Determination of Tocopherols and Sterols by Capillary Gas Chromatography

H.T. SLOVER, R.H. THOMPSON JR. and G.V. MEROLA, USDA, S&E, Agricultural Research Service, Beltsville Human Nutrition Research Center, Nutrient Composition Laboratory, Bldg. 161, BARC-East, Beltsville, MD 20705

ABSTRACT

A method is described for simultaneously determining tocopherols and sterols in fats and oils by quantitative capillary gas chromatography. Samples containing ca. 100 mg of lipid were saponified in capped tubes with aqueous KOH by heating for 8 min at 80 C; the unsaponifiable fraction was extracted with cyclohexane, freed of solvent, derivatized to form the trimethylsilyl ethers of both tocopherols and sterols, and chromatographed on a 50 m × 0.25 mm glass capillary column coated with Dexsil 400. Most of the individual tocopherols and common sterols were well separated, although interfering peaks were seen in some samples, which for better specificity should be subjected to an initial purification. For most samples, however, the simplified sample preparation, without preliminary purification, was adequate when combined with capillary gas chromatography. Recovery, method and gas liquid chromatographic precision, and applications are discussed.

INTRODUCTION

Tocopherols and sterols are among the lipids of nutritional interest that are frequently determined in fats and oils and other foods. The two species are commonly determined separately, but when data on both are required it is possible to determine them in the same analysis, since they are both found in the unsaponifiable fraction, have a hydroxyl group, and are separable by gas liquid chromatography (GLC) under similar conditions (1-5). Further simplification can be achieved by eliminating any initial purification and relying on the superior separation efficiency of capillary gas chromatography to separate the tocopherols and sterols from each other and from the numerous extraneous compounds. We have developed a method for the simultaneous determination of tocopherols and sterols in the same sample, using capillary gas chromatography, and featuring the further simplifications of decreased sample size, saponification and unsaponifiable extraction in a single capped tube, and the derivatization and analysis of the entire unsaponifiable fraction. The method has the advantage of eliminating both the usual reflux step for saponification and the use of separatory funnels for the extraction of the unsaponifiables.

The method has been used extensively in our laboratory for the analysis of a variety of foods; typical results for a number of fats and oils are presented, although any lipid extract may be analyzed. Quantitative recovery and the precision both of the GLC analysis and the entire method have been evaluated.

EXPERIMENTAL

General Description

The procedure described is applicable to the determination of the major tocopherols and sterols in fats, oils and lipid extracts of foods. Samples consisting of ca. 100 mg of lipid were combined with a known weight of an internal standard (5,7-dimethyltocol) and saponified with aqueous KOH in the presence of BHA and pyrogallol. Unsaponifiables were extracted into cyclohexane, solvent was removed with a stream of nitrogen, and trimethylsilyl ethers were formed by the use of *bis*(trimethylsilyl)-trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) with pyridine. The derivatized total unsaponifiable fraction was chromatographed.

Standards

Internal standard. 5,7-Dimethyltocol, synthesized from phytol and trimethylhydroquinone as described by Karrer (6), was used as the internal standard. This standard is now available from Supelco, Inc. (Bellefonte, PA). In the procedure described here a solution in isooctane containing ca. 40 μg/mL, plus ca. 50 μg/mL of butylated hydroxyanisole as an antioxidant, was used.

Reference standards. The reference standard, in isooctane, contained 8 μg/mL each of α-, γ- and δ-tocopherols and 18 μg/mL each of campesterol, stigmasterol, sitosterol and cholesterol. Five mL of this standard was processed as a sample with each group of samples analyzed and was the basis for quantitative calculations.

Solutions

Pyrogallol: 3% in absolute ethanol. KOH: saturated aqueous.

