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Elucidation of the Chemical Structure of a Novel Antioxidant, Rosmaridiphenol, Isolated from Rosemary

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ABSTRACT

A novel antioxidant compound has been isolated and identified from the leaves of the *Rosmarinus officinalis L.* The compound, named rosmaridiphenol, is a diphenolic diterpene. When tested in lard, the antioxidant activity of this compound was superior to BHA. Structural elucidation of rosmaridiphenol was accomplished by infrared spectroscopy (IR), mass spectroscopy (MS), ^{1}H-NMR (nuclear magnetic resonance) and ^{13}C-NMR spectroscopy.

INTRODUCTION

In the 1950's, Chipault and coworkers (1-3) evaluated the antioxygenic properties of several herbs. Although these tests were performed using a variety of fat products, 2 herbs, rosemary (*Rosmarinus officinalis L.*) and sage (*Salvia officinalis L.*) consistantly demonstrated superior antioxidative effects.

Over the years, several reports have appeared on the preparation of rosemary extracts, which were very effective in retarding lipid autoxiation (4-7).

In addition to the production of extracts, several studies have been aimed at isolating and identifying active antioxidant compounds in rosemary. In 1964, Brieskorn et al. (8) isolated a phenolic diterpene, carnosol, from rosemary leaves. Later, Wu et al. (9), using a different isolation method, also identified carnosol from the leaves of the *Rosmarinus officinalis L.* plant. They reported that when carnosol was added to lard, its antioxidative effectiveness was comparable to BHT (9). Recently, Inatani et al. (10) isolated another antioxidant compound, rosmanol, from the leaves of the same plant. Rosmanol was also a phenolic diterpene and possessed a structure closely related to that of carnosol. In a subsequent study, Inatani et al. (11) reported that rosmanol was a fine antioxidant in several fat substrates with activity similar to that of carnosol.

The present paper reports the isolation and characterization of a new antioxidant, rosmaridiphenol, from rosemary leaves.

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EXPERIMENTAL

Isolation of the New Antioxidative Compound

A rosemary antioxidant extract was obtained from dried, ground rosemary leaves following a procedure described by Wu et al. (9). Following a vacuum steam distillation process, this extract was fractionated using a 5 cm × 122 cm glass column packed with activated silicic acid. Activation of this adsorbent was accomplished by a procedure set forth by Sahasrabudhe and Chapman (12). The column was eluted by step-by-step gradient using 100% hexane as the initial eluent and then employing the following solutions of diethyl ether in hexane (E/H): 5% E/H, 10% E/H, 15% E/H, 25% E/H, 50% E/H, and 75% E/H. The final eluent of this separation was 100% methanol. A total of 15 fractions resulted from this elution pattern.

Spectroscopic Procedures

The infrared (IR) spectrum of this antioxidant compound was obtained using a KBr pellet on a Beckman Acculab 4 Infrared Spectrophotometer. A mass spectrum (MS) was acquired using a duPont 21-490 Mass Spectrometer. The source temperature was held at 200 C with the ionization voltage at 70 eV. All of the proton and carbon-13 NMR (nuclear magnetic spectra) spectra was obtained using a Bruker WM-250 NMR Spectrometer. Quantitative elemental analysis of carbon and hydrogen was performed by Galbraith Laboratories, Inc., Knoxville, TN.

Antioxidant Activity Analysis

The antioxidant activity of each compound tested was based on its ability to prevent the formation of peroxides in prime steam lard samples. The samples were kept at 60 C without light for 4 weeks. Peroxide values were determined by Official Method Cd 8-53 of the American Oil Chemists' Society (13).

RESULTS AND DISCUSSION

Isolation of the New Antioxidant

On chromatographic separation of the vacuum steam distilled rosemary extract, the 75:25 diethyl ether/hexane fraction was found to be composed of many white crystals dispersed in a thick yellow liquid. Using a cold 75:25 ether/hexane solution, the white crystals were separated from the viscous portion of this fraction and filtered using a Buchner funnel. The crystals were then recrystallized twice using 100% ethanol. After the second recrystallization, these white, fluffy crystals were filtered with cold ethanol and collected.

Identification of the New Antioxidative Compound

Through spectroscopic methods, the isolated white crystals were identified and named rosmaridiphenol (structure shown in Fig. 1), $C_{20}H_{28}O_3$, m.p. 182-184 C. The elemental analysis of rosmaridiphenol by Galbraith Laboratories concluded that this compound was 75.75% carbon and 8.73% hydrogen. These percentages were almost identical to the values calculated from this compound's molecular formula of 75.81% carbon and 8.91% hydrogen.

