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Evaluation of the antioxidant and prooxidant properties of several commercial dry spices by different analytical methods

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Abstract

The antioxidant capacity of 12 different types of dry spices was determined using the following methods for each of them: the ORAC method, a recent biosensor method and the OXY-Adsorbent test. Furthermore, a biosensor method for the measurement of the polyphenol pool was also applied. The d-ROMs test for the measurement of hydroperoxides and a well known spectrophotometric test for the measurement of lipoperoxides were also applied to evaluate the presence of prooxidant species in the samples examined.

Keywords: Antioxidant capacity; Peroxides; Dry spices

1. Introduction

It is a widely known fact that spices play a non-negligible role in human diet. In recent years, it has been acknowledged that many spices not only have properties that make food more pleasant and tastier but also important preservative and antioxidant properties (Madsen et al., 1995; Shobana & Akhilender Naidu, 2000).

The antioxidant properties of many spices are well known, while their prooxidant properties less so; it is even less well known that a relationship, for example an inverse one or of another type, exists between the former and the latter.

The aim of this research was to some extent to fill in the gaps in our knowledge regarding the more commonly used dry spices.

Wide-ranging analytical research was therefore performed, not only with different methods, but also from various points of view, to evaluate the antioxidant properties of a good number of dry spices: anise, fennel, basil, mint, tarragon, marjoram, rosemary, thyme, parsley, juniper, laurel and black pepper. The antioxidant properties of equal weights of all the samples were measured using a superoxide dismutase biosensor (SOD) (Campanella, Favero, & Tomassetti, 1999). The analyses were performed both after simple homogenization in phosphate buffer and after homogenization and subsequent centrifugation at 3500 rpm for 15 min (Campanella, Favero, Persi, & Tomassetti, 2000, 2001). The total antioxidant capacity values as found by the biosensor method for the homogenized and centrifuged samples were then compared with those obtained with the classical spectrofluorimetric (ORAC) method, which was chosen as reference method (Cao, Alessio, & Cutler, 1993; Cao, Verdon, Wu, Wang, & Prior, 1995).

In addition the comparison was extended to the OXY-Adsorbent test (Brambilla et al., 2002; Gerardi et al., 2002), which evaluates the antioxidant power of the sample by measuring its capacity to oppose the massive oxidative action of hypochlorous acid. Lastly, also the concentration of the polyphenol "pool" was determined using a tyrosinase

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biosensor (Campanella, Sammartino, & Tomassetti, 1992). It is not necessary to remember that polyphenols frequently represent the principals responsible for the antioxidant capacity of several plants, which contain them. The same dry spices were also analysed to evaluate the concentration of the prooxidant species, with the so-called d-ROM test (Alberti, Bolognini, Macciantelli, & Carratelli, 2000) (''Colorimetric determination of Reactive Oxygen Metabolites'', generated by the free radicals), i.e., a method by which, the concentration of the so-called ROMs (reactive oxygen metabolites) is determined, in practice the hydroperoxide ''pool''.

Lastly, lipoperoxides were analysed to evaluate also the prooxidant species contained in the lipidic fraction of samples. They were extracted by isopropyl alcohol from the lipidic fraction, and all samples were then analysed by a method based on their capacity to oxidise iron (II) to iron (III), which is complexed by thiocyanate, generating a complex species which absorbs at $\lambda = 505$ nm (Alberti et al., 2000).

2. Materials and methods

2.1. Reagents and chemicals

Xanthine (2,6-dihydroxy purine) sodium salt, ethylenediamine tetracetic acid (EDTA), superoxide dismutase 4980 U mg^{-1}, dialysis membrane (art. D-9777), tyrosinase 6050 U mg^{-1}, formic acid, Teflon membrane, phenol and β-phycoerythrin were supplied by Sigma (Milan, Italy).

Xanthine oxidase 0.39 U mg^{-1} and cellulose acetate were supplied by Fluka AG, Buchs (Switzerland). 2,2′-Azobis(2-amidinopropan)dihydrochloride (ABAP) was supplied by Waco Chem (Richmond, VA, USA).

