various common edible oils and fats, viz., coconut, sesame, rapeseed, mustard, groundnut, cottonseed, soybean, palm-olein, RBD palm oil, nigerseed, rice bran, safflower oils, vanaspati and pure ghee, have been screened following the usual procedure (5) along with castor oil as the internal reference standard. Spots were visualized under iodine vapors and were finally confirmed by charring the components on a hot plate after spraying with perchloric acid (5%). No spot in any of the oils was found within the range (R_f 0.44-0.47) of castor triricinolein, indicating that the TLC method is reliable if the oils under investigation are fresh and free from rancidity. The chromatography was repeated using benzene as developing solvent, but showed very slow migration of triricinolein (0.02), diricinolein (0.07), and monoricinolein (0.1) from castor oil compared to the other oil samples.

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Elucidation of the Chemical Structures of Natural Antioxidants Isolated from Rosemary[1,2]

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ABSTRACT

A natural antioxidant extract with activity greater than BHA and equal to BHT was isolated from rosemary leaves. The extract was separated into 7 primary fractions with liquid chromatography, using silicic acid as an adsorbent followed by gradient elution. Each fraction was rechromatographed to yield a total of 16 subfractions. Two compounds, carnosol and ursolic acid, were identified by infrared, mass and nuclear magnetic resonance spectrometry. Carnosol was shown to be one of the active antioxidant components in rosemary. Ursolic acid was not an effective antioxidant. Further fractionation of the most active antioxidant subfractions by high performance liquid chromatography and the elucidation of the chemical structures of these fractions are now in progress.

INTRODUCTION

About 20 million lb of synthetic antioxidants is used annually by food manufacturers in the United States (1). The compounds currently used—primary butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and tert-butylhydroquinone (TBHQ) are added to a variety of foods.

In recent years, there has been increasing concern over the safety of many synthetic food additives. Antioxidants have been one of the major food additive groups which have come under the review of regulatory agencies. In feeding tests, high levels of the antioxidants BHA, BHT and TBHQ cause significant enlargement of the liver (2). BHT, but not BHA and TBHQ, also increases liver microsomal enzyme activity (2). BHT was removed from the FDA Generally Recognized as Safe (GRAS) list (3).

The use of extract from rosemary spice as a natural antioxidant was first reported by Rac and Ostric-Matijasevic (4). Later, Chang et al. (5) reported a patented process for the extraction of rosemary and sage, followed by a vacuum steam distillation of the extract in an edible oil or fat to obtain an odorless and flavorless natural antioxidant. Its antioxidant activity was demonstrated in both animal fats and vegetable oils. Furthermore, it was able to improve the flavor stability of soybean oil, as well as the flavor stability of potato chips. More recently, Bracco et al. (6) also reported the use of double-step, falling film molecular distillation to obtain an active antioxidant from rosemary extract.

This paper reports the fractionation and identification of one of the active antioxidant components in the extract of rosemary.

EXPERIMENTAL

Preparation of Rosemary Antioxidant

The scheme for the preparation of rosemary antioxidant (RA) is shown in Figure 1.

Three kg of rosemary leaves which had been ground to a fine powder was extracted with 18 L of methanol at 60 C for 2 hr. The mixture was filtered and the residue was extracted again with 12 L of fresh methanol. The combined filtrate was bleached with 600 g of active carbon and then filtered to yield a light-brown filtrate. The methanol solution was then concentrated to ca. 2 L by rotary evaporator and then filtered to remove the precipitates (A). The fil-

