Relevance of Carnosic Acid Concentrations to the Selection of Rosemary, *Rosmarinus officinalis* (L.), Accessions for Optimization of Antioxidant Yield

CAROL R. L. WELLWOOD* AND ROSEMARY A. COLE

The National Herb Centre, Banbury Road, Warmington, nr. Banbury,
Oxfordshire OX17 1DF, United Kingdom

Methods were developed to identify and select accessions of rosemary, *Rosmarinus officinalis* (L.), producing optimum antioxidant activity. Extracts from 12 different rosemary accessions, using three solvents of varying polarity, were assayed for their antioxidant activity, and their major antioxidant compounds were identified and quantified by high-performance liquid chromatography (HPLC). Carnosic acid concentrations were correlated with (i) the free radical scavenging activity of these extracts, as measured by the 2,2-diphenyl-1-picrylhydrazyl assay (adjusted $R^2 = 77.3\%$) and (ii) their inhibition of linoleic acid oxidation, as measured by the β-carotene assay (adjusted $R^2 = 44.1\%$). The correlation was broadly confirmed by the production of volatile aldehydes as measured by the hexanal assay. The variation of carnosic acid concentrations in extracts of 29 accessions, grown in field trials at three sites in England, was determined.

INTRODUCTION

Antioxidants are added to food, cosmetic, and pharmaceutical products in tiny amounts to inhibit the oxidation of lipids, thereby greatly extending the shelf life of lipid-rich products (*1*). Equivocal data on the long-term toxicology of commonly used synthetic antioxidants, such as butylated hydroxytoluene (BHT) and propyl gallate (*2*), have combined with consumer preference for "natural" ingredients to motivate extensive research into effective antioxidants derived from plants. Rosemary has proved to be a rich source of antioxidants, many of which have been isolated and their structures identified (*3*, *4*). Antioxidant compounds in rosemary extracts have been isolated by high-performance liquid chromatography (HPLC), characterized by nuclear magnetic resonance (NMR) (*5*), mass spectrometry (MS), infrared, and UV (*6*) and quantified by HPLC (*7*, *8*). Cuvelier et al. (*8*) identified 22 antioxidant compounds, including carnosic acid, carnosol, and rosmarinic acid, in rosemary and sage extracts using HPLC/UV/MS. Hidalgo et al. (*9*) ascribe the variation of carnosic acid in rosemary to genetic origins, cultivation conditions, and photoperiods. No research has been published on the selection of rosemary accessions for antioxidant yields.

Different studies conflict in their ranking of rosemary antioxidants, possibly owing to differences in evaluation methodologies. The abietane diterpenes carnosic acid and carnosol, along with rosmarinic acid, are generally accepted as the most antioxidant active compounds (*8*, *10*, *11*). Carnosic acid is the major phenolic diterpene present in rosemary leaves. Other antioxidant compounds such as carnosol, rosmanol, and rosmariquinone are formed as it undergoes an oxidative degradation and rearrangement cascade within the plant (*12*) and in vitro (*5*). Rosemary also yields substantial quantities of the polyphenolic antioxidant, rosmarinic acid.

To assess the potential antioxidant yield of an accession, with its complex of antioxidant compounds and their possible synergies, an evaluation methodology is needed that can provide reproducible and realistic results. The evaluation of antioxidants depends on measuring their ability either to scavenge free radicals or to inhibit the oxidation of a lipid-rich substrate, i.e., to delay the onset of oxidative rancidity. Lipid oxidation is characterized by three stages: initiation, forming lipid radicals; propagation via peroxides, forming hydroperoxides; and termination, mainly by the decomposition of hydroperoxides. As antioxidation can involve several possible routes or mechanisms, depending on the oxidizing medium, antioxidant, and temperature (*13*), researchers have stressed the importance of determining inhibition at more than one stage (*14*) and in more than one oxidizing substrate (*11*, *15*).

This paper reports the ranking of rosemary accessions by the relative antioxidant activity of their extracts, determined by reproducible assays at the first two stages of oxidation. The results of those assays are correlated with the concentrations of carnosic acid, carnosol, and rosmarinic acid, determined by HPLC and then confirmed by results from an assay at the third stage of oxidation. The correlation enabled the selection of accessions with a high antioxidant potential on the basis of HPLC analysis.

* To whom correspondence should be addressed. Carol Wellwood, 21 Collapark, Totnes, Devon TQ9 5LN, United Kingdom. E-mail: wellwood@globalnet.co.uk.

MATERIALS AND METHODS

Reagents. Rosmarinic acid was obtained from ICN (Hampshire, United Kingdom), and carnosic acid was obtained from A. G. Scientific (San Diego, CA). All other reagents were obtained from Sigma-Aldrich (Dorset, United Kingdom).

Plants. Plant accessions, including named cultivars, were collected throughout the United Kingdom and Europe, to include plants from as wide a genetic base as possible. All accessions were clonally propagated in order to ensure genetic uniformity, with the exception of numbers 24–27, which were initially raised from seed.

The preliminary trial sampled 12 English accessions, planted in September 1998 at the National Herb Centre (NHC) in Oxfordshire. The plants were well grown by March 2000, when the sampling began.

Six replicates of 33 accessions were planted in September 1999 at each of the three trial sites, in Oxfordshire, Norfolk, and Cornwall; plants of 29 accessions survived and grew sufficiently to provide samples in summer 2000. A randomized block pattern was used to minimize local environmental effects. Plants at the Oxfordshire site were sampled between 2/6/00 and 12/6/00. Samples and extracts were taken from an entire replicate on the same day. Each extract was analyzed separately, and at least three extracts of each accession were analyzed. Plants at the Cornwall site were sampled on 6/7/00, and those at the Norfolk site were sampled on 25/7/00. The plant material for each accession from these sites was pooled for extraction and analysis, generating one sample per accession for each site, with the exception of replicates of accession 11 from the Norfolk site, each sample of which was extracted and analyzed separately to confirm within accession variability.