Isopropylic acid, potassium dihydrogenphosphate and sodium hydrogenphosphate were supplied by Carlo Erba (Milan, Italy).

Polyvinylacetate, acid-2-carboxy-6-hydroxy-2,5,7,8-tetramethylchroman (Trolox) and cellulose triacetate were supplied by Aldrich (Germany).

Kit Diacron for lipoperoxide assay, kit Diacron OXY-Adsorbent test, kit Diacron d-ROMs test and ROM-Diacron Standard were supplied by Diacron (Grosseto, Italy).

2.2. Apparatus

Crison pH meter mod. GLP 22; spectrofluorimeter Perkin–Elmer, mod. LS-5, provided with Perkin–Elmer recorder, mod. 561; electrode mod. 4000-1 by Universal Sensor Inc. New Orleans, LA, USA, coupled with an Amel potentiostat mod. 551, connected to an Amel differential electrometer, mod. 631 and to an Amel analog recorder, mod. 868; Ultra-Turrax homogenizer mod. T8 by Ika Labortechnik; microplate spectrophotometer Bio-Rad 550; analyser Synchron Clinical CX5 Delta Beckmann Coulter Italia (Milan, Italy); mulino, A 10 Yellow line IKA Works Inc.

2.3. Samples and treatment

All the analysed dry species were bought in a local market from a specialized retailer of spices for human consumption. Each sample was contained in a small glass bottle sealed with a plastic screw cap. The products were in different forms: minced desiccated leaf samples: basil, rosemary, tarragon, thyme, parsley, laurel, mint, marjoram; powdered samples: black pepper; samples of seeds and berries: fennel, juniper, anise.

Those samples, which were not already powdered, were ground and homogenized immediately before analysis.

2.4. ORAC method

The spectrofluorimetric method (ORAC) (Oxygen Radical Absorbance Capacity) is well known and extensively described in literature (Cao et al., 1993, 1995): in the presence of free radicals or oxidising species, the protein β-phycoerythrin (β-PE) loses more than 90% of its fluorescence within 30 min. The addition of antioxidant species, which react with the free radicals, inhibits the fluorescence of this protein. This inhibition can be correlated with the sample's antioxidant capacity. In particular, 2,2-azobis-(2-amidinopropane)dihydrochloride (ABAP) was used to generate peroxide radicals ($ROO^{\bullet}$). To perform the measurements, the wavelengths were set at $\lambda = 540$ nm for excitation and 565 nm for emission. Initially 80 μl of sample were placed in the cuvette together with 20 μl of phosphate buffer (75 mmol/l, pH 7), and 1.46 ml of β-phycoerythrin (18.3 mmol/l in phosphate buffer), prepared and allowed to stand at 37 °C for 15 min before use. The cuvette was placed in the spectrofluorimeter and the initial fluorescence (f_0) read off after 30 s. Then a further 20 μl of phosphate buffer was added to the solution in the cuvette together with 20 μl of ABAP (0.32 mmol/l in phosphate buffer). After stirring, the fluorescence was read off after 0.5 s and then every 2 min, for a total time of 70 min. A similar procedure was also carried out using a 20 μmol/l solution of trolox instead of sample.

The final results are expressed in ''ORAC units'' (micromoles of Trolox equivalent per litre of sample):

$$\text{ORAC value} = 20\, k(S_{\text{sample}} - S_{\text{white}})/(S_{\text{Trolox}} - S_{\text{white}})$$

k = dilution factor for the sample; S = integral of the fluorescence curve of the sample, of the Trolox, or of the ''blank''.

