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Screening of antioxidative activity of spices. A comparison between assays based on ESR spin trapping and electrochemical measurement of oxygen consumption

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Spices belonging to the Labiatae family were used to develop assays for evaluation of antioxidative activity of spices and spice extracts. Two different principles for detection of antioxidative activity were used: (i) an ESR spin trapping technique in which hydroxyl radicals were generated by the Fenton reaction and trapped by 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) in competition with spice extract constituents, (ii) electrochemical measurement of oxygen depletion rate in a heterogeneous lipid/water emulsion with lipid oxidation initiated by metmyoglobin. The ESR free radical method relates to the effect of antioxidant on the initiation of oxidation, while the oxygen depletion method relates to the effect of antioxidant on the propagation of oxidation. For both methods, marjoram and basil showed the lowest activity, while winter savory showed the highest activity as measured by the ESR method, and Turkish oregano and Chilean oregano showed the highest activity as measured by the oxygen depletion method. Total phenol content in the extract correlates linearly with the antioxidant activity as measured by oxygen depletion, but not with the free radical scavenging effect. It is concluded that extracts of the investigated spices contain components with at least two different antioxidative mechanisms. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Certain spices and spice extracts contain components with antioxidative effects (Gerhardt and Schröter, 1983), and it has been shown that addition of spices in sensorially acceptable amounts improves the oxidative stability of cooked meat products (Huisman *et al.*, 1994). The components responsible for the antioxidative effect have been identified for a number of spices such as rosemary (Inatani *et al.*, 1983), sage (Cuvelier *et al.*, 1994), thyme (Miura and Nakatani, 1989) and summer savory (Bertelsen *et al.*, 1995), and it has been recognized that the antioxidative activity in spices is mainly due to phenolic compounds (Herrmann, 1973).

The increasing restriction in the use of synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole and tertiary butylated hydroxyquinone in foods has increased the interest in natural antioxidants, including those present in spices, and during the last decade spice extracts have been marketed as antioxidants for use in the food industry. The optimal substrates for measuring antioxidative activity are the actual food items, as large variation exist between different products. In experiments with foods, spices should be evaluated at concentrations which are sensorially acceptable and with all interfering compounds present, and the food should be stored under realistic storage conditions (Huisman *et al.*, 1994). Storage experiments are, however, time consuming and have to be repeated with different kinds of foods since the antioxidative activity depends on the substrate (Chipault *et al.*, 1956). The development and use of effective screening systems are clearly needed in order to piece together the picture of the antioxidative characteristics of different spices, and in order to reveal which spice should be used in a particular food for an optimal oxidative stability and flavour.

Antioxidative effect is the result of the capacity of the antioxidants to (i) inhibit the initiation of free radical processes, or (ii) to interrupt the chain reactions in the

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propagation of oxidation. The present study used two different methods simultaneously in order to develop an assay, which could provide an overall evaluation of the antioxidative activity of spice and spice extracts. While spin trapping based on electron spin resonance spectroscopy focuses on the early stages of oxidation, the other method is based on electrochemical detection of oxygen depletion with a Clark electrode in an emulsion of an unsaturated lipid model system. The antioxidative activity of nine different spices, all belonging to the Labiatae family, was investigated by both methods.

MATERIALS AND METHODS

Spices and chemicals

Dried leaves of sage (*Salvia officinalis* L.), Turkish oregano (*Origanum vulgare* L.), Chilean oregano (*Origanum onites* L.), rosemary (*Rosmarinus officinalis* L.), summer savory (*Satureja hortensis* L.), winter savory (*Satureja montana* L.), basil (*Ocimum basilicum* L.), hyssop (*Hyssopus officinalis* L.) and marjoram (*Origanum majoranae* L.) were obtained from Paul Müggenburg, GmbH and Co. (Alveslohe, Germany). The authenticity and purity of the herbs were confirmed by microscopy at the Department of Pharmacognosy, Royal Danish School of Pharmacy, Copenhagen. Horse heart metmyoglobin (MMb, type III), methyl linoleate and Tween 20 were obtained from Sigma (St. Louis, Missouri). Analytical grade hydrogen peroxide 30% and analytical grade iron(II) sulphate heptahydrate were from Merck (Darmstadt, Germany), 5,5-dimethyl-1-pyrroline-*N*-oxide 97% (DMPO) was from Aldrich Chemical Co. (Milwaukee, Wisconsin). All other chemicals were of analytical grade, and water was purified through a Millipore Q-plus purification train.

