Evidence for the Probable Presence of Sulfur-Containing Fatty Acids as Minor Constituents in Canola Oil[1]

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Three isomeric epithio stearic acids have been concentrated as possible minor sulfur-bearing components of unprocessed canola oil. Chromatographic and mass spectral evidence is presented in support of these novel fatty acid structures, tentatively identified as isomeric 9, 12; 8, 11; and 7, 10 epithio stearic acids, each with a methyl substituent on the ring.

The occurrence of small amounts of sulfur compounds in rapeseed oil has long been known (1,2). These compounds have been implicated as hydrogenation catalyst poisons (3,4) and also have been held responsible for certain unpleasant odors in heated rapeseed oil (5). Their chemical composition is not fully understood, but they generally are believed to arise from the hydrolysis of the glucosinolates present in the seed (3). The hydrolysis products transform into a variety of sulfur compounds which include isothiocyanates, thiocyanates, nitriles and oxazolidinethiones (6).

The introduction of new rapeseed cultivars (referred to as "canola") with low glucosinolate content has helped to produce oils with less sulfur content. However, sulfur levels as low as one mg/kg of oil are capable of interfering with the catalytic hydrogenation of canola oil (4,7), and there is a need to further reduce the level of these compounds in canola oil. To this end, a thorough understanding of the chemical composition of all the sulfur compounds present in the oil has become necessary. It is possible that not all the sulfur compounds occurring in canola are derived from glucosinolates. Indeed, Devinat et al. (8) showed that the sulfur compounds in rapeseed oil are of three types, volatile, thermolabile and nonvolatile. The isothiocyanates, which have been studied extensively (6,9), belong to the volatile type. The present study was directed specifically toward the identification of the nonvolatile components by the general procedure outlined in Figure 1.

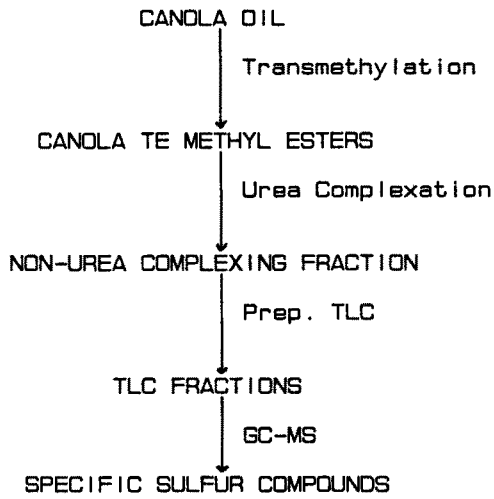

FIG. 1. General methods of isolation of methyl esters of fatty acids used for identification of sulfur compounds in canola oil.

EXPERIMENTAL

Source of material. Commercially expelled but unrefined canola oil (clear, greenish brown in color) was supplied by J.M. deMan of the University of Guelph. The oil had been stored at 5 C in an amber-colored glass bottle for ca. 15 mo before analysis.

Preparation of methyl esters by transesterification (TE). Canola oil (50 g) was agitated with benzene (62.5 ml) and sodium methoxide in methanol [0.5 M, 125 ml, prepared by dissolving 27 g of sodium methoxide powder (methanol sodium derivative, 'Baker') in reagent grade methanol (one l)] for two hr at room temperature and allowed to stand overnight. Brine (5%, 150 ml) was added to the reaction mixture, which was then extracted with diethyl ether (150 ml × 3). The combined ether extracts were washed with brine solution (25 ml), and the solvent was removed under vacuum. The TE methyl esters were further heated (60 C) with benzene under vacuum to remove the last traces of water (yield 47 g).

Urea fractionation of methyl esters. The TE methyl esters (10.0 g) were crystallized overnight at 0 C from a solution of methanol (300 ml) and urea (50 g). The precipitate was filtered under suction and washed with cold methanol saturated with urea (30 ml). Most of the methanol was removed from the filtrate under vacuum and after dilution with water (250 ml) the esters were extracted with diethyl ether (150 ml × 5) until the yellow color was transferred completely into the ether layer. The combined ether extracts were then worked up in the usual manner to obtain (Fig. 2) the nonurea crystallizing fraction, $NUCF_1$ (1.18 g). Mixing the precipitate with water (400 ml), followed by extraction with diethyl ether (100 ml × 3) furnished the urea crystallizing fraction, UCF_1 (8.13 g).