2.5. Superoxide dismutase (SOD) biosensor method

The superoxide radical is determined using a biosensor obtained by coupling a transducer (an amperometric electrode for hydrogen peroxide) with the superoxide dismutase enzyme immobilised in kappa-carrageenan gel (Campanella et al., 1999). The gel containing the enzyme is sandwiched between a cellulose acetate membrane and a dialysis membrane. The whole assembly is secured to

the electrode with an O-ring. A constant potential of +650 mV with respect to an Ag/AgCl/Cl^- cathode is applied to the platinum anode. The dialysis membrane is used to support the gel and to prevent attack by the enzyme. The superoxide radical is produced by the oxidation of xanthine in aqueous solution to uric acid in the presence of the xanthine oxidase enzyme free in solution. The disproportion reaction of the superoxide radical, catalysed by the superoxide dismutase immobilised on the electrode, produces oxygen and hydrogen peroxide. The hydrogen peroxide produced is oxidised at the anode, generating an amperometric signal (in nA) that is proportional to the concentration of the superoxide radical present in solution. The addition of a sample possessing antioxidant properties produces a decrease in the signal as, by reacting with the superoxide radical, the concentration of these species in solution is lowered. As a consequence, both released H_2O_2 and intensity of the amperometric current diminished. In practice the electrochemical biosensor was placed in a cell thermostated at 25 °C containing 15.0 ml of phosphate buffer 0.05 mol/l at pH 7.5 and allowed to stabilise under constant stirring. After addition to the same solution of a fixed quantity of the xanthine oxidase enzyme (1.2 mg), two successive additions of 200 μl of the solution of xanthine 0.01 mol/l were added, waiting for the signal to stabilize between successive additions. The values of the recorded current variations were thus plotted versus the xanthine concentration and the slope value calculated.

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

$$\text{(RAC) ``Relative antioxidant capacity''} = 1 - (m_c/m_x)$$

m_x = slope of the straight line obtained by means of successive additions of xanthine; m_c = slope of the straight line obtained by means of successive additions of xanthine, but in the presence of the sample possessing antioxidant properties.

The superoxide dismutase assembling and SOD immobilization in kappa-carrageenan were described in detail in previous papers (Campanella et al., 1999; Campanella, Favero, Persi, & Tomassetti, 2001).

2.6. Tyrosinase biosensor method

Also the tyrosinase biosensor method to investigate polyphenols has been extensively described in a previous paper by the present authors (Campanella et al., 1992). Phenol content was determined according the following enzymatic reaction, which involved the phenol –OH group:

$$\text{phenol} + O_2 \xrightarrow{\text{tyrosinase}} o\text{-quinone} + H_2O \quad (1)$$

The biosensor used is obtained by coupling a Clark electrode, as transducer, with the tyrosinase enzyme immobilised in kappa-carrageenan gel (Campanella, Bonanni, Finotti, & Tomassetti, 2004). The gel containing the enzyme is sandwiched between a teflon membrane and a dialysis membrane. The internal solution of the oxygen electrode consists of KCl 0.1 mol/l and phosphate buffer 0.06 mol/l at pH 6.6. The electrode is made of a platinum cathode and a Ag/AgCl/Cl^- anode between which a constant potential of −650 mV is applied.

Phenol content is determined by plotting variations in the recorded amperometric signal against the consumption in dissolved oxygen in the solution, due to the enzymatic reaction. Signal variation due to O_2 consumption is correlated with the concentration of the phenol ''pool'' contained in the sample. In performing the measurement the biosensor is placed in a cell thermostated at 25 °C containing 15.0 ml of TRIS buffer 0.1 mol/l at pH 8.5 and allowed to stabilise under constant stirring. Two hundred microlitres of the sample are added and the signal variation recorded. Likewise, 200 μl of a standard phenol solution 0.1 mol/l are added and the signal variation again recorded. Comparison between signal variation due first to the addition of the sample and then to that of a phenol standard solution allows the phenol content of the sample, expressed as mol/l of phenol, to be determined.

2.7. OXY-Adsorbent test method

The sample is subjected to massive oxidation through HClO; the antioxidant substances contained in the sample react with the acid and can be quantified by measuring the excess of HClO.