The IR spectrum of Rosmaridiphenol revealed the presence of hydroxy groups by absorptions at 3520 cm^{-1}, 3480 cm^{-1} and 3250 cm^{-1}. Also, indications were found of an aromatic ring corresponding to peaks at 3020 cm^{-1} and 1580 cm^{-1}. In addition, a conjugated keto group band was seen at 1680 cm^{-1}. The other IR absorptions of this molecule were at 2950 cm^{-1}, 2850 cm^{-1}, 1580 cm^{-1}, 1500 cm^{-1}, 1465 cm^{-1}, 1450 cm^{-1}, 1380 cm^{-1}, 1280 cm^{-1}, 1160 cm^{-1}, 1100 cm^{-1}, 1025 cm^{-1}, 1000 cm^{-1}, 960 cm^{-1}, 880 cm^{-1}, 700 cm^{-1} and 650 cm^{-1}.

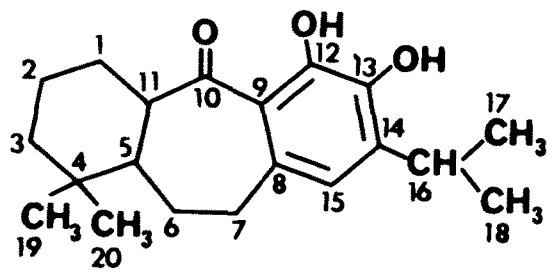

FIG. 1. Structure of rosmaridiphenol.

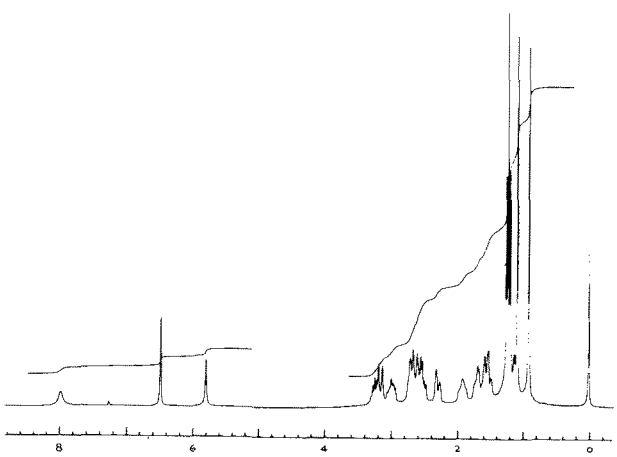

FIG. 2. Proton NMR spectrum of rosmaridiphenol.

Rosmaridipherol's MS showed a very stable molecular ion at m/z 316 (100%). Other fragmentation ions appeared at m/z (relative intensity) 301 (25%), 283 (7%), 273 (5%), 260 (10%), 247 (10%), 217 (14%) and 179 (12%). The peak at m/z 301 demonstrated the loss of a methyl group from the parent ion. The small peak appearing at m/z 273 suggested the loss of the isopropyl group from this molecule.

The proton NMR spectrum of rosmaridiphenol appears in Figure 2. As the ^1H NMR spectrum shows, the phenolic protons appeared as singlets at 7.90 ppm and 5.80 ppm. The hydrogen of the phenolic group of carbon No. 12 was shifted downfield because of intramolecular hydrogen bonding with the keto group of this molecule. The only aromatic proton of rosmaridiphenol was seen characteristically as a singlet at 6.50 ppm.

The gem-dimethyl hydrogen atoms appeared as singlets at 1.1 ppm and 0.9 ppm. The protons of the methyl groups of the isopropyl structure gave confusing spectroscopic results. In the ^1H NMR spectrum of this molecule, the absorption at 1.2 ppm appeared to be a triplet. In reality, these 2 methyl groups exhibited 2 closely spaced doublets that appeared as a triplet because of overlapping. This conclusion was proven when the methine hydrogen of the isopropyl group (which appeared as a septet at 3.3 ppm) was irradiated and 2 narrowly spaced singlets resulted from the triplet at 1.2 ppm. The other aliphatic protons appeared as multiplets between 1.6 and 3.2 ppm.

The ^{13}C NMR spectrum of rosmaridiphenol is shown in Figure 3. Other ^{13}C NMR experiments were performed, including an off-resonance type (Figure 4) and a J-modulation study (Figure 5). The phenolic carbon atoms appeared characteristically in the ^{13}C NMR spectrum at 143 ppm and 141 ppm. The expanded off-resonance spectrum between 110-150 ppm (not presented here) indicated a single aromatic C-H group at 118 ppm. which corresponded to C 15:0. The other aromatic carbon atoms absorbed at 121 ppm, 131 ppm and 133 ppm.