Sample Preparation

Saponification. About 100 mg of sample (or 5 mL of reference standard) was placed in a 25 × 150 mm screw-capped test tube (Catalog 14-930-10J, Fisher Scientific Co.). One mL of the internal standard (IS) containing ca. 40 μg of 5,7-dimethyltocol was added to each sample and to the reference standard. Solvent was removed with a stream of nitrogen while the tubes were heated to 45-50 C in a water bath. The removal of all traces of solvent, particularly chloroform, was essential to avoid destruction of unsaponifiables, especially sterols. After solvent removal, the tubes were removed from the heat and 8 mL of 3% ethanolic pyrogallol added, while the tubes remained under a nitrogen atmosphere. The tubes were flushed with nitrogen for an additional 2 min, and, without interrupting the nitrogen flow, 0.5 mL of saturated aqueous KOH was added and the tubes quickly sealed with Teflon-lined screw caps. The samples were vigorously mixed in a Vortex mixer for ca. 5 sec, then heated for 8 min in a water bath at 80 C. The tubes were shaken vigorously by hand three times, after 1, 2 and 4 min of heating. After exactly 8 min in the water bath, the tubes were removed and cooled in cold tap water for 15 sec.

Extraction of unsaponifiables. Cyclohexane (20 mL) was added, followed by 12 mL of degassed distilled water, then the tubes were recapped, shaken vigorously for exactly 2 min, and centrifuged at 1300 G for 5 min. (Distilled water may be degassed by boiling or by vigorous sonication for 15 min). The clear upper layer of cyclohexane was transferred to a second 25 × 150 mm silanized screw-capped test tube. Great care was taken during this transfer to avoid accidental contamination of the cyclohexane layer by traces of the lower layer. Since a quantitative transfer was unnecessary, a few mL of the cyclohexane extract were left behind to ensure that no lower phase was carried over. The lower aqueous phase was reextracted with a second 20 mL of cyclohexane and the two extracts pooled. After concentration to 5 mL or less with a stream of nitrogen, the pooled extract was transferred to a 16 × 125 mm silanized screw-capped test tube and the remaining solvent removed.

Derivatization. Pure dry pyridine (50 μL) and 50 μL of BSTFA containing 1% TMCS were added and the tubes

securely capped with Teflon-lined caps and mixed thoroughly. The samples were held at room temperature for at least 15 min before GLC analysis. The reaction mixture in pyridine was injected directly into the chromatograph with no discernible deterioration of either the splitter insert or the column. The reaction mixture was stable for several days in test tubes or in vials sealed with Teflon-lined caps, but was analyzed on the same day whenever possible.

Gas Chromatography

The gas chromatograph was a Hewlett-Packard Model 5840 modified to accommodate glass capillary columns (7). The instrument was equipped with an automatic liquid sampler (Hewlett-Packard Model 7671A), a flame ionization detector, and a glass splitter system (J&W Scientific, Inc., Rancho Cordova, CA).

The columns were 50 m × 0.25 mm glass capillary columns coated with Dexsil 400 (Quadrex Corporation, New Haven, CT) and were stable for several months without deterioration. The chromatographic conditions were: split ratio, 1/20; sample size, 1.7 μL; carrier gas, helium at 1 mL/min; average linear velocity, 34 cm/sec; injection port temperature, 270 C; column temperature, 260 C; detector temperature, 300 C; auxilliary (detector makeup) gas, nitrogen at 40 mL/min. The column when operated in this way gave 29,000 effective plates for α-tocopherol TMS ether, with a capacity factor of 17 for α-tocopherol. (Note: Capillary columns of comparable efficiency coated with other phases, such as SE-30, have been found suitable for this analysis.)

Calculation

A 5-mL aliquot of the reference standard solution (see above) was treated in the same way as the samples, including the addition of internal standard, saponification and derivatization. It was chromatographed at the beginning of each group of samples analyzed, and the peak areas used to calculate response factors (F) relative to 5,7-dimethyltocol as follows:

$$F(X) = \frac{\text{area(IS)/amt(IS)}}{\text{area (X)/amt(X)}}$$

where: area(IS) = area of the internal standard peak; amt (IS) (μg) = amount of the internal standard added to the reference mixture; area(X) = area of peak of compound X; and amt(X) = amount of compound X (μg) in the reference mixture.

Using these response factors, we calculated the amounts of each tocopherol and sterol in the samples as follows:

$$\text{wt(X)} = \frac{\text{F(X)} \times \text{area(X)} \times \text{amt(IS)} \times \text{DF}}{\text{area (IS)} \times \text{sample wt (in g)}}$$

where: wt(X) = μg of X per g of sample; area(X) = area of compound X in the chromatogram of the sample; amt(IS) = amount of the internal standard (μg) added to the sample; area(IS) = area of the internal standard in the chromatogram of the sample; and DF = dilution factor (reciprocal of the fraction of the original sample extract taken for saponification).