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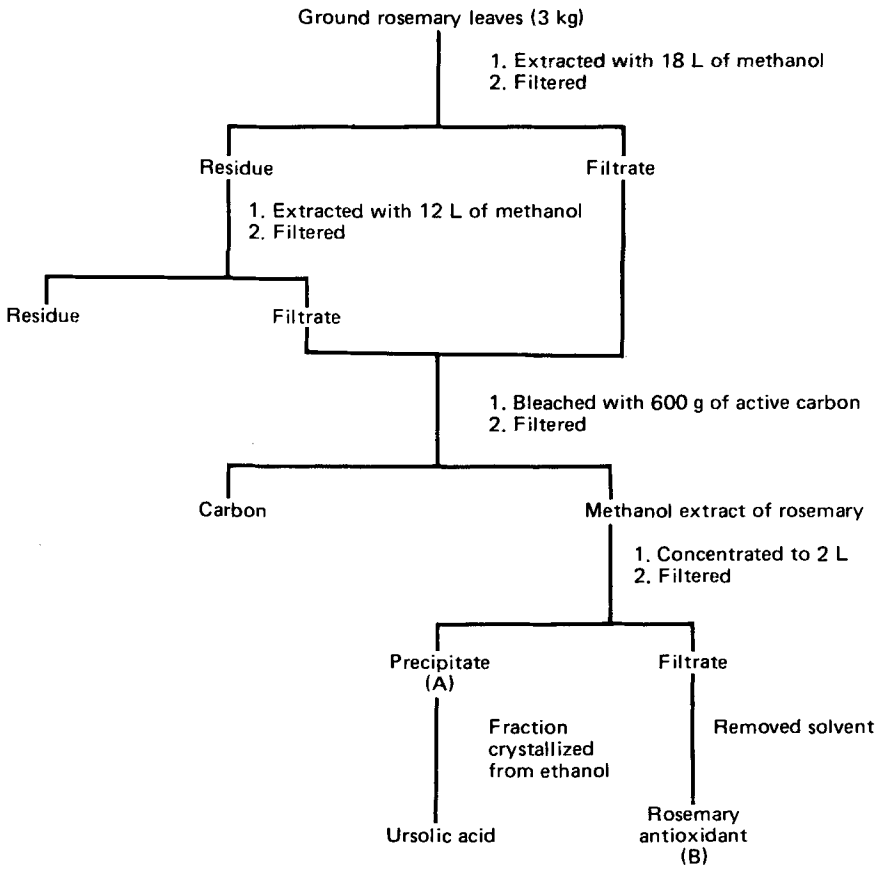

FIG. 1. Scheme for the preparation of rosemary antioxidant.

trate was freed of solvent to yield 3.5% of RA (B).

Fractionation of Rosemary Antioxidant

RA (10 g) was first separated into 7 fractions using a glass column (id, 1.75 in.; length, 23 in.) packed with activated silicic acid (7). The column was eluted by stepwise gradient elution, using 5% ether in hexane, 10% ether in hexane, 25% ether in hexane, 50% ether in hexane, 75% ether in hexane, pure ether and pure methanol.

Each fraction was then rechromatographed on the same silicic acid column to yield a total of 16 fractions (Fig. 2).

Isolation of Unknown Crystal 1 (UC-1) and Unknown Crystal 2 (UC-2)

As shown in Figure 2, fraction 3 was rechromatographed in a silicic acid column using stepwise gradient elution. The initial amount used was a 10% ether-in-hexane solution and the final amount was a 25% ether-in-hexane solution. Four subfractions, 3A, 3B, 3C and 3D were obtained. UC-1 obtained from the last subfraction, 3D, was recrystallized twice from methanol.

UC-2 was obtained by fractional crystallization from precipitate A, as shown in Figure 1. It was recrystallized from methanol to obtain a pure sample for analysis.

Identification of UC-1 and UC-2

Infrared (IR) spectra were obtained in a KBr pellet on a Beckman Acculab-4 infrared spectrophotometer. Mass spectra were obtained by using a DuPont 490 mass spectrometer with a direct insertion probe technique. Source temperature was held at 200 C, ionization voltage was 70 eV and ion current was 300 μA for all analyses. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian Associates Model T-60 NMR spectrometer.

Peroxide Values

Peroxide values were determined by Official Method Cd 8-53 of the American Oil Chemists' Society (8).

RESULTS AND DISCUSSION

Preparation of the Rosemary Antioxidant— A Modified Method

In 1977, Chang et al. (5) reported a patented method for the extraction of an active antioxidant from rosemary and sage. In order to elucidate the chemical structures of the active components in RA, a modified method was used to prepare the RA, which had excellent antioxidant activity. The flow diagram for this method is shown in Figure 1.

