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Evaluation of radical scavenging properties of several plants, fresh or from a herbalist's, using a superoxide dismutase biosensor

L. Campanella *, G. Favero [1], L. Persi, M. Tomassetti

Department of Chemistry, University of Rome 'La Sapienza', Piazzale le Aldo Moro, 5-00185 Rome, Italy

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Abstract

The experimental evaluation of properties of defence against free radicals represents an extremely interesting heuristic and applicational objective. Research was carried out to experimentally evaluate the scavenging properties of several fruits and plants, fresh or from a herbalist's using an amperometric superoxide dismutase (SOD) biosensor recently developed by the present authors. The superoxide radical was produced in solution using the xanthine/xanthine oxidase system; measurements were carried out by comparing biosensor response to superoxide radical both in the presence and absence of the sample considered. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

It has now been extensively demonstrated that radical reactions are detrimental to human health as they are responsible for ageing processes and the cause of numerous diseases. The same type of reaction is involved in the processes leading to the breakdown of lipids contained in most foodstuffs and their rapid deterioration. This is the cause of considerable economic loss and potential problems for health itself.

Therefore, the use of antioxidants in the pharmaceutical and food field has grown constantly. In particular in the food sector, synthetic compounds are the most frequently used as they are effective and cheaper than natural ones. Recent findings have, however, cast serious doubts on their alleged non toxicity and research in this sector is now being addressed to finding safer, more effective and mainly natural antioxidants.

Different screening tests have been developed to determine the antioxidant properties of natural and synthetic antioxidant compounds. Several studies to this end have been reported involving organic solvents, micelles and liposomes [1–5],

* Corresponding author. Tel.: +39-6-49913744; fax: +39-6-490375.

E-mail address: campanella@axrma.uniroma1.it (L. Campanella).

[1] Grant holder of Consorzio Interuniversitario Nazionale 'La Chimica per l'Ambiente' (INCA).

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sometimes using the rate of oxygen uptake via the pressure transducer method [6,7], or measured by an oxygen electrode. Successively, methods based on conjugated diene formations, which have a higher sensitivity than oxygen uptake and allow spectrophotometric or fluorimetric measurements, are proposed [8–10].

Recently, we studied a biosensor able to determine the superoxide radical obtained by coupling a transducer consisting of an amperometric electrode for hydrogen peroxide with superoxide dismutase enzyme immobilised in κ-carrageenan gel [11].

As a first application of this biosensor the scavenging properties in vitro of important molecules such as cysteine, gluthathione, melatonin, β-carotene and acetyl salicylic acid as scavenger molecules of great biochemical and pharmaceutical interest were studied, including also pharmaceutical formulations containing acetyl salicylic acid [12].

We are currently addressing this problem through the study of the antiradical properties of fresh vegetal and fruit tissues, or else of preparations and extracts of the same fruit and vegetals present on the market and readily available in the chemists' or herbalists' shops; lastly, the investigation was extended to include several active principles contained in the vegetal tissues examined.

In practice, the antiradical action of some homogenised natural plant tissues (garlic, tomato, shallot, onion, carrot, aloe, etc.) was investigated by means of a completely original approach involving the use of a superoxide dismutase biosensor of recent fabrication, the response of which to the superoxide radical was modulated by the scavenging properties of the homogenised vegetal tissue or extract present in the measuring cell. The antiradical properties of some preparations or extracts obtained from some of the same plants, which are commonly sold in chemists' shops or by specialised herbalists for therapeutic use, were also tested. Lastly, similar experiments were performed also on the homogenised pulp or juice of different types of fresh fruit (lemon, orange, kiwi, strawberry).

2. Experimental

2.1. Reagents and apparatus

Hydrogen peroxide, potassium chloride, potassium phosphate, sodium hydroxide and all the other reagents of analytical reagent grade were supplied by Carlo Erba (Milan, Italy).

κ-Carrageenan, xanthine oxidase (XOD) from buttermilk 0.39 $U \cdot mg^{-1}$ was supplied by Fluka (Buchs, Switzerland); sodium carbonate was provided by Merck (Darmstadt, Germany); superoxide dismutase (SOD) was from bovine erythrocytes 4400 $U \cdot mg^{-1}$, xanthine sodium salt was supplied by Sigma (Norfolk, MO, USA); cellulose acetate was supplied by Aldrich (Steinheim, Germany).