Spice extract preparation

Ethanol (80%, 20 ml) and the dried leaves (2.0 g) were homogenized in an Ultra Turrax homogenizer (13 500 rpm, 1 min). After rinsing the knife of the homogenizer with 5 ml 80% ethanol and centrifugation (2 300 ***g***, 10 min), the supernatant was removed and the residue resuspended in 5 ml 80% ethanol. The suspension was centrifuged and the supernatant was removed. The procedure of resuspending, centrifugation and removing the supernatant were repeated once more. The supernatants were combined to yield approximately 35 ml, and evaporated in a rotary evaporator at 40°C to a final volume of approximately 5 ml, during which process a precipitate was formed. The suspension was transferred with 2×2 ml water and after centrifugation (2 300 ***g***, 10 min), the supernatant was removed. Centrifugation was repeated after resuspension in 4 ml water. The aqueous extract from first and second centrifugation were combined and freeze-dried. The resulting dry material was stored under nitrogen at −18°C until use. The extraction procedure resulted in different amounts of water extractable compounds for the nine species (see Table 1). In both the assay based on ESR spectrometry, and the assay based on oxygen consumption, equal amounts of the extracted material from each spice were resuspended in water. Phenolic compounds in the extracts were quantified using the Folin–Ciocalteu reagent with phenol as standard (Amarine and Ough, 1980) (see Table 1.)

Assay based on ESR spectrometry

The ESR assay was based on competition between DMPO and the antioxidants present in the extracts in scavenging of hydroxyl radicals. Hydroxyl radicals were generated either (i) by the Fenton reaction (H_2O_2/Fe(II)) or (ii) photochemically by homolytical cleavage of H_2O_2 by UV light.

Fenton-based assay

A 0.10 M aqueous solution of DMPO was prepared by direct dissolution and was stored for up to one week at 5°C. The DMPO concentration was controlled prior to use by measuring the absorbance at 228 nm (HP 8452A diode array spectrophotometer, Hewlett-Packard, Palo Alto, California). The DMPO solution itself was found to be ESR-silent. 0.083 M H_2O_2, as determined by

Table 1. Extractables of spices, phenolic compounds in spice extracts and antioxidative index of spice extracts evaluated from initial oxygen consumption rate of metmyoglobin initiated oxidation of a pH 5.8, aqueous methyl linoleate o/w emulsion at 25°C

Spice	Extract[a] (%)	Phenols[b] (meq/g extr.)	Extractable phenols[b] (meq/g spice)	I_{spice}[c]	Significance[d]
Basil	7.5	1.53 ± 0.02	0.11	0.317	A
Marjoram	13.1	3.1 ± 0.1	0.41	0.315	A
Hyssop	11.1	2.61 ± 0.01	0.29	0.242	B
Sage	8.3	3.36 ± 0.03	0.28	0.177	C
Summer savory	16.6	2.9 ± 0.2	0.48	0.158	C
Winter savory	5.0	3.9 ± 0.1	0.20	0.156	C
Rosemary	6.4	5.43 ± 0.08	0.34	0.089	D
Turkish oregano	13.5	4.3 ± 0.2	0.58	0.064	D
Chilean oregano	19.0	5.2 ± 0.2	0.99	0.050	D

[a]Freeze-dried, 80% ethanolic extract based on dried weight. [b]Phenol equivalent determined using the Folin–Ciocalteu reagent. [c]Defined in Equation 2. A common standard deviation of 0.05 is calculated (Fisher's least significant difference). [d]Different letters indicate significance ($p < 0.05$) between I_{spice} for different spices.