In this method, the ground rosemary leaves were extracted twice with methanol. The methanol solution was bleached directly with active carbon. As there was no "water washing" in this method, the RA obtained had a brown color and a slightly spicy flavor. The yield of RA (3.5%) was lower than the RA (10%) obtained by the previously reported method.

Antioxidant Activity of Rosemary Antioxidant

The RA prepared showed an excellent antioxidant activity when added at a concentration of 0.02% into prime steam

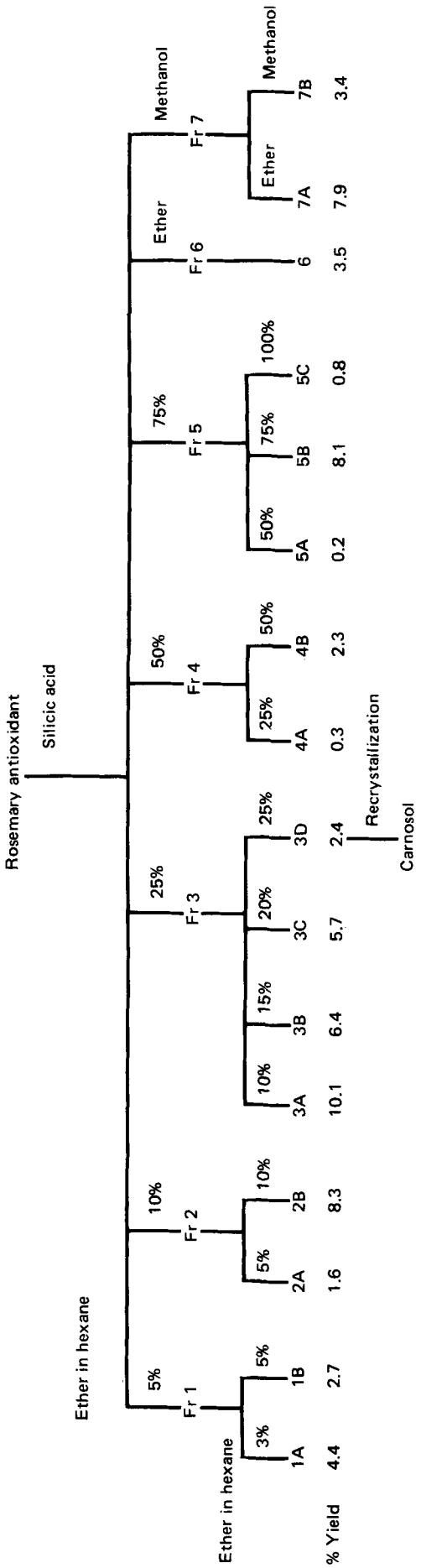

FIG. 2. Chromatographic fractionation of rosemary antioxidant.

TABLE I

Antioxidant Property of Rosemary Antioxidant

Additive	Peroxide value (meq/kg) at 60 C after days: 6	14	21	28	36
None	1.7	7.4	23.0	99.4	220.0
BHT 0.02%	0.9	2.0	2.7	3.6	4.3
BHA 0.02%	1.2	4.0	7.4	12.4	18.1
RA[a] 0.02%	0.8	1.4	2.1	2.5	3.9

[a]RA: rosemary antioxidant.

TABLE II

Antioxidant Activity of Chromatographic Fractions of Rosemary Antioxidant

Additive (0.02%)	Peroxide value (meq/kg) Prime steam lard stored for weeks at 60 C: 0	1	2	3	4
Control, no additive	0.2	1.4	5.1	10.6	31.0
BHT	0.2	0.6	1.2	1.6	2.0
BHA	0.2	1.0	3.0	7.0	7.8
1A	0.2	1.1	1.8	2.3	2.6
1B	0.2	1.0	1.3	1.6	2.4
2A	0.2	–	1.7	2.1	2.8
2B	0.2	1.0	1.5	2.0	2.6
3A	0.2	0.8	1.8	2.5	3.6
3B	0.2	1.1	2.6	3.3	4.8
3C	0.2	0.8	2.1	3.0	4.4
3D	0.2	0.3	2.1	2.5	3.6
4A	0.2	–	–	8.8	13.7
4B	0.2	1.7	3.0	3.8	5.1
5A	0.2	–	–	8.4	13.4
5B	0.2	1.1	2.0	2.6	3.2
5C	0.2	–	5.3	8.9	22.9
6	0.2	1.0	2.2	3.2	8.2
7A	0.2	1.0	2.4	4.2	7.2
7B	0.2	–	–	7.3	15.0