The quantification of the unreacted acid is carried out by the spectrophotometric method (reading at $\lambda = 490$ nm), after addition of suitable buffered chromogenous agent, an aromatic alkyl diamine (*N*,*N*-diethylparaphenylendiamine). The concentration of the coloured complex is directly proportional to the concentration of HClO and is indirectly related to the antioxidant capacity. The results are expressed as 100 (l/μmol) of non-reacted HClO.

Practically, 1 g of homogenized sample is added to 4 ml of water into a Falcon tube. The suspension is sequentially sonicated (15 min) and centrifuged (15 min, 4000 rpm). The supernatant is filtered through MILLEX GV filter (0.45 μm).

To perform the calibration curve for the quantitative analysis, different solutions of ROMs DIACRON stabilized Standard (340 μmol/l as antioxidant) are used. The concentrations of those standard solutions are, respectively: 6.8, 3.4, 1.7, 0.85 and 0.43 μmol/l of antioxidant.

Measurements are performed simultaneously both on the standards and the samples. Two hundred microlitres of oxidant solution (hypochlorous acid), 5 μl of sample, or of water for the blank, or of standard solution for the construction of calibration curve, are placed in each well of the microtitre plate. The plate is incubated at room tem-

perature for 10 min, then 5 μl of chromogenous reagent are added to each well. After gently mixing the analysis is carried out by immediately reading the absorbance at $\lambda = 490$ nm. Sample absorbance values are corrected for reagent blanks and concentrations calculated using the standard calibration curve.

2.8. Colorimetric determination of peroxides

Peroxides are determined both after aqueous extraction and after alcoholic extraction using two different colorimetric methods. Two methods are, respectively, called the "d-ROMs test", to check the peroxides in aqueous phase, and "lipoperoxide test" to check peroxides in the alcoholic phase.

2.8.1. Principle of method for aqueous extracts (d-ROMs test)

The d-ROM test is a spectrophotometric test that is based on the capacity of transition metal ions to generate in vitro alkoxyl and peroxyl radicals in the presence of hydroperoxides. A chromogenic reagent (*N*,*N*,-diethylparaphenylen-diamine) is then added to this solution. This chromogen compound possesses the feature of being oxidized by hydroperoxyl and alkoxyl radicals and transformed into a pink to red coloured cation. The concentration of the coloured complex is directly related to the hydroperoxide levels of the sample.

A calibration curve is constructed to quantify the peroxide concentration. To this end some standard solutions are prepared by dissolving different volumes of the standard

