Minor Components of Olive Oils. II. *trans*-9:10-Epoxystearic Acid in Orujo Oil[1,2]

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An orujo oil (sulfur olive oil) has been shown to contain several classes of oxygenated fatty acids amounting to more than 10% of the total oil. Epoxy acids amount to 3.6% of the fatty acids of this oil and monohydroxy acids constitute a further 6%. The major epoxy acid has been proven to be *trans*-9:10-epoxystearic acid.

Introduction

ORUJO OIL, or sulfur olive oil, is obtained by solvent extraction of pressed olive pulp. Gracián *et al.* (1) have studied the changes in the chemical and physical properties of the oil extracted from the pressed pulp after various periods of storage between pressing and extraction. They found an increase of free acids during this storage, due to enzymatic or microbiological hydrolysis. The same workers also determined that as the viscosity of the oil and the proportion of "oxy-acids" increased there was a decrease in the total unsaturation. The formation of the "oxy-acids" was evidenced by an increasing proportion of petroleum ether insoluble residue in the oil. This residue was shown by Gracián *et al.* (2) to contain polymeric material which was considered to have derived from polymerization of "oxy-acids." The proportion of "oxy-acids" in the oil is subject to wide variation. Gracián and Ventura (3) have studied the effects of various factors on the amount of "oxy-acids" in the orujo oils, and found that their proportion depended on the elapsed time between pressing of the olives and extraction of the pulp and upon the nature of the solvent used in the extraction.

Little work has been carried out to determine the nature of the individual oxy-acids so produced. Desnuelle and Burnet proposed a procedure for the separation of the oxygenated acids as a class (4). This chromatographic technique appears to be more specific than the usual method for their determination, which depends on their insolubility in petroleum hydrocarbon. That precipitation from light petroleum is not a very specific procedure for determination of oxygenated fatty acids is obvious in view of the relatively high solubility of epoxy- and mono-hydroxy fatty acids in this solvent, particularly in the presence of a high proportion of non-oxygenated acids and glycerides. Paquot and Querolle (5) examined the "oxy-acids" from orujo oil and reported the presence of a 9:10-dihydroxystearic acid and a Diels-Alder type of dimer of 9-hydroxyoctadeca-10,12-dienoic acid. The structures of the individual oxygenated acids in orujo oil must be determined as a preliminary to an understanding of the oxidation process and other changes occurring in pressed olive pulp. This paper describes the isolation and identification of *trans*-9:10-epoxystearic acid from orujo oil.

Experimental

The orujo oil used in this study was obtained in the laboratories of the Instituto de la Grasa, Seville, Spain, by extraction of pressed olive cake with trichloroethylene. The elapsed time between the pressing of the olives and the extraction of the cake was three months. The characteristics of the orujo oil follow; values for olive oil obtained from the same olives by pressing are given in parentheses: Free acids, determined by alkalimetry and calculated as oleic acid, 8.8% (0.9%); Conjugated diene, calculated as C_{18}-diene from absorptivity at 234 mμ, 1.2% (trace); Hydroperoxides determined by A.O.C.S. Method CD