lard (Table I). The peroxide value of the lard was determined after the samples were aged at 60 C in the dark for 6, 14, 21, 28 and 36 days, respectively. The decrease in the rate of the formation of peroxide was used as a measurement of the antioxidant activity.

At concentrations of 0.02%, the antioxidant activity of the RA was comparable to that of BHT.

Fractionation of the Rosemary Antioxidant

In order to elucidate the chemical structures of the components responsible for the antioxidant properties of RA, it was fractionated by repeated column chromatography with silicic acid using stepwise gradient elution. As shown in Figure 2, a total of 7 primary fractions and 16 subfractions was obtained.

The antioxidant activities of the 16 subfractions of RA obtained from the column chromatography were compared with those of BHT and BHA as shown in Table II. The values of the peroxide number of this experiment were comparably lower than the other experiments, probably because this batch of prime steam lard had some antioxidant added during processing.

The most active fractions were those with lower peroxide values, including 1A, 1B, 2A, 2B, 3A, 3D and 5B.

Isolation and Identification of Unknown Crystal 1 (UC-1) as Carnosol

UC-1 was isolated by fractional crystallization from one of

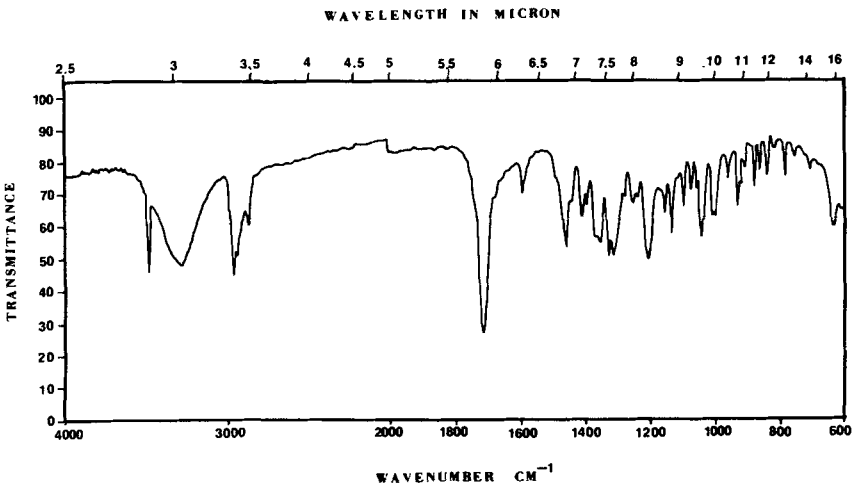

FIG. 3. Infrared spectrum of UC-1.

