentration of 5% of a given organic solvent for a given period of time in order to extract the antioxidant substances contained in them. The measure was repeated on set days until a maximum antioxidant capacity value of the sample was obtained that tended to diminish in the days that followed. In this experiment, the measure of the antioxidant capacity was performed using cyclic voltammetry as the mixtures used, each containing 5% of a given organic solvent, were found to be detrimental to the activity of the enzymes used in the biosensor method.

In these voltammetric analyses, 10 mL of the solution obtained by placing 0.5 g of alga in an aqueous solution containing 5% of a suitable solvent was added to 10 mL of phosphate buffer. The solvents used were ethanol, methanol and acetone. These solvents were selected because, according to literature reports [39], they allow the antioxidant substances contained in alga samples to be extracted and solubilized. Also in this case it was observed how the antioxidant capacity varies with time (Figs. 6 and 7). Of course, the solutions of these solvents mostly extract compounds that differ in their characteristics. Methanol and ethanol are protic polar solvents and thus extract compounds having medium polarity while acetone, which is an aprotic polar solvent, extracts non-polar compounds. According to the literature [40], a mixture of methanol and water is able to extract the liposoluble antioxidant pigments from cellular lipids, i.e. β-carotene and

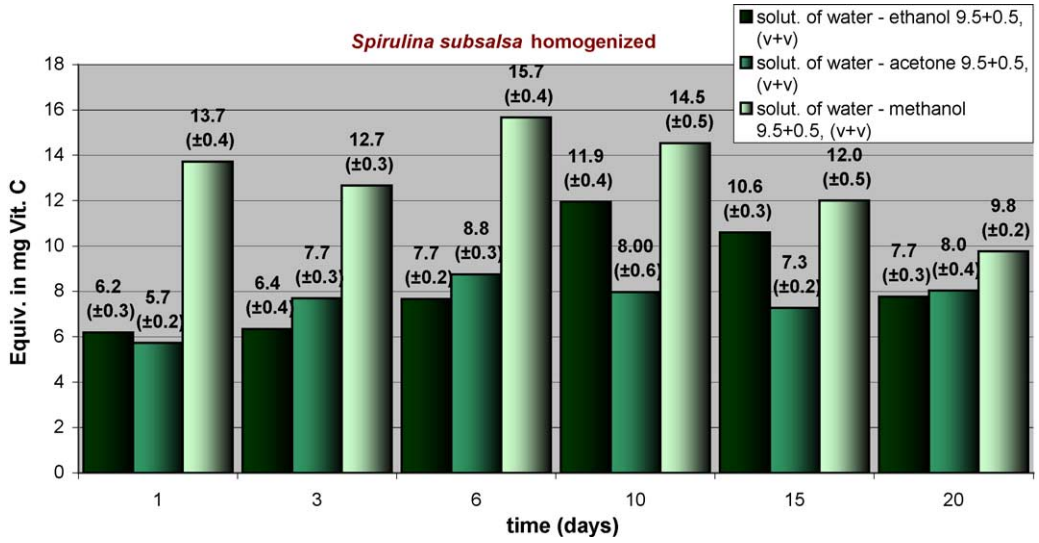

Fig. 7. Histogram, obtained using cyclic voltammetry, referring to the variation over time in the antioxidant capacity of extracts obtained using suitable solvent mixtures from the powdered alga *Spirulina subsalsa*.