RESULTS AND DISCUSSION

The method was tested by evaluating the precision of the GLC analysis alone, the linearity of the entire procedure, recovery after saponification, and the precision of the entire procedure. Both oils and pure standards were used, as appropriate. In addition, several vegetable oils were analyzed to illustrate the application of the procedure.

Precision of the GLC Analysis

The TMS ether derivative of a soybean oil sample was chromatographed six times sequentially. The variability, as the coefficient of variation, was highest for α-tocopherol at 1.4%, and ranged from 0.2 to 0.6% for the other compounds (Table I).

Precision of the Method

Replicate samples of soybean oil were put through the entire procedure (Table I). The precision of the method was not as high as that of the GLC step alone; CV ranged from 2.0 to 2.7%. The increase in CV may be attributed to the additive effects of the variabilities introduced by individual steps in the analysis.

Linearity of the Method

The effect of sample size on response is a common concern in all GLC analysis. Since all the steps in the procedure may be affected by sample size, we evaluated the linearity of the entire procedure. α-Tocopherol, which was used for this evaluation, was added in varying amounts to ca. 100 mg of lard that contained no native α-tocopherol. The same amount of internal standard (40 μg) was added to each sample to normalize the results to a common basis, since the amounts injected onto the column vary, even with an automatic sampler. A plot of the ratio of the peak area of α-tocopherol to the peak area of 5,7-dimethyltocol vs their weight ratios (Fig. 1) was essentially a straight line over a range of 0.25-125 μg of α-tocopherol. The slope of this line is the correction factor (F) of α-tocopherol relative to 5,7-dimethyltocol discussed above.

Recovery after Saponification

Recoveries of tocopherols and sterols from the saponifica-

TABLE I

Analytical Precision of Soybean Oil Analysis

	GLC precision (N = 5)		Method precision (N = 5)	
	Mean (mg/100g)	CV (%)	Mean (mg/100g)	CV (%)
α-Tocopherol	8.6	1.4	8.6	2.0
γ-Tocopherol	66.4	0.2	74.8	2.3
δ-Tocopherol	27.8	0.3	28.0	2.7
Campesterol	64.9	0.3	70.0	2.7
Stigmasterol	65.3	0.2	70.5	2.3
Sitosterol	175.9	0.6	188.5	2.2

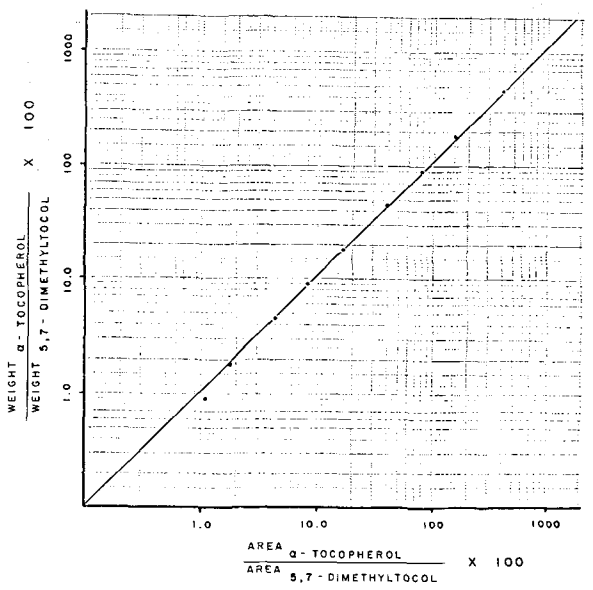

FIG. 1. Linearity of response for α-tocopherol. Results obtained by analyzing samples containing lard as a matrix and varying amounts of α-tocopherol. Samples consisted of: lard, 100 mg; internal standard (5,7-dimethyltocol), 40 μg; and α-tocopherol, 0.25-125 μg.