Urea fractionation of the hydrogenated TE methyl esters. TE methyl esters (20 g) were dissolved in methanol (100 ml) and agitated with platinum oxide catalyst (2 mg) in the presence of a hydrogen atmosphere (continuous flow) for 3 hr.

The hydrogenated product (20 g) was crystallized from methanol (600 ml) and urea (100 g) in the usual manner to obtain the nonurea crystallizing fraction, $NUCF_2$ (0.460 g).

Saponification and removal of unsaponifiable material. Canola oil (500 g) was refluxed with potassium hydroxide in 95% ethanol (2M, one l) for one hr. The mixture was allowed to cool, diluted with water (2.5 l) and extracted

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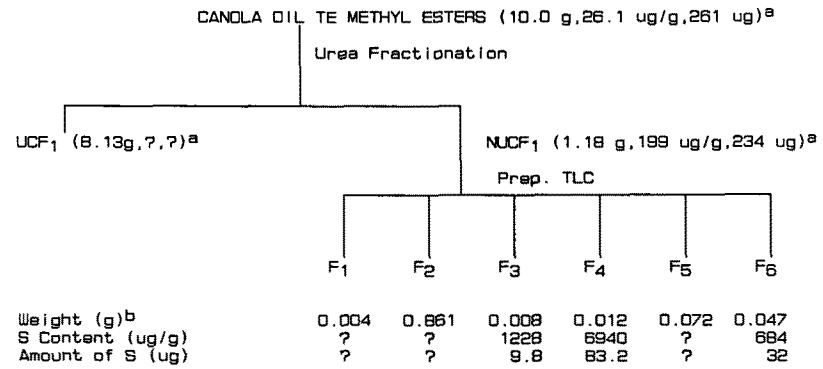


FIG. 2. Distribution of sulfur in canola oil fractions from transesterification. The three figures in parentheses (a) represent the weight of the material in grams, the sulfur content in μg/gram and the absolute amount of sulfur in μg, respectively. Weights (b) are adjusted for 1.18 g $NUCF_1$. The (?) represents < 0.1 μg/g, which is the lower level of sulfur detection by the method used.

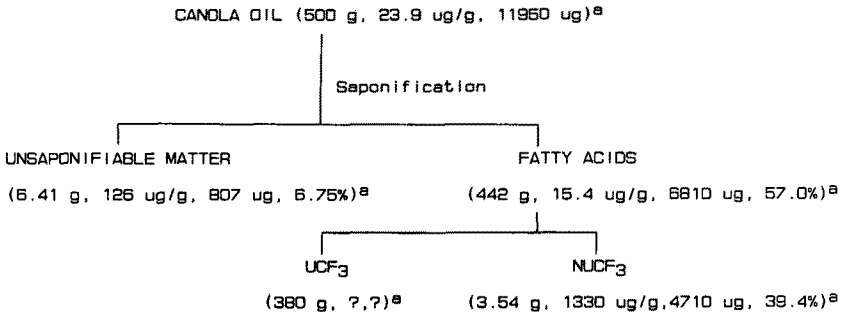


FIG. 3. Distribution of sulfur in unsaponifiables and fatty acids of canola oil. The figures in parentheses (a) show the mass of the material in grams, its sulfur content in μg/gram, the absolute sulfur content in μg, and the percent of original sulfur, respectively. The (?) represents < 0.1 μg/g, which is the lower level of sulfur detection by the method used.

with diethyl ether (2.5 l). These quantities of water and ether were necessary for a good phase separation. The ether extraction was repeated twice more (one l and 0.5 l, respectively). The combined ether extracts were washed with distilled water (250 ml × 3) and allowed to stand overnight. The ether was removed under vacuum at 40 C and the residue dried by evaporation with acetone to obtain the unsaponifiable matter (6.41 g, 1.3%, Fig. 3).

Saponifiable matter was recovered from a portion (100 ml) of the aqueous layer from above (1250 ml) by acidification with HCl (6N, 20 ml), followed by extraction with diethyl ether (100 ml × 3). The combined ether extracts were worked up to obtain the saponifiable matter (35.42 g, 88.5%). In Figure 3 these fatty acids are recalculated from the volumes to show a recovery of 442 g.