A Mod. 332/P electrode supplied by Amel (Milan, Italy) and connected to an Amel model 551 potentiostat, was used as the 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.

2.2. Samples analysed and treatment

The samples of fresh fruit and plants tested (garlic, shallot, onion, Japanese garlic, chilli, aloe, cauliflower, radish, carrot, tomato, kiwi fruit, mandarin, orange, lemon, grapefruit, strawberry), were purchased at the local market. Test samples were prepared by weighing out 0.5 g fresh substance, which if necessary had been peeled beforehand. The crushed pulp was homogenized for 5 min at 10000 rpm in a homogenizer after addition of 3 ml phosphate buffer (0.05 M, pH 7.5). The homogenates thus obtained were analysed as such, after adding 0.5 ml of each one to the measuring cell. Alternatively, the homogenates were centrifuged for 15 min at 3000 rpm and 0.5 ml of the supernatant added to the measuring cell.

The other samples (garlic tablets, granules and capsules, aloe based integrator and aloe juice) were purchase at a chemist's or from a specialized herbalist; their declared composition was as follows:

Garlic tablets: Concentrated garlic pills; ingredients: dried garlic bulb, lactose, cellulose, silicon dioxide, magnesium stearate, castor oil, methylhydroxypropyl cellulose, polyethyleneglycol, saccharose, talcum, gelatine, polyvinylpyrrolidone, carnauba wax and beeswax.
Garlic perles: Garlic perles with mistletoe, hawthorn and natural vitamin E; ingredients: garlic macerated in oil, hawthorn macerated in oil, mistletoe, natural vitamin E.
Garlic capsules: ingredients: dry extract of *Allium sativum*.
Aloe based integrator: Aloe complex herbal preparation; ingredients: water, saccharose, mango pulp, papaya pulp, gel extract of Aloe vera, dry extract of angelica, flavouring, sodium benzoate, potassium sorbate, citric acid.Aloe juice: Aloe vera, stabilized pure juice for drinking; ingredient: liquid gel of Aloe vera, water, sodium benzoate, potassium sorbate, citric acid.

Depending on the form in which they are marketed, the samples were subjected to different treatments before testing: the liquid samples (garlic perles after rupture of the outer shell, aloe based integrator and aloe juice) were diluted (0.5 g in 3 ml) with phosphate buffer; the solid samples (garlic tablets, after grinding in a mortar and pestle, and garlic capsules after opening) were treated in the same way as fresh samples. Of course, samples already in the liquid state were not centrifuged after dilution (0.5 g in 3 ml).

2.3. Biosensor assembly and enzyme immobilisation

The assembly of the SOD/H_2O_2 biosensor is described in previous papers [11,12]: the transducer consisted of an amperometric electrode for hydrogen peroxide (Pt anode and an Ag/AgCl cathode; between the two electrodes a constant potential of + 650 mV was applied). The absence of possible interferents, such as ascorbic acid, is guaranteed by a cellulose acetate membrane.

The enzyme immobilisation is by entrapment in a κ-carrageenan gel membrane and the construction of the latter is reported in the same Refs. [11,12].

2.4. Method and test execution

The superoxide dismutase biosensor was allowed to stabilise in 20 ml of phosphate buffer 0.05 M, pH 7.5, containing xanthine oxidase (0.12 $mg \cdot ml^{-1}$) in homogenous solution, maintained under constant magnetic stirring in a 25 ml thermostatted glass cell; after signal stability was achieved a series of additions of 100 μl of a xanthine solution 0.01 M were made: superoxide radical production occurred in a homogenous solution according to the following enzymatic reaction catalysed by xanthine oxidase [13]:

(1)

The superoxide radical produced was detected by the superoxide dismutase biosensor: from the superoxide radical, hydrogen peroxide was obtained through the reaction catalysed by the superoxide dismutase enzyme, immobilised on the biosensor, according to the following reaction [14]:

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

The variation of the signal (of the order of 10 nA) provided by the amperometric electrode for hydrogen peroxide, which represented the indicator electrode of the biosensor due to the addition of xanthine was then recorded.