tion mixture were evaluated by saponifying mixtures of standards, extracting twice with cyclohexane, and analyzing each extract separately. Seven 5-mL aliquots of the reference standard mixture (each aliquot containing 40 μg each of α-, γ- and δ-tocopherols and 90 μg each of campesterol, stigmasterol, sitosterol and cholesterol) were prepared. To six of the aliquots we added 100 mg of lard; none was added to the seventh aliquot. The seven reference standard mixtures were saponified and extracted with cyclohexane as described above. After the first extraction, the lower aqueous phase was carefully recovered and freed of all traces of the first cyclohexane extract before extracting for the second time. A known amount of 5,7-dimethyltocol was then added to both the first and second extracts which were then derivatized, chromatographed, and the amounts of tocopherols and sterols in each extract calculated. The results of this evaluation (Table II) show that one extraction removed more than 94% of all compounds except δ-tocopherol. Two extractions removed 93% of the δ-tocopherol and 100% of the other compounds. A third extraction to recover more of the δ-tocopherol was considered unnecessary. The aliquot that contained no added fat was included to demonstrate that the presence of fat in the saponification mixture, presumably in the form of soaps, affected the extraction of both tocopherols and sterols, and must be considered when evaluating methods for the quantitative recovery of unsaponifiables.

TABLE II

Recovery of Tocopherols and Sterols After Saponification by Extraction with Cyclohexane

	With added fat (N=6)		Without added fat (N=1)
	One extraction (%:x + SD)	Two extractions (%: x + SD)	One extraction (%)
α-Tocopherol	100.0 + 0.4	101.9 + 0.8	102.4
γ-Tocopherol	94.6 + 0.6	100.2 + 0.9	99.0
δ-Tocopherol	76.9 + 2.2	93.0 + 1.3	99.1
Campesterol	96.6 + 1.5	102.5 + 1.9	100.6
Stigmasterol	96.1 + 2.7	101.9 + 3.3	99.9
Sitosterol	96.9 + 2.6	102.6 + 3.3	98.8

TABLE III

Tocopherols and Sterols in Selected Oils (mg/100g)

Oil	Label info.	Tocopherols			Sterols		
		α	γ	δ	Campesterol	Stigmasterol	Sitosterol
Apricot	Cold pressed	32.2	5.8	–	11.8	9.8	177.0
		32.6	6.2	–	11.4	9.1	178.8
Corn	Pure corn oil	13.3	34.1	1.3	148.7	67.6	587.3
		13.7	35.1	1.4	154.2	70.0	605.8
Cottonseed	Cold pressed	39.6	41.4	0.4	29.2	5.2	349.0
		40.4	41.4	0.5	29.3	5.3	349.9
Olive	Virgin olive oil	15.4	2.5	–	6.6	3.1	156.9
		14.5	2.1	–	6.1	3.1	165.7
Peanut	Pure peanut oil	17.1	13.6	0.6	42.7	25.5	171.0
		17.0	13.6	0.6	44.7	27.0	179.7
Safflower	Nonhydrogenated	37.8	–	2.3	27.3	18.1	129.0
		28.0	0.3	1.9	23.1	15.3	109.2
Sesame	Cold pressed	8.8	16.7	–	67.4	202.9	193.6
		8.2	15.5	–	66.2	199.1	190.3
Soybean	Nonhydrogenated	8.4	75.8	28.7	75.9	67.4	176.8
		8.2	66.2	27.6	75.9	67.7	176.7
Sunflower	Cold pressed	49.7	1.4	–	30.5	33.1	209.8
		46.7	1.4	–	29.1	31.1	[a]
Walnut	Cold pressed	6.6	17.4	4.7	10.2	5.7	137.8
Unknown	Pure vegetable oil	8.2	54.8	25.1	51.9	48.6	135.4
		8.0	60.3	26.4	51.7	48.4	135.1
Unknown	Buttery flavor oil	9.1	55.2	27.3	61.0	64.2	177.6
		10.4	68.9	28.2	60.6	63.4	175.3
Unknown	Pure vegetable oil	8.9	74.5	28.9	73.1	69.5	183.2

[a]Not determined.