titration (AOCS official method Cd 8.53), was used as stock solution. 0.010 M $FeSO_4$ was freshly prepared and used within 4 h. Aqueous extracts of each of the spices were prepared by resuspension of the freeze-dried powders in water. DMPO solution, Fe(II) solution and aqueous spice extract were mixed, and the reaction was initiated by addition of H_2O_2, giving [DMPO]= 9.8 mM, $[Fe^{2+}]=4.9$ μM and $[H_2O_2]=8.3$ μM in the final solution. For each of the spice extracts a concentration of 0.042 mg/ml was used. For Turkish oregano, the effect of extract concentration was further investigated as part of a kinetic analysis. Immediately after mixing, the reaction mixture was transferred to a flat quartz cell (WG-813-TMS) for measurement of aqueous solutions (0.3×13×115 mm, Wilmad Glass Company Inc., Bueno, New Jersey) in the cavity of a Bruker ECS 106 spectrometer (Bruker, Karlsruhe, Germany). Measurements were carried out at room temperature (20 ± 1°C) after exactly 2 min and repeated for the same reaction mixture after exactly 5 min with the following spectrometer settings: centre field 3475.60 G; sweep width 55 G; microwave power 20 mW; modulation frequency 100 kHz; modulation amplitude 1.01 G; receiver gain 1.00×10^5; conversion time 81.92 ms; time constant 163.84 ms and sweep time 83.89 s.

UV light based assay

The reaction mixture, which was obtained by mixing 0.10 M DMPO solution, 0.083 M H_2O_2 solution and each of the spice extracts to obtain a final concentration of 1.0 mM for DMPO, of 1.0 mM for H_2O_2 and 0.125 mg/ml for spice extract, was transferred to a thermostatted (20 ± 0.5°C) quartz cuvette (10×10 mm) and illuminated for 30 or 50 s at 254 nm (Oriel 6035 low-pressure mercury–argon pen-light equipped with an Oriel 6041 short-wave filter as diffuse light source with an approximate intensity of 10^{-7} (einstein/l)/s, Oriel Corporation, Stratford, Connecticut). The irradiated reaction mixture was immediately transferred to the flat quartz cell in the cavity of the ESR spectrometer, and the spectra were recorded using the same instrumental settings as described for the Fenton-based assay.

Assay based on oxygen consumption

To 5.0 ml of a 1.33 mM methyl linoleate emulsion in 5.0 mM aqueous phosphate buffer with pH 5.8 and 0.2 w/w % Tween 20 as emulgator (Jørgensen and Skibsted, 1993) was added 50 μl of spice extract (0.50 mg/ml) and 50 μl 0.2 mM MMb to yield final concentrations of 4.9 μg/ml spice extract and 2.0 μM MMb. Immediately after MMb addition, measurement of the oxygen consumption was started by injection of the sample into a thermostatted (25.0 ± 0.1°C) 70 μl measuring cell (Chemiware, Viby J., Denmark) with no headspace. Oxygen consumption was measured with a Clark electrode (Radiometer, Copenhagen, Denmark), and the relative oxygen concentration was recorded for approximately 20 min at time intervals of 30 s with a PC-based data collecting system.

Statistical analysis

The ESR peak heights reported are the average values of duplicate determinations performed on the same day in two blocks using a freshly prepared Fe(II) solution for each block of analysis. No significant effect of this block was found ($p>0.05$). Results were analysed by a one-way analysis of variance of peak height after 2 min (ph_2) and peak height after 5 min (ph_5). The effect of the spice extracts was compared by using Fisher's least significant difference (LSD). Values of the index of reduction of oxygen consumption (I_{spice}) were analysed by a one-way analysis of variance of I_{spice}, with at least three repetitions for each spice. The two methods were compared by multiple regression analyses of I_{spice} in relation to peak height ph_2, peak height ph_5 and the difference (ph_5-ph_2).

RESULTS

Both the radical scavenging assay and the oxygen consumption assay confirmed that extracts of each of the nine spices belonging to the Labiatae family had antioxidative properties. However, the antioxidative activity was clearly different for the different spices as shown by both methods. The ranking of the antioxidative effects as evaluated by the two methods was moreover different, especially for the spices with strong antioxidative effects.