From the off-resonance and J-modulation spectra, we determined that there were 4 methyl carbon atoms. The CH_3 groups of the isopropyl structure appeared at 22.3 ppm and 23.0 ppm. The gem-dimethyl carbon atoms, numbered 19 and 20, were assigned absorptions at 29.2 ppm and 20.1 ppm. Also, the off-resonance spectrum (Figure 4) showed 1 methine group and 1 quaternary carbon atom. From this information we concluded that C16:0 appeared at 27.0 ppm and C4:0 was responsible for the signal at 34.4 ppm. The peaks at 50.2 ppm and 49.3 ppm were assigned to the "fused" carbon atoms numbered 5 and 11. The other 5 aliphatic carbon atoms resulted in signals that appeared between 23.0-42.0 ppm.

The keto group carbon atom appeared at 217 ppm. This absorption was again shifted downfield because of intramolecular hydrogen bonding.

All of the spectroscopic data obtained accord with the proposed structure of rosmaridiphenol.

Antioxidant Activity of Rosmaridiphenol

Rosmaridiphenol was tested for antioxidative effectiveness at a concentration of 0.02% in prime steam lard. This fat substrate was maintained at 60 C in the dark for the duration of the experiment. As already mentioned, antioxidant activity was based on the peroxide-inhibiting capacity of the compound tested. The peroxide values obtained from rosmaridiphenol and other standard antioxidants appear in Table I. The antioxidant activity of rosmaridiphenol surpassed BHA and approached the effectiveness of BHT.

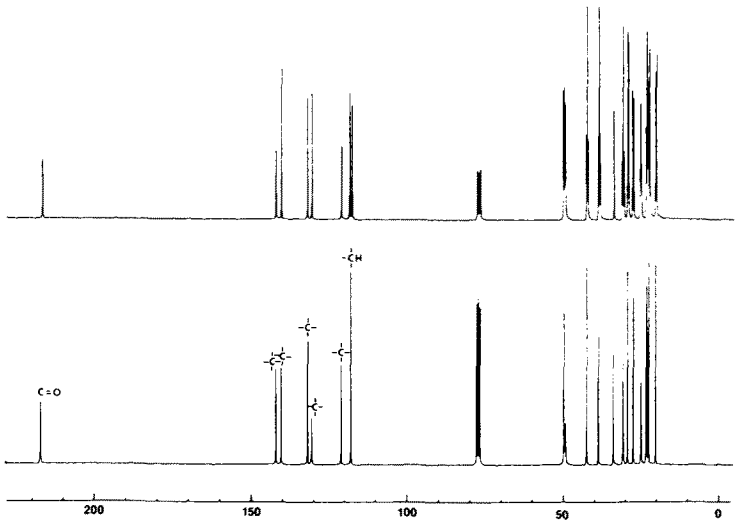

FIG. 3. C-13 NMR spectra of rosmaridiphenol; upper: off-resonance decoupled; bottom: noise-decoupled.

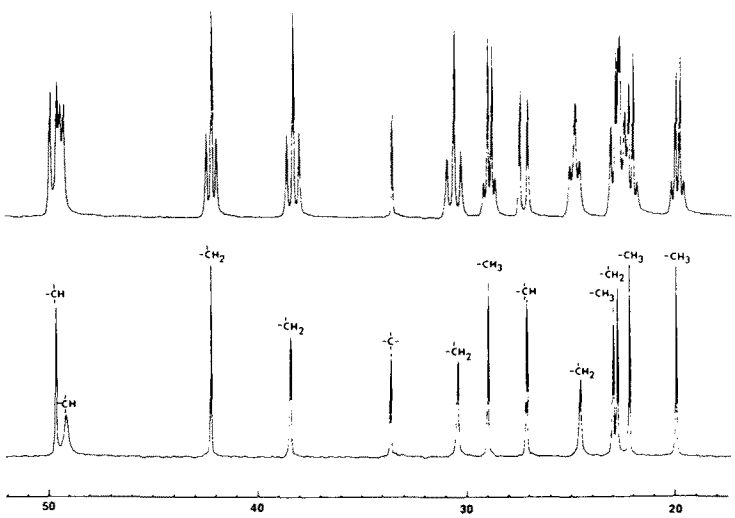

FIG. 4. Expanded off-resonance decoupled C-13 NMR spectrum of rosmaridiphenol.

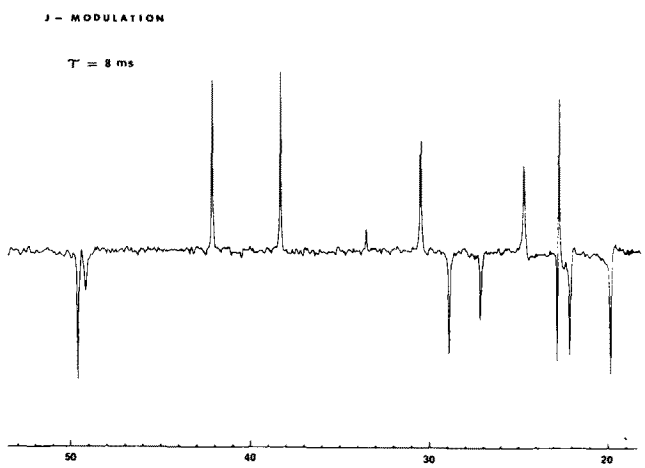

FIG. 5. J-modulation study in ^{13}C NMR spectrum of rosmaridiphenol.