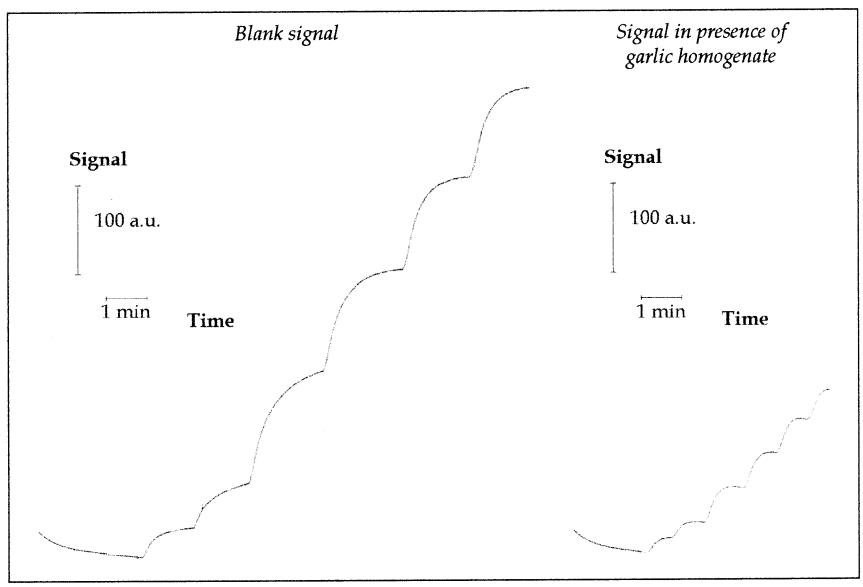
A calibration curve was thus recorded; the latter was compared with the calibration curve obtained under the same experimental conditions, but in the presence of 0.5 ml of the homogenised plant under test or of its aqueous extract.

The scavenging properties of the vegetal considered were evaluated from the percent ratio of slope values of calibration graph, both in the absence and presence of the scavenger vegetal considered. The results have been presented as a histogram.

3. Results

Typical experimental responses obtained using the SOD/H_2O_2 biosensor in phosphate buffer, are plotted in Fig. 1, which shows the trend in the H_2O_2 production in solution as a function of the successive additions of xanthine, both in the presence and absence of homogenised plant.

In the same figure a summary of all the working conditions optimised in a previous paper [12] and



Main working conditions and analytical data for the calibration curve in the absence of any sample in solution (i.e. "blank curve")

Buffer	Phosphate 0.05 M, KCl 0.01 M, EDTA 0.5 mM
pH	7.5
Temperature	25.0±0.5 °C
Equation of calibration curve (Y = a.u.; X = [Xanthine] (mM)) *Confidence interval*: t=2.23 , (1-α)=0.95	Y = (328.9 ± 6.4) X - (2.3 ± 1.3)
Correlation coefficient	0.9996
Linear range (mM)	0.02 – 2.0

Fig. 1. Recording of the experimental signal produced by the biosensor for successive additions of 100–200 μl xanthine (5.8 mM), i.e. final concentrations in solution in the range 0.03–0.3 mM, in the absence (blank) and presence of the sample (garlic homogenate), the superoxide radical scavenging properties of which are to be measured.

Table 1
Evaluation of scavenging properties of homogenates and extracts of several vegetables using the superoxide dismutase biosensor working in aqueous phosphate buffer solution 0.05 M at pH 7.5[a]

Sample added	Slope value (%) for homogenate (R.S.D.% ≤ 5.5)	Slope value (%) for the extract (R.S.D.% ≤ 5.0)
Buffer only	100	100
Tomato	90.4	77.7
Pepper	73.8	69.4
Carrot	53.7	60.9
Radish	47.2	58.4
Cabbage	13.9	8.1

[a] Behaviour of the percent sensitivity of the biosensor in the absence (buffer only) and presence of different vegetables (0.5 ml in 20 ml phosphate buffer).

the main analytical data obtained for the mean calibration curve, without any sample in solution, are also reported.

A number of tests were performed to investigate the antiradical action of different homogenised vegetal tissues, comparing the responses given by the superoxide dismutase biosensor, working in buffer solution (pH = 7.5), in the presence and absence of homogenised tissues, respectively.