Plants were sampled by cutting fresh growth, leaves, and tips. Damaged leaves were discarded, and any soil present was washed from the plant material, which was wiped dry before weighing for extraction. Samples from Cornwall and Norfolk were stored at 4 °C for the period (<24 h) between harvesting and extraction.

Sample Extraction. For samples from the preliminary trial, three extraction solvents with a range of polarities were investigated as follows: petroleum ether 40–60, dichloromethane (DCM), and ethanol. For samples from the field trials, a mixture of DCM and ethanol (75:25 v/v) was chosen. The solvent (15 mL) was added to a sample (1 g) of fresh rosemary leaves in a 100 mL Duran bottle. The bottle was incubated in a water bath at 35 °C for 3 h, after which the extract was collected and taken down to dryness at 40 °C with a rotary evaporator. The residue was redissolved in ethanol and made up to 2 mL. The extracts were stored in tightly stoppered brown glass bottles at −20 °C, which allowed samples to be kept for any delay incurred between extraction and analysis, a maximum of 21 days, with no significant loss of compounds.

Antioxidant Activity Assays. *2,2-Diphenyl-1-picrylhydrazyl (DPPH•) Assay.* DPPH• assays were used to measure the free radical scavenging capacity of extracts. DPPH• (0.010 g) was dissolved in methanol (100 mL) to make a stock solution, which was stored in the dark at −20 °C. Aliquots (10 mL) of this stock solution were made up to 50 mL in methanol as required to provide the DPPH• solution for assays, which was stored in the dark and discarded after 3 days. The method was adapted from Bersuder et al. (*16*) and used four mixtures: (i) DPPH• blank, containing aliquots of DPPH• (0.04 mg/mL) solution, ethanol, and deionized water; (ii) DPPH•/antioxidant sample, containing aliquots of DPPH• (0.04 mg/mL) solution, rosemary extract, and deionized water; (iii) control antioxidant sample, containing aliquots of methanol, rosemary extract, and deionized water; and (iv) solvent/spectrophotometer zero, containing aliquots of methanol, ethanol, and deionized water.

These mixtures were shaken and put to stand in a water bath at 25 °C for 30 min, after which their absorbance was measured, using the last mixture to confirm zero on the spectrophotometer. The free radical scavenging activity of a sample was expressed as the percentage disappearance of DPPH•, calculated by:

$$100 \times \{\text{absorbance of DPPH• blank} - (\text{absorbance of DPPH• sample} + \text{absorbance of control sample})\}/\text{absorbance of DPPH• blank}$$

All assays were conducted in triplicate. The reproducibility was initially evaluated by assaying in triplicate the commonly used synthetic antioxidant BHT and both DCM and petroleum ether 40–60 extracts of rosemary.

β-Carotene Assay. β-Carotene assays were used to measure the formation of hydroperoxides in a model emulsion. The method used was that adapted by Shimoni et al. (*17*) from Marco (*18*), with minor modifications: Tween 80 was substituted for Tween 40 and rosemary extracts, or a 95% ethanol control blank (20 μL), were added to the model linoleic acid/β-carotene emulsion (5 mL); aliquots (2 mL) were withdrawn for absorbance measurements and diluted with 95% ethanol (0.5 mL), and the absorbance at 450 nm was measured with a Cecil CE2202 spectrophotometer immediately after the addition of the rosemary extract and again after exactly 3 h of incubation at 50 °C. The relative bleaching of β-carotene was calculated by:

$$(\text{initial sample absorbance} - \text{final sample absorbance})/(\text{initial control absorbance} - \text{final control absorbance})$$

The controls were run concurrently with each set of samples, and all assays were conducted in duplicate. The reproducibility was evaluated by assaying petroleum ether 40–60, ethanol, and DCM extracts of rosemary in triplicate.

Hexanal Assay. The rosemary extract in ethanol (5 μL) or ethanol alone (5 μL) was put into scrupulously clean sample vials and rolled around until the ethanol had evaporated to leave a green powdery residue. The extracts from four accessions, chosen for their differing concentrations of carnosic acid, carnosol, and rosmarinic acid, were used. Fresh beef fat (5 g), obtained from a local supermarket, was transferred into the vials, melted, sealed with a septum cap, and then shaken well before being placed in an oven at 50 °C. The hexanal evolved was measured after 15, 23, and 30 days. The sample vials were taken from the oven and placed into a water bath at 65 °C for 30 min to equilibrate, after which a small hole was pierced in the septum to allow an SPME fiber tip [carbowax (30 μm)/divinylbenzene (50 μm)/poly(dimethylsiloxane)] to be exposed to the headspace for 20 min. This tip was then desorbed for 2 min (splitless injection) onto a Perkin-Elmer 8320 capillary GC with Supelcowax-10 fused silica column (30 m × 0.25 mm × 0.25 μm film thickness). Hydrogen was used as the carrier gas with the injector and detector set to 250 °C. The temperature was programmed from 37 to 125 °C at 6 °C/min, then 125 to 250 °C at 20 °C/min, and 5 min at 250 °C. New SPME tips were conditioned at 250 °C for 4 h and cleaned at 250 °C for 2 min before each sampling. Hexanal standards in 1 mL of nonoxidized almond oil were calibrated between 0.01 and 0.05 μL/mL oil. A standard of 0.01 μL hexanal in 1 mL of oil, equilibrated and collected by SPME as for the samples, was run each day. The oxidation of each oil sample was considered to be proportional to the concentration of hexanal in the headspace above it.