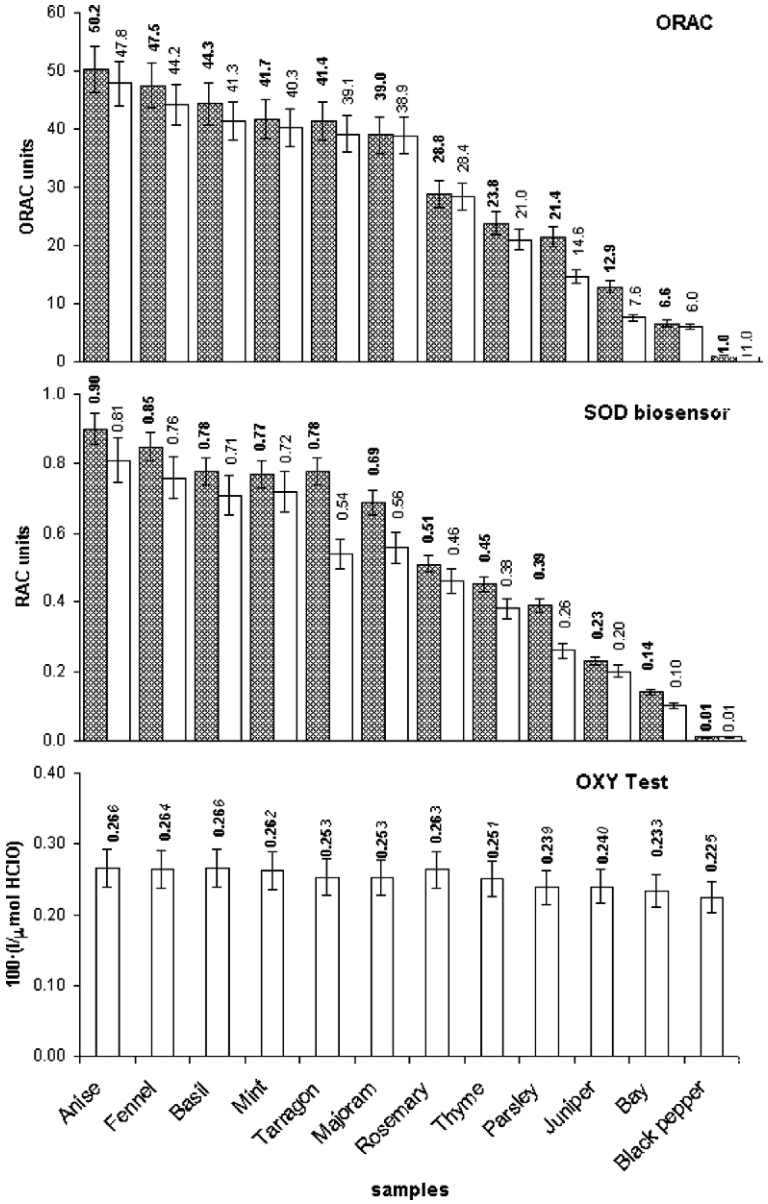

Fig. 1. Trends of the antioxidant capacity values, of dry common spices obtained, respectively, by: the ORAC method, the biosensor method and the OXY-Adsorbent test method, using the same amounts in weight of the homogenized samples (black histograms), or homogenized and then centrifuged samples (white histograms).

(ROM-DIACRON standard, title: 5.88 mmol/l as H_2O_2) in 1 ml of water. The calibration range is (2.94–0.37) mmol/l of H_2O_2.

Briefly, 1 g of homogenized sample is added to 4 ml of water in a Falcon tube. The suspension is previously sonicated, centrifuged and filtered as previously described in the OXY-Adsorbent test method. At the same time, a working mixture is prepared by mixing R1 reagent and R2 reagent in the ratio of 1:100. The preparation of the microplate is carried out as follows: 200 μl of work mixture, 5 μl of sample, or of water for the blank reagent, or of standard solution for the construction of the calibration curve, are placed in each well of the microtitre plate. The plate is incubated at 37 °C for 75 min and then the analysis is carried out by immediately reading at $\lambda = 490$ nm. Absorbance values of the samples are corrected for reagent blanks and concentrations calculated using the standard calibration curve. The results are expressed as mmol/l of H_2O_2.

2.8.2. Principle of method for alcoholic extracts (lipoperoxide test)

The method is based on the capacity of peroxides to catalyse the oxidation of Fe^{2+} to Fe^{3+}. The Fe^{3+} produced is linked to the thiocyanate anion, yielding a red complex, which is measured spectrophotometrically. The increase of the absorbance is directly proportional to the concentration of the peroxide present in the samples.

The sample (1 g in 4 ml of isopropyl alcohol) is processed (sonicated, centrifugated and filtered) as described above except that sonication is performed at 40 °C.

Peroxide quantification is carried out by means of a calibration curve. Several standard solutions, with concentrations of 375, 750, 1500 and 3000 μeq/ml of peroxides (DIACRON's standard), are prepared by dissolving different volumes of standard in 1 ml of isopropyl alcohol.

The analyses are conducted as follows: 1000 μl of R1 reagent (Fe^{2+} salt) are added to 10 μl of each standard solution, then 10 μl of R2 reagent (chromogenous agent) are added and, after gentle mixing, allowed to stand for 5 min. The spectrophotometric analysis is carried out at $\lambda = 505$ nm. The real samples undergo the same procedure, but the working volumes used are different, i.e., 400 μl of alcoholic extract, 600 μl of R1 reagent, 10 μl of R2 reagent.

Readings are corrected for the blank reagent absorbance. The results are expressed as μeq/l of lipoperoxides.