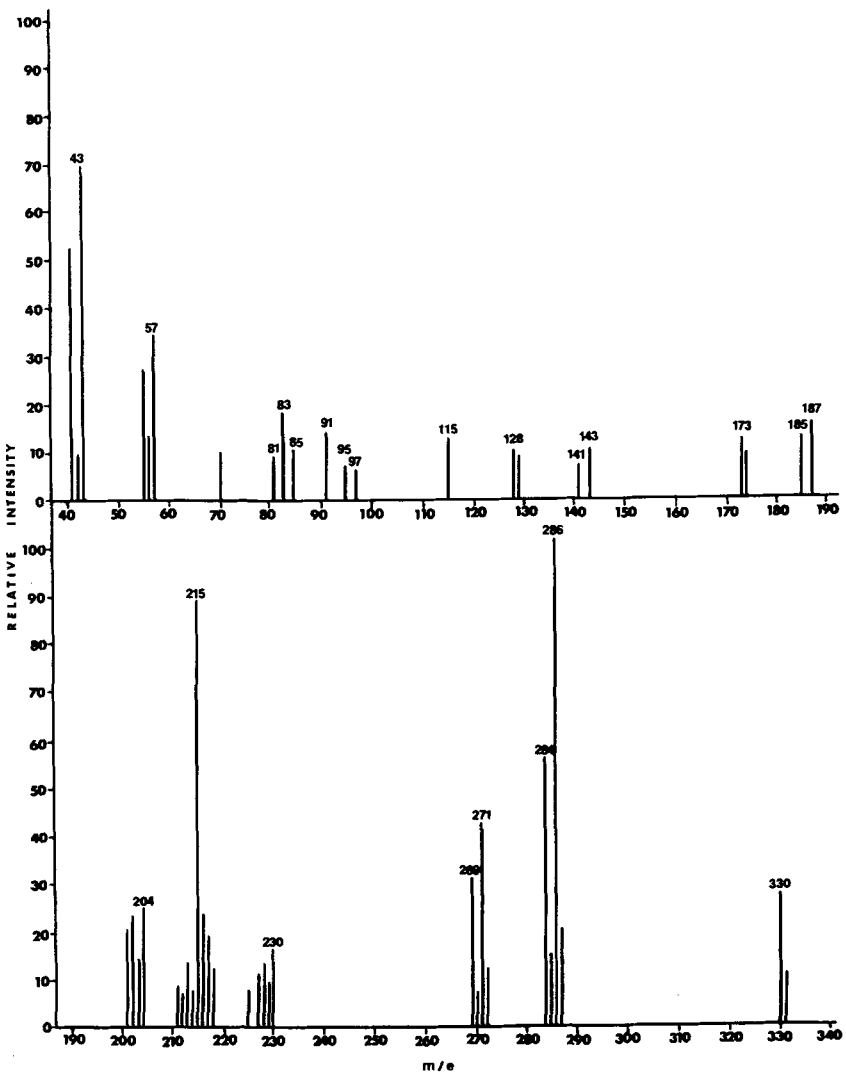

FIG. 4. Mass spectrum of UC-1.

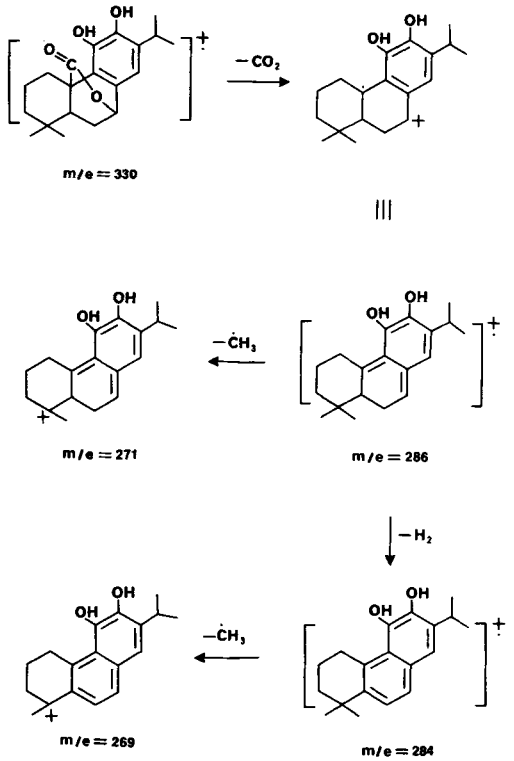

FIG. 5. Interpretation of mass spectrum of UC-1.

the 16 subfractions obtained, i.e., 3D, and was recrystallized from methanol. The yield of these crystals was 2.4% of the RA.

UC-1 was identified as carnosol—a triterpene. It was identified by comparing its IR, mass and NMR spectra with that of an authentic sample of carnosol. The IR spectrum of UC-1 is shown in Figure 3. The sharp absorption at 3495 cm^{-1} and broad absorption at 3300 cm^{-1} indicated the phenolic OH group. Absorption at 1720 cm^{-1} and 1210 cm^{-1} are characteristic bands of lactone, -C-O- absorptions.