HPLC Analysis. For HPLC analyses, a method adapted from Cuvelier et al. (*8*) was performed on a reversed phase C18 Hypersil-ODS column (25 cm × 4.6 mm, 5 μm pore size; Supelco, Dorset, England) using a C18 guard column. Twenty microliters of sample was injected. The mobile phase was programmed with a linear gradient from 90% A (840 mL of deionized water with 8.5 mL of acetic acid and 150 mL of acetonitrile), 10% B (methanol), to 100% B in 30 min, with a flow rate of 1.5 mL/min. The system was left to stabilize for 3 min between consecutive injections. The samples were detected by UV at 284 nm (Gilson Holochrome UV detector). Duplicate analyses were initially run to establish reproducibility. The compounds were identified by comparison with the relative retention time of standards and by reference to a published chromatogram (*8*). The absorbance at 284 nm of carnosic acid relative to that of rosmarinic was determined by analysis of both standards together at 0.5 mg/mL each. Carnosic acid was calibrated between 0.5 and 15 mg/mL, and rosmarinic acid was calibrated between 0.2 and 1.0 mg/mL. Rosmarinic acid (1 mg/mL) was used daily as an external standard, and quantification was by comparison with it.

Statistical Analysis. All statistical analyses including coefficient of variation (CV), regression analysis, analysis of variance, and standard deviation (SD) were performed using Genstat for Windows 6.1.

Selection of Rosemary for Antioxidant Yields

J. Agric. Food Chem., Vol. 52, No. 20, 2004   **6103**

Table 1. Concentrations of Carnosic Acid, Rosmarinic Acid and Carnosol (mg/g Fresh Weight, Determined by HPLC Analysis) in Extracts from Fresh Growth of One Plant, Using Three Different Solvents

extraction solvent	carnosic acid	rosmarinic acid	carnosol
petroleum ether 40–60	1.41	0.00	3.01
DCM	16.82	0.12	9.31
ethanol	29.77	2.19	5.03

RESULTS AND DISCUSSIONS

Analysis of Extracts. Different solvents extracted noticeably dissimilar levels of antioxidant compounds from identical plant materials (**Table 1**). HPLC analysis of the DCM and ethanol (75:25 v/v) rosemary extracts (**Figure 1**) was shown to be reproducible with a 4% CV. The relative UV absorbance of rosmarinic acid was determined as 13.9 ± 0.005 (SD) times that of carnosic acid (**Figure 2**). The identity and quantification of carnosic acid and carnosol were confirmed using HPLC with photodiode array and by MS (not reported here).

The sampling strategy frequently required extracting from 30 or more samples in a day. Fresh plant material was used because of the labile nature of carnosic acid and the risk of artifacts being formed during the drying process. While using fresh plant material creates unacceptable variability for most herbaceous plants, the water content of which can range from 80 to 95% when fully turgid to 50% or less when droughted (*19*), it is much less of a problem with sclerophylls such as rosemary, the leaves of which have a significantly lower water content, typically around 70% when fully turgid to 60% when droughted (*20*). Preliminary extractions showed that a 3 h 35 °C waterbath extract had an antioxidant activity (determined by DPPH• assay) comparable with that of a 3 h Soxhlet extract from identical samples. Steeping in the waterbath at 35 °C allowed good solvent penetration of the leaves, yet was gentle enough to avoid heat degradation of the target phenolic antioxidants. Reported antioxidant activities of rosemary extracts have generally related inversely to the extraction temperature (*21*) and directly to the polarity of the solvent used (*22*). However, carnosic acid was known to degrade rapidly in methanol (*21*), especially in the presence of active carbon (*5*), so methanol was not used.

The three solvents investigated in the preliminary antioxidant assessment were chosen to draw out as great a range of antioxidant compounds as possible and determine the most appropriate extraction solvent for the evaluation of accessions. Ethanol extracts contained the highest levels of both carnosic acid and rosmarinic acid, but these did not correlate well with their β-carotene assay results (**Table 2**). Petroleum ether 40−60 extracts performed very poorly in the antioxidant assays and contained negligible amounts of the target phenolics. DCM extracted more carnosol than did ethanol but almost no rosmarinic acid, possibly because rosmarinic acid, being hydrophilic, dissolved preferentially into the of water component of the fresh leaves and remained there when the solvent was removed from their residue. Carnosic acid in DCM extracts provided a sufficiently good correlation with both assay results for the selection of accessions with high antioxidant potential on the basis of HPLC analysis alone, avoiding the need to carry out several antioxidant assays on each extract. However, HPLC analysis revealed that DCM extracted very little rosmarinic acid, which, although it did not correlate well to measured antioxidant activity, has repeatedly been identified as an effective and desirable antioxidant (*23, 24*). As the carnosic acid content of

both DCM and ethanol extracts correlated well with DPPH• assay results, a mixture of 75% DCM and 25% ethanol was used for extractions in the field trials, enabling the gathering of data on rosmarinic acid concentrations without affecting carnosic acid concentrations.

The optimal wavelength for detecting carnosic acid was 284 nm, while that for rosmarinic acid was reported as 328 nm (*8*). However, rosmarinic acid also showed very good absorbance at 284 nm with only a slight shoulder to the peak so this was used as the detection wavelength. Rosmarinic acid was considerably cheaper and more stable than carnosic acid and was therefore used as the daily reference standard, with the UV response ratio (**Figure 2**) of 13.9 being used to calculate concentrations of carnosic acid and the structurally similar carnosol.