The histograms in Fig. 2 show that the high or low signal variation of the biosensor is significantly different after addition of different homogenised tissue or extract, which indicates that vegetal tissue contains different amount of superoxide radical scavengers.

The experimental results shown in Fig. 2 refer to the antiradical action exerted by different types of bulb, such as garlic, shallot and onion, which are known to contain different compounds capable of exerting a strong scavenging action.

Owing to these properties, these vegetables are believed to exert a possible therapeutic action on various forms of cancer. For this reason, the same figure also contains the data obtained for two other plants (dried red chilli and aloe) which are quite different from the above-mentioned bulbs but also often cited recently for their scavenging properties and, in the case of aloe, also for their alleged anti-cancer properties. Furthermore, Fig. 2 also displays two sets of histograms: Fig. 2a showing the experimental results referring to the homogenates of these vegetals, Fig. 2b those referring to their aqueous extracts obtained after centrifugation of homogenates. The same criterion was used to obtain the results, collected in Table 1, to show the experimental results referring to several vegetable products such as cabbage, carrot, radish, tomato, or in Table 2 those referring to the antiradical properties of different kinds of fruit (kiwi, strawberry and different kinds of citrus fruit). Moreover, Fig. 3 shows the results of the same test performed on different kinds of commercial pharmaceutical or herbal products containing garlic extracts or dry garlic powder, or else aloe extracts.

Lastly, Table 3 shows the results obtained using different varieties of the same vegetable (garlic) or fruit (grapefruit), while in Table 4 the results obtained with the same vegetable or fruit preserved by different methods, are depicted.

4. Discussion

Using the superoxide dismutase biosensor, the scavenging superoxide radical properties of several plant tissues or fruit pulp were studied in vitro. First of all, the response of the biosensor to the superoxide radical in the presence and absence of

Table 2
Evaluation of scavenging properties of homogenates and extracts of different kinds of fruit using the superoxide dismutase biosensor working in aqueous phosphate buffer solution 0.05 M at pH 7.5[a]

Sample added	Slope value (%) for homogenate (R.S.D.% ≤ 5.0)	Slope value (%) for the extract (R.S.D.% ≤ 5.0)
Buffer only	100	100
Mandarin	93.7	86.0
Lemon	78.1	94.2
Orange	66.2	74.2
Grapefruit	54.8	86.1
Kiwi fruit	40.5	66.4
Strawberry	39.8	52.5

[a] Behaviour of the percent sensitivity of the biosensor in the absence (buffer only) and presence of different kinds of fruit (0.5 ml in 20 ml phosphate buffer).