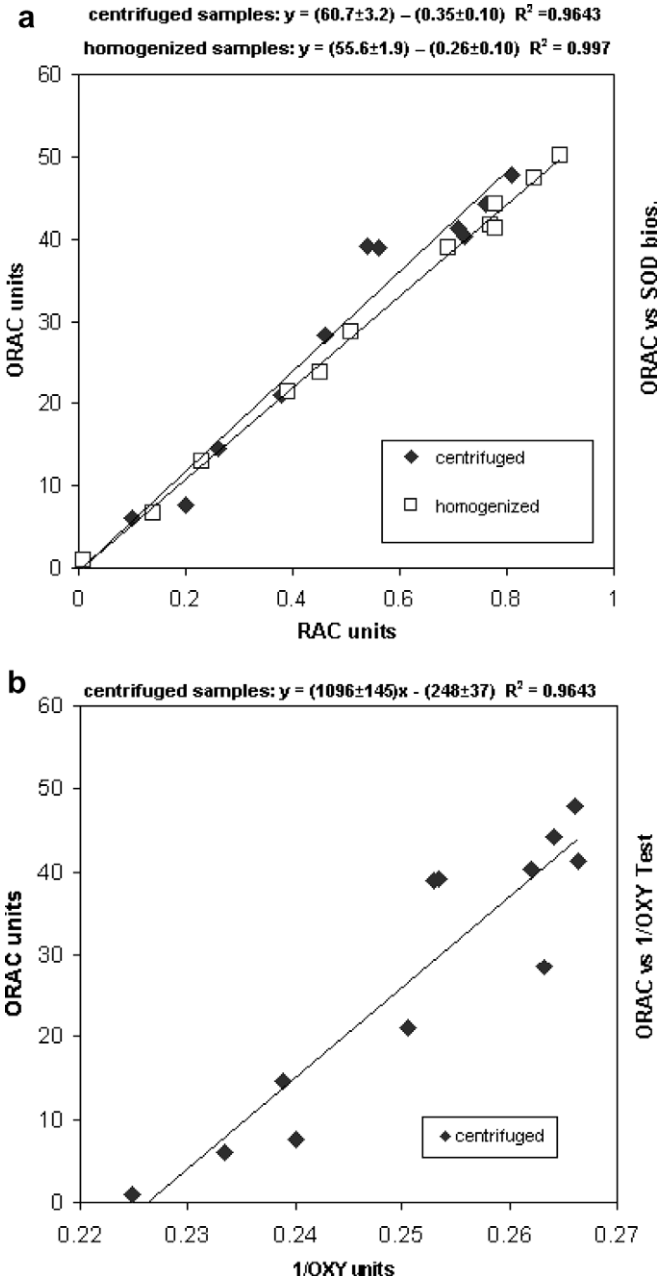

Fig. 2. (a) Correlation straight lines between the spectrofluorimetric method (ORAC) and the SOD biosensor method (RAC), both for the homogenized samples and for the homogenized and then centrifuged samples. (b) Correlation straight line between the spectrofluorimetric method (ORAC) and the OXY-Adsorbent test method for the homogenized and then centrifuged samples.

3. Results and discussion

It is first necessary to underline that the comparison of the different methods to check the antioxidant capacity obviously was made not on the basis of the absolute values found, as every method has its own value scale, but on the basis of trends of the values obtained for the same weight (1 g) of the different samples, analyzed using different methods. Values of the antioxidant capacity, obtained with the spectrofluorimetric reference method, for homogenized samples alone, or for homogenized and centrifuged ones, are, respectively, shown in the histograms in Fig. 1; also those obtained with the biosensor method, are shown in the histograms of the same figure. The trend of values obtained with both methods for similar samples (only homogenized, or homogenized and then centrifuged) is seen to be practically the same. Therefore, the correlation between these two methods is very good (in this regard see also the correlation straight lines shown, respectively,

Table 1
Comparison of obtained values for homogenized and homogenized and then centrifuged samples by ORAC method, SOD biosensor method and OXY-Adsorbent test method (only for centrifuged samples)