The IR of UC-1 matched perfectly that of the authentic carnosol.

The mass spectrum of UC-1 is shown in Figure 4 and its interpretation is shown in Figure 5. A molecular ion appears at m/e 330. The base peak observed at m/e 286 is a result of the loss of CO_2. Further loss of a hydrogen molecule from m/e 286 resulted in the formation of a more stable ion, m/e 284, with an aromatic resonance structure. Loss of one each of the terminal methyl groups from fragment m/e 286 and fragment m/e 284 led to the formation of stable, tertiary carbonium ions of fragment m/e 271 and fragment m/e 269, respectively.

The NMR spectrum of UC-1 is shown in Figure 6. The broad singlet absorption at $\delta 8.2$ clearly indicated the presence of 2 phenolic protons. The NMR spectrum of UC-1 matched that of the authentic carnosol.

Isolation and Identification of Unknown Crystal 2 (UC-2) as Ursolic Acid

UC-2 was isolated by fractional crystallization from the precipitate (A) during concentration of methanol-extracted rosemary solution (Fig. 1). The crystals obtained were recrystallized twice from methanol.

UC-2 was identified as ursolic acid—a pentacyclic triterpene monocarboxylic acid. The identification was accomplished by comparing its IR, mass and NMR spectra with that of an authentic sample of ursolic acid.

The IR spectrum of UC-2 is shown in Figure 7. The strong absorption at 1720 cm^{-1} and broad absorption between 3100 cm^{-1} and 3500 cm^{-1} were characteristic of a carboxylic group. The IR absorption frequencies of the UC-2 matched perfectly those of the authentic ursolic acid.

The mass spectrum of UC-2 (Fig. 8) showed a molecular ion at m/e 456. The base peak at m/e 248 and the peak at m/e 207 were results of the retro-Diels-Alder reaction, as shown in Figure 9.

The NMR spectrum of UC-2 is shown in Figure 10 and matched that of the authentic ursolic acid.

Antioxidant Properties of Carnosol and Ursolic Acid

Table III shows the antioxidant activity of carnosol compared to ursolic acid, BHT and RA. It demonstrated that carnosol is one of the active antioxidant components in rosemary extract.

Carnosol has been claimed to be a bitter principle of rosemary and sage extracts (9,10). However, the pure carnosol isolated in our laboratory was sensory evaluated to

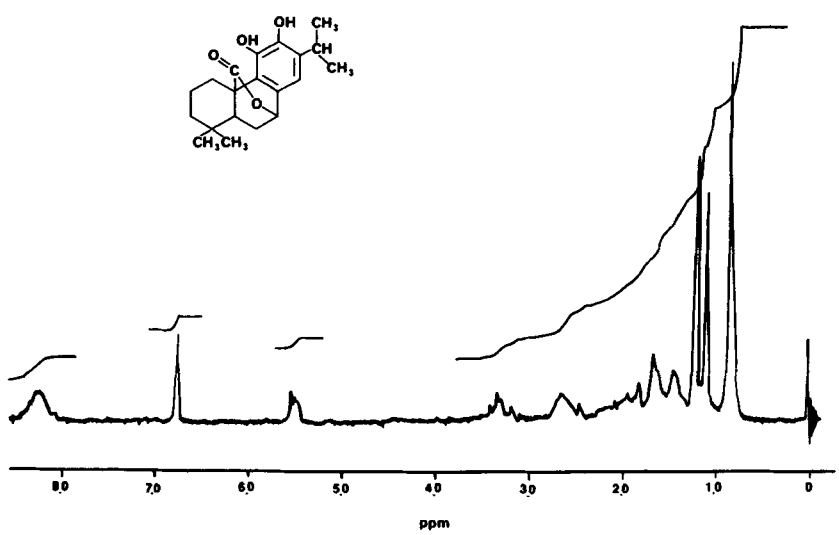

FIG. 6. Nuclear magnetic resonance spectrum of UC-1.