Antioxidant Assays. Extracts in each of the three solvents, from the 12 accessions planted in 1998, were assayed for antioxidant activity by the DPPH• and β-carotene assays. Preliminary experiments ascertained that absorbances in the DPPH• assay ceased to change rapidly after 30 min and those in the β-carotene assay after 2 h (*20*). The DPPH• assay was rapid and reproducible and yielded consistent results with both DCM and ethanol extracts. However, petroleum ether 40−60 extracts showed much lower and much less consistent levels of free radical scavenging in the assay and so were abandoned. The β-carotene assay took longer and yielded consistent results for the majority of extracts.

The use of crude extracts not only enabled rapid comparison of the antioxidative potential of accessions but also avoided the loss or degradation of carnosic acid during refinement processes (*5, 21*). The extract control used in the DPPH• assay allowed for any absorbance at 516 nm by pigments such as anthocyanins or carotenoids in the antioxidant extract. Interference from other compounds in the crude extracts may account for some of them not yielding consistent results in the β-carotene assay, despite several attempts.

Some official standard methods of measuring antioxidant activity, including peroxide value (PV) and 2-thiobarbituric acid reactive substances (TBARS) were tried but failed to deliver meaningful results. In the case of PV, Reblova et al. (*25*) suggested that rosemary antioxidants stabilized hydroperoxides against decomposition into free radicals, which, while it partially contributed to antioxidant efficiency, would compromise the assay's validity. The TBARS assay appears unsuitable for crude extracts, again owing to interference from other compounds in them. Described by Frankel and Meyer (*26*) as "notoriously unspecific and unreliable", it did not correlate well with flavor panel assessments (*27*), unlike the measurement of headspace volatiles, including hexanal.

Extracts from four accessions, chosen for their differing concentrations of rosmarinic and carnosic acid (**Table 3**), were evaluated by the hexanal assay (**Figure 3**). Because of problems with SPME tips, which were expensive to replace, reproducibility was assessed for hexanal standards only, which were calibrated between 0.01 and 0.05 μL/mL (R^2 adjusted = 96.0%).

Hexanal has been shown to correlate well with the oxidation time of oils and to be the most consistent GC peak among replicates (*14*). It is a breakdown product of linoleic acid. In vegetable oils, fatty acids are usually stabilized by naturally occurring tocopherols, which have been shown to interact in vitro with abietane diterpene antioxidants (*28, 29*), affecting their antioxidant activity. Animal fats have very low tocopherol levels. Beef fat evolved small amounts of hexanal, sufficient for detection by SPME/GC. Lard, almond, corn, safflower, soy, and
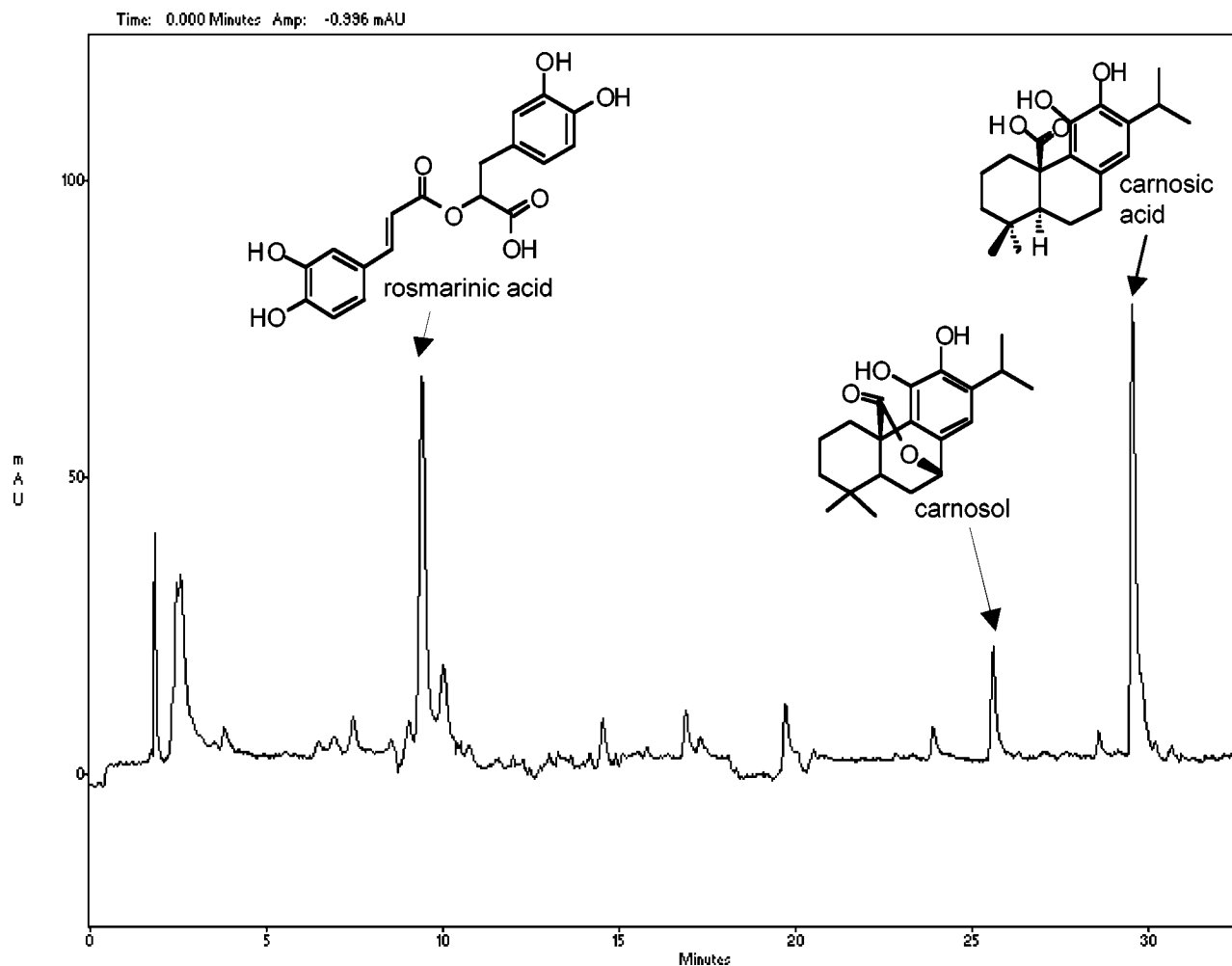
Figure 1. HPLC chromatogram of an ethanol/DCM mixture (25/75 v/v) extract of fresh rosemary leaves and structures of rosmarinic acid, carnosol, and carnosic acid (showing absorbance peaks at 284 nm).
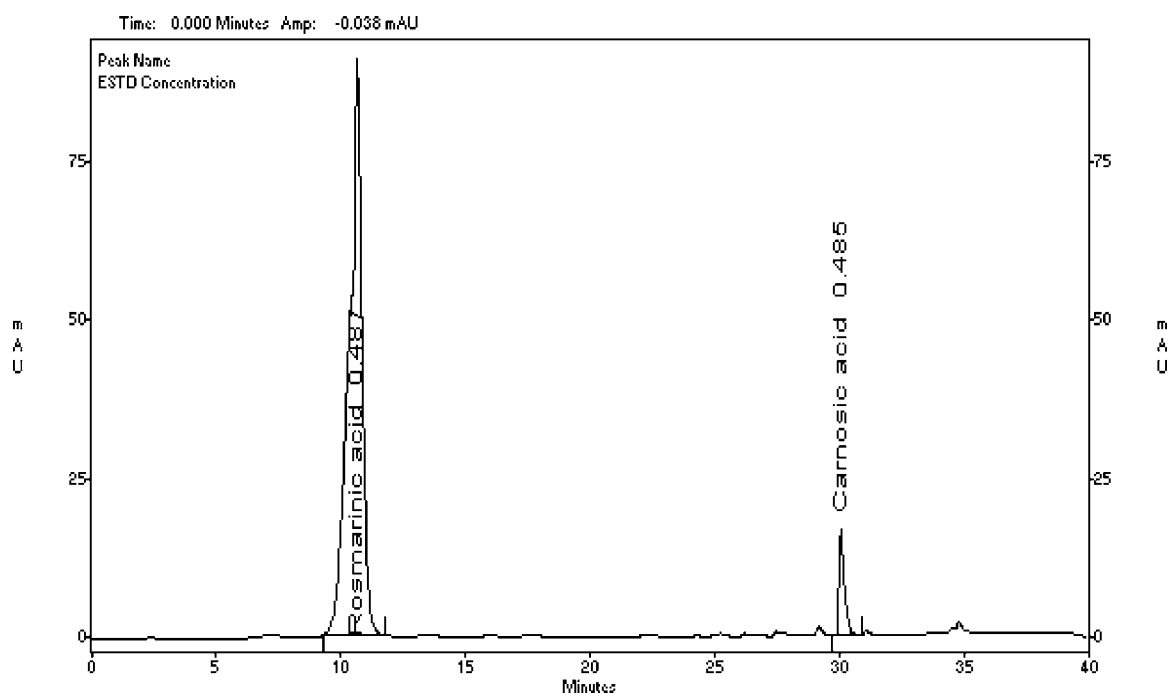