Samples	Homogenized		Centrifuged		
	Biosensor method (RSD% < 5)	Spectrofluorimetric method (RSD% < 8)	Biosensor method (RSD% < 5)	Spectrofluorimetric method (RSD% < 8)	OXY-Adsorbent test method (RSD% < 10)
Anise	50.00	50.20	48.82	47.83	43.61
Fennel	47.28	47.50	46.08	44.21	41.42
Basil	43.05	44.33	32.37	39.14	43.61
Mint	42.61	41.67	43.05	41.33	39.22
Tarragon	42.38	41.40	43.11	40.27	29.36
Marjoram	38.37	39.02	33.70	38.91	29.36
Rosemary	28.10	28.75	27.69	28.42	40.32
Thyme	24.76	23.83	22.72	21.03	27.17
Parsely	21.20	21.43	15.19	14.61	14.02
Juniper	12.81	12.89	11.55	7.64	15.11
Bay	7.69	6.62	5.90	6.02	7.44
Black pepper	0.29	1.00	0.26	1.00	≈0

All these results are expressed in ORAC units ($n \geqslant 5$).

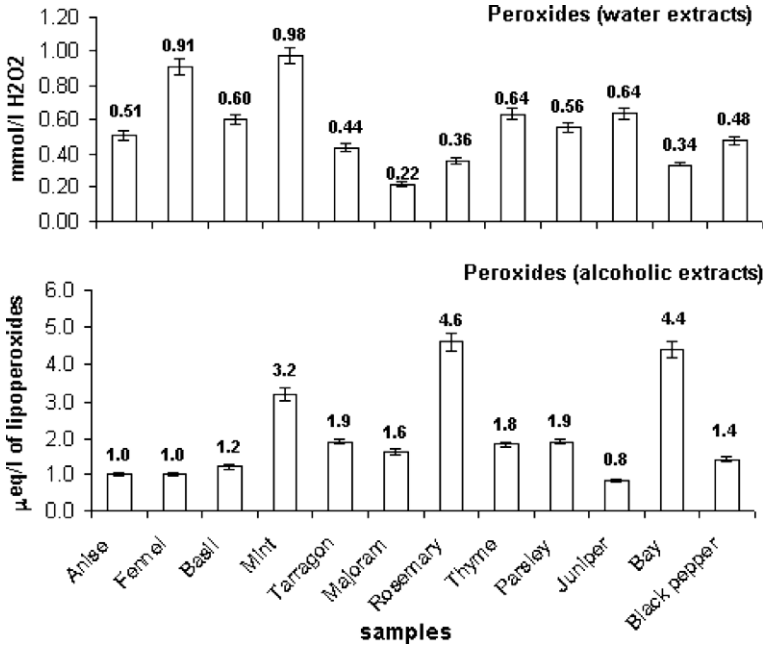

Fig. 3. Trends of peroxides values of dry commercial spices, obtained by the d-ROMs test method (water extracts), and "lipoperoxide test" method (alcoholic extracts), using the same amounts in weight of the homogenized and then centrifuged samples.

in Fig. 2a), this has two very positive implications: the first is that the good correlation between the methods is a further confirmation of the antioxidative capacity scale obtained for the matrixes examined, the second is that, using the equations shown in Fig. 2a, it is possible to convert into ORAC units also the values obtained in RAC units by the biosensor method (to this end, in Table 1, see the comparison of the values determined by this method, with those obtained directly in ORAC units by the spectrofluorimetric method).

Moreover, making a comparison between the, respectively, black histograms and white histograms in Fig. 1, it is found that the antioxidant capacity of the homogenized samples alone is always higher than that of the homogenized and then centrifuged ones. This confirms the observation made in previous works (Campanella et al., 2001; Campanella, Bonanni, & Tomassetti, 2003), i.e., in the case of vegetal matrixes, after the centrifugation operations, that a certain percent of the real antioxidant sample capacity, probably related to antioxidant compounds contained in the micelles in suspension, and which are precipitated during the centrifugation, is lost, as it is not determined later. Lastly, we can still observe how the trend of the values obtained for the homogenized samples is practically the same for the homogenized and centrifuged ones, with the single clear exception of dry basil.