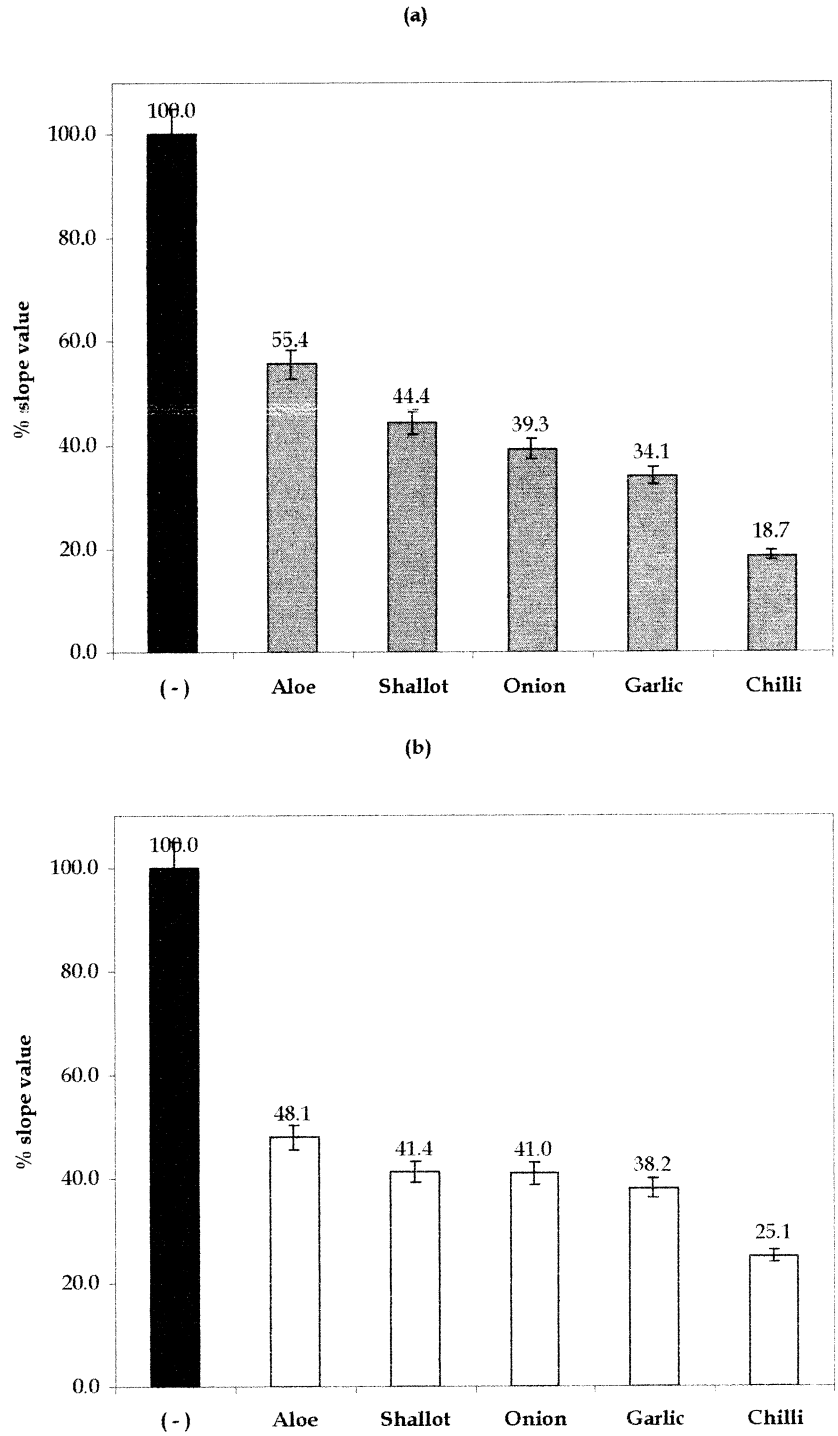


Fig. 2. Evaluation of scavenging properties of (a) homogenates and (b) extracts of several bulbs and other vegetals using the superoxide dismutase biosensor working in aqueous phosphate buffer solution 0.05 M at pH 7.5; behaviour of the percent sensitivity of the biosensor in the absence ((−) = buffer only) and presence of different bulbs and other vegetals (0.5 ml in 20 ml phosphate buffer).

