Oxygenated Fatty Acids of Oil From Sunflower Seeds After Prolonged Storage[1]

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ABSTRACTS

Chemical analysis of a number of sunflower *(Helianthus annuus)* seed oil samples revealed a low and variable percentage of hydrogen bromide-reactive material. To characterize the compounds responsible for this reactivity, oil was extracted from selected introductions from Uruguay, Turkey, and Yugoslavia that had been subjected to prolonged storage. Two epoxy fatty acids and two conjugated dienolic acids were isolated from the methyl esters derived from these sunflower seed oils by using a combination of column chromatography and countercurrent distribution. The epoxy acids are *cis*-9,10-epoxystearic acid (0.5%) and *cis*-9,10-epoxy-*cis*-12-octadecenoic (coronaric) acid (2.2%). Characterization of the dienols revealed that they are 9-hydroxy-*trans*-10,*cis*-12-octadecadienoic acid (1.2%) and 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid (1.3%). Fresher seed of some of these introductions contained less of the oxygenated components.

Oils from recently produced seed of selected high-oil Russian sunflower varieties, including some currently grown in the United States, contained no more than trace amounts of oxygenated acids. Though the relative contributions of genetic and environmental factors toward genesis of oxygenated acids are not established, increase of those acids in some sunflower lines as a result of storage has been demonstrated.

INTRODUCTION

SUNFLOWER *(Helianthus annuus)* has not achieved major crop status in the United States because of its susceptibility to attack by insects and diseases. Availability of other good oilseeds such as soybeans, cottonseed, flaxseed, peanuts and safflower has lessened the incentive to develop improved sunflower varieties. In the Soviet Union, however, sunflower is the major oilseed crop, and this status is probably the direct result of a successful program of plant selection and breeding to develop varieties that have desirable agronomic characteristics and that produce seeds of high oil content. The success of these high-oil Russian varieties has spurred interest in sunflower as a potential United States crop.

Presumably, a large proportion of the sunflower seed oil produced would be utilized by the edible oil industry. Previous reports (1-3) have indicated that sunflower seed oil contains small amounts of oxygenated acids, which might adversely affect its stability and nutritional properties. Morris and co-workers (1,2) obtained evidence for both hydroxy and epoxy acids in sunflower oil but did not isolate or characterize them structurally. We wish to report the isolation and identification of four oxygenated fatty acids from sunflower seed oil. The oil samples used in this study and the sample Morris used for his research were derived from the same group of stored seeds. As far as we can determine, these samples of sunflower seeds are the only ones known to yield oils containing oxygenated fatty acids. Samples of four Russian high-oil varieties harvested in 1966 were screened for oxygenated fatty acid content and were found to contain little, if any, of these acids.

EXPERIMENTAL PROCEDURES

Materials and Methods

Infrared (IR) spectra were determined with 1% solutions in carbon disulfide on a Perkin-Elmer Infracord, Model 137, and ultraviolet (UV) measurements were made on cyclohexane solutions with a Beckman DK-2A spectrophotometer. A Cary Model 60 spectropolarimeter was used for the optical rotatory dispersion (ORD) studies, and a Fisher-Johns block was used to determine melting points (uncorrected).

Thin-layer chromatographic (TLC) analyses of the oxygenated fatty acid esters were done on Silica Gel G developed with ether-hexane-acetic acid (30:70:1 or 20:80:1). Dihydroxy acid methyl esters were analyzed on Silica Gel

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TABLE I
Analysis of Sunflower (*H. annuus*) Seed Oils

Identity (crop year)	HBr equiv.[a] 0 C	25 C	55 C	UV[b]
Russian variety				
Armavirec (1966)	0.2	....	0.3	0.0
Peredovik (1966)	0.1	....	0.3	0.0
Smena (1966)	0.2	....	0.4	0.0
VNIIMK (1966)	0.2	....	0.3	0.0
Introductions from[c]				
Uruguay (1956) PI 162454	....	7.1 (2.9)[d]	....	2.3 (1.2)
Turkey (1956) PI 173704	....	7.5 (3.3)	....	2.7 (....)
Turkey (1956) PI 182777	....	9.6 (4.2)	....	2.9 (....)
Seed increase crop samples[c]				
Turkey (Ames, Iowa; 1966) PI 173704	0.5	....	1.2	0.0
Turkey (Ames, Iowa; 1966) PI 181994	0.5	....	1.0	0.0
Canada (Ames, Iowa; 1966) PI 201812-10	0.6	....	1.0	0.0

[a] Expressed as per cent epoxyoleate.

[b] Expressed as per cent *cis-trans*-conjugated diene, $A_{max}^{cyclohexane}$ 234 mμ.

[c] A limited number of representative samples from each group have been listed. Only PI 162454 was included in the group of seeds used for our characterization work.

[d] Parentheses contain values found when seed was received in 1956; other values represent oil extracted recently from the same seed accession.

G impregnated with boric acid (4) and developed with ether-hexane-acetic acid (40:60:1). TLC of the esterified diimide reduction products was done on Silica Gel G impregnated with $AgNO_3$ (5) and developed with ether-hexane (40:60). The spots on all analytical TLC plates were visualized by charring the plates at 120 C after they had been sprayed with a saturated solution of chromium trioxide in 50% aqueous H_2SO_4. Preparative plates were sprayed with 2',7'-dichlorofluorescein and viewed under UV light; then the separated components were recovered by the usual procedure.

Analysis of methyl ester samples by gas-liquid chromatography (GLC) was done as described previously (6) and free acid samples were analyzed on a polyester column.

Description of Seeds and Oils

Seeds of four high-oil Russian sunflower varieties (Armavirec, Peredovik, Smena, and VNIIMK) that were grown in northern United States or southern Canada in 1966 were obtained from Cargill, Inc. Dehulled seeds of these varieties were ground and analyzed for oil content by overnight extraction with petroleum ether (bp 30-60 C) in Soxhlet extractors. Solvent was removed in vacuo at 40 C, and the oils were titrated with HBr according to the procedure of Harris et al. (7) which utilizes the Durbetaki reagent (8) at two different temperatures. The results of these analyses, as well as those for other oil samples, are given in Table I. Titration values of the magnitude shown for the Russian varieties mean little because, as stated previously (7), a few tenths of a per cent of interfering autoxidation products are usually found in most oils. UV spectra of these four oils showed no maximum at 234 mμ; therefore, no attempt was made to isolate HBr-reactive materials from these samples. The petroleum ether extract of the hulls from one of the four seed samples had no unusual components as determined by IR and UV analysis.

A large group of sunflower seed samples that originated in a number of different countries were furnished by the North Central Regional Plant Introduction Station, Ames, Iowa, in 1956. Our screening results (3) indicated that oil from these seeds (dehulled) contained 2-4% of HBr-reactive materials. For our current work we selected four of these seed samples from Uruguay, Turkey, and Yugoslavia [plant introduction (PI) numbers 162454, 170390, 175725 and 184049]. The oils were extracted from these four seed samples (including hulls) and combined. This combined oil sample gave a UV spectrum with a maximum at 234 mμ equivalent to 2.3% of conjugated *cis,trans* diene (9).

Oxygenated Methyl Ester Concentrate

Sunflower seed oil was dissolved in a 3:1 mixture of methanol and diethyl ether, and this solution was stirred 4 hr at room temperature with sodium methoxide catalyst. The methyl esters were recovered by the usual ether extraction method. Separation of these mixed methyl esters into an oxygenated ester fraction and an ordinary ester fraction was accomplished on a 2.2 × 30-cm column of Adsorbosil CAB (100-140 mesh, Applied Science Laboratories, Inc.). Esters were applied to the column in 1.5-2.0 g batches dissolved in benzene; the column was then eluted with benzene to remove methyl esters of ordinary fatty acids and finally with benzene-methanol (9:1) to remove oxygenated methyl esters.

The oxygenated methyl ester concentrate (2.70 g from 37.0 g of mixed methyl esters) had infrared bands indicating hydroxyl (2.75 μ), conjugated *cis,trans* (10.18 and 10.54 μ), and epoxide (11.8 and 12.1 μ) absorption. Analysis of this concentrate by TLC revealed

three major spots, R_f 0.33, 0.36 and 0.60. Another relatively strong spot at R_f 0.45 turned pink within 5 min after spraying with acid and was, presumably, due to triterpene alcohols. Under the same conditions, methyl dimorphecolate (methyl 9-hydroxy-*trans,trans*-10,12-octadecadienoate) (10) had R_f = 0.31; methyl vernolate (methyl *cis*-12,13-epoxy-*cis*-9-octadecenoate (11), R_f = 0.67; methyl *cis*-9,10-epoxystearate, R_f = 0.62; and methyl coriolate (methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate) (12), R_f = 0.35. GLC analysis of the oxygenated methyl ester concentrate revealed approximately 10% of epoxystearate, 48% of epoxyoctadecenoate and 40% of conjugated octadecatrienoates. The conjugated trienes are presumably formed during GLC by dehydration of the original hydroxydienes (13). Titration of the concentrate with HBr at room temperature indicated 81% of reactive materials calculated as methyl epoxyoleate.

Countercurrent Distribution

The oxygenated methyl ester concentrate (2.70 g) was dissolved in 120 ml of acetonitrile (lower phase) and 120 ml of hexane (upper phase), and the solution was placed in the first three tubes of a 200-tube countercurrent distribution (CCD) apparatus. Lower phase (40 ml) was added to each of the remaining tubes, and the instrument was set to deliver 40 ml of upper phase at each transfer. After the 200 fundamental transfers had been completed, fraction collection was begun and was continued until 800 transfers had been made (600 fractions collected).

A fast-moving fraction (0.360 g) in transfers 214-263 apparently contained the sterol and triterpene components as indicated by the pink coloration it gave after TLC when sprayed with H_2SO_4/CrO_3 reagent. An epoxide, shown to be saturated by TLC on $AgNO_3$-impregnated Silica Gel G, was recovered from transfers 322-400 (0.250 g), and the corresponding monoenoic epoxide was recovered from transfers 412-520 (0.812 g). At the beginning of the run, a 1-in. piece of plastic tubing was used for an emergency repair to the CCD apparatus and this repair resulted in contamination of the solvent system with a plasticizer. The saturated epoxide fraction was the only one seriously affected; the contaminant could not be removed by column chromatography or crystallization.

The solvent in the CCD instrument was removed from all tubes except those containing dienol material and was replaced with fresh solvent. This technique was used to remove minor constituents which could contaminate the dienols during subsequent operations. The instrument was then operated on automatic recycle until 2,800 transfers had been made. In this technique, upper phase is not collected but passes directly from the last tube back to the first tube of the instrument. At this stage, the two dienols were partially resolved, 0.092 g of dienol I (higher R_f on TLC) was recovered from the leading edge of the peak (tubes 75-110), and 0.156 g of dienol II was recovered from the trailing edge (tubes 180-199 and 0-10). The vacated tubes were refilled with solvent, and the recycling operation was continued until a total of 5,500 transfers were completed. Additional quantities of dienol I (0.201 g) and dienol II (0.170 g) were recovered from tubes 70-125 and from tubes 160-199 and 0-30, respectively. Tubes 31-69 contained about 0.200 g of unseparated dienol mixture. Overall recovery from the CCD operation was 2.61 g, including a small, but undetermined, amount of contaminant.

Characterization of Saturated Epoxide

Analysis of the saturated epoxide fraction by GLC and by HBr titration demonstrated that it was about 60% pure. The ORD spectrum (c = 3.9, hexane) showed this fraction was dextrorotatory, but this result has little significance since the optical properties of the contaminant are not known. Saponification of a portion of this mixture, followed by crystallization of the products from ethyl acetate, yielded 40% of an epoxy acid, mp 54.0-55.0 C. No depression of the melting point was observed on admixture with authentic *cis*-9,10-epoxystearic acid, mp 53.0-54.0 C. The epoxy acid was converted to a dihydroxy acid, mp 92.5-93.5 C, by acetolysis followed by saponification (11). An admixture with authentic DL-*threo*-9,10-dihydroxystearic acid had an mp of 92.0-93.0 C. Comparison of the methyl esters of the unknown diol with those of the standard diol by TLC on boric acid-impregnated Silica Gel G (4) also demonstrated that both had the *threo* configuration.

The crude dihydroxy acid preparation (before crystallization) was cleaved by the periodate-permanganate method of von Rudloff (14) to determine the location of the original epoxy group and to detect other positional isomers, if present. Analysis of the monobasic cleavage products as free acids by GLC showed 92% nonanoic acid and small amounts of unidentified components. The dibasic fragments, analyzed by GLC of their methyl esters, contained 96% of nonanedioate but no dodecanedioate.

Characterization of Unsaturated Epoxide

The unsaturated epoxide fraction had a purity of 96% by HBr titration; GLC confirmed this result. IR showed no *trans* absorption. Analysis by TLC revealed that this epoxide was probably methyl coronarate since it migrated slower than methyl vernolate on Silica Gel G (2). This epoxy fraction gave a plain positive ORD spectrum, $[\alpha]_D^{24.5°}$ + 3.9° (c = 11.7, hexane), in contrast with the spectrum of methyl vernolate (15). A portion of the unsaturated epoxide was first subjected to acetolysis and then saponified (11), yielding a dihydroxy acid, mp 60.5-61.0 C (from ethyl acetate at −18 C). Oxidative cleavage (14) of this dihydroxy acid before crystallization yielded only hexanoic and nonanedioic acids as shown by GLC. The unsaturated diol consumed 0.97 mole equivalents of hydrogen (Pt catalyst) to yield a saturated diol, mp 92.5-93.0 C. No depression of the melting point was observed on admixture with authentic DL-*threo*-9-10-dihydroxystearic acid, mp 92.0-93.0 C. The saturated diol methyl ester, mp 67.0-68.0 C, had the same R_f value by TLC on boric acid-impregnated Silica Gel G as the methyl ester of the known *threo*-dihydroxy acid. Periodate-permanganate cleavage of the saturated diol gave nonanoic and nonanedioic acids as the major products (94%) and no other products in amounts larger than 1.5%. If vernolic acid had occurred in the unsaturated epoxy fraction, hexanoic and dodecanedioic acids would have been found in the cleavage products; these fragments were not observed.

Characterization of Dienol I

Dienol I gave an IR spectrum that showed hydroxyl (2.73 μ) and conjugated *cis,trans* (10.16 and 10.53 μ) absorption. The UV spectrum revealed $\lambda_{max}^{cyclohexane}$ 234 mμ (ε 29, 100), which also is indicative of a *cis,trans*-conjugated diene, although the absorption is slightly stronger than reported previously (16). Dienol I gave a plain negative ORD curve, $[\alpha]_{400}^{25°}$ −0.55° ± 0.10° (c = 2.9, hexane). Powell et al. (16) reported essentially the same spectrum for the 13-hydroxyoctadecadienoate from *Xeranthemum annuum* seed oil.

Periodate-permanganate (14) cleavage of dienol I (0.016 g) yielded hexanoic and nonanedioic acids as indicated by GLC analysis of the cleavage products as free acids and as methyl esters.

When hydrogenated in absolute ethanol over Adams catalyst, dienol I (0.0182 g) consumed 1.9 mole equivalents of H_2 to yield a saturated methyl ester (0.0151 g), mp 51.0-52.0C (from petroleum ether). The saturated hydroxy ester (0.012 g) was treated with 0.245 g of chromium trioxide (17) in 2.0 ml of acetic acid and 0.2 ml of water for 1 hr at room temperature. Pentanoic, hexanoic, dodecanedioic and tridecanedioic acids comprised the major portion (80%) of the cleavage products according to GLC analysis. Small quantities of numerous degradation products were also evident.

Partial diimide reduction of dienol I (0.120 g) with potassium azodicarboxylate (18) as the diimide source (19) was accomplished by utilizing reactants in the exact proportions described by Powell et al. (16). Analysis of the partial reduction mixture (0.109 g) by TLC on $AgNO_3$-impregnated Silica Gel G revealed four spots with R_f values of 0.31, 0.38, 0.52 and 0.68. This mixture was separated by preparative TLC on $AgNO_3$-impregnated Silica Gel G (1 mm thick), but because of overloading, only three discrete fractions could be isolated. The first fraction (highest R_f) was a saturated ester (0.052 g) having an IR band at 2.76 μ (OH), mp 51.0-51.5 C [lit. mp of methyl hydroxyoctadecanoates; racemic 13-hydroxy, 53.3-53.5 C; 13D-hydroxy, 56-57 C; racemic 9-hydroxy, 50.3-50.6 C; 9D-hydroxy, 53.0-53.5 C (12,20,21)]. No depression in mp was observed on admixture with authentic (±) methyl 13-hydroxyoctadecanoate. The IR spectrum of the next fraction (0.010 g), R_f 0.52, showed hydroxyl (2.74 μ) and isolated *trans* (10.34 μ) absorption. Hexanoic and undecanedioic acids were the only products obtained by oxidative cleavage of this fraction as demonstrated by GLC analysis of the fragments as free acids and also as methyl esters. The last fraction (0.032 g) contained both a *cis*-monoene and starting material as shown by IR (*cis,trans*-conjugation) and by two spots, R_f 0.37 and 0.32, on TLC. Oxidative cleavage of this fraction and analysis of the products as described above, indicated that the fragments were hexanoic and nonanedioic acids and a γ-lactone (5.64 μ IR band). The equivalent chain lengths (22) of the lactone were 10.7 in an Apiezon L column (nonpolar) and 15.9 in an LAC-2 R-446 (polyester) column (16).

Characterization of Dienol II

The IR spectrum of II showed hydroxyl (2.74 μ) and conjugated *cis,trans* (10.16 and 10.54 μ) absorption, and the UV spectrum indicated *cis,trans*-conjugated diene absorption, $\lambda_{max}^{cyclohexane}$ 234 mμ (ε 27,400). Dienol II

gave a plain positive ORD spectrum, $[\alpha]_{400}^{25°}$ + 2.3° ± 0.6° (c = 3.0, hexane). Oxidative cleavage of II yielded only hexanoic and nonanedioic acids. The product resulting from hydrogenation of dienol II (2.1 mole equivalents of H_2 consumed) had mp 48.0-49.0 C and was cleaved to nonanoic, decanoic, octanedioic, and nonanedioic acids by chromium trioxide.

Analysis of the partial diimide reduction products (0.145 g) from dienol II by TLC on $AgNO_3$-impregnated Silica Gel G indicated three spots, R_f 0.30, 0.48, and 0.64. The partially reduced mixture was separated as before by preparative TLC. The first fraction, R_f 0.64, contained a saturated hydroxy ester (0.055 g) which melted at 47.5-48.5 C. This mp was undepressed by admixture with authentic (±) methyl 9-hydroxyoctadecanoate. Residual starting material from the reduction was found in the second fraction (0.047 g), R_f 0.48, along with a *trans*-hydroxymonoene as demonstrated by IR bands at 2.76 μ (OH), 10.34 μ (isolated *trans*), and 10.17 and 10.54 μ (conjugated *cis,trans*). The permanganate-periodate cleavage products from the second fraction were hexanoic and nonanedioic acids, derived from the starting dienol, and octanoic and nonanedioic acids, derived from the *trans*-monoene. Hexanoic acid was the only monobasic acid fragment from cleavage of the last fraction (0.027 g) R_f 0.30, which was the *cis*-hydroxymonoene. A dibasic acid fragment (after esterification) was not observed, but a fragment giving strong γ-lactone (5.64 μ) and ester carbonyl (5.77 μ) absorption in the IR was one of the products and was shown by GLC to have equivalent chain lengths of 17.0 on a nonpolar column and 26.0 on a polyester column (16).

DISCUSSION

The dihydroxy acids derived from the epoxy acids of sunflower oil were identified as *threo*-9,10-dihydroxystearic and *threo*-9,10-dihydroxy-*cis*-12-octadecenoic acids. These results demonstrate conclusively that the original epoxy acids are *cis*-9,10-epoxystearic and *cis*-9,10-epoxy-*cis*-12-octadecenoic acids. Previous work in our Laboratory (15) revealed that the 9,10-epoxy acids from *X. annuum* seed oil both have the (9*R*,10*S*) configuration (23). Since the corresponding epoxides isolated here are also both dextrorotatory, they too must have the (9*R*,10*S*) configuration. None of the *cis*-12,13-epoxy-*cis*-9-octadecenoic (vernolic) acid was detected, either by TLC or by cleavage fragments, although Morris et al. (2) suggested on the basis of TLC results that a small amount of this acid occurs in sunflower seed oil. Powell et al. (15) also found a mixture of vernolic and coronaric acids in *Xeranthemum* seed oil.

Characterization of the diimide partial reduction products from the two dienols definitely establishes the location and configuration of the double bonds. Dienol I is 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid and dienol II is 9-hydroxy-*trans*-10,*cis*-12-octadecadienoic acid. Both of these isomeric fatty acids were previously isolated from *Xeranthemum* oil (16), and the 13-hydroxy isomer is a major constituent of *Coriaria nepalensis* seed oil (12). The optical rotations of these dienols, while measurable, are lower than those of optically pure compounds (12) and this characteristic suggests that they are extensively racemized. However, since both the dienols and the epoxy acids are optically active, we conclude that they are the results of biological processes and not autoxidation.

The γ-lactones resulting from cleavage of the *cis*-hydroxymonoenes give retention characteristics (on GLC) that differ slightly from those previously reported (16), but this difference is probably caused by variations in column parameters.

As shown in Table I, earlier work (1-3) indicated that oils of sunflower seeds from Uruguay and Turkey contained HBr-reactive materials and also gave UV maxima at 234 mμ. The same was true of oils from seeds introduced from Yugoslavia and Canada. Analysis of recently extracted oil from the same seed accessories revealed that both the HBr equivalent and the UV absorption had more than doubled since the first analysis. Since seed kernels only were used in the original work, whereas whole seeds were used in the current work, one might suspect that the hull extract was responsible for the variation in amounts of HBr-reactive materials. However, our most recent information indicates that the hull extract contains little, if any, HBr-reactive material. The apparent increase in HBr-reactive materials during storage of the seeds introduced in 1956 is most interesting and we are investigating this aspect of the problem in more detail.

Seed oils from recently grown Russian varieties contain very low percentages of HBr-reactive materials by titration at either 0 C or 55 C (Table I). To establish whether the much larger quantities of such components found in other sunflower lines were due to age of seed or to genetic makeup, several selected introductions from Turkey and Canada that pro-

vided seed oils of substantial HBr reactivity were regrown in 1966 at Ames, Iowa, to produce fresh seed. Results of HBr titration of the oils from the Ames seed increases provided equivocal results. Their HBr uptake of 0.5-1.2% was several times that of the Russian varieties grown in the same crop year, yet below that found initially in the older seed from Turkey and Uruguay. Thus, further work is required to assess the relative importance of storage time and genetic composition in governing the amount of oxygenated acids in sunflower seed oil.

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