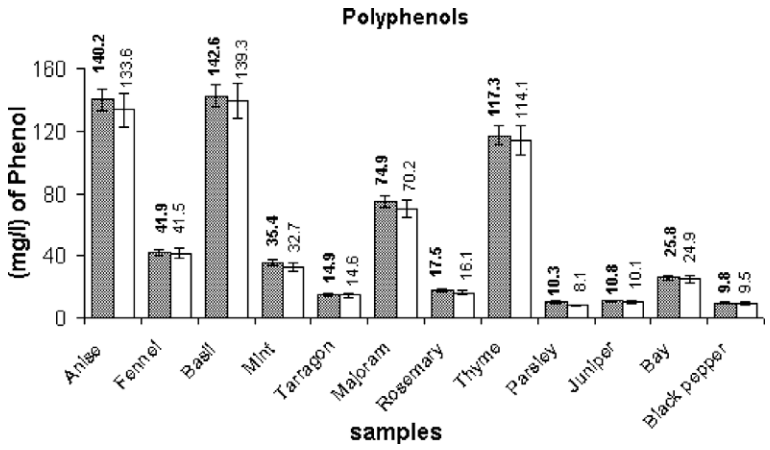

Fig. 4. Trends of polyphenols content values of dry commercial spices, obtained by the tyrosinase biosensor method, using the same amounts in weight of the homogenized samples (black histograms), or homogenized and then centrifuged samples (white histograms).

The OXY-Adsorbent test, even if some inversion is observed (Fig. 1), shows a satisfactory agreement with the biosensor method too (see the correlation straight lines shown in Fig. 2b and the last column of Table 1).

Conversely, the trends obtained, both for the concentration of the ROMs, and for lipoperoxide concentration (Fig. 3), are clearly substantially different from that of the total antioxidant capacity. This was partly to be expected. However, it might also have been expected to find, for example, a reverse correlation, at least between certain limits, but this was not the case. This is probably due to the diversity of the vegetal samples analysed and therefore to the different composition of their vegetal tissues.

On the other hand, the considerable difference found in the polyphenol trend (Fig. 4) with respect to that of total antioxidant capacity (shown in Fig. 1), was less obvious considering our previous experiments on several real matrices (Campanella et al., 2001, 2003; Campanella, Bonanni, Favero, & Tomassetti, 2003). It must also be considered that the trend found was (little, or not at all) affected by the fact of whether the polyphenol test was carried out on the homogenized and therefore centrifuged solutions or on the homogenized ones alone. This represents a further confirmation of the substantial diversity from the point of view of the composition of the vegetal tissue of the spices considered.

4. Conclusion

Taking into consideration the data in the RAC units in particular, the antioxidant capacity analysis provided by the biosensor method indicated that the antioxidant capacity is especially high for anise, fennel, basil, mint, tarragon and marjoram.

Furthermore, the OXY-Adsorbent test also showed a high value for rosemary; while the polyphenol pool turned out to be especially high for anise and mint, thyme and quite high for marjoram.

On the other hand, hydroperoxides (i.e., which are generally the principal reason for the prooxidant properties of the sample) determined in the aqueous extracts, proved especially high in fennel and tarragon, while those determined in the alcoholic extracts turned out to be high in tarragon, rosemary and basil.

What seems less convincing in the experimental data thus obtained, is the finding of a very high antioxidant capacity accompanied by a high hydroperoxide concentration for fennel and tarragon. In the latter case, a non-negligible lipoperoxide concentration was also highlighted. On the other hand, should it be possible to attribute to the inaccuracy of one of the experimental methods used, or to the presence of possible interference in the measurement method, the possible causes of this inaccuracy would have to be sought in the methods of hydroperoxide evaluation, since three different methods were used to evaluate antioxidant capacity, and the same experimental results were always obtained for practically all the dry species samples considered.

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