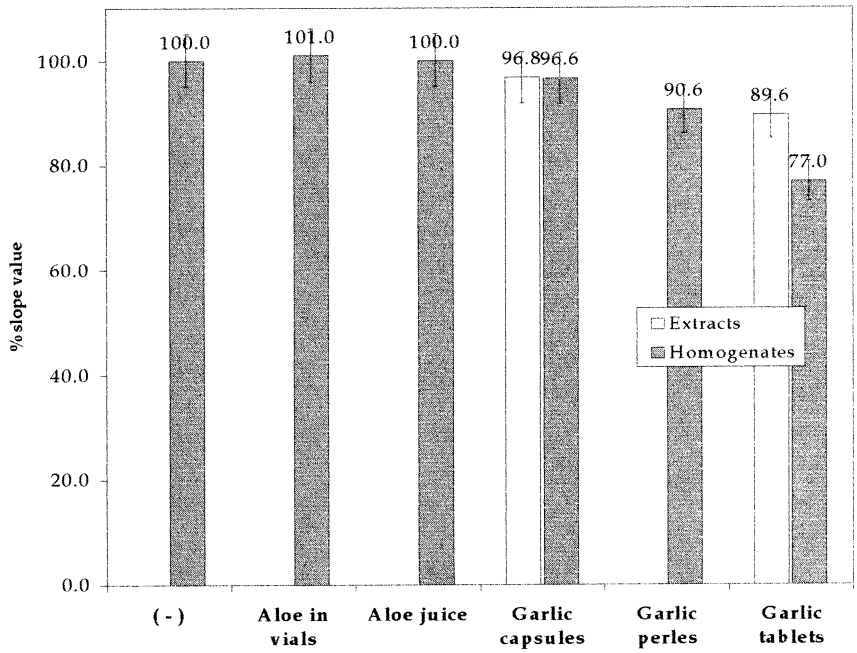


Fig. 3. Evaluation of scavenging properties of homogenates and extracts of different kinds of commercial pharmaceutical or herbal products using the superoxide dismutase biosensor working in aqueous phosphate buffer solution 0.05 M at pH 7.5; behaviour of the percent sensitivity of the biosensor in the absence ((−) = buffer only) and presence of different products (0.5 ml in 20 ml phosphate buffer).

vegetal bulb homogenates or extracts was investigated.

The results, displayed in the form of histograms in Fig. 2, confirm the good free radical scavenging function of all the bulbs (garlic, onion, shallot).

Biosensor response to standard superoxide radical concentration is reduced by more than 50% in the presence of these vegetables, and in particular by about 66% in the presence of garlic homogenate, which amply confirms the literature reports on the subject.

It was also observed that the homogenates and extracts obtained after centrifugation have a quite comparable antiradical action. Also aloe may be said to possess good scavenging properties (it actually reduces biosensor response by about 50% on average), while dried red chilli was found to be one of the most active of the vegetals tested, causing an 80% reduction in biosensor response.

It is also important to point out how, unlike all the commercial garlic-based preparations (from the chemist's or the herbalist's) display very slight, practically negligible, scavenging activity, and those containing aloe none at all (Fig. 3).

It should be noted, however, that these chemist's or herbalist's preparations are not usually recommended or administered as specific antiradical preparations, for example, in the case of products containing aloe extracts and their use is recommended more for gastric problems.

It should also be noted that the comparison between the scavenging activity of the different products examined was carried out in a way that appeared to us to be the most correct, and in any case proved to be practically the only one feasible, namely to compare the activity of equal quantities by weight (0.5 g) of the various products examined, whether or not it was a vegetable product or fruit pulp, or else a herbal product or a solid pharmaceutical product, after, of course, having homogenized all the products in the same way in the same volume of buffer solution. The more compact samples, such as tablets, were, of course, ground up before being placed in the homogenizer.

Indeed the sole cases in which the comparison among equal quantities of weighed product might not be completely homogeneous were those in which pharmaceutical or herbal products had to be compared in the liquid state, for which, moreover, the manufacturers had not communicated the quantity by weight of plant product from which the commercial speciality had been obtained.

Clearly, in this case the evaluation we obtained consisted only in an indication of the greater or lesser effectiveness as a radical scavenger of 0.5 g of liquid product as marketed by the producer.

Of the plants tested (Table 1) green cabbage displayed very strong antiradical properties both as homogenate and extract. Radish and carrot exert an appreciable action (50 or 40% reduction in biosensor response as homogenate or extract, respectively).

Lastly, as far as the scavenging properties of the various species of fruit pulp are concerned, strawberries were confirmed (Table 2) as having a strong effect, which is often cited in the literature, but also various kinds of citrus fruit (kiwi, grapefruit, orange) display good antiradical properties, especially in the form of homogenates; indeed, in the case of fruit, the extracts obtained after centrifugation generally display weaker antiradical properties and furthermore the intensity trend for the different kinds of fruit is not always the same if homogenates or filtrates (Table 2) are used.

Table 3
Evaluation of scavenging properties of homogenates and extracts of different varieties of the same plant or fruit using the superoxide dismutase biosensor working in aqueous phosphate buffer solution 0.05 M at pH 7.5[a]

	Slope value (%) for homogenate (R.S.D.% ≤ 5.0)	Slope value (%) for the extract (R.S.D.% ≤ 5.0)
Garlic sample added		
Buffer only	100	100
Garlic	34.1	38.2
Japanese garlic	76.9	77.0
Grapefruit sample added		
Buffer only	100	100
Yellow grapefruit	54.8	86.1
Red grapefruit	32.4	58.8

[a] Comparison of the percent sensitivity of the biosensor in the absence (buffer only) and presence of different varieties of the same vegetal or fruit (0.5 ml in 20 ml phosphate buffer).