Figure 2. HPLC chromatogram of 0.5 mg/mL standard solutions of rosmarinic acid and carnosic acid (showing absorbance peaks at 284 nm).

sunflower oils were tried as oxidizing media, but beef fat provided the most consistent results. The problems experienced

with SPME tips made the replication necessary to establish sample reproducibility impossible, although the hexanal con-

Selection of Rosemary for Antioxidant Yields

J. Agric. Food Chem., Vol. 52, No. 20, 2004 **6105**

Table 2. Percentage of Antioxidant Activity Accounted for by Concentrations (mg/g Fresh Weight) of Antioxidant Compounds (Adjusted R^2), as Measured by Regression Analysis

solvent	assay	carnosol (%)	carnosic acid (%)	rosmarinic acid (%)	sum of all three peaks (%)
DCM	DPPH•	63.3	77.3	33.1	74.0
	β-carotene	11.7	44.1	a	28.6
ethanol	DPPH•	1.7	66.0	a	21.7
	β-carotene	24.7	a	a	a

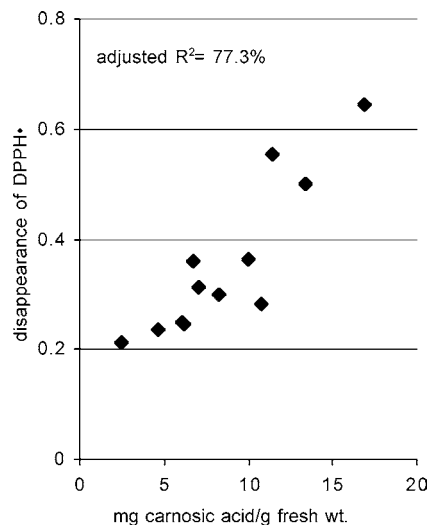[a] Residual variance exceeded variance of response variate.