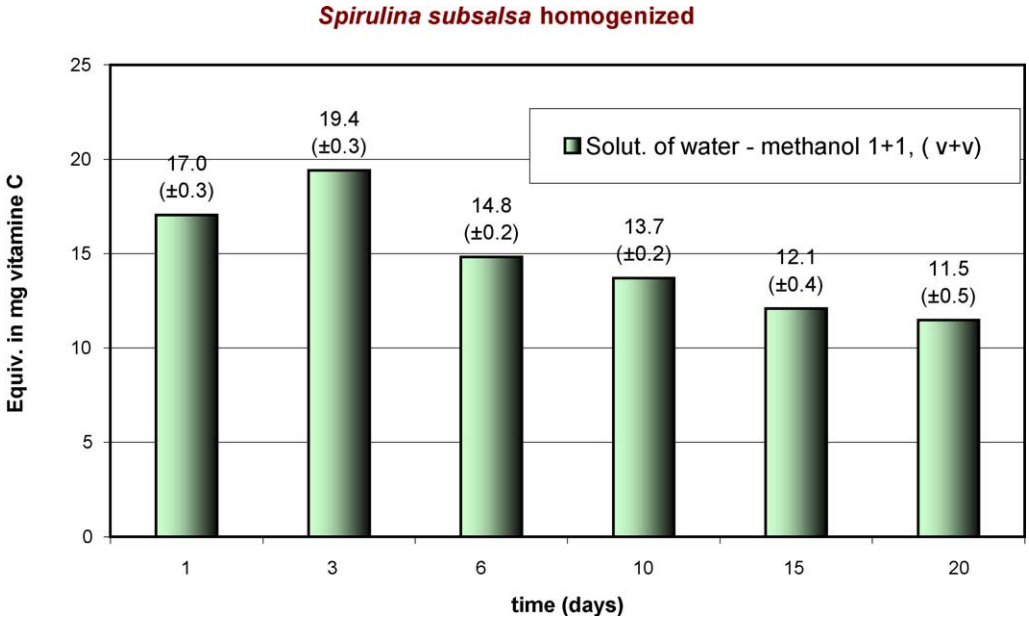

Fig. 8. Histogram, obtained using cyclic voltammetry, referring to the variation over time in the antioxidant capacity of extracts obtained using a mixture of methanol 1 + 1 (v + v) in water from the cultivated alga *Spirulina subsalsa*.

Vitamin E. On the other hand, acetone has the capacity to extract substances such as α-tocopherol, which has a strong antioxidant power. It is, therefore, the solvent most frequently used for the extractive treatment of algae; however, for blue-green algae (including the Spirulinas), it is found to have a lower extractive power than the alcohols [41]. On the basis of the experimental data obtained in our investigation, as shown in Figs. 6 and 7, it may be inferred that the best results are obtained by extraction with acetone and water solutions in the case of the alga *S. maxima* and with methanol and water solutions in the case of the alga *S. subsalsa*.

It should also be noted that, in solutions containing ethanol or methanol, the sample of *S. maxima* alga behaves in roughly the same way. Its extracts are able to provide their maximum antioxidant capacity after 38 days while, in the case of extraction with acetone mixtures, the highest value is found after 33 days, as shown in Fig. 6. The behaviour of the extracts of the *S. subsalsa* alga was instead recorded over a shorter period of time (20 days), as the results obtained, shown in Fig. 7, indicate a diminution of antioxidant capacity already after only 6–10 days. The 5% acetone and ethanol solutions give less satisfactory results for the living alga *S. subsalsa*. In the case of *S. subsalsa* (Fig. 7), the sample, placed in contact with a 5% methanol solution, displays twice the antioxidant capacity of the samples placed in contact with other solvents, with the peak values being observed after 6 days of contact. The solutions containing methanol were thus found to be the best and most effective for extraction in the case of the living alga *S. subsalsa* (Fig. 7). Lastly, we decided to determine the maximum antioxidant capacity of the cultivated alga *S. subsalsa*, again using a methanol mixture (found to be the most satisfactory in the preceding tests) but at a much higher methanol concentration. In practice, 0.5 g of *S. subsalsa* alga, taken from the culture flask, was placed in 5 mL of distilled water, homogenized completely (at 10,000 rpm for 5 min). The mixture was then heated at 60 °C for 15 min and then the homogenizer was placed in a 50% methanol solution, i.e. in the solvent displaying the best extractive capacity in previous tests. In this case, in addition to the mechanical homogenization treatment, the alga also underwent thermal treatment, which ensured the highest extractive capacity by the solvent, thus facilitating the solubilization of the antioxidants, as shown in Fig. 8.

5. Conclusions

In the present investigation, the algae are confirmed to have a high antioxidant capacity and rightly to be considered as a foodstuff of the future. It was also confirmed that the SOD biosensor method is a valid one for measuring antioxidant capacity, also in the case of alga samples. This is because the method allows the antioxidant capacity to be measured in the case of both cultivated and powdered algae, and also both when the alga tested is whole or after homogenization treatment, as well as after homogenization and centrifuging. Furthermore, it was also possible to plot the variation in antioxidant capacity over time for cultivated algae and to correlate variations in antioxidant capacity over time with factors related both to the chronological ageing of the algal strains and to experimental factors (homogenization and centrifugation treatment). The voltammetric method generally confirmed the SOD electrochemical biosensor results, although we also managed to carry out interesting additional work on the processes, such as the extraction of soluble antioxidant substances in given solvent mixtures, the

simultaneous degradation of cellular structures (living alga) by the organic solvents used themselves. In conclusion, it must also be pointed out that the commercially available diet integrator investigated in the present research based on the alga *S. maxima* proved to have a strong antioxidant capacity, which was often not the case in other non fresh vegetal materials examined in previous research, even when sold in herbalist shops or drugstores [20,42].

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