Effects of Rosemary Extracts and Major Constituents on Lipid Oxidation and Soybean Lipoxygenase Activity

Qinyun Chen, Huang Shi and Chi-Tang Ho*

Department of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State University of New Jersey, New Brunswick, New Jersey 08903

Rosemary (*Rosemarinus officinalis* L.) leaves were extracted with three different solvents, namely hexane, acetone and methanol. A reverse-phase high-performance liquid chromatography system in combination with a mass detector was used to quantitate the content of carnosol, carnosic acid and ursolic acid in the rosemary extracts. All rosemary extracts showed strong inhibitory effects on lipid oxidation and soybean lipoxygenase activity.

KEY WORDS: Antioxidant, HPLC analysis, lipoxygenase inhibition, Rancimat, *Rosmarinus officinalis* L.

Antioxidants are added to fats and oils or foods containing fats to prevent the formation of various off-flavors and other objectionable compounds that result from the oxidation of lipids. Butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), the most widely used synthetic antioxidants, have—besides their high stability, low cost, and other practical advantages—unsurpassed efficacy in a variety of food systems (1). However, their use in food has been decreasing because of their suspected action as promoters of carcinogenesis, as well as the general consumer rejection of synthetic food additives (1).

The most important natural antioxidants being exploited commercially are tocopherols. Tocopherols have a potent ability to inhibit lipid peroxidation *in vivo* by trapping peroxyl radicals (2). Unfortunately, tocopherols are much less effective than BHA or BHT as food antioxidants. The search for and development of other antioxidants of natural origin are, therefore, highly desirable. Such new antioxidants might also play a role in combatting carcinogenesis as well as the aging process.

Rosemary leaves are commonly used as a spice and a flavoring agent. The use of the extract from rosemary leaves as an antioxidant was first reported by Ostric-Matijasevic in 1955 (3). Over the years, several reports have appeared on the preparation of rosemary extracts for retarding lipid oxidation (4–7). Several phenolic compounds with antioxidant activities have been isolated and identified from rosemary leaves. Carnosol (I), rosemanol (II), carnosic acid (III) and rosemaridiphenol (IV) (Fig. 1) have the same structural backbone of vicinal diphenol (8–12).

The Rancimat method recently has been used to measure the antioxidant activities of synthetic and natural antioxidants (13,14). This method is both simpler and easier compared to the traditional active oxygen method (AOM). The Rancimat method measures the conductivity changes caused by formation of small free fatty acid molecules when fats and oils are oxidized under elevated temperatures and accelerated aeration. Experimental results (13,14) showed that the Rancimat method and the AOM method correlated well over a range of temperatures (100–120°C) on a variety of fats and oils.

In addition to their antioxidant activities, rosemary extracts inhibit carcinogenesis. Huang *et al.* (15) reported that rosemary extract had strong inhibitory effects on 12-0-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation, ornithine decarboxylase activity and tumor promotion, as well as on arachidonic acid-induced inflammation. The major component of this rosemary extract, carnosol, demonstrates inhibitory activities similar to those of rosemary extract (15). Singletary *et al.* (16) reported that oral administration of 1% crude rosemary extract in the diet to a group of female Sprague-Dawley rats for three weeks before a single i.g. dose of 7,12-dimethylbenz[a]anthracene (DMBA) reduced mammary gland tumor incidence by 47% for 16 weeks after DMBA treatment. In subsequent studies, dietary supplementation with 0.5% and 1% rosemary extract inhibited the binding of DMBA *in vivo* to mammary epithelial cell DNA, as well as the formation of two major DNA adducts (16).

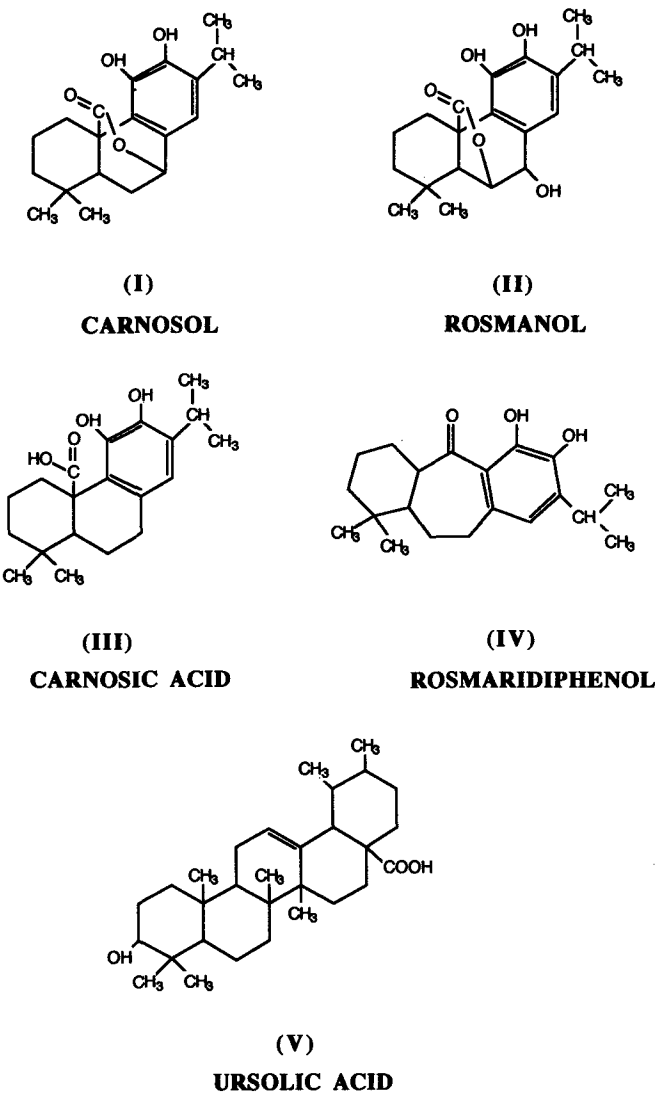

FIG. 1. Structures of five major components in rosemary extracts.

*To whom correspondence should be addressed.

The lipoxygenase pathways of arachidonic acid metabolism produce reactive oxygen species, and these reactive forms of oxygen and other arachidonic acid metabolites may play a role in inflammation and tumor promotion (17,18). Inhibitors of arachidonic acid metabolism also inhibited tumor promotion in animal models (18,19). The most physiologically important mammalian lipoxygenase has been shown to be the arachidonate 5-lipoxygenase (20). There are structural as well as mechanistic similarities between soybean lipoxygenase and mammalian lipoxygenase (21,22). The inhibition of soybean lipoxygenase was, therefore, used by scientists as an *in vitro* method for the screening of the antitumor promotion activity of natural products (23).

The aim of this research was to develop a general procedure for determining major antioxidative components in various rosemary extracts. The inhibitory effects of various rosemary extracts on lipid oxidation and soybean lipoxygenase activity also were studied.

EXPERIMENTAL PROCEDURES

Materials. Rosemary (*Rosemarinus officinalis* L.) leaves were supplied by General Spice, Inc. (South Plainfield, NJ). Solvents were purchased from Fisher Scientific, Inc. (Springfield, NJ). Prime steam lard with no antioxidant added was purchased from Hatfield Quality Meats, Inc. (Hatfield, PA). Lipoxygenase and linoleic acid were purchased from Sigma Chemical Company (St. Louis, MO). Carnosic acid was a gift from Nestec, Ltd. (Vevey, Switzerland). Carnosol and ursolic acid were purified in our laboratory according to previously reported procedures (7).

Preparation of rosemary extracts. Fifty grams of ground rosemary leaves were extracted twice with 250 mL of solvent (hexane, acetone or methanol) at 60°C for 2 h, and the samples were filtered after each extraction. Solvent was removed from the combined extract with a vacuum rotatory evaporator to obtain crude rosemary extracts.

Preparation of bleached rosemary extract. Bleached rosemary extract was prepared according to the previously reported method of Wu *et al.* (7). Fifty grams of ground rosemary leaves were extracted twice with 250 mL of methanol at 60°C for 2 h, and the samples were filtered after each extraction. The combined extract was bleached with 10 g of active carbon at 60°C and then filtered to yield a light brown filtrate. The methanol filtrate was then concentrated to a final volume of 50 mL by vacuum rotatory evaporator and then filtered to remove the precipitates. Water (75 mL) was then added to the filtrate. The precipitates formed after the addition of water were filtered and dried to yield 2.5 g the bleached rosemary extract.

High-performance liquid chromatography (HPLC) analysis of rosemary extracts. HPLC analysis was performed by using a Varian 5000 Liquid Chromatograph (Varian Associates, Inc., Walnut Creek, CA) with an ACS model 750/14 mass detector from Peris Industries (State College, PA). In this detector, the flow of the column moves through a nebulizer and then passes through a tube held at a temperature at which the solvent vaporizes and the solute forms small droplets. The light scattered by the droplets is measured by a photomultiplier (24). The detector was operated at a temperature of 50°C and 20 psig air pressure. The column was a Whatman PartiSphere (Whatman Inc., Clifton, NJ) C_{18} column (12.5 × 0.46 cm i.d.). The column was run with the following ternary solvent system: solvent A, water with 1% acetic acid; solvent B, acetonitrile; and solvent C, methanol with 1% acetic acid. The mobile phase was programmed linearly from 30% A, 70% C (T_o) to 5% A, 5% B, 90% C (T_{30}) in 30 min and then to 100% B (T_{35}) in another 5 min, and held at this final condition for 15 min. The flow rate was 0.7 mL/min.

Evaluation of the antioxidant activity by the Rancimat method. Pure lard (pork fat) without any additives was used as the substrate to evaluate the antioxidant activity of rosemary extracts and three of their components. The test samples were prepared in duplicate by mixing the rosemary extract or component with lard in 0.02% concentration on a weight basis. A 670 Rancimat (Metrohm AG, Herisau, Switzerland) was used. A 2.5-g portion of each test sample was loaded into the reaction vessel cylinder. Six different samples were conducted in one batch. The air supply was maintained at 20 mL/min and the heating temperature was kept at 110°C throughout the experiment.

Lipoxygenase assay. Lipoxygenase (Sigma; Type V) activity was analyzed according to the method of Block *et al.* (23). Linoleic acid (Sigma; final concentration, 1.2 mM) was used as the substrate in a 0.1 M, pH 8.5, Tris buffer at 22°C. The absorbance at 234 nm was recorded as a function of time on a Hitachi U-3110 UV spectrophotometer (Hitachi Co., Danbury, CT). A sample containing all of the reagents except the enzyme solution was used as the blank control. Various concentrations of inhibitor were added to the enzyme and the mixture was incubated for 5 min. The residual enzyme activity was then measured as described above. All treatments were run in triplicate.

RESULTS AND DISCUSSION

Effect of solvent on yield and composition of rosemary extract. Crude and refined extracts of rosemary (*Rosemarinus officinalis* L.) are now commercially available. Manufacturing procedures generally involve two steps (25). In the first step, the essential oils of rosemary are removed by steam distillation. The residue containing the active antioxidant principals is extracted with different solvents, *e.g.*, methanol, ethanol, acetone or hexane. Depending on the degree of purification required, the crude rosemary extracts can be further refined to obtain preparations with a relatively bland odor and color (25).

Reverse-phase HPLC in conjunction with the light-scattering mass detector was developed to quantitatively measure the major composition of rosemary extracts prepared from different solvents, *e.g.*, hexane, acetone or methanol. Figure 2 shows the HPLC chromatograms of hexane, acetone and methanol extracts of rosemary leaves. Table 1 lists the contents of carnosic acid, carnosol and ursolic acid in these extracts. Carnosol and carnosic acid are well-known active antioxidant principals of rosemary (7,25–27). Ursolic acid (V, Fig. 1), on the other hand, is not an active antioxidant (7). Although hexane extract had the highest content of carnosic acid and carnosol (11%, sum of both compounds), due to the low yield of hexane extract, acetone was probably the most efficient solvent to extract the antioxidants from rosemary leaves. Methanol has been considered the solvent of choice by both

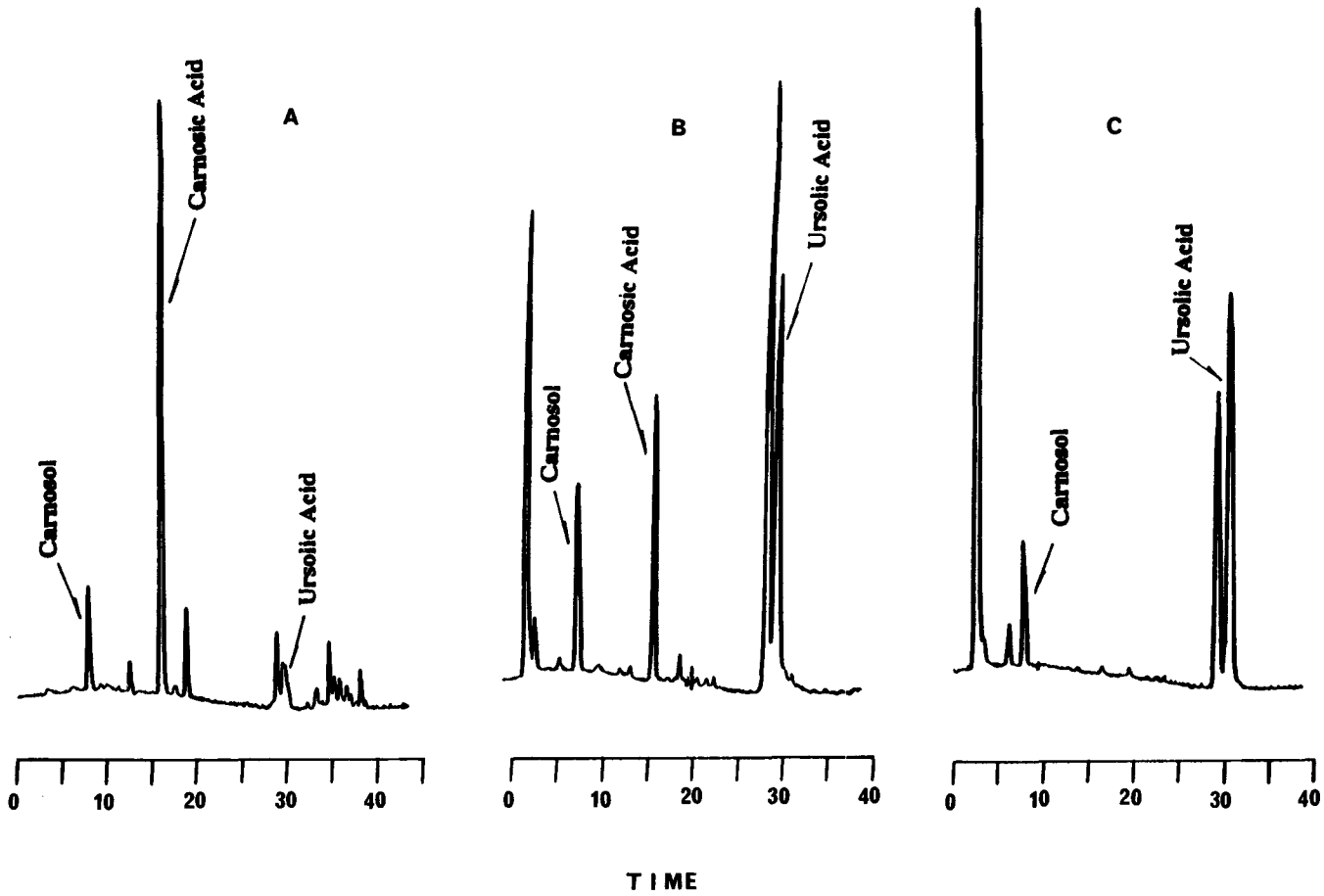

FIG. 2. Reverse-phase high-performance liquid chromatography profiles of rosemary extracts: A, hexane extract; B, acetone extract; and C, methanol extract.

TABLE 1

Yields and Major Antioxidant Components of Rosemary Extracts[a]

Extraction solvent	Yield of extract (%)	Carnosic acid (mg/g)	Carnosol (mg/g)	Ursolic acid (mg/g)
Hexane	4.2 ± 0.5	100.3 ± 2.4	16.4 ± 0.8	13.7 ± 1.7
Acetone	13.8 ± 1.3	58.4 ± 2.1	36.5 ± 2.3	88.5 ± 8.6
Methanol	26.0 ± 1.8	trace	24.1 ± 1.9	76.6 ± 9.4
Methanol followed by bleaching	5.0 ± 0.7	trace	42.7 ± 4.5	180.0 ± 11.8

[a]Data are the mean value of duplicate determinations.

Chang *et al.* (4) and Wu *et al.* (5) for the extraction of rosemary antioxidants, due to its high extraction efficiency. It is interesting to note that methanol extract of rosemary contains 2.41% of carnosol but only a trace amount of carnosic acid.

Table 1 also includes the yield and composition of bleached rosemary extract prepared as reported by Wu *et al.* (5). The active carbon bleaching followed by water precipitation concentrated the components carnosol and ursolic acid.

Antioxidative activities of rosemary extracts. The antioxidant activities of rosemary extracts and purified components were measured by the Rancimat method. The induction times of lard with rosemary extract or with its purified component added are shown in Table 2. Longer induction times suggest stronger antioxidant activities. Both carnosol and carnosic acid had stronger antioxidant activities than the commonly known antioxidants BHT and BHA. The antioxidant activities of hexane extract and acetone extract of rosemary also showed stronger antioxidant activities than did both BHA and BHT. The methanol extract had a longer induction time than did BHA, but had a slightly shorter induction time than that of BHT. It seems that the antioxidant activities of rosemary extracts depend mainly on their content of carnosic acid and carnosol.

TABLE 2

Antioxidant and Lipoxygenase-Inhibitory Activities of Rosemary Extracts and Three Major Components

Sample	Induction time[a] (h)	Lipoxygenase inhibition[b] (IC_{50})[c] (μM)	(μg)
Control (pure lard)	1.65 ± 0.02		
Butylated hydroxytoluene	5.48 ± 0.05	0.6	0.1[d]
Butylated hydroxyanisole	9.02 ± 0.07		
Hexane extract	18.90 ± 0.12		1.25 ± 0.03
Acetone extract	14.40 ± 0.09		2.32 ± 0.03
Methanol extract	7.10 ± 0.04		2.59 ± 0.02
Methanol followed by bleaching	6.35 ± 0.05		4.92 ± 0.04
Carnosol	25.40 ± 0.22	2.24 ± 0.04	0.71 ± 0.01
Carnosic acid	30.60 ± 0.20	6.82 ± 0.09	2.31 ± 0.03
Ursolic acid	2.47 ± 0.02	28.61 ± 0.09	13.00 ± 0.04

[a]Average of duplicate measurements.
[b]Average of triplicate measurements.
[c]Concentration of compound added that inhibits 50% of lipoxygenase activity.
[d]Value from Imai *et al.* (ref. 28).

Lipoxygenase inhibitory effects of rosemary extract. The lipoxygenase-inhibitory activities of rosemary extracts and their isolated components were measured. Because the rosemary extracts are a mixture of compounds, we can only obtain the IC_{50} (concentration at which 50% of enzymic activity is inhibited) values in μg. The rosemary extract displayed IC_{50} values toward soybean 15-lipoxygenase enzyme ranging from 1.3–2.6 μg (Table 2). Carnosol was a more effective lipoxygenase inhibitor than was carnosic acid, and the ursolic acid also showed a great inhibitory effect.

ACKNOWLEDGMENTS

New Jersey Agricultural Experiment Station Publication No. D-10205-4-92, supported by State Funds. We thank Joan Shumsky for her secretarial aid.

REFERENCES

1. Namiki, M., *CRC Crit. Rev. Food Sci. Nutr. 29*:273 (1990).
2. Burton, G.W., and Ingold, K.U., in *Vitamin E: Biochemistry and Health Implications*, edited by A.T. Diplock, L.J. Machlin, L. Packer and W.A. Pryor, The New York Academy of Sciences, New York, 1989, pp. 7–22.
3. Rac, M., and B. Ostric-Matijasevic, *Rev. Fr. Corps Gras 2*:796 (1955).
4. Berner, D.L., and G.A. Jacobson, U.S. Patent 3,732,111 (1973).
5. Chang, S.S., B. Ostric-Matijasevic, O.A.-L. Hsieh and C.-L. Huang, *J. Food Sci. 42*:1102 (1977).
6. Bracco, U., J. Löliger and J.-L. Viret, *J. Am. Oil Chem. Soc. 58*:686 (1981).
7. Tateo, F., M. Fellin, L. Santamaria, A. Bianchi and L. Bianchi, *Perfumer & Flavorist 13(6)*:50 (1988).
8. Wu, J.W., M.-H. Lee, C.-T. Ho and S.S. Chang, *J. Am. Oil Chem. Soc. 59*:339 (1982).
9. Nakatani, N., and R. Inatani, *Agric. Biol. Chem. 45*:2385 (1981).
10. Inatani, R., N. Nakatani, H. Fuwa and H. Seto, *Ibid. 46*:1661 (1982).
11. Nakatani, N., and R. Inatani, *Ibid. 48*:2081 (1984).
12. Houlihan, C.M., C.-T. Ho and S.S. Chang, *J. Am. Oil Chem. Soc. 61*:1036 (1984).
13. Laubli, M.W., and P.A. Bruttel, *Ibid. 63*:792 (1986).
14. Zhang, K.Q., Y. Bao, P. Wu, R.T. Rosen and C.-T. Ho, *J. Agric. Food Chem. 38*:1194 (1990).
15. Huang, M.T., C.T. Ho, S.J. Cheng, J.D. Laskin, K. Stauber, C. Georgiadis and A.H. Conney, *Proc. Amer. Assoc. Cancer Res. 30*:189 (1989).
16. Singletary, K.W., and J.M. Nelshoppen, *Cancer Lett. 60*:169 (1991).
17. Wagner, H., M. Wierer and B. Fessler, *Planta Med. 53*:305 (1987).
18. Huang, M.-T., T. Lysz, T. Ferraro, T.F. Abidi, J.D. Laskin and A.H. Conney, *Cancer Res. 51*:813 (1991).
19. Belman, S., J. Solomon, A. Segal, E. Block and G. Barany, *J. Biochem. Toxicology 4*:151 (1989).
20. Needleman, P., J. Turk, B.A. Jakshik, A.R. Morrison and J.B. Lefkowith, *Ann. Rev. Biochem. 55*:69 (1986).
21. Percival, M.D., *J. Biol. Chem. 266*:10058 (1991).
22. Hamberg, M., and B. Samuelsson, *Ibid. 242*:5329 (1967).
23. Block, E., R. Iyer, S. Grisoni, C. Saha, S. Belman and F.P. Lossing, *J. Am. Chem. Soc. 110*:7813 (1988).
24. Grossberger, T., and E. Rothschild, *LC.GC 7*:439 (1989).
25. Löliger, J., in *Free Radicals and Food Additives*, edited by O.I. Aruoma and B. Halliwell, Taylor & Francis, Ltd., London, 1991, p. 121.
26. Brieskorn, C.H., and H.J. Dömling, *Z. Lebensm. Unters. Forsch. 141*:10 (1969).
27. Schuler, P., in *Food Antioxidants*, edited by B.J.F. Hudson, Elsevier Applied Sciences, Ltd., London, 1990, p. 99.
28. Imai, S., M. Morikiyo, K. Jurihata, Y. Hayakawa and H. Seto, *Agric. Biol. Chem. 54*:2367 (1990).

[Received March 10, 1992; accepted July 16, 1992]