Table 4
Evaluation of scavenging properties of homogenates and extracts of the same plant or fruit preserved by different methods using the superoxide dismutase biosensor working in aqueous phosphate buffer solution 0.05 M at pH 7.5[a]

	Slope value (%) for homogenate (R.S.D.% ≤ 5.0)	Slope value (%) for the extract (R.S.D.% ≤ 5.0)
Strawberry sample added		
Buffer only	100	100
Fresh strawberry	39.8	52.5
Frozen strawberry	66.5	67.0
Kiwi fruit sample added		
Buffer only	100	100
Fresh kiwi fruit	40.5	66.4
Frozen kiwi fruit	89.9	87.9
Garlic sample added		
Buffer only	100	100
Fresh garlic	34.1	38.2
Dried garlic	58.4	59.3

[a] Comparison of the percent sensitivity of the biosensor in the absence (buffer only) and presence of vegetal or fruit (0.5 ml in 20 ml phosphate buffer) preserved by different methods.

In this case it was also possible to compare our results for several of the fruit species tested with those reported in the literature by Wang et al. [15], obtained using the automated oxygen radical absorbance capacity (ORAC) assay. The order found by Wang et al. [15] using fruit pulp homogenized with water and centrifuged was: strawberry > orange > kiwi fruit > grapefruit, that is, very similar to our results: strawberry > kiwi fruit > orange > grapefruit: in other words, with only one inversion of sequence, that between orange and kiwi fruit.

On the other hand, in the course of the tests it was observed that the scavenging properties of different varieties of the same vegetable or fruit were often very different (see Table 3). Furthermore, the method of preservation also exerted a strong influence on the intensity of the scavenging

properties, especially in the case of fruit, as can be seen from the examples in Table 4.

Also, the results shown in Table 2 refer to samples of fresh fruit purchased at the local market, although it was obviously not possible to determine, for example, whether and for how long the oranges, grapefruit, kiwis and other citrus fruits were actually kept in cold storage prior to sale (we are certain at least that they were not stored on ice or deep frozen).

Lastly, it may also be pointed out that, when comparing the scavenging activity observed for homogenized products and for the extracts of the same vegetable species examined, the order of activity of the former is nearly always the same as that of the latter. However, in the case of fruit, the order is not always the same in both cases.

Moreover, for both vegetables and fruit, the activity observed for a given homogenate is sometimes found to be higher than the extract, while on other occasions the opposite is true; this would seem to indicate that, depending on the product tested, the procedure for obtaining a solution with the highest activity could be one or other of the two we tested, that is, simple homogenization in buffer solution, or else homogenization plus centrifuging and separation of the centrifuged material, thus obtaining the extract.

At the present state of our research it can only be conjectured that this may be dependent on the different chemical and structural characteristics of the vegetable or plant considered.

5. Conclusions

Several in vitro tests have recently been proposed for measuring the antioxidant properties of food products, based on the inhibition of human low-density lipoprotein oxidation [16], or by using the oxygen radical absorbance capacity (ORAC) [9,15].

Methods such as ORAC are used to determine the total antioxidant activity, while the method described in this paper determines the scavenging activity to superoxide radical of the sample tested. It is thus closer to spectrophotometric methods such as RANSOD, in which the superoxide radical reacts with a derivative of phenyltetrazolium chloride (INT) to form a red formazan dye [17], or to those that measure the absorbance of the reduced nitro blue tetrazolium compound [18].

On the other hand, the test essentially determines the action of scavengers on the superoxide radical generated in situ in the measuring cell using the xanthine/xanthine oxidase system. However, taking into account the good agreement found, at least in the case of fresh fruit, with the results of Wang et al. [15], who determined the total antioxidant activity, it would thus seem to be fairly reasonable to affirm that, generally speaking, fresh vegetal tissues, for which the experimental results show greater scavenging properties than commercial herbal products, probably possess greater scavenging properties against the total concentration of free radicals, and not just the superoxide radical, than commercial products.

From these results, it seems possible to conclude that the superoxide dismutase biosensor can be used to evaluate in a very simple and rapid way the antiradical capacity of plant and fruit tissues and, in practice, the level of scavenger molecules contained in them.

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