Table 3. Carnosic Acid, Rosmarinic Acid, and Carnosol Concentrations (mg/g Fresh Weight) of Rosemary Extracts (from Numbered Accessions) Used in the Hexanal Assay

antioxidant: extract from	carnosic acid	rosmarinic acid	carnosol
accession 3	8.91	4.28	1.06
accession 5	23.02	1.90	1.42
accession 12	27.91	3.58	3.51
accession 31	7.50	0.99	0.00
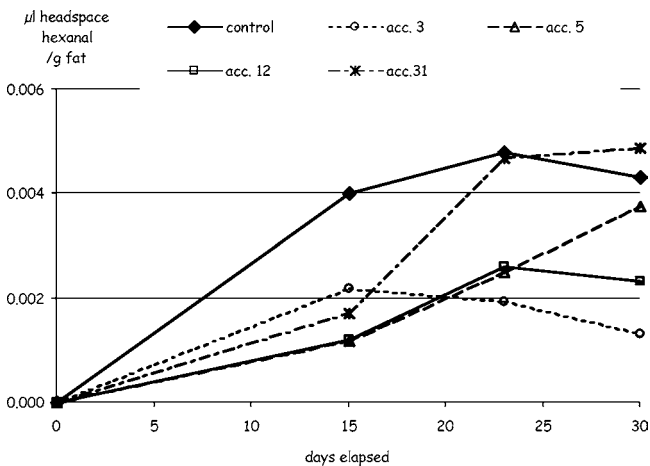
Figure 3. Headspace hexanal evolved over 30 days in beef fat samples treated with extracts from four different rosemary accessions. Lower readings indicate a higher antioxidant activity of extracts.

Figure 4. Correlation between carnosic acid in DCM extracts, measured by HPLC, and their antioxidant activity as measured by the DPPH• assay.
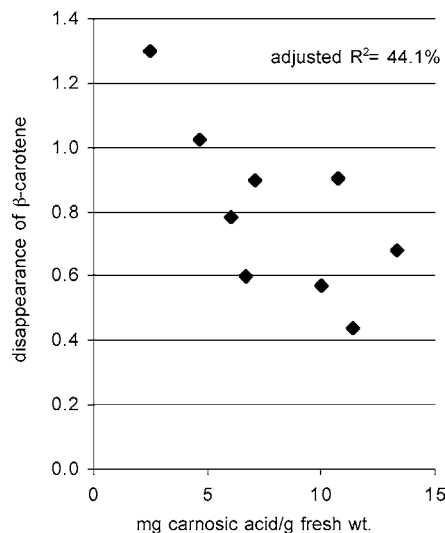
Figure 5. Correlation between carnosic acid in DCM extracts, measured by HPLC, and their antioxidant activity as measured by the β-carotene assay.

centrations in the headspace above the oxidizing fat broadly corresponded to combined levels of rosmarinic and carnosic acids. It is possible that tiny amounts of hexanal escaped through the hole in the septum during oxidation, causing the slight decrease in volumes of hexanal measured after 30 days. It is also likely that the use of crude extracts added to the difficulties encountered in the hexanal assay. As a rule, researchers have used purified antioxidant compounds in purified triglycerides or fatty acid methyl esters when determining the extent of oxidation by measuring hexanal and other volatiles (*8, 30*). This appeared somewhat removed from the "true-to-life" criteria aspired to by the authors but appears to be required for consistency and reproducibility.

Care was taken to choose assays that did not require temperatures in excess of 60 °C, thereby minimizing side reactions that could give misleading results. Methods of accelerating oxidation, necessary for many assays, have been rigorously criticized for failing to use realistic conditions, as have those for determining its progress (*26, 31, 32*). The authors included assays using both bulk oil and emulsion substrates because oxidizing media as well as temperature have been shown to affect the mechanisms involved in antioxidative activity (*13*).

There is as yet no standardized methodology for the evaluation of natural antioxidants, especially in crude extracts.

Statistical Analyses. In the preliminary assessment, each reproducible antioxidant assay yielded a single value relating to the antioxidative activity of an extract. Regression analysis was used to correlate these values with concentrations of rosmarinic acid, carnosol, and carnosic acid in the extracts (**Table 2**). The carnosic acid content of the DCM extracts (**Figures 4** and **5**) proved to be the best predictor of antioxidant activity as measured by both the DPPH• (R^2 adjusted = 77.3%) and the β-carotene (R^2 adjusted = 44.1%) assays. The high degree of correlation between antioxidant activity and carnosic acid concentrations confirmed the literature evaluations of it as the principal antioxidant compound of rosemary (*5, 11, 33*). Ranking of rosemary accessions for antioxidant production potential, by HPLC analysis of their extracts, was therefore based on the carnosic acid content of DCM extracts.

In the field trials, analysis of variance on carnosic acid concentrations of each of the 29 accessions sampled at the Oxfordshire site showed that these concentrations were accession-dependent ($P < 0.001$). The distribution of carnosic acid content was continuous across accessions and its variability
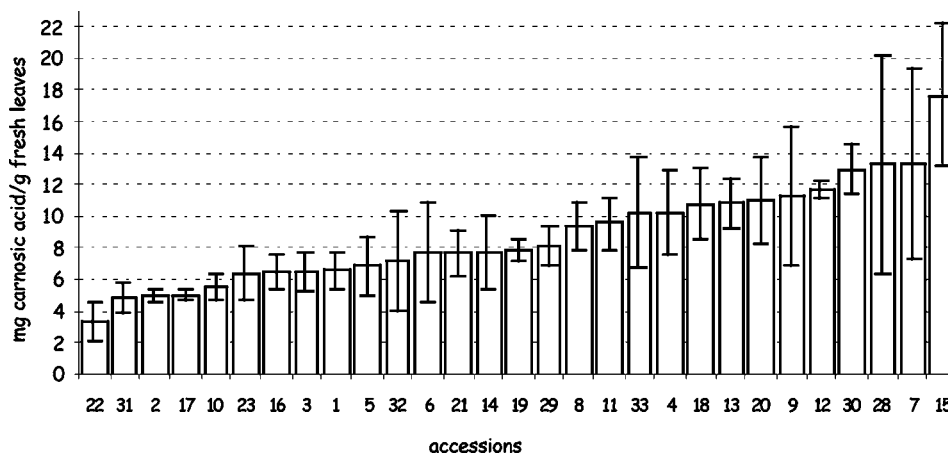
Figure 6. Range of mean concentrations of carnosic acid determined in extracts from 29 rosemary accessions at the Oxfordshire site in summer 2000. Data represent the mean and SD of extracts of at least three replicate plants.