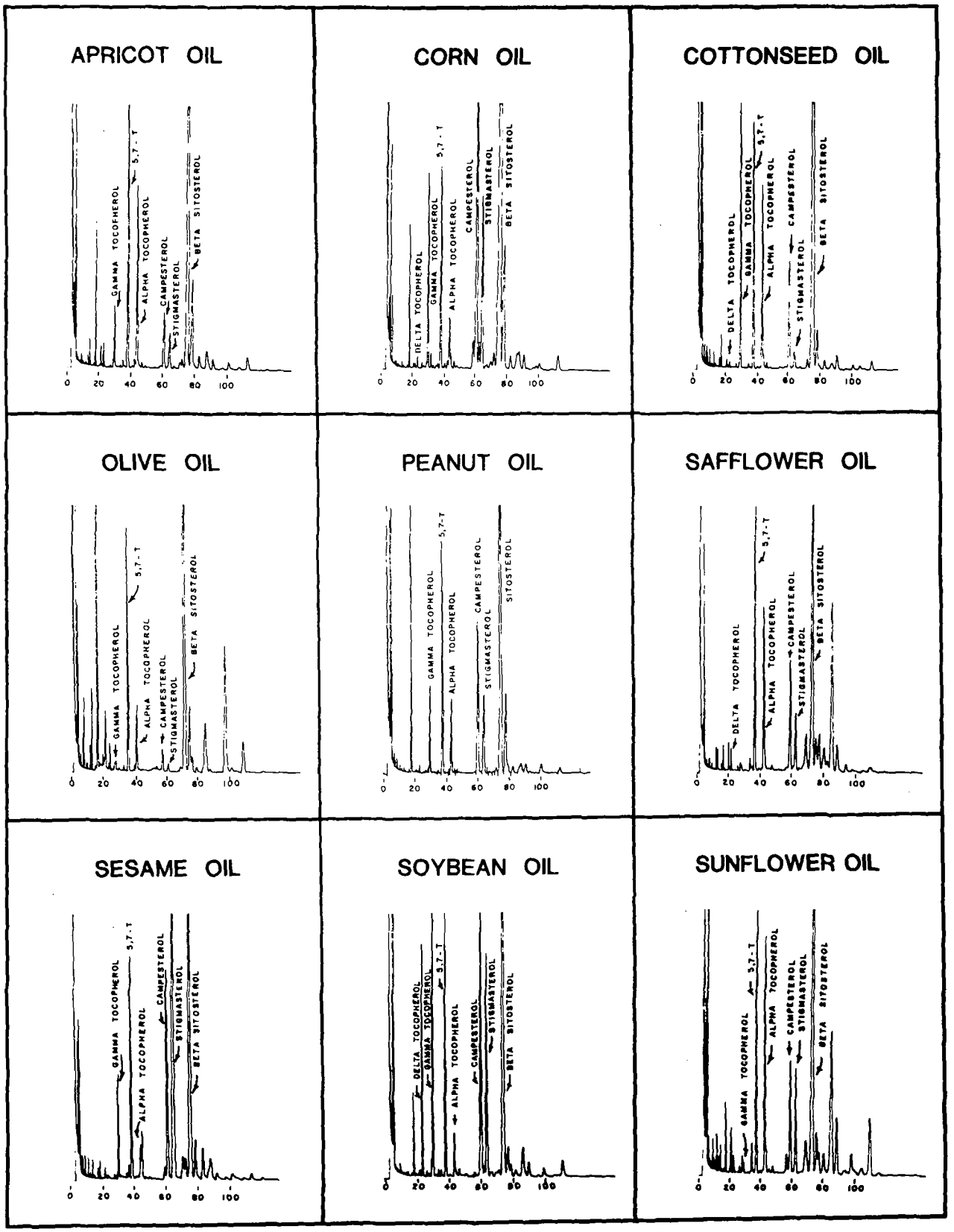

FIG. 2. Representative chromatograms of vegetable oil unsaponifiables as their TMS ether derivatives. Conditions are given in the text.

Many solvents were tested for extracting the unsaponifiables after saponification. Cyclohexane was selected because it is not toxic, does not form peroxides as does diethyl ether, and above all because it does not form stable emulsions if the procedure is followed exactly.

Vegetable Oil Analysis

We analyzed 13 vegetable oils purchased in the Washington DC area for their tocopherol and sterol contents, to evaluate the applicability of the method and possible separation problems. With a few exceptions, duplicate analyses agreed well (Table III). The chromatograms of 9 of these oils appear in Figure 2. Not all peaks were completely separated but the major sterols and tocopherols were either completely separated or separated well enough for quantitative estimation. A peak that interfered with α-tocopherol appeared in some of the chromatograms, and was most prominent in sesame oil. A complete separation of the two peaks could be obtained by using temperature programming and lowering the carrier gas linear velocity.

GLC Retention Data

Kovats retention indices and retention ratios relative to 5,7-dimethyltocol were determined or calculated for tocopherols, tocotrienols and the major sterols (Table IV). Separation would be possible for all compounds listed, except for α-tocotrienol and campesterol.

TABLE IV

Retention Indices and Relative Retention Times of Tocopherols and Sterols

	Retention indices	Relative retention time
Internal standard		
5,7-Dimethyltocol	3129.7	1.000
Tocopherols		
α	3183.9	1.161
β	3022.9	0.746
γ	3034.7	0.771
δ	2935.6	0.587
Tocotrienols		
α	3309.6	1.632
β	3147.4[a]	
γ	3160.2	1.082
δ	3058.2	0.822
Sterols		
Cholesterol	3209.7	1.245
Campesterol	3310.2	1.634
Stigmasterol	3333.0	1.738
Sitosterol	3389.3	2.024

[a]Calculated.

ACKNOWLEDGMENT

This work was supported partly by funds from the National Institutes of Health, Interagency Cooperative Agreement No. 2 Y1 HB60041-05.

REFERENCES

1. Sheppard, A.J., D.R. Newkirk, W.D. Hubbard and T. Osgood, JAOAC 60:1302 (1977).
2. Nelson, J.P., A.J. Milun and H.D. Fisher, JAOCS 47:259 (1970).
3. Govind Rao, M.K., and E.G. Perkins, J. Agric. Food Chem. 20: 240 (1972).
4. Hartman, K.T., JAOCS 54:421 (1977).
5. Mordret, F., A. Prevot, N. Le Barbanchon and C. Barbati, Rev. Fr. Corps Gras 24:467 (1977).
6. Karrer, P., and H. Fritzsche, Helv. Chim. Acta 21:1234 (1938).
7. Slover, H.T., and E. Lanza, JAOCS 56:933 (1979).

[Received December 29, 1980]

♣Participation of Sesamol in Stability of Sesame Oil

K. KIKUGAWA, M. ARAI and **T. KURECHI**, Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan

ABSTRACT

Sesame oil is known to be the most resistant to oxidative rancidity. Constituents of sesame oil such as sesamolin, sesamol and sesamol dimer (a possible intermediate of oxidative degradation of sesamol) were determined by high performance liquid chromatography using a reverse-phase column. Sesamol was specifically determined in an alternative way by use of hydrogen peroxide/horseradish peroxidase. Sesamolin was relatively stable but sesamol and sesamol dimer were unstable when irradiated in benzene, and the final degradation products were identical. Whereas sesamolin was inactive, sesamol and sesamol dimer showed significant antioxidant activity in several kinds of fat and oils. Stability of Japan Pharmacopoeia sesame oil free from sesamol was relatively low; antioxidant activity of sesamol incorporated in the oil was unexpectedly low and was rapidly lost in the oil activated by oxygen. Edible sesame oil with intrinsic sesamol was highly stable. Activation of the edible oil gradually increased the sesamol content with concomitant decrease of sesamolin. High stability of edible sesame oil could not be ascribed to sesamol, but it could be explained by another powerful antioxidant(s) which might stabilize both the oil and unstable sesamol.

INTRODUCTION

Among the several vegetable oils, sesame oil is known to be most resistant to oxidative rancidity (1,2). It was suggested that sesamol, one of the constituents of sesame oil, might be responsible for the stability of sesame oil, and the comparative antioxidant activity of sesamol and other antioxidants in certain fats and oils has been evaluated (3-7). It was found that certain processing treatments resulted in the formation of free sesamol from its bound form (sesamolin) (8,9). We have demonstrated that sesamol was readily oxidized into sesamol dimer and successively into its quinone by mild treatment with hydrogen peroxide/horseradish peroxidase (10). It is readily conceivable that sesamol is converted into its dimer during the progress of peroxidation of sesame oil (see Scheme 1), since butylated hydroxyanisole was oxidized into the dimers during the peroxidation of oils (11,12).

Selective determination of the constituents of sesame oil hitherto reported has been very troublesome (1,2,13-15). Due to the lack of an excellent method for determination of the constituents of sesame oil, it is difficult to monitor the changes of these constituents during the peroxidation of sesame oil. We attempted to separate sesamolin, sesamol and the possible oxidation product, sesamol dimer, in sesame oil by high performance liquid chromatography (HPLC), and to elucidate the relevance of these constituents