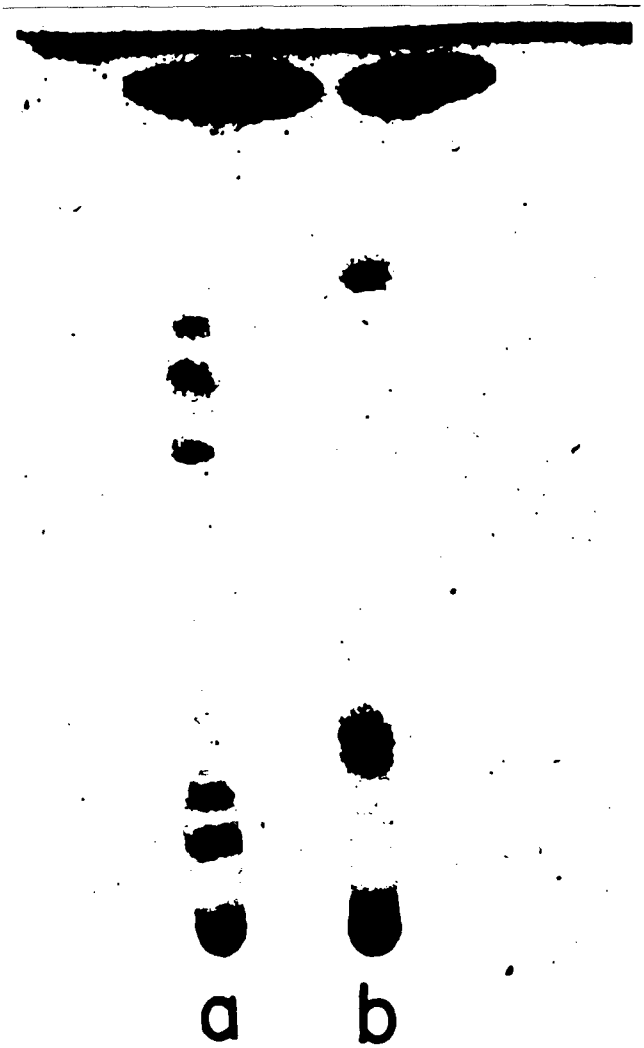

FIG. 1. Thin-layer chromatogram of the methyl esters of orujo oil fatty acids. Sample a: esterified with diazomethane. Sample b, esterified with methanol containing 2% of hydrogen chloride. The chromatogram was developed with 10% diethyl ether in hexane, the spots were located by charring after spraying with 50% sulfuric acid, and the reproduction was obtained by direct photocopy of the plate.

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8–53 and calculated as hydroperoxyoleic acid, 1.8% (1.7%); Epoxy acids estimated by near-infrared spectrophotometry (6), 1.5%, 2.3% (trace); Reactive hydroxy acids also estimated by near-infrared spectrophotometry (6, 7) and calculated as C_{18}-hydroxydiene, 0.4% (trace). The reactive hydroxy acid and epoxy acid values are at best an estimate because of high interfering background absorption in the 2.8 μ region. Two values are given for epoxy acids in orujo oil, the former is calculated as *cis*-epoxystearic acid, and the latter as *trans*-epoxystearic acid (6).

The oil was saponified at room temperature by standing overnight with excess 5*N* ethanolic potassium hydroxide. Unsaponifiable matter was extracted from the hydrolysate with diethyl ether. The soap solution was then made just acid, with the calculated amount of 2*N* hydrochloric acid and the precipitated fatty acids were immediately extracted with diethyl ether, washed with water, dried over Na_2SO_4, and recovered. This procedure minimizes alteration of epoxy or reactive hydroxy components (8, 9).

To check whether epoxy or reactive hydroxy acids were indeed present, a small portion of the mixed fatty acids was esterified with anhydrous methanolic hydrogen chloride, the bulk of the mixed acids was esterified with diazomethane in ether, and the two ester preparations were compared by thin-layer chromatography (10, 11). The results are shown in Fig. 1. In addition to the usual esters near the solvent front, the sample obtained with diazomethane *(a)* showed three spots in the area to be expected for epoxy esters plus two corresponding to monohydroxy esters and some more polar material remaining at the starting point. The spots designated as epoxy esters and monohydroxy esters migrated with known esters of these types when chromatographed on the same plate. The esters prepared with methanol in the presence of HCl, on the other hand, showed neither the epoxy nor the monohydroxy components *(b)*. Two spots appeared in new positions demonstrating that some alteration of the original components had occurred during the acid catalyzed esterification. This test was a good indication that epoxy acids were present in the mixed acids and that the HCl in methanol and split them to either chlorohydrins or, more likely, to hydroxymethoxy compounds which would be more polar than epoxy esters. The test also indicated that reactive hydroxy acid was present which was altered in the acid medium to a less polar product, probably a methoxy ester (cf. 12). The probable reactions and possible products are delineated in Fig. 2.

Separation of the Mixed Esters

Fractionation of the mixed esters prepared with diazomethane was effected by adsorption chromatography on silica gel. A portion of the esters (14.5 g.) was applied to a 80 x 2.4 cm. column of 60–200 mesh silica gel [4] (270 g.) and eluted with petroleum ether (b.p. 35–40) containing successively increasing proportions of diethyl ether. Fractions of 50 ml. each were collected, monitored by thin-layer chromatography and fractions containing similar components were combined and weighed. A thin-layer chromatogram of the fractions so obtained is shown in Fig. 3.

Fraction 1 (sample a) was eluted with 3% diethyl ether in petroleum ether and consisted of normal nonoxygenated esters accounting for most of the total mixed esters. Gas-liquid chromatography showed this fraction was similar in composition to the mixed esters of olive oil (Table 1) but contained less linoleate.