within each accession (as measured by $\pm$ SD) was, for most accessions, far less than that over the range of accessions (**Figure 6**); as expected, SDs of the means tended to be greater with higher concentrations. Mean accession carnosic acid concentrations ranged from 3.4 to 17.7 mg/g fresh weight, i.e., the highest mean concentration was five times that of the lowest, with a least significant difference of 4.5 mg/g. The results of the field trials in Oxfordshire show that the variability of foliar carnosic acid concentrations within accessions was small enough for selection by accession to have a significant effect on yields of the compound per plant.

The six replicates of accession 11 at the Norfolk site, each sample of which was extracted and analyzed separately, had a mean carnosic acid concentration of 7.70 mg/g fresh weight, with a SD of 1.25, confirming the low within-accession variability established in the Oxfordshire trial. Analysis of variance in carnosic acid concentrations in each of the 29 accessions sampled at the Cornwall and Norfolk trial sites (using the mean value for accession 11 at the latter site) and the accession means of the Oxfordshire site showed that they were both accession- and site-dependent ($P < 0.001$ in both cases). Accessions were ranked by their mean carnosic acid content at each site and by the mean for all three sites. The Spearman's rank correlation coefficients for each site as compared to the ranks for means of all three sites were 0.94 for Oxfordshire, 0.96 for Norfolk, and 0.90 for Cornwall ($P = 0.000$), confirming that rank was largely independent of environment.

Plants. Overall plant survival and growth in the field trials was good; plant vigor and survival rates appeared accession specific. Not all accessions proved sufficiently hardy to thrive in the English climate. Some losses were experienced during the winter, although all of the accessions sampled at the Oxfordshire site had at least three survivors. Accession samples from the Cornwall site were pooled before extraction because of the low within-accession variability of carnosic acid demonstrated by the NHC analyses and the larger scale of the losses in Cornwall, where the trial field became waterlogged in the winter.

The low within-accession variability and the similarity in ranking of plants with respect to their carnosic acid content confirmed the validity of selection, independent of environmental factors, as a means of maximizing antioxidant yields per unit biomass. Growers are likely to be paid on carnosic acid rather than biomass yield, so further selection will be for antioxidative activity per hectare, based on a combination of carnosic acid content, annual biomass production, and habit of

growth. Plants with a high carnosic acid concentration but little vigor would not be suitable for commercial cultivation, nor would plants of prostrate habit, which could not be harvested mechanically.

Recent research (*34*) has indicated that water, light, and heat stress negatively affect carnosic acid concentrations. The high levels of carnosic acid in our trial plants suggest that the English climate favors the production of carnosic acid more than the warmer, more arid environment found in Mediterranean countries whence rosemary is typically sourced.

LITERATURE CITED

(1) Coppen, P. P. The use of antioxidants. In *Rancidity in Foods*, 2nd ed.; Allen, J. C., Hamilton, R. J., Eds.; Elsevier Applied Science: London and New York, 1989; pp 83−104.

(2) Barlow, S. M. Toxicological aspects of antioxidants used as food additives. In *Food Antioxidants*; Hudson, B. J. F., Ed.; Elsevier Applied Science: London and New York, 1990; pp 253−307.

(3) Wenkert, E.; Fuchs, A.; McChesney, J. D. Chemical artifacts from the family Labiatae. *J. Org. Chem.* **1965**, *30*, 2931−2934.

(4) Nakatani, N.; Inatani, R. Two antioxidative diterpenes from Rosemary (*Rosmarinus officinalis* L.) and a revised structure for rosmanol. *Agric. Biol. Chem.* **1984**, *48*, 2081−2085.

(5) Richheimer, S. L.; Bailey, D. T.; Bernart, M. W.; Kent, M. C.; Vininski, J. V.; Anderson, L. D. Antioxidant activity and oxidative degradation of phenolic compounds isolated from Rosemary. *Recent Res. Dev. Oil Chem.* **1999**, *3*, 45−48.

(6) Cuvelier, M.-E.; Berset, C.; Richard, H. Antioxidant constituents in Sage (*Salvia officinalis*). *J. Agric. Food Chem.* **1994**, *42*, 665−669.

(7) Schwarz, K.; Ternes, W. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. I. Determination of phenolic diterpenes with antioxidative activity amongst tocochromanols using HPLC. *Z. Lebensm.-Unters. Forsch.* **1992**, *195*, 95−98.

(8) Cuvelier, M.-E.; Richard, H.; Berset, C. Antioxidative activity and phenolic composition of pilot-plant and commercial extracts of Sage and Rosemary. *J. Am. Oil Chem. Soc.* **1996**, *73*, 645−652.

Selection of Rosemary for Antioxidant Yields

J. Agric. Food Chem., Vol. 52, No. 20, 2004 **6107**

(9) Hidalgo, P. J.; Ubera, J. L.; Tena, M. T.; Valcarcel, M. Determination of the carnosic acid content in wild and cultivated Rosmarinus officinalis. *J. Agric. Food Chem.* **1998**, *46*, 2624–2627.

(10) Chen, Q.; Shi, H.; Ho, C.-T. Effects of Rosemary extracts and major constituents on lipid oxidation and soybean lipoxygenase activity. *J. Am. Oil Chem. Soc.* **1992**, *69*, 999–1002.

(11) Frankel, E. N.; Huang, S.-W.; Aeschbach, R.; Prior, E. Antioxidant activity of a Rosemary extract and its constituents, carnosic acid, carnosol and rosmarinic acid, in bulk oil and oil-in-water emulsion. *J. Agric. Food Chem.* **1996**, *44*, 131–135.

(12) Munné-Bosch, S.; Alegre, L. Subcellular compartmentation of the diterpene carnosic acid and its derivatives in the leaves of Rosemary. *Plant Physiol.* **2001**, *125*, 1094–1102.

(13) Denisov, E. T.; Khudyakov, I. V. Mechanisms of action and reactivities of the free radicals of inhibitors. *Chem. Rev.* **1987**, *87*, 1313–1357.

(14) Satue, M. T.; Huang, S.-W.; Frankel, E. N. Effect of natural antioxidants in virgin olive oil on oxidative stability of refined, bleached and deodorized olive oil. *J. Am. Oil Chem. Soc.* **1995**, *72*, 1131–1137.

(15) Huang, S.-W.; Frankel, E. N.; Aeschbach, R.; German, J. B. Partition of selected antioxidants in corn oil–water model systems. *J. Agric. Food Chem.* **1996**, *45*, 1991–1994.

(16) Bersuder, P.; Hole, M.; Smith, G. Antioxidants from a heated histidine-glucose model system. I. Investigation of the antioxidant role of histidine and isolation of antioxidants by high-performance liquid chromatography. *J. Am. Oil Chem. Soc.* **1998**, *75*, 181–187.

(17) Shimoni, E.; Armon, R.; Neeman, I. Antioxidant properties of deferoxamine. *J. Am. Oil Chem. Soc*. **1994**, *71*, 641–644.

(18) Marco, G. J. A rapid method for evaluation of antioxidants. *J. Am. Oil Chem. Soc.* **1968**, *45*, 594–597.

(19) Street, H. E.; Öpik, H. Water relations. In *The Physiology of Flowering Plants: Their Growth and Development*, 3rd ed.; Willis, A. J., Sleigh, M. A., Eds.; Edward Arnold (Publishers): London, United Kingdom, 1984; pp 47–73.

(20) Wellwood, C. R. L. Potential of selection and cultivation for improving yields of antioxidants from rosemary (*Rosmarinus officinalis*, L.) grown in the UK. Ph.D. Thesis, University of Reading, 2004.

(21) Schwarz, K.; Ternes, W. Antioxidative constituents of *Rosmarinus officinalis* and *Salvia officinalis*. II. Isolation of carnosic acid and formation of other phenolic diterpenes. *Z. Lebensm.-Unters. Forsch.* **1992**, *195*, 99–103.

(22) Chang, S. S.; Ostric-Matijasevic, B.; Hsieh, O. A. L.; Huang, C.-L. Natural antioxidants from Rosemary and Sage. *J. Food Sci.* **1977**, *42*, 1102–1106.

(23) Holzmannova, V. Rosmarinic acid and its biological activity. *Chem. Listy* **1996**, *90*, 486-496.

(24) Chen, J. H.; Ho, C. T. Antioxidant activities of caffeic acid and its related hydroxycinnamic acid compounds. *J. Agric. Food Chem.* **1997**, *45*, 2374–2378.

(25) Reblova, Z.; Kudrnova, J.; Trojakova, L.; Pokorny, J. Effect of rosemary extracts on the stabilization of frying oil during deep fat frying. *J. Food Lipids* **1999**, *6*, 13–23.

(26) Frankel, E. N.; Meyer, A. S. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. *J. Sci. Food Agric.* **2000**, *80*, 1925–1941.

(27) Waltking, A. E.; Goetz, A. G. Instrumental determination of flavor stability of fatty foods and its correlation with sensory flavor responses. *Crit. Rev. Food Sci.* **1983**, *19*, 99–132.

(28) Hopia, A. I.; Huang, S.-W.; Schwarz, K.; German, J. B.; Frankel, E. N. Effect of different lipid systems on antioxidant activity of Rosemary constituents carnosol and carnosic acid with and without α-tocopherol. *J. Agric. Food Chem.* **1996**, *44*, 2030–2036.

(29) Huang, S.-W.; Frankel, E. N.; Schwarz, K.; Aeschbach, R.; German, J. B. Antioxidant activity of carnosic acid and methyl carnosate in bulk oils and oil-in-water emulsions. *J. Agric. Food Chem.* **1996**, *44*, 2951–2956.

(30) Marinova, E. M.; Yanishlieva, N. V. Effect of temperature on the antioxidative action of inhibitors in lipid autoxidation. *J. Sci. Food Agric.* **1992**, *60*, 313–318.

(31) Ragnarsson, J. O.; Labuza, T. P. Accelerated shelf life testing for oxidative rancidity in foods—A review. *Food Chem.* **1977**, *2*, 291–308.

(32) Frankel, E. N. In search of better methods to evaluate natural antioxidants and oxidative stability in food lipids. *Trends Food Sci. Technol.* **1993**, *4*, 220–225.

(33) Aruoma, O. I.; Halliwell, B.; Aesbach, R.; Loliger, J. Antioxidant and pro-oxidant properties of active Rosemary constituents: Carnosol and carnosic acid. *Xenobiotica* **1992**, *22*, 257–268.

(34) Munné-Bosch, S.; Alegre, L. Changes in carotenoids, tocopherols and diterpenes during drought and recovery, and the biological significance of chlorophyll loss in Rosmarinus officinalis plants. *Planta* **2000**, *210*, 925–931.