The extraction procedure resulted in different amount of extract for the nine different spices investigated. The amount of phenolic compounds in the extract likewise showed variation corresponding to extractable phenolic compounds in the dried spices ranging from 0.11 meq/g for basil to 0.99 meq/g for Chilean oregano (Table 1). Extracts of the different spices had different effects on the oxygen depletion in the assay based on metmyoglobin initiated oxidation of the methyl linoleate. The initial oxygen consumption rate $v(O_2)$ in (μmol/l)/s was calculated from:

$$v(O_2) = -\text{slope}[O_2]_{initial} \cdot 10^6/100 \qquad (1)$$

where the slope (percent per second) was calculated from the oxygen consumption in the 80–60% interval of the initial oxygen concentration corresponding to water saturated with air, $[O_2]_{initial}=2.6\times10^{-4}$ M at 25°C. The influence of each of the spice extracts on the initial rate of oxygen consumption was expressed as an antioxidative index relative to the rate in the absence of spice extract:

$$I_{spice} = v(O_2) \text{ with spice present} \; / v(O_2) \text{ without spice present.} \qquad (2)$$

The antioxidative index depends on the content of phenol equivalent in the extract as shown in Fig. 1, and a linear model provides an adequate description ($p<0.01$).

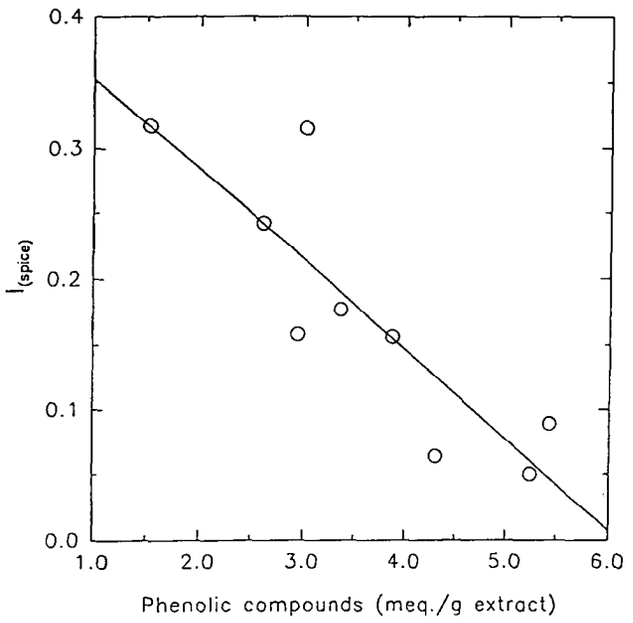

Fig. 1. Dependence of antioxidative index of spices determined from oxygen consumption rate of the content of phenolic compounds of spice extracts. The linear model $I_{spice} = 0.42–6.9 \times 10^{-2}$ meq (phenol) provides ($p < 0.01$) a satisfactory description. Each spice may be identified from Table 1 by the content of phenols.

The antioxidative effect of the spice extracts was further investigated by the ability of the extract to scavenge hydroxyl radicals. Hydroxyl radicals were generated photochemically by homolytic cleavage of H_2O_2 in the presence of the spin trap DMPO under conditions similar to those used by Wolfrum *et al.* (1994). However, illumination of solutions of DMPO without H_2O_2 gave ESR signals with an intensity of 1/3 of the signal for conditions with H_2O_2 present. Similar observations have been reported by Chignell *et al.* (1994) and rationalized on the basis of photochemical formation of an intermediate DMPO cation radical as a precursor for the stable DMPO–OH adduct. Although the assay based on UV light provided some qualitative results, the photochemical degradation of DMPO precluded a more quantitative use. The spin-trapping method based on generation of hydroxyl radicals by the Fenton reaction