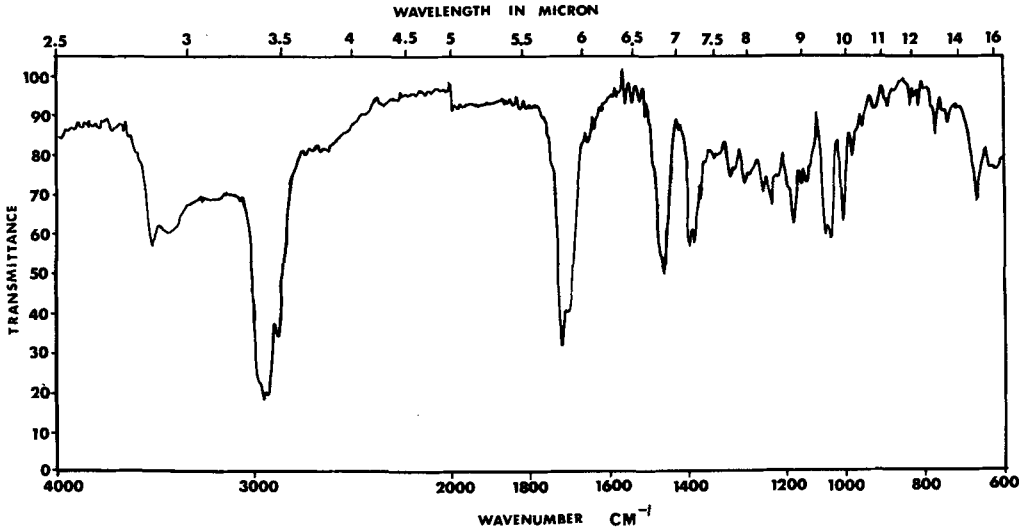

FIG. 7. Infrared spectrum of UC-2.

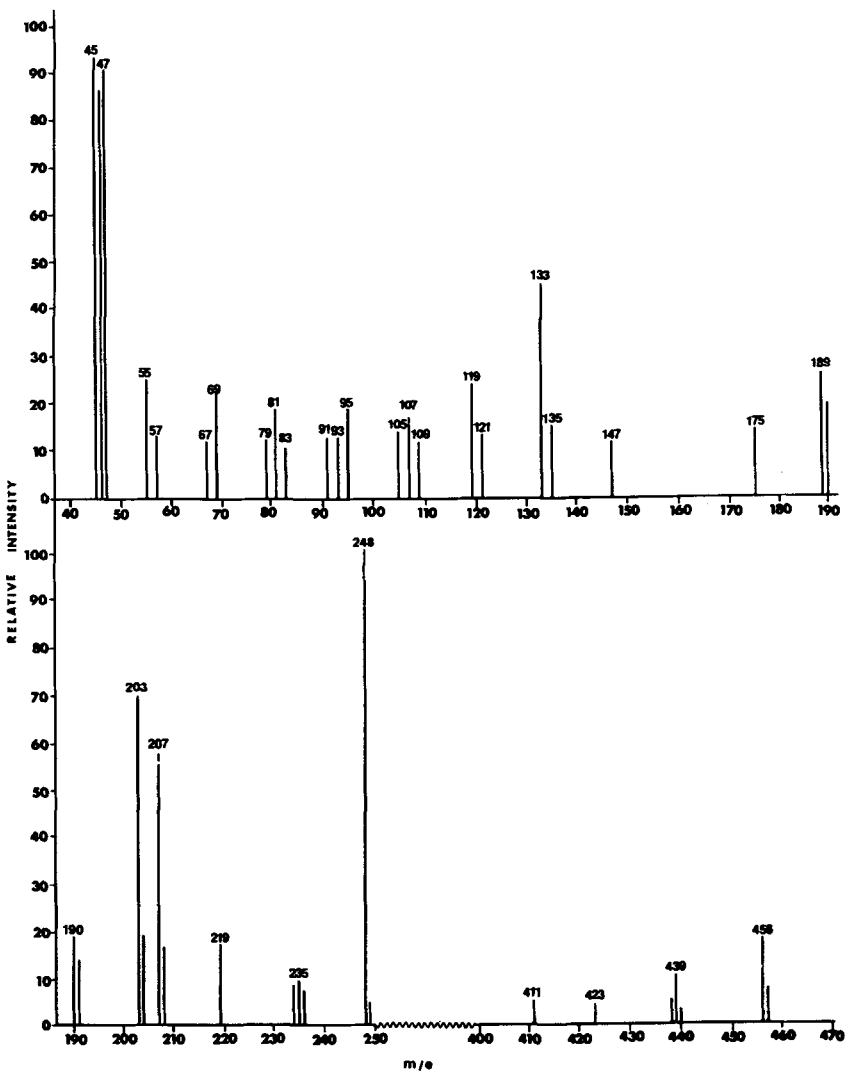

FIG. 8. Mass spectrum of UC-2.

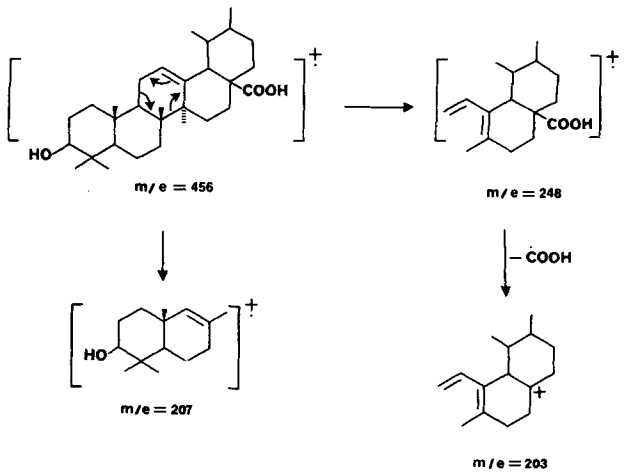

FIG. 9. Interpretation of mass spectrum of UC-2.

TABLE III

Antioxidant Activities of Carnosol and Ursolic Acid

Additive (0.02%)	Peroxide value (meq/kg) Prime steam lard stored for weeks at 60 C				
	0	2	3	4	5
Control, no additive	0.2	5.0	9.0	84.7	193.6
BHT	0.2	1.3	2.3	3.0	3.9
Carnosol	0.2	2.8	3.0	3.5	6.8
Ursolic acid	0.2	4.0	8.0	27.6	102.8
RA[a]	0.2	1.0	1.2	2.0	2.6

[a]RA: rosemary antioxidant.

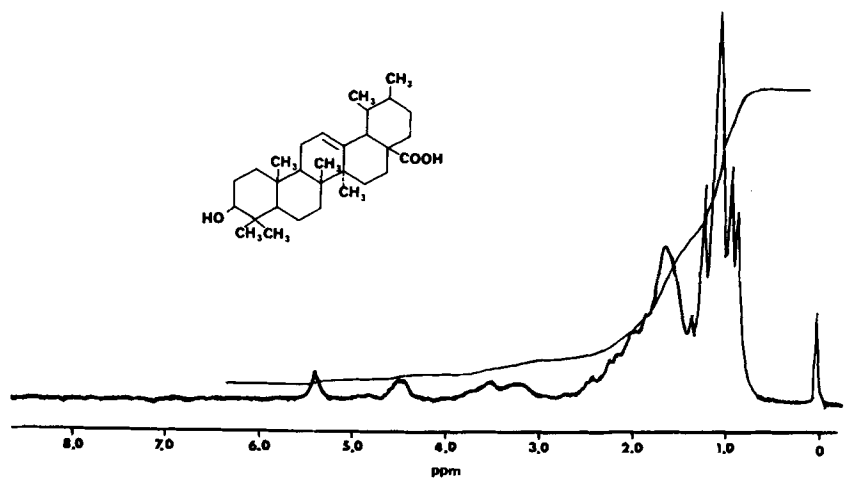

FIG. 10. Nuclear magnetic resonance spectrum of UC-2.

be odorless and tasteless.

As shown in Table III, ursolic acid is not as active an antioxidant component as carnosol. Due to the poor solubility of ursolic acid in oil, it is beneficial to remove the ursolic acid during the preparation of the RA.

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