TABLE I

Antioxidant Activity of Rosmaridiphenol and Other Commercial Antioxidants

Additive (0.02%)	Peroxide value (meq/kg) in prime steam lard at 60 C after days: (average of 2 samples)			
	7	14	21	28
Control—no additive	4.70	10.08	29.93	119.67
BHT	1.26	1.86	2.71	3.37
BHA	2.72	6.54	12.10	17.01
Rosmaridiphenol	1.57	2.30	3.10	4.09

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Preliminary Studies on Processing of Sunflower Seed to Obtain Edible Protein Concentrates

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ABSTRACT

A process for obtaining edible-grade sunflower protein concentrate has been standardized. Dehulling the seed and removing polyphenols were accomplished using a centrifugal sheller and treating the kernels with acidified sodium chloride solution. The sunflower protein concentrate had a protein content of 60%, available lysine of 3.4 g/16 g N and was free from polyphenols. The protein efficiency ratio (PER) of the concentrate was 2.4 and in vitro digestibility was 90%.

INTRODUCTION

Sunflower cultivation was introduced on a national scale in 1971-72 as an oilseed crop in India. Since then considerable progress has been made by various states to promote this crop. The current estimate of production is nearly 300,000 MT on 720,000 ha (1). Sunflower meal is rich in protein and free from antinutritional factors (2). Chlorogenic acid and caffeic acids are the 2 polyphenolic constituents present in sunflower proteins that have undesirable effects, not only from the standpoint of nutrition, but also from that of technical application (3). Specifically, the polyphenolic oxidation products bring about an irreversible blocking of ϵ-amino and sulfhydryl groups, inactivating the gastrointestinal enzymes and reducing the biological value of proteins. In addition, during the production of protein concentrates and isolates from oilseeds, the properties that determine the utility value of the proteins are negatively influenced by the covalent bonding of oxidized phenolic acids to proteins and impart a dark green or yellow color to the product (4). Various methods have been reported in the literature for removing polyphenols from sunflower seed (5-14). However, most of these methods suffer from a combination of incomplete extraction, loss of proteins or the use of expensive reagents. In this paper, a method using acidic sodium chloride solution to extract polyphenols is described and the subsequent nutritional evaluation of the resultant processed meal is presented.

EXPERIMENTAL PROCEDURES

Precleaning

Black hybrid (EC-68414) sunflower seeds produced by Agro Seeds Corporation, Mysore, had 8-10% moisture content, and contained shriveled and unfilled seeds and other extraneous matter. The seeds were dried at 40 C in a through-flow dryer for 30 min to bring the moisture content to 4%. After cooling, they were passed through air-classifier equipment to remove unfilled, shriveled and lighter seeds.

Dehulling

After initial cleaning, the seeds were graded using BS-3 and BS-5 screens to separate small, medium and large seeds. These graded seeds were dehulled by a centrifugal sheller followed by air-classification. The sheller consisted of a circular metal housing lined with a hard rubber and PVC ring and had an inlet at the center for feeding in sunflower seed. The material fell on an impeller, which hurled the seed in a curved path against the rubber lining of the sheller by centrifugal force. As a result of the impact, the seeds were dehulled. The sheller housing had an 8-in. diameter and the impeller speed was 3,300 rpm. It had a capacity of dehulling 150 kg of seed per hr.

Partial Defatting

Kernels obtained after dehulling and air-classification were pressed in a laboratory Carver press at 5,000-6,000 psi for 2 hr and ca. 30% of the total oil was recovered. The resultant partially defatted kernels were brushed on a wire-mesh screen (30 mesh) and the translucent layer (testa) was removed by air-classification. The kernels free from the translucent layer were used for further processing.

Acidic Sodium Chloride Extraction

Three extractions of 100 g samples of partially defatted kernels were conducted at room temperature in plastic buckets with continuous slow stirring. The water-to-kernel ratio was 12:1 (V/W) with 1% NaCl for 2nd extraction and 2:1 (V/W) for 3rd extraction with water only. During these extractions, protein losses were minimized by adjusting the pH to 5.0 with 0.01 N HCl. Second and third washings were reused for fresh extraction of kernels. The extracts were separated from the kernel slurries by cloth filtration after each extraction. For comparison studies, water was used as an extractant for the partially defatted kernels. The wet, washed kernels were dried at 50 C for 40 min in a through-