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO\cdot \qquad (3)$$

showed no similar complications as the photochemical method, as confirmed by a kinetic analysis for extracts for Turkish oregano. Hydroxyl radicals react readily with DMPO (Janzen, 1980) to give a spin adduct with a characteristic 1:2:2:1 ESR spectrum, as seen in Fig. 2. The second-order rate constant, k_{RETO}, for the reaction between reactants in an extract of Turkish oregano and hydroxyl radicals was estimated by

$$F/(1-F) = k_{RETO}/k_{DMPO} \cdot [RETO]/[DMPO] \qquad (4)$$

in accordance with the analysis of Yoshikawa *et al.* (1993), based on two competing reactions for deactivation of hydroxyl radicals and an independently determined value for the second-order rate constant k_{DMPO} for reaction between DMPO and hydroxyl radicals. [RETO] and [DMPO] are the concentration of reactants in extract of Turkish oregano and the concentration of DMPO, respectively, and F is the fraction of inhibition of formation of hydroxyl adducts of DMPO formed in the presence of extract relative to the reference without extract ($0 \leq F \leq 1$).

For an increasing amount of extract added, the ESR signal gradually became smaller in the concentration range from 0.0042 to 0.17 mg extract/ml, while at concentrations of 0.33 mg and above, the ESR signal

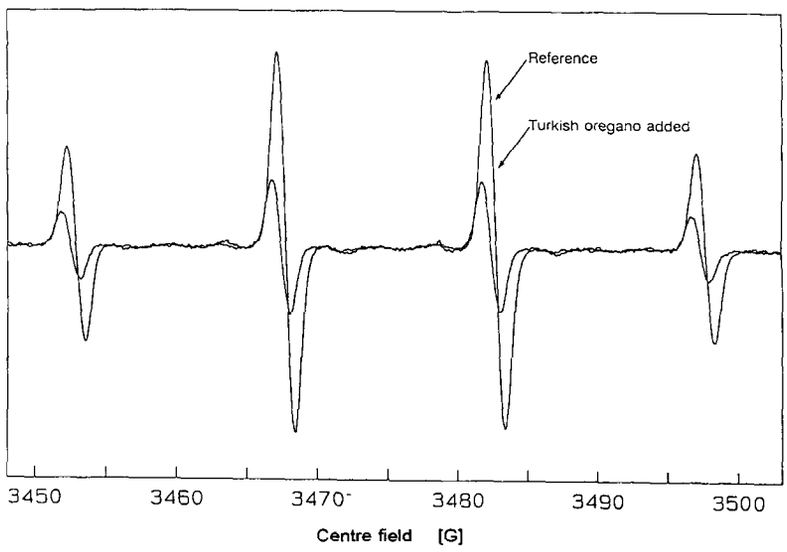

Fig. 2. Effect of extract of Turkish oregano on the formation of a DMPO-hydroxyl adduct in a spin trapping experiment. ESR spectrum marked Reference is recorded 5 min after mixing of an aqueous solution with [DMPO] = 9.8 mM, $[Fe^{2+}] = 4.9$ μM and $[H_2O_2] = 8.3$ μM. ESR spectrum marked Turkish oregano is recorded for the same conditions as Reference but with 0.050 mg/ml extract of Turkish oregano added.