Fraction 2 (sample b) immediately following this main ester fraction, was also eluted with 3% ether in petroleum ether and amounted to 150 mg. or 1.0% of the total. This material showed the color development pattern characteristic of a conjugated polyunsaturated ester when the thin-layer chromatogram was

[4] W. R. Grace & Co., Davison Chemical Division, Baltimore, Maryland.

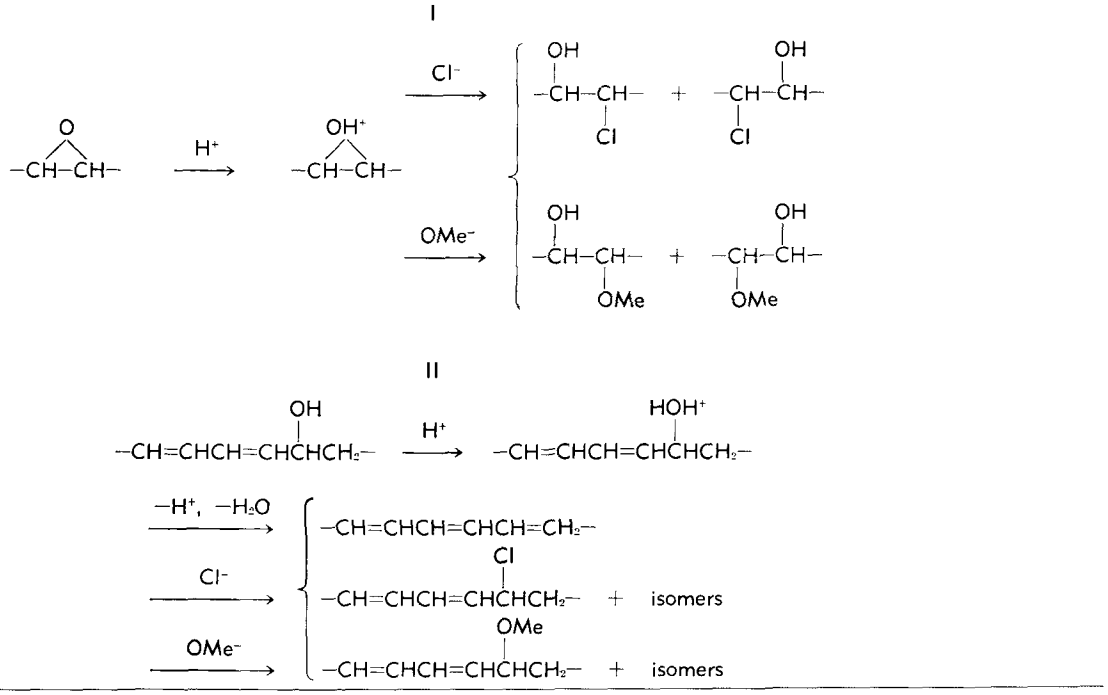

FIG. 2. Probable reactions of epoxy and vicinally unsaturated hydroxy compounds in methanol containing anhydrous hydrogen chloride.

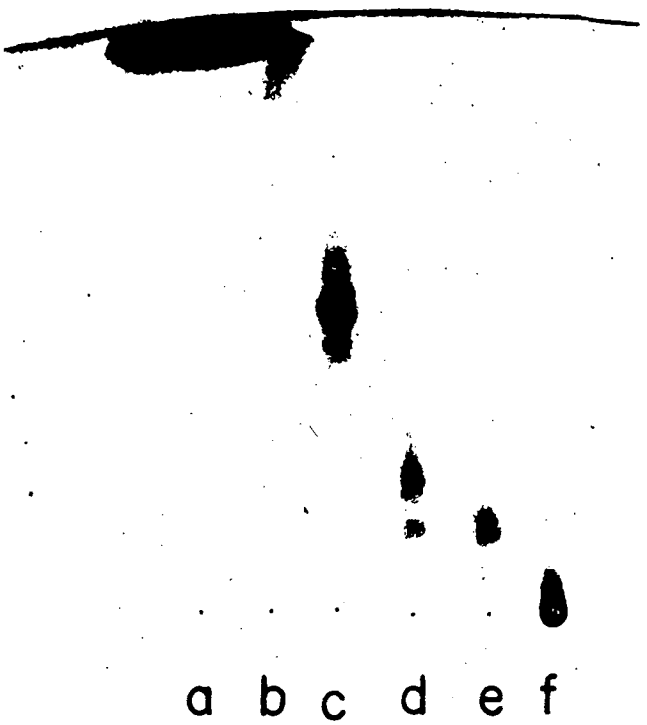

FIG. 3. Thin-layer chromatogram of fractions obtained by adsorption chromatography on silica gel of the methyl esters of orujo oil fatty acids. Samples a–f correspond to Fractions 1–6 and are described in the text. The chromatogram was developed with 10% diethyl ether in hexane, the spots were located by charring after spraying with 50% sulphuric acid, and the reproduction was obtained by direct photocopy of the plate.

heated after spraying with sulfuric acid (7). Conjugated dienes were also indicated by the strong absportion of this fraction at 230 mμ.

Fraction 3 (sample c) eluted with 5% ether in petroleum ether, amounted to 520 mg. or 3.6% of the sample and had similar migration characteristics to authentic epoxy esters on a thin-layer chromatogram.

Fractions 4 and 5 (samples d and e) were eluted with 10% ether in petroleum ether and migrated like monohydroxy esters on thin-layer chromatography. Together they amounted to 870 mg. or 6.0% of the total mixed esters.

Fraction 6, the final fraction (sample f) was eluted with pure ether and consisted of very polar material. This fraction has not been studied further but was shown to include three triterpenoid acids, two of which appeared to be identical to those described in the preceding paper (13).

TABLE I

GLC Analysis[a] of Fraction 1 of the Chromatography of Orujo Oil Methyl Esters and of Olive Oil Methyl Esters

Major component acids	Composition: as % of total area under curves[b]	
	Orujo esters Fraction 1	Olive esters
16:0	16.2	16.7
16:1	1.8	2.0
18:0	3.2	2.4
18:1	74.9	69.3
18:2	3.9	9.6

[a] Analysis was carried out on a 180 cm. x 3.5 mm. I.D. column packed with diethyleneglycol succinate polyester (25%) on a support of Chromosorb W (80–100 mesh). The column was operated at 193°C. with argon as carrier gas maintained at a flow rate of 60 ml./minute (calculated to S.T.P.) under a head pressure of 10 p.s.i. The detector was a β-ionization cell (Barber-Coleman Model 10) operated at 500 volts.

[b] These data are not presented as absolute values but serve for comparison.

Further Characterization of Some Fractions

Fractions 4 and 5, in carbon tetrachloride solution, showed hydroxyl absorption near 2.76 μ in the near infrared.[5] After treatment with anhydrous hydrogen chloride in ether, the strong hydroxyl absorption of Fraction 4 disappeared. We have demonstrated that this phenomenon is shown by vicinal unsaturated hydroxy esters (6, 7) and this type of reaction has been studied by Lohmar, Smith and Wilson (12). The strong conjugated diene absorption of this fraction at 230 mμ suggested that it contained some $\alpha\beta$, $\gamma\beta$-unsaturated hydroyl esters. These may have been present in the original oil or may have been produced from linoleate hydroperoxides by the hydrolysis procedure. Conversion of hydroperoxides to hydroxy acids by hydrolysis has recently been demonstrated (14), and even our relatively mild hydrolysis procedure reduced the peroxide value of a concentrate of oleate peroxides from 4000 to 200, showing almost complete degradation of the hydroperoxides.

Fraction 3 was shown to consist of epoxy esters by the near infrared spectrophotometric method of Morris and Holman (6). This method consists of treating the esters with anhydrous hydrogen chloride in ether and measuring the increase in hydroxyl absorption at 2.795 μ, due to chlorohydrins produced by cleavage of the epoxy groups. The fact that the peak produced, in this case, had its maximum at 2.792 μ instead of 2.795 μ, and showed a distinct shoulder at 2.760 μ, indicated that a considerable proportion of *trans*-epoxide was present, and prevented any accurate quantitative determination (cf. 6).

On thin-layer chromatography of Fraction 3, alongside authentic epoxy esters, the upper, middle, and lower of the three major spots were found to have migration characteristics identical to *cis*-12:13-epoxyoleate, *trans*-9:10 epoxystearate and 10-epoxystearate respec tively. The smearing effect between these three spots (cf. Fig. 3, sample c) could be duplicated by chromatography of a mixture of these three known epoxy esters. In addition, gas-liquid chromatography (cf. 10) showed the presence of components with "carbon numbers" identical to *cis*- and *trans*-9:10 epoxystearates and *cis*-12:13-epoxyoleate, the major peak again corresponding to the saturated *trans* isomer.

A portion (161 mg.) of the epoxy ester fraction was saponified at room temperature, acidified with the theoretical amount of 2*N* hydrochloric acid, and the epoxy acids extracted and recovered. Three crystallizations from hexane gave a product of m.p. 52–53° which was not raised by further crystallization from the same solvent. This product depressed the melting point of authentic *cis*-9:10-epoxystearic acid (m.p. 57.5–58°) but gave no depression on admixture with authentic *trans*-9:10-epoxystearic acid (m.p. 52–54°). The infrared spectrum of this acid[6] showed a strong band at 11.44 μ, which is the wavelength characteristic of a *trans*-epoxide (15). Further evidence that this acid was *trans*-9:10-epoxystearic acid was obtained by conversion of a portion (29 mg.) to the corresponding dihydroxy acid by acetylation and subsequent hydrolysis (16). The product, after three crystallizations from ethyl acetate, had a melting point of 130.5°

[5] Beckman Model DK-2 Spectrophotometer.

[6] Obtained on a microscale with a thin film on a silver chloride plate, using a modified Perkin Elmer 12 C Spectrophotometer with Model 81 Microscope and a Reeder thermocouple. The authors are grateful to P. R. Edmondson and H. Dinsmore, of the Dept. of Medicine of this University, for carrying out this analysis.

which was undepressed on admixture with authentic *erythro*-9,10-dihydroxystearic acid (m.p. 132°).

Final verification of the structure of the epoxy acid was obtained by oxidative cleavage of this dihydroxy derivative with periodate-permanganate reagent by the method of von Rudloff (17). Oxidation at pH 8–9 was allowed to continue for 20 hours and, after acidification and decolorization with bisulfite, the products were recovered by exhaustive extraction with ether. These were then separated into a fraction soluble in petroleum ether and one soluble in water. The petroleum ether-soluble product was esterified with diazomethane, and shown to consist only of methyl nonanoate by gas-liquid chromatography. The water soluble product was crystallized and was shown, by melting point and mixed melting point, to be azelaic acid.

Discussion

It has been demonstrated that an orujo oil (sulfur olive oil) contains several classes of oxygenated acids amounting to more than 10% of the component fatty acids. A major component of these oxy-acids has been shown to be *trans*-9:10-epoxyoctadecanoic acid which constitutes about 2% of the mixed acids of the oil. Thin-layer chromatography and gas-liquid chromatography indicate that this acid is accompanied by smaller amounts of its *cis*-isomer and of *cis*-12:13-epoxyoctadec-9-enoic acid. Of the hydroxy acids present, some are vicinal unsaturated and some may contain a conjugated dienol structure, which has been shown to occur naturally in several seed oils (7, 18, 19).

The presence of a *trans*-epoxy acid in this oil is of some interest since all naturally occurring epoxy acids thus far described have been *cis*. This acid and the other oxygenated acids however are unlikely to be true natural components of the olives, at least in the proportions found in the orujo oil; but it should be noted that the olive oil used for comparison apparently contained much smaller amounts of some of them. Even if these acids are produced in the interval between pressing the olive and extracting the cake, as previous work indicates, they must still be considered as being naturally occurring provided their production is due to enzymatic or bacterial oxidation and not merely to chemical autoxidation.

Ellis (20), Gold (21), and others have shown that *trans*-9:10-epoxystearic acid is a major secondary product in the autoxidation of oleic acid. However these workers used heavy metal salts as catalysts for their oxidations and Ellis (20) has stated that very little epoxystearic acid is formed in the absence of a metal catalyst. Moreover Gold (21) showed that, during autoxidation, hydroperoxide formation preceded formation of epoxy acids and her results indicate that some 15–20% of hydroperoxy products would be present along with a proportion of epoxy acids similar to that present in our orujo oil. The orujo oil however had a peroxide value corresponding to only 1.8% of hydroperoxides, i.e., less than the proportion of epoxy acids present, indicating that the epoxy acids were not produced by an autoxidative mechanism as described by Gold and Ellis. Further indication that these acids were not produced by autoxidation was the fact that, whereas the orujo and olive oils contained almost identical amounts of hydroