Antioxidant Activity of Lipid-Soluble Phenolic Diterpenes from Rosemary

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ABSTRACT: A high-performance liquid chromatography method for analyzing the phenolic diterpenes present in rosemary (*Rosmarinus officinalis* L.) and commercial rosemary extracts is reported. Carnosic acid was the major phenolic diterpene present in rosemary leaves, with lesser amounts of 12-methoxycarnosic acid and carnosol. Several commercial rosemary extracts also were analyzed by this method, and in addition to these three compounds, other phenolic diterpenes, such as 7-methoxyrosmanol, 7-methoxy-epirosmanol, and rosmanol, were found in some samples. These latter three compounds seem to be artifacts, produced from carnosic acid by oxidation and cyclization. The major phenolic diterpenes were isolated, and their relative antioxidant activities in soybean oil were measured by the Rancimat. The potency of carnosic acid was more than twice that of any other compound. The antioxidant activity of pure carnosic acid was compared to butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butylhydroquinone (TBHQ) and was several times greater than BHT and BHA but less than TBHQ. Nuclear magnetic resonance data for several of the compounds that were incompletely characterized in previous literature are reported. *JAOCS 73*, 507–514 (1996).

KEY WORDS: Antioxidant, carnosic acid, carnosol, diterpene, HPLC, NMR, rancidity, Rancimat, rosemary, soybean oil.

The antioxidant properties of rosemary, *Rosmarinus officinalis* L. (Labiatae), have been known for centuries. Extracts of rosemary are used to prevent the oxidation of fats and the resulting formation of objectionable compounds that produce various off-flavors (1–3). Several phenolic abietane diterpenes have been isolated from rosemary (4–13) (see Fig. 1), and several reports have been written that address the antioxidant potency of pure compounds and extracts by methods that generally measure the prolongation of induction periods for the onset of rancidity (14–19). There also are several reports that identify the compounds that are chiefly responsible for the antioxidant properties of rosemary extracts and that establish carnosic acid (CA) as the major phenolic diterpene present in fresh rosemary (7,14,15,19–21). It also is known that CA is converted to carnosol (CAR) upon heating and that

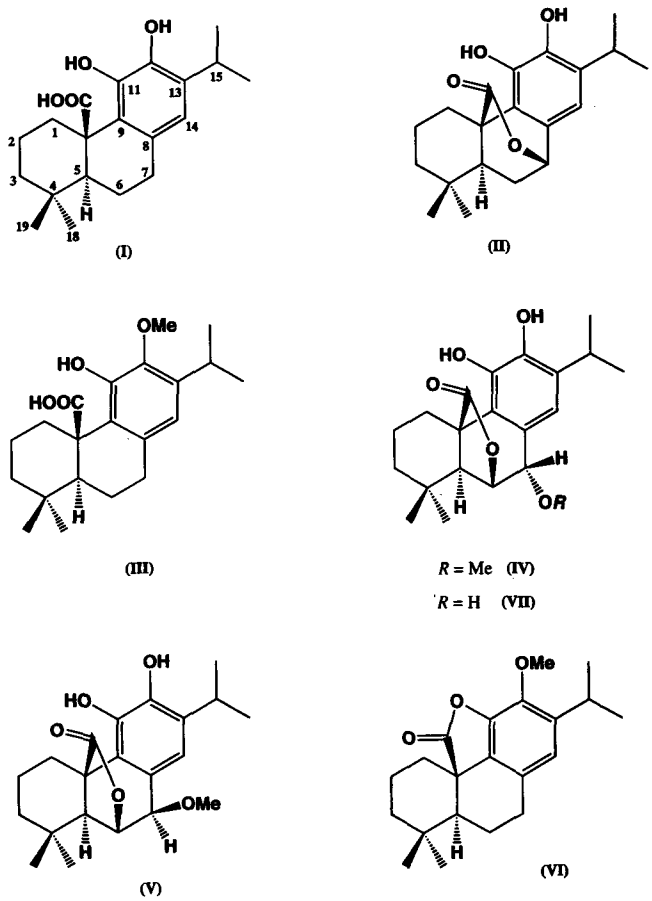

FIG. 1. Chemical structures of phenolic diterpenes from rosemary: (I), carnosic acid; (II), carnosol; (III), 12-methoxycarnosic acid; (IV), 7-methoxyrosmanol, (VII), rosmanol; (V), 7-methoxy-epirosmanol; (VI), carnosic acid, 12-methoxy-γ-lactone.

CAR can degrade further to produce other compounds, such as rosmanol and 7-oxy derivatives of rosmanol (18–20,22, 23). Because some commercial rosemary preparations contain significant amounts of these CA by-products, it is important to know the relative antioxidant activity of all phenolic diterpenes present in rosemary products, so that a total antioxidant index can be determined for products containing a mixture of several different antioxidant compounds.

The Rancimat method has been used successfully to mea-

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sure the antioxidant activities of synthetic and natural antioxidants (1,15,24). The method measures the increase in conductivity that arises when fats and oils are oxidized to smaller free acids under accelerated conditions of heat and aeration. Chen *et al.* (15) reported that this method correlated well over a range of temperatures with the active oxygen method.

The purpose of this research was to develop a method for assaying the major antioxidant phenolic diterpenes present in rosemary and commercial rosemary extracts, and to determine the relative antioxidative activities of these compounds and how they compare to synthetic antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), and tertiary butylhydroquinone (TBHQ). We also report the ^{1}H and ^{13}C nuclear magnetic resonance (NMR) assignments for these molecules where they are lacking in the literature.

EXPERIMENTAL PROCEDURES

Materials. Samples of dried and fresh rosemary (*R. officinalis*) were obtained from growers and collectors worldwide. Samples of commercial rosemary extracts that were derived from *R. officinalis* were obtained from manufacturers. Soybean oil was Wesson brand and was used "as is" without stripping. Solvents were Burdick and Jackson brand from Baxter Scientific Products (Bedford, MA). Phosphoric acid, ethylenediaminetetraacetic acid (EDTA), disodium salt, dihydrate, and trifluoroacetic acid (TFA) were purchased from J.T. Baker (Phillipsburg, NJ). Methanol and reagent alcohol (90:5:5 mixture of ethanol, methanol, and isopropanol) were S/P grade and purchased from Baxter Diagnostics, Inc. (Deerfield, IL), and water was from the output of a Barnsted Model D4754 NANOpure® water system (Dubuque, IA).

Extraction of rosemary biomass. About 10 g of dried whole rosemary leaves was extracted with approximately 75 mL of reagent alcohol on a Buchi Model 810 soxhlet apparatus (Westbury, NY). The apparatus was cycled until the extract appeared colorless (about 15 cycles). After cooling, the extract was transferred quantitatively to a 100-mL volumetric flask with the aid of reagent alcohol and then diluted to volume with reagent alcohol. A portion of each sample was filtered through a 0.45-μm nylon membrane filter before being injected on the high-performance liquid chromatograph (HPLC). Experiments (data not shown) indicated that an equal quantity of antioxidant compounds was extracted from whole rosemary as compared to ground rosemary.

HPLC analyses of rosemary extracts and commercial products. The method was similar to that used by Schwarz and Ternes (7) and Schwarz *et al.* (14). The HPLC system consisted of a Model L-6200 pump, a Model AS-4000 autosampler equipped with a 100-μL loop, and a Model L-4500A diode array detector (Hitachi Instruments, Inc., Fremont, CA). For the selective detection of readily oxidizable analytes, the system also was equipped with a Bioanalytical Systems LC-4B amperometric detector (West Lafayette, IN) set with an applied voltage of +0.8 V. The HPLC system was equipped with a 486 computer and DAD System Manager HPLC software (Hitachi Instruments, Inc., Tokyo, Japan). The column was a 4.6-mm × 25-cm Hypersil ODS, 5μ (Alltech Associates, Deerfield, IL). The column was run at either 1 or 1.5 mL/min with a 65:35 mixture of acetonitrile and water that contained 0.5% phosphoric acid and 1 mM EDTA. The sample in alcohol (10 μL) was injected on the column, and the primary detection wavelength was 230 nm, but spectral data obtained over the range of 215–500 nm were useful in identifying compounds of interest. Because the phenolic diterpenes are not commercially available, and because it was not practical to isolate and purify standards for each of the phenolic diterpenes, an external standard of CA (0.5 mg/mL) in reagent alcohol with 0.5% H_3PO_4 and 1 mM EDTA was used to estimate the concentrations of CA, CAR, 12-methoxycarnosic acid (MCA), rosmanol, and 7-methoxyrosmanol (MR) present in ethanol extracts of rosemary. The ultraviolet (UV) spectra of the various compounds were similar; therefore, the use of CA as an external standard on the HPLC was used to give a good estimate of the concentration of the phenolic diterpenes.

Liquid chromatography/mass spectrometry (LC/MS) analyses. The system consisted of a Fison VG Platform quadrupole mass spectrometer equipped with an electrospray ionization source operated in positive ion mode (Manchester, United Kingdom) and a Hewlett-Packard 1050 liquid chromatograph (Avondale, PA). The mobile phase consisted of a 65:35 mixture of acetonitrile and water with 0.15% TFA flowing at 1 mL/min that was split 80:920 μL/min between the electrospray interface and the UV detector at 230 nm.

NMR spectra. Both ^{1}H and ^{13}C NMR spectra were obtained on a JEOL Eclipse 400-MHz NMR spectrophotometer (Peabody, MA) equipped with a Silicon Graphics Indigo workstation and DELTA NMR software.

*Isolation of CA (**I**).* The method of Paris *et al.* (25) was used. Dried leaves (500 g) were extracted with ethanol at room temperature, and the ethanol was evaporated to dryness under reduced pressure. The dried extract then was mixed with 1 L of *n*-hexane for 70 h and filtered. The extract was concentrated to one-third of its volume and extracted three times with 0.5 L of 5% $NaHCO_3$ solution. The combined bicarbonate fractions were treated with H_3PO_4 to achieve pH 2.2. The acidic solution was then reextracted with *n*-hexane, dried over Na_2SO_4, and concentrated under reduced pressure until off-white crystals appeared. Evaporative recrystallization from *n*-heptane yielded off-white crystals of CA with a melting point of 190°C and purity greater than 95% as indicated by HPLC and NMR (see also Tables 3 and 4).

*Isolation of MCA (**III**).* The combined heptane mother liquors from the crystallizations of CA were evaporated to dryness under reduced pressure and dissolved in a small amount of methanol. MCA was purified *via* semipreparative HPLC on a 2.5-cm × 20-cm NovaPak C-18 cartridge, installed in an RCM 25-cm × 20-cm radial compression holder (Waters Chromatography, Milford, MA). The system was equipped with an Eldex high-pressure pump operating at 15 mL/min, a 290-nm UV detector, and an integrator. The mo-

bile phase was a 65:35 mixture of acetonitrile and water with 0.15% TFA. The methanolic solution (1–3 mL) containing MCA was injected. The eluant from the MCA peak was evaporated under reduced pressure, and the remaining water and TFA were removed *via* lyophilization to give a white powder that was chromatographically and spectroscopically pure. Electrospray LC/MS gave a molecular weight (MW) of 346. Proton and ^{13}C NMR spectra (see also Tables 3 and 4) indicated that this compound was 12-MCA, which was previously isolated from sage by two other groups (26,27). MCA was distinguished from a compound with similar spectrochemical characteristics (28), methyl carnosate, by the fact that it dissolved in sodium bicarbonate solution, it was much less susceptible to oxidation than CA, and upon heating with acetic anhydride, it was converted to a compound that was spectroscopically identical to CA 12-methoxy-γ-lactone (**VI**) [previously reported to be a natural product isolated from sage by Djarmati *et al.* (29)]. The conversion of MCA to the lactone (**VI**) proved conclusively that the methoxy group was located on the 12-position. The ^{1}H and ^{13}C NMR spectra of MCA are given in Tables 3 and 4.

*Isolation of CAR (**II**), 7-MR (**IV**), and 7-methoxy-epirosmanol* (epi-MR) (**V**). Commercial rosemary extract A (3.0 g) was mixed with 75 mL of methanol and filtered. The filtrate then was evaporated to dryness under reduced pressure and mixed with 100 mL boiling *n*-hexane and filtered. The filtrate was evaporated to dryness and dissolved in a minimal volume of methanol. The methanol solution (1–3 mL) was injected on the semipreparative HPLC described above, except that the mobile phase was a 55:45 mixture of acetonitrile and water with 0.15% TFA. Peaks corresponding to 7-epi-MR (MW = 360 by LC/MS), MR (MW = 360 by LC/MS), and CAR eluted between 10 to 15 min in the order listed, and the eluants were separately collected, evaporated, and lyophilized as described above for MCA.

Antioxidant potency by the Rancimat method. A 670 Rancimat (Metrohm AG, Herisau, Switzerland) was used with the air supply maintained at 20 L/h and the heating temperature maintained at 100°C. Four samples plus two controls of soybean oil without added antioxidant were run simultaneously. Each of the four purified rosemary antioxidant compounds (CA, CAR, MCA, and MR; 25.0 mg) was dissolved in 10.0 g soybean oil at 60°C. The four stock solutions were then diluted with soybean oil to make working samples that were 25, 50, 75, and 100 ppm in the soybean oil. Each sample was run in triplicate on the Rancimat. The tests for the CA vs. synthetic antioxidants (BHT, BHA, and TBHQ) were run similarly, except that all four antioxidants were tested at levels of 50, 100, 150, and 200 ppm in soybean oil.

RESULTS AND DISCUSSION

Reversed-phase HPLC method for the assay of the phenolic diterpenes. In the presence of metal ions (iron and copper), dilute solutions of CA readily undergo autoxidation to the orthoquinone derivative and CAR (19,20). For this reason, EDTA was added to the CA standard solutions and to the mobile phase. Figure 2 shows the HPLC chromatograms obtained from an ethanol extract of whole, dried rosemary leaves by both UV detection at 230 nm and an electrochemical detector operating in oxidative mode at +0.8 V applied voltage. The redox detector selectively detects readily oxidizable phenolic compounds (Fig. 2B), most of which also show strong absorbance at 230 nm (Fig. 2A).

Although CA is the principal antioxidant compound found in rosemary, there have been previous reports that identified CAR as the major antioxidant component of rosemary (7,14,30). These investigators may have inadvertently overestimated the amount of CAR as a result of its formation from CA during extraction or from the analytical procedures used. The largest peak in a typical chromatogram of rosemary biomass by either UV or electrochemical detection is CA, followed by CAR and 12-MCA. In addition, some samples have a minuscule amount of rosmanol (**VII**), and other unknown compounds may be observed in the chromatogram, such as the peak with a relative retention time of 1.8 compared to CA (see Fig. 2). The UV spectra of the peaks eluting before rosmanol were not like CA or CAR but were similar to caffeic acid, which suggested that they were derivatives of caffeic acid and other similar phenolic compounds. The UV spectra of most of the other minor unidentified peaks in chromatogram 2A suggested that they were flavonoids. Examination of the chromatograms obtained for rosemary leaves from worldwide sources showed that the principal antioxidant constituents were CA, CAR, and MCA.

Analysis of rosemary biomass and commercial rosemary extracts. Wenkert *et al.* (20) demonstrated that CAR is an ox-

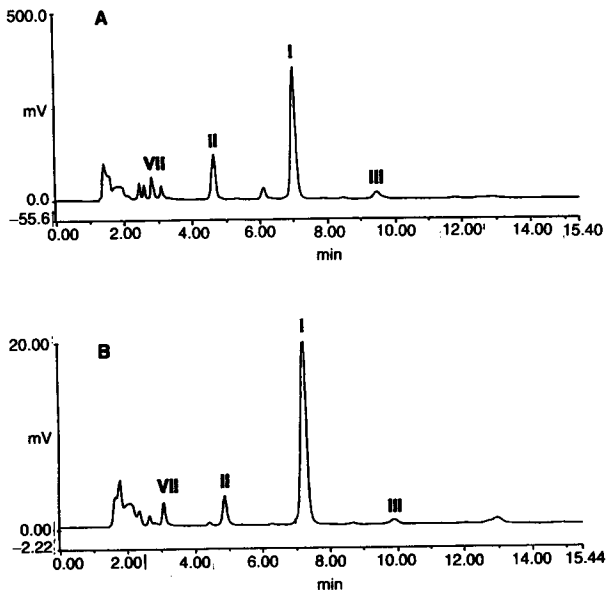

FIG. 2. High-performance liquid chromatography chromatograms of an ethanolic rosemary extract by using (A) ultraviolet detection at 230 nm and (B) electrochemical detection at an applied voltage of +0.8 V: (**I**) = carnosic acid, (**II**) = carnosol, (**III**) = 12-methoxycarnosic acid, and (**VII**) = rosmanol.

TABLE 1
Antioxidant Components in Samples of Rosemary Biomass[a]

Description and grade[b]	Country	Carnosic acid (%)	Carnosol (%)	12-Methoxycarnosic acid (%)	CAR/CA ratio[c]
Dried, spice	United States	3.9	0.4	0.2	10
Undried, fresh spice (leaves)	United States	3.0	0.2	0.1	7
Undried, fresh spice (stems)	United States	0.1	0.1	0.0	62
Dried, wild	Spain	3.5	0.4	0.1	11
Dried, wild	Spain	3.4	0.4	0.1	12
Dried, deoiled, wild	Spain	1.1	0.8	0.1	73
Undried, fresh, wild	Spain	5.5	0.4	0.6	7
Dried, wild	Morocco	4.0	0.3	0.5	8
Dried, wild	Morocco	3.8	0.4	0.7	11
Dried, wild	Albania	1.7	0.4	0.1	24
Dried, wild	Turkey	2.2	0.2	0.1	9
Dried, wild	Tunisia	2.7	0.2	0.1	7

[a]Average for duplicate assays of undried, fresh biomass were corrected for loss on drying, whereas dried samples are reported on an as is basis.
[b]Wild rosemary is usually harvested for its oil content, whereas spice grade is generally cultivated.
[c]Carnosol (CAR) content divided by carnosic acid (CA) content times 100. A measure of CA degradation (see also text).

idative artifact of CA. Aeschbach and Philippossian (21) reported that freshly cut leaves of rosemary do not contain CAR, and that it may be reasonably assumed that rosmanol, rosmaridiphenol (4), and other phenolic diterpenes that are reported present in rosemary or rosemary extracts are artifacts formed from the oxidation of CA. Table 1 lists the amounts of the principal antioxidant compounds found in various samples of rosemary biomass from different geographical regions. The data indicate that CAR is a minor constituent of rosemary leaves. We found that, when the freshly cut leaves from a rosemary plant were assayed before and after air drying, the CAR increased from 1 to 2% (as percentage of total antioxidants). The ratio of CAR to CA in biomass also was higher in stems and in leaves that were commercially deoiled. Hence, our results agree with those of Aeschbach and Philippossian (21) and indicate that CAR is at best a trace constituent of fresh rosemary leaves. CAR may increase at the expense of CA as the biomass is dried, stored, extracted, steam-distilled, etc., to remove rosemary oil.

The amounts of the three principal rosemary antioxidants found in several commercial rosemary products are listed in Table 2. Some of these products are powders that are designed to be mixed with oils, whereas others are dispersions of rosemary extract in vegetable oil plus other inactive ingredients that may make the product dispersible in water. The chief phenolic diterpene found in product **A** was 7-MR (see also Fig. 3). Commercial rosemary extracts largely are used as natural alternatives for synthetic antioxidants. Most of these rosemary extracts are deodorized by removing most or all of the rosemary oil and are bleached to reduce their color (31). Most of the commercial deodorizing processes involve heating the biomass, which converts CA to CAR. Some of the

TABLE 2
Antioxidant Components in Commercial Rosemary Products[a]

Product	Description	Recommended usage level (%)[b]	Carnosic acid (%)	Carnosol (%)	12-Methoxycarnosic acid (%)	7-Methoxy-rosmanol (%)	ECAC[c] (%)
A	Oil-soluble powder	0.02	1.0	9.0	0.2	14.7	12.3
B	Oil-soluble liquid	0.06	4.0	0.7	0.4	ND[d]	4.3
C	Water-dispersible powder	0.06	3.4	0.6	0.2	ND	3.6
D	Oil-dispersible powder	0.04	0.7	9.1	0.2	ND	4.4
E	Water and oil-dispersible liquid	0.2	0.0	0.2	0.0	1.2	0.7
F	Powder for direct use on foods	0.2	0.1	1.7	0.1	ND	0.8
G	Powder for direct use on foods	0.2	1.1	1.7	0.1	ND	1.8
H	Water-dispersible solid	[e]	0.7	0.2	0.1	ND	0.8
I	Water-dispersible liquid	[e]	3.5	0.6	0.2	ND	3.7
J	Water-dispersible liquid	0.4	0.3	0.5	0.0	ND	0.5
K	Oil-soluble powder	0.07	3.6	3.3	0.1	ND	4.9
L	Oil-soluble liquid	0.2	2.3	1.0	0.1	ND	2.7

[a]Percentages are averages for duplicate assays and are on an "as is" weight basis.
[b]Average of range based on fat content as recommended by manufacturer.
[c]ECAC = effective carnosic acid content = (% carnosic acid × 1.0) + (% carnosol × 0.40) + (% 7-methoxyrosmanol × 0.52).
[d]None detected.
[e]No recommended use level provided.

TABLE 3
^{1}H NMR Assignments[a] for Carnosic Acid (I), 12-Methoxycarnosic Acid (III), 7-Methoxyrosmanol (IV), and 7-Methoxy-Epirosmanol (V)

	Carnosic acid (I)			12-Methoxycarnosic acid (III)			7-Methoxyrosmanol (IV)			7-Methoxy-epirosmanol (V)		
H#	δ ^{1}H	Multiplet	*J* (Hz)	δ ^{1}H	Multiplet	*J* (Hz)	δ ^{1}H	Multiplet	*J* (Hz)	δ ^{1}H	Multiplet	*J* (Hz)
1 α	1.14	*ddd*	13,13,3.7	1.10	*ddd*	13,13,4	1.96	*ddd*	13.6,13,5.5	1.92	*ddd*	14,14,5.1
1 β	3.46	*ddd*	13,3.3,3.3	3.69	*m*		3.26	*m*		3.27	*m*	
2 α	1.97	*ddddd*	13,13,13,3.7,3.3	2.35	*ddddd*	13,13,13,4.4	1.47	*ddddd*	13,13,13,3.5,2.6	1.46	*m*	
2 β	1.50	*dddd*	13,3.6,3.3,1.7	1.50	*m*		1.58	*dddd*	13,5.5,3.5,3.5	1.56	*m*	
3 α	1.35	*ddd*	13,13,3.6	1.31	*ddd*	13,13,4	1.27	*ddd*	13,13,3.5	1.24	*ddd*	13,13,3.3
3 β	1.57	*dd*	13,1.7	1.47	*m*		1.42	*br d*	13	1.40	br d	13
5	1.55	*dd*	13,2	1.53	*br d*	12.5	2.17	*s*		1.96	*s*	
6 α	2.42	*dddd*	13,13,8,8	2.26	*m*		4.78	*d*	3.3	5.04	*d*	2.6
6 β	1.85	*dddd*	13,5,5,2	1.81	*br d*	13.2	—			—		
7	2.81	2nd order *dd*		2.81	2nd order *dd*		4.24	*d*	3.3	4.45	*d*	2.6
14	6.52	*s*		6.49	*s*		6.82	*s*		6.89	*s*	
15	3.22	*qq*	6.8,6.8	3.18	*qq*	7,7	3.26	*m*		3.27	*m*	
16	1.17[b]	*d*	6.8	1.17[b]	*d*	7	1.18[b]	*d*	7.0	1.17	*d*	6.8
17	1.18[b]	*d*	6.8	1.18[b]	*d*	7	1.17[b]	*d*	7.0	1.16	*d*	6.8
18	1.01	*s*		0.98	*s*		1.01	*s*		1.04	*s*	
19	0.94	*s*		0.91	*s*		0.91	*s*		0.93	*s*	
–OMe	—			3.67	*s*		3.64	*s*		3.54	*s*	

[a]All spectral data are reported in ppm using acetone-d_6 and are referenced to the central peak of the CHD_2OCD_3 residual signal at 2.04 ppm. Assignments were determined by ^{1}H-^{1}H-correlation spectroscopy, ^{1}H-^{13}C-heteronuclear correlation, and Overhauser effect-difference experiments.
[b]Assignments may be interchanged.

bleaching processes utilize activated charcoal, which was observed to rapidly convert CA to CAR. Therefore, it is not surprising to find that many of the commercial products contain more CAR than CA. Both 7-MR and 7-epi-MR (V) previously were isolated from sage and rosemary (6,7,9,14), but Schwarz and Ternes (19) postulate that these compounds are artifacts formed when CA is heated in the presence of methanol. Djarmati *et al.* (32) isolated a homologous artifact, 7-ethoxyrosmanol, from an ethanolic/supercritical CO_2 extract of sage. We found that, when a small amount of either CA or CAR was heated with methanol in a sealed vial at 100°C for 2 h, most of the starting material disappeared while MR and epi-MR were formed, with the major product being MR. These reports and data suggest that compounds MR and its epimer are artifacts produced when extracts containing CA or CAR are heated in the presence of methanol.

Analysis of compounds by NMR. The NMR spectra for CA, CAR, MR, and epi-MR are listed in Tables 3 and 4. The use of acetone-d_6 provided well-dispersed spectra, separating otherwise overlapping carbon resonances and allowing comparison of the spectra to spectra of related compounds (8). Although the compound was reported decades ago, adequate NMR spectra for CA are lacking in the literature. Moreover,

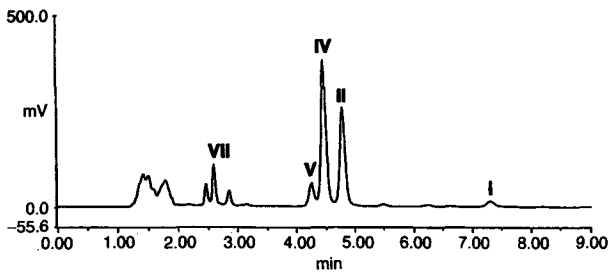

FIG. 3. High-performance liquid chromatography chromatogram of commercial rosemary product A (oil-soluble powder; see Table 2) by ultraviolet detection at 230 nm: **I** = carnosic acid, **II** = carnosol, **IV** = 7-methoxyrosmanol, **V** = 7-methoxy-epirosmanol, and **VII** = rosmanol.

TABLE 4
^{13}C Nuclear Magnetic Resonance Assignments[a] for Carnosic Acid (I), 12-Methoxycarnosic Acid[b] (III), and 7-Methoxy-Epirosmanol (V)

Carbon number	I	III	V
1	35.3	35.0	28.3
2	21.0	20.8	19.7
3	42.5	42.2	38.8
4	34.8	35.0	32.5
5	54.8	54.8	55.7
6	19.7	19.4	79.3
7	32.5	32.9	74.3
8	129.7	134.8	128.8
9	124.9	127.9	124.5
10	49.1	48.4	48.4
11	144.4	149.2	144.4
12	141.7	143.8	142.2
13	134.5	140.0	136.1
14	119.0	118.2	118.8
15	27.6	27.1	27.5
16	23.0[c]	24.0[c]	23.1[c]
17	22.7[c]	23.8[c]	22.9[c]
18	33.0	33.1	31.8
19	21.7	20.6	22.2
20	179.1	177.0	178.0
–OMe	—	61.7	56.3

[a]All spectral data are reported in ppm using acetone-d_6 and are referenced to the central peak of the solvent methyl group at 29.8 ppm. Assignments were determined by ^{1}H-^{1}H-correlation spectroscopy, ^{1}H-^{13}C-heteronuclear correlation, and Overhauser effect-difference experiments and by comparison with literature values (Refs. 8,26).
[b]Spectrum acquired at 35°C.
[c]Assignments may be interchanged.

the carbon data of Schwarz and Ternes (19) erroneously assign C-6 of CA at 56 ppm.

Our isolate of CAR was spectrochemically identical to the published values (8). Our data for 12-MCA correct the inadvertent transposition of the C-5 and –OMe ^{13}C assignments published by Al-Hazimi *et al.* (27) and are in close agreement with the data published for the semisynthetic methyl ester derivative of MCA (26).

A series of nuclear Overhauser effect (nOe) difference experiments (data not shown) enabled us to unequivocally assign 7-methoxyrosmanol and its epimer. When MR was irradiated at H-5 (2.17 ppm), no nOe enhancement was observed for H-7 (4.24 ppm) in the difference spectrum. Moreover, irradiation at H-7 gave no enhancement at H-5. However, when epi-MR was irradiated at H-5 (1.96 ppm), a marked nOe enhancement was observed for H-7 (4.45 ppm). The converse experiment, in which we irradiated at H-7, showed a strong nOe for H-5. These data support a 1,3-diaxial relationship between H-5 and H-7 in epi-MR (12); thus, epi-MR (**V**) is assigned as 7-methoxy-epirosmanol, and MR (**IV**) has the same stereochemistry as rosmanol. The ^{13}C NMR spectrum of compound MR matched that reported by Arisawa *et al.* (9) for this compound. However, when our isolate of MR was dissolved in methanol-d_4, all of its carbon resonances (data not shown) coincided within 0.8 ppm of those that Schwarz and Ternes (19) reported for epi-MR. Together, these data indicate that the German group (7,14,19) misassigned the epimer, and had, in fact, isolated MR (**IV**), not epi-MR (**V**).

Relative antioxidant activities of pure compounds. The antioxidant activities of the purified components of rosemary and MR were measured by the Rancimat method. The method measured the induction time for the onset of rancidity in soybean oil at 100°C. The longer the induction time, the stronger the antioxidant activity. CA, CAR, MCA, and MR each were tested in triplicate at 0, 25, 50, 75, and 100 ppm, and the data are represented graphically in Figure 4. The relative antioxidant activity of the compounds, as compared to CA, can be obtained from the slopes of the curves. If CA is assigned an activity of 1.0, then CAR is 0.40, MR is 0.52, and MCA is zero. Hence, the antioxidant activity of CA in soybean oil was more than twice that of MR and about three times that of CAR. The high activity of CA is somewhat surprising, considering its instability in aqueous solution, especially in the presence of metal ions. Apparently, in hot oil, it is much more resistant to autoxidation than in aqueous solution, and upon oxidative rearrangement it is largely converted to CAR, which is also a potent antioxidant.

Chen *et al.* (15) reported that, in lard, CA had about 1.2 times greater activity than CAR in delaying rancidity in the Rancimat. In our tests, MCA had no antioxidant activity (or perhaps a slight prooxidant effect). This could result from the 12-methoxy group, which prohibits the molecule from easily forming the orthoquinone structure upon oxidation. Each of the potent antioxidants in this group has the *ortho* diphenolic structure adjacent to the isopropyl group.

The antioxidant activities in soybean oil of CA and the

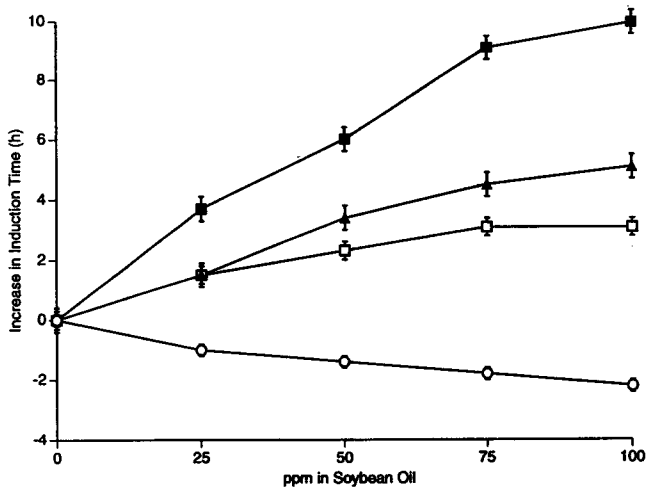

FIG. 4. The effects of —■—, carnosic acid; —□—, carnosol; —▲—, 7-methylrosmanol; and —○—, 12-methoxycarnosic acid on the Rancimat induction time in soybean oil. The induction time of each sample is corrected for the induction time of the control without added antioxidant, which was run concurrently. Bars represent ± SD.

synthetic antioxidants BHT, BHA, and TBHQ were also measured by the Rancimat methodology at levels of 0, 50, 100, 150, and 200 ppm for each compound. The data are represented graphically in Figure 5 and indicate that CA had approximately seven times the antioxidant activity of BHT and BHA (0.14 vs. 1.0) and a little less than half the activity of TBHQ (2.26 vs. 1.0). These results are similar to those reported by Chen *et al.* (15) for the relative activities of these compounds in lard at the 200-ppm level and are considerably higher than those reported by Cuvelier *et al.* (33), who measured antioxidant activities by the disappearance of methyl linoleate under oxidizing conditions (34). The high antioxi-

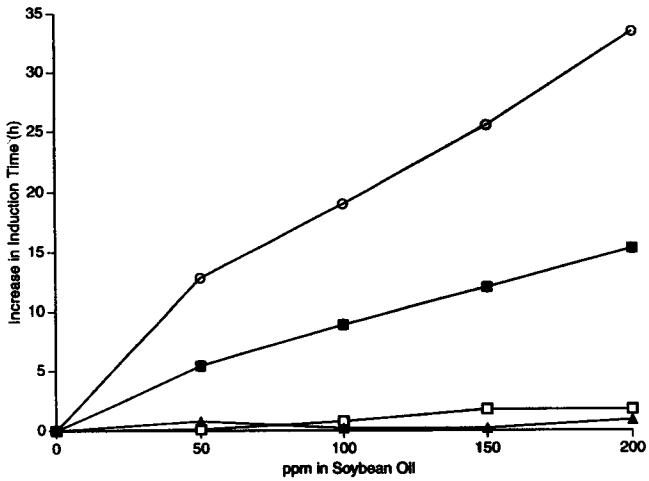

FIG. 5. The effects of —■—, carnosic acid; —□—, butylated hydroxytoluene; —▲—, butylated hydroxyanisole; and —○—, tertiary butylated hydroquinone on the Rancimat induction time in soybean oil. The induction time of each sample is corrected for the induction time of the control without added antioxidant, which was run concurrently.

dant activity of CA would account for the high antioxidant activity of rosemary extracts that is reported by numerous researchers and literature from commercial producers of rosemary extract.

We have shown that the dried leaves of rosemary contain approximately 2–3% CA, a small amount of MCA, and traces of other antioxidant compounds such as CAR and rosmanol. The other phenolic diterpenes that are reported in rosemary extracts (besides CA and MCA) are probably artifacts that are produced during the extraction or purification process (20,21). Hence, CA and 12-MCA are the only phenolic diterpenes that can lay undisputed claim to being natural constituents of rosemary. Of these two compounds, only CA has antioxidant activity and abundance in the plant. There are numerous commercial rosemary extracts that are marketed as natural alternatives to synthetic antioxidants. However, commercial and laboratory extraction, bleaching, and deodorization procedures apparently produce additional quantities of CAR and other artifacts, such as rosmanol, epirosmanol, MR, epi-MR, and rosmaridiphenol (4–9,12,15). CA has much greater antioxidant activity than the other phenolic diterpenes present in rosemary extracts. The total antioxidant potency of such products can be estimated by adjusting the assayed amounts of the other antioxidant potencies to that of CA and determining a total effective carnosic acid content (ECAC). Such an analysis was performed on various commercial rosemary extract products and is included in Table 2. The ECAC is perhaps the best measure of product potency and can be used to rank different products according to total antioxidant activity.

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