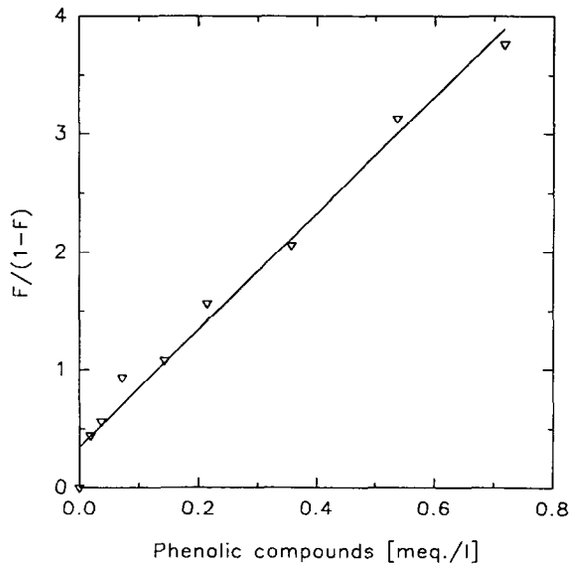

Fig. 3. Dependence of $F/(1-F)$ on concentration of phenolic compounds from extract of Turkish oregano. F is the fraction of hydroxyl adducts of DMPO (9.8 mM) formed by the Fenton reaction in the presence of extract relative to the reference without extract at 20 ± 1°C as measured by ESR spectroscopy. From the linear relationship, an estimate of the rate constant for the reaction of hydroxyl radicals with phenolics in the extract relative to the rate constant for the reaction of hydroxyl radicals with DMPO may be calculated according to Equation 4. Each point is an average of two experiments.

almost vanished, indicating an efficient scavenging activity of extract. In Fig. 3, a linear relationship is demonstrated for extract concentrations up to 0.17 mg/ml corresponding to 0.72 meq phenols/l, as calculated by the result of the phenol analysis of Turkish oregano extract (Table 1). Under the assumption that the reactants of Turkish oregano can be identified as phenols, a second-order rate constant of $k_{RETO} = 9 \times 10^{10}$ M^{-1} s^{-1} is calculated from Equation 4 based on the previously reported value of 2.1×10^9 M^{-1} s^{-1} for k_{DMPO} (Finkelstein *et al.*, 1980).

In order to compare the free radical scavenging activity of the different spice extracts by the spin trapping technique, a series of competition experiments was made each with 0.042 mg extract/ml added. For Turkish oregano, 0.042 mg/ml corresponds to an F-value around 0.5, which provides optimal sensitivity for determination of inhibition activity (Fig. 3). Two different reaction times were used (2 and 5 min, respectively), and some differences in the scavenging activity were noted (Fig. 4). However, for both reaction times a similar pattern is noted with winter savory showing significantly more efficient scavenging activity compared to the other spices, and with basil and marjoram being the least efficient of the tested spices. While the latter observation is the same for both the spin-trapping assay and the assay based on inhibition of oxygen consumption rate, the ranking of the other spices according to the two different assays are different, as may be seen in Fig. 5A, inviting further speculation.

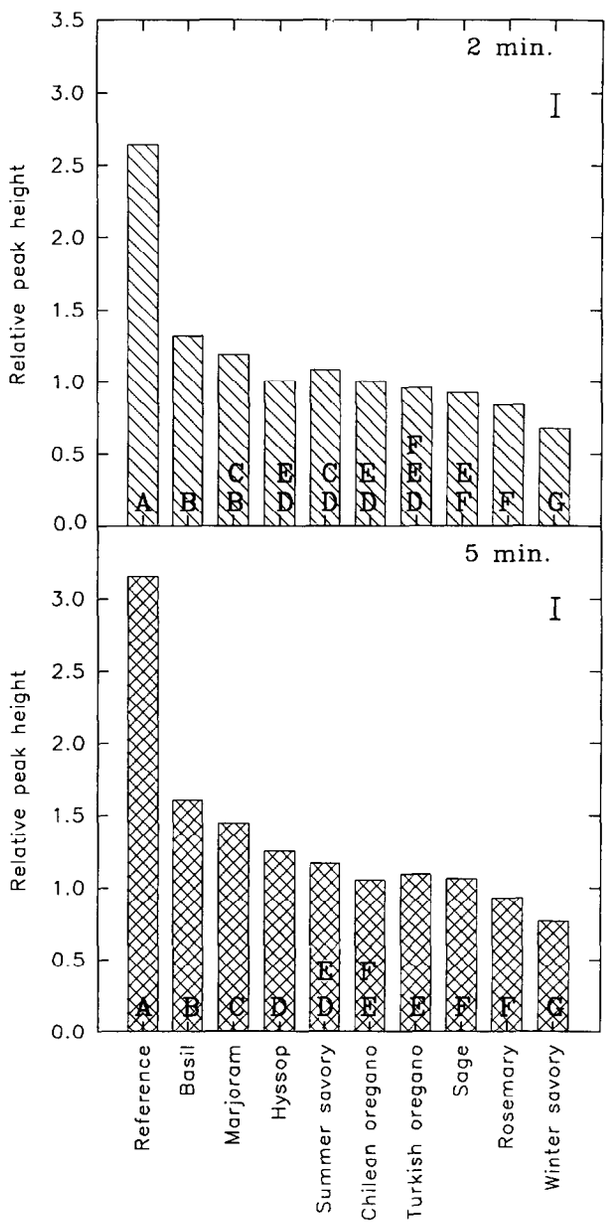

Fig. 4. Peak height of ESR spectra in arbitrary units for competition experiments between spin trap DMPO and spice extract after 2 min and 5 min of reaction time, respectively. Reference: [DMPO] = 9.8 mM, $[Fe^{2+}] = 4.9$ μM and $[H_2O_2] = 8.3$ μM. All other samples as reference but with 0.042 mg/ml of spice-extract added. Columns with different letters are significantly different ($p < 0.05$). A common standard deviation is shown as a bar (Fisher's least significant difference).

DISCUSSION

The ESR method was based on a hydroxyl generating system, and the presence of spice extract diminished the ESR signal (Fig. 2), indicating that compounds in the extracts compete efficiently for the hydroxyl radicals. The relatively high activity and hence small selectivity seen for all of the nine spices could be due to the fact that the hydroxyl radicals are very aggressive and also react with organic compounds other than phenols. A similar spin trapping technique has been used to

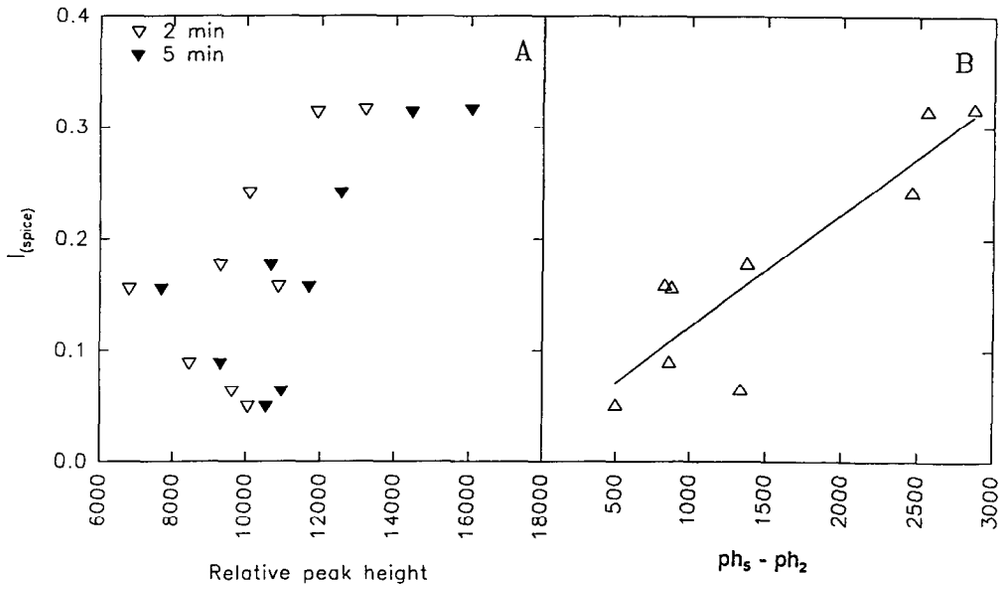

Fig. 5. Antioxidative index of spice extracts evaluated from initial oxygen consumption rate as function of (A) relative peak height obtained from ESR spectra after 2 min and after 5 min, and (B) the difference between the relative peak height after 5 and 2 min; ph_5-ph_2. For B a linear regression line is indicated. Each spice may be identified by the information in Fig. 4 or Table 1.