Methylation of the saponifiable matter. Of the saponifiable fatty acids 20 g was methylated by refluxing with methanol (90 ml) and 10% methanolic boron trifluoride (10 ml) for 20 min. The esters were recovered in the usual manner.

Urea fractionation of the saponifiable matter methyl esters. The methyl esters prepared from the saponifiable matter (20.0 g) were crystallized from methanol (600 ml) and urea (100 g) in the usual manner to obtain a nonurea complexing fraction, $NUCF_3$ (0.16 g) and urea complexing fraction, UCF_3 (17.20).

Preparative thin layer chromatography. Preparative TLC was carried out on precoated 0.25 mm silica gel plates (Prekote, Applied Science, Deerfield, IL). The plates were precleaned with ethyl acetate and activated at 130 C for 1/2 hr before use.

The TE non-urea crystallizing fraction from the unhydrogenated canola oil methyl esters, $NUCF_1$ (0.800 g), was chromatographed on 10 TLC plates with hexane, diethyl ether and acetic acid (80:20:1) as the developing solvent and separated into six fractions (Fig. 2). These were located on the plates by viewing under ultraviolet light after spraying with 0.2% methanolic 2′,7′-dichlorofluorescein. The separated components were extracted from the silica with diethyl ether.

The nonurea crystallizing fraction of the TE hydrogenated methyl esters $NUCF_2$ (280 mg) was chromatographed using hexane, diethyl ether and acetic acid (70:30:1) as the developing solvent and also separated into six fractions as described above.

The methyl esters purified by removal of unsaponifiable matter from the acids prior to urea complexation ($NUCF_3$, Fig. 3) were also separated by preparative TLC into the same six fractions.

Gas chromatography. The canola oil fractions were examined by gas chromatography on a Perkin Elmer Model 900 instrument. Fused silica capillary columns (J&W Scientific Inc., Rancho Cordova, California) coated with bonded polyethylene glycol (DB-WAX, 30 m, 0.24 mm i.d.) as well as methyl silicone (DB-1, 3 m, 0.24 mm i.d.) were used. Helium (26 psig) was used as carrier gas and the column temperature was programmed from 150-240 C and 200-300 C, respectively, at the rate of 8 C/min for both columns.

Gas chromatography/mass spectrometry. The gas chromatography was carried out on a fused silica capillary column (28 m, 0.24 mm i.d., J&W Scientific Inc.) coated with methyl silicone (DB-1). Helium was used as the carrier gas at a pressure of 10 psig, and the column temperature was programmed from 150-300 C at the rate of 8 C/min. For ITD mass spectra, the column outlets were connected (by direct insertion) to an Ion Trap Detector (Finnigan Mat Model 700), which was controlled by an IBM personal computer XT (PC XT). For CI (CH_4, 0.9 torr, 100 C) and conventional EI spectra the same gas chromatographic conditions were used, but the spectra were recorded on a FINNUP instrument in the Halifax Laboratories of the National Research Council of Canada.

RESULTS AND DISCUSSION

Basically the approach used in this work to identify the nonvolatile sulfur compounds was to concentrate, by various fractionation techniques, amounts of methyl esters of fatty acids, or other oil components, sufficient for detection of sulfur-bearing fatty acids by GC/MS. The fractionation procedure (Fig. 1) was based on adduct formation with excess urea, designed primarily to remove the straight long-chain components. The nonurea complexing fraction would thus be enriched in any short-chain, branched-chain or cyclic compounds. Substances such as alkyl sulfides, alkyl disulfides, thiols and sulfur-containing heterocyclics were considered potential candidates for nonvolatile sulfur. We expected to find some of these, if present, in the nonurea complexing fraction.

Initially, canola oil was transesterified with sodium methoxide and methanol at ambient temperature to avoid structural alterations, and the resultant esters were subjected to urea complexation both before and after catalytic hydrogenation with platinum oxide. The nonurea complexing fractions were recovered and were further fractionated by preparative thin layer chromatography (preparative TLC) on silica gel. Each of these sub-fractions was then analyzed by GC/MS. In a slightly different approach, the oil was first saponified, freed of unsaponifiables, and the saponifiables (fatty acids) were esterified, treated with urea, and examined as described in Fig. 1.

The distribution of sulfur in the different fractions was followed by the method of combustion and ion chromatography previously reported by us (10). Fractions containing a few micrograms of sulfur can be detected by this technique. We found that after urea fractionation of canola TE methyl esters, practically all of the sulfur was retained in the nonurea complexing fraction, $NUCF_1$ (Fig. 2). Preparative TLC of $NUCF_1$ afforded six further fractions F_1 to F_6 (numbered in the order of decreasing mobility). F_1 contained pigments. F_2, the biggest of the six $NUCF_1$ fractions, was comprised predominantly of methyl linoleate and linolenate. No sulfur was detected in these two fractions. Fractions F_3 and F_4 were both found to contain sulfur accounting for 3.7% and 31.8% of the sulfur originally present in the oil. We did not find any sulfur in fraction F_5, which was comprised almost entirely of sterols. F_6, which was polar material recovered from the origin of the chromatogram, contained 12.3% of the original sulfur. In all, 47.8% of the original canola oil sulfur was recovered. The apparently poor recovery of sulfur is probably due to loss of volatile or water-soluble compounds in early steps, or of polar components during the TLC separation and band recovery isolation procedures.

Figure 3 shows the distribution of canola sulfur after initial saponification of the oil. It is noteworthy that a substantial portion of the sulfur is retained in the saponifiable matter, confirming the probable presence of sulfur-containing fatty acids. Urea complexation of the methyl esters prepared from the saponifiables furnished an adduct (UCF_3) which was virtually free from sulfur, while the nonurea complexing fraction ($NUCF_3$) was enriched in sulfur. This showed that the sulfur is contained in a noncomplexing fatty acid, most

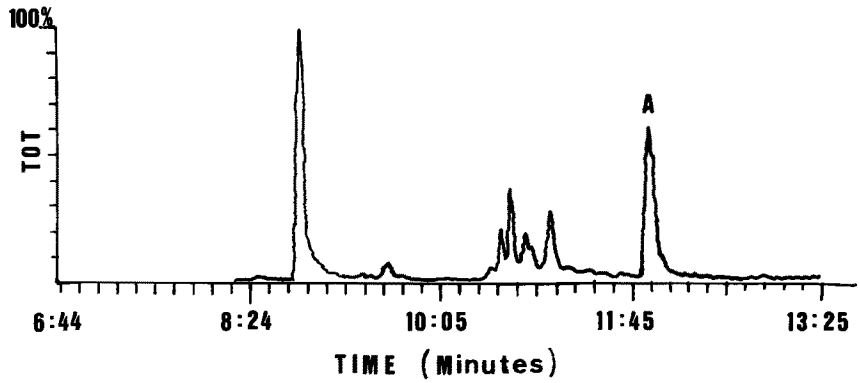


FIG. 4. The Ion Trap Detector recombinant ion chromatogram of $NUCF_1$ Fraction 4. Separation on a 28 m × 0.24 mm i.d. fused silica capillary column coated with methyl silicone (DB-1), temperature programmed from 150-300 at 8 C/min.

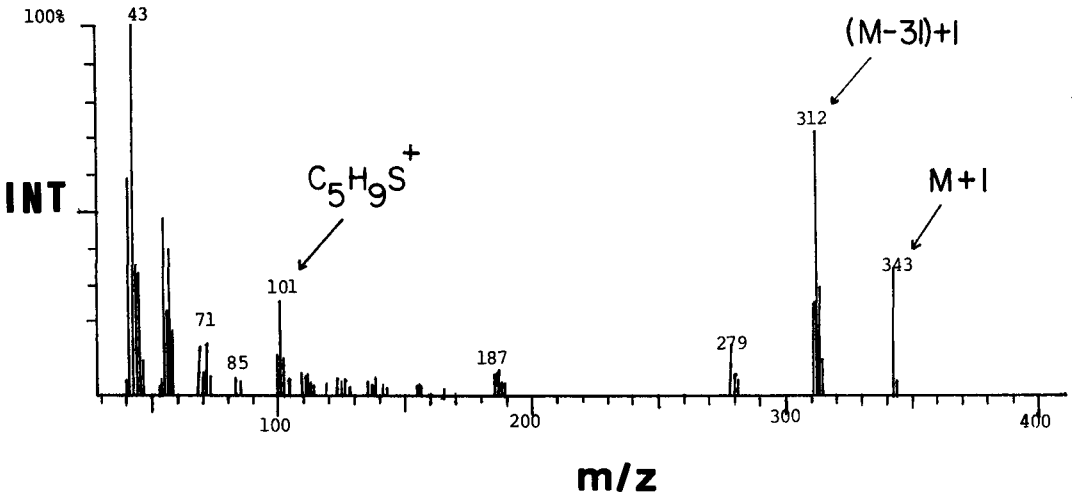


FIG. 5. The Ion Trap Detector mass spectrum of $NUCF_1$, Fraction 4, Component A.

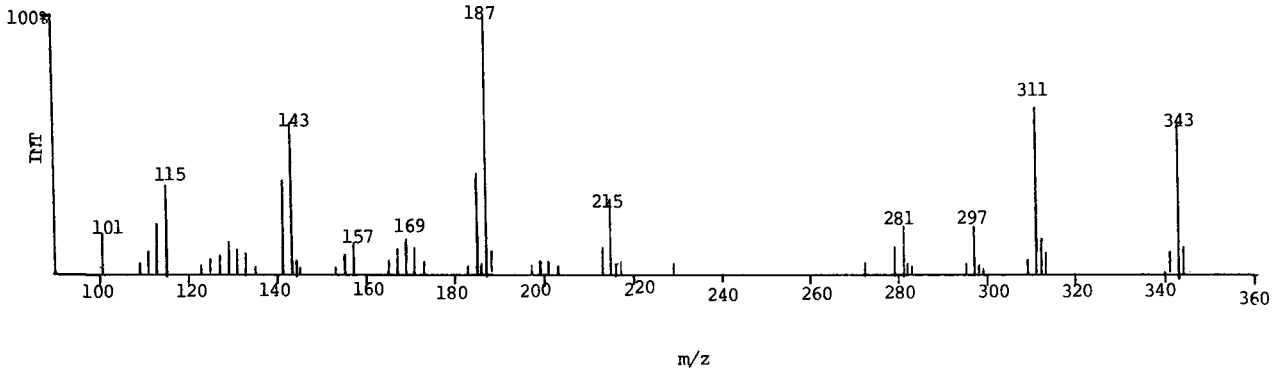
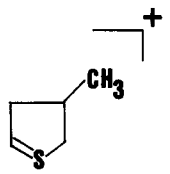


FIG. 6. The conventional equipment CI mass spectrum of $NUCF_1$, Fraction 4, Component A.

probably with either a branched-chain or a cyclic structure. Parallel concentration of sulfur in the nonurea complexing fraction, when either fatty acids freed of unsaponifiables or the directly prepared TE methyl esters of whole unrefined canola oil were treated with urea (Fig. 2), provides evidence for the occurrence of sulfur-containing fatty acids of complex structures in the original oil.

The ITD recombinant ion chromatogram of fraction F_4 obtained by preparative TLC of the nonurea complexing fraction ($NUCF_1$) from canola methyl esters is shown in Figure 4. We paid particular attention to peak A, which had an ECL value of 21.3 because it was found not only in F_4 from the TE methyl esters, but also in the corresponding TLC fractions from the hydrogenated TE methyl esters ($NUCF_2$) as well as from the saponifiable product ($NUCF_3$).

The ITD mass spectrum of Figure 4, peak A (Fig. 5), contained a prominent ion peak at m/z 343. Mass spectra obtained with the ITD almost always exhibit a strong $(M+1)^+$ ion peak instead of M^+ (11); thus, the molecular weight of A is 342. The CI mass spectrum of A (Fig. 6) had the highest ion peak at 343, while its EI mass spectrum (Fig. 7) had the highest ion peak at 342, confirming the molecular weight of 342. The presence of a series of ion peaks in the latter spectrum, at m/z 55, 69, 83 and 97 associated with C_nH_{2n-1} ions, coupled with an ion peak at 311 ($M-OCH_3$), furnished evidence for a fatty acid methyl ester structure. The ion peak at 327 (M-15) in the Figure 7 spectrum suggested the presence of a methyl substituent. Structures shown in Figure 8 are consistent with the rest of the mass spectral data. The two sets of fragment ions (Fig. 7) at m/z 185, 199, and 213 (arising from fission α to the ring on the ester side) and at m/z 257, 243 and 229 (arising from α-fission on the alkyl side of the ring) strongly suggest that the ring system is at three positions respectively 9,12; 8,11; and 7,10. Simultaneous α-cleavage on either side of the ring gives the fragment of m/z 101 due the ion



which is prominent in the ITD spectrum (Fig. 5) as well as in the conventional EI spectrum (Fig. 7).

The CI as well as the ITD spectra contain prominent ions at m/z 187 in addition to 185. Presumably they arise from the diprotonation of the sulfur-containing

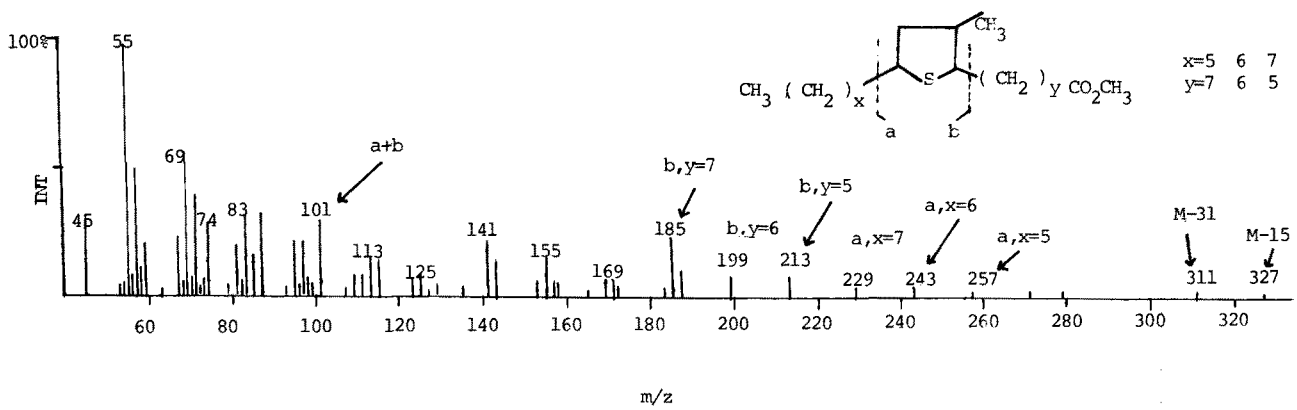
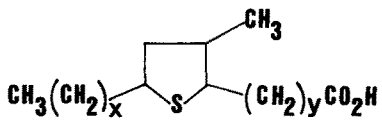


FIG. 7. The conventional equipment EI mass spectrum of NUCF$_1$, Fraction 4, Component A.



	x	y
1	5	7
2	6	6
3	7	5

FIG. 8. Structures proposed for three sulfur-containing fatty acids in canola oil.

fragment. Such ions have been observed for epithio stearic acids even in the case of EI spectra (16).

According to the proposed structures, the epithio stearic acid should contain 93 μg S for each milligram of acid. Thus, the 83 μg S in Fraction 4 represent about one milligram of the acid, which accounts for only a portion of F_4 (12 mg). This discrepancy probably arises from the presence in F_4 of nonsulfurous components, or of some containing sulfur which may not be volatile in GLC. Also, contamination with silica gel used for the chromatography cannot be ruled out in such small masses.

The epithio stearic acid structure may be related to the furanoid fatty acids (oxygen in the ring in lieu of sulfur) which are known as plant products but limited to the seed of *Exocarpus cupressiforimis* (12) and the latex of the rubber tree *Hevea brasiliensis* (13). A series of furanoid fatty acids with one or two methyl substituents on the furan ring also have been found in certain fish lipids (14,15). The three epithio isomers proposed would be unlikely to separate with the gas chromatographic conditions employed.

Thus, it is shown that unrefined canola oil contains low levels of sulfur-containing fatty acids. They contribute sulfur to the oil in the stable form of triglycerides and resist refining procedures. The unsaponifiable matter did not contain an important proportion of the total sulfur in the oil.

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