document *in vitro* antioxidant properties of rebamipide, a novel antiulcer agent (Yoshikawa *et al.*, 1993), a scavenging effect of Chinese herbs for superoxide anions and hydroxyl radicals (Xin *et al.*, 1990) and scavenging activity of carnosine and related dipeptides (Chan *et al.*, 1994). Deighton *et al.* (1993) found that the volatile fraction of thyme, summer savory and oregano contain the compounds carvacrol and thymol, which both have the ability to form stable free radicals with characteristic ESR spectra upon reaction with potassium superoxide. Two of these spices, summer savory and oregano (identical to the Turkish oregano used in the present experiments) also showed a high antioxidative activity in our investigation, even though carvacrol and thymol in the ethanol/water extract would hardly be dissolved in the resuspension step in water. This observation could indicate that antioxidative activity is due to a variety of components including both hydrophilic and lipophilic compounds in those spices. For Turkish oregano, a dose-dependent reduction of the ESR signal of the DMPO–hydroxyl adduct was demonstrated, from which a rate constant for deactivation of hydroxyl radicals could be estimated (Finkelstein *et al.*, 1980). When this kinetic analysis was based on the concentration of phenolic equivalents in the extract, a second-order rate constant slightly above the diffusion limit for water was found, confirming that other compounds in the extract also react with the hydroxyl radicals in competition with phenols and DMPO.

The key role of the phenolic compounds as antioxidants was, however, demonstrated by the oxygen depletion method. The antioxidative index for the different spices, as determined by the reduction in rate of oxygen consumption, was found to depend linearly on equivalents of phenolics present in the extract (Fig. 3).

A comparison of the antioxidative effect of the different spices as evaluated by the two methods may relate to the different mechanisms by which individual compounds present in the herbs influence oxidative processes. Both methods showed a poor antioxidative effect of basil and marjoram. Winter savory had significantly the highest effect on the lowering of the number of hydroxyl radicals captured by DMPO, while this spice only had an intermediate activity as antioxidant as evaluated in the oxygen consumption assay. Rosemary and sage, spices which both have well-documented properties as antioxidants, were able to scavenge hydroxyl radicals, while sage only slightly reduced the rate of oxygen consumption. The rationale behind the difference between the two methods may be that the ESR method also relates to the early stages in oxidation in which radicals are generated, while the oxygen consumption method is a direct measure of the effect of the antioxidants on the progression of lipid oxidation during which alkoxyl and peroxyl radicals are chain carrying. A similar difference has been observed by Hanasaki *et al.* (1994) for flavonoids, which have strong antioxidative activity without having hydroxyl radical or superoxide radical scavenging effect. Both for the flavonoids and the compounds present in the spices belonging to the Labiatae family, further investigation of the mechanisms is clearly needed in order to optimize the use of natural antioxidants in foods. In this context it is interesting to note that while the antioxidant index based on oxygen consumption shows only poor correlation with the free radical scavenging effect after 2 min or after 5 min, the difference ph_5-ph_2 appears to be linearly correlated with the index I_{spice} (cf. Figure 5). The spices which are most effective in scavenging the hydroxyl radicals, i.e. winter savory and rosemary, may on the basis of the ESR assay be expected to have a number of active compounds in common, cf. Bertelsen *et al.* (1995). These active components are not solely phenols as especially winter savory only have a moderate

amount of extractable phenols (Table 1). The observation that the difference ph_5-ph_2, but not ph_2 and ph_5, correlates with I_{spice} points to a differentiation between two groups of compounds active as antioxidants in the spices. One group of non-phenolic compounds act as scavengers of free radicals or metal chelators preventing the Fenton reaction and are effective in early stages of oxidation, and a group of phenols are effective in interrupting the chain processes responsible for oxygen consumption by a mechanism similar to that for the tocopherols. While the antioxidative capacity measured by the ESR assay as ph_2 or ph_5 covers both groups of compounds, the difference ph_5-ph_2 apparently is corrected for the activity of the non-phenolic antioxidants active as initial radical scavengers or as metal chelators halting the Fenton reaction through binding of Fe(II). For practical use, combinations of spices showing high activity in both methods, i.e. rosemary combined with Chilean oregano, may result in better protection of foods from oxidation, and we are currently exploring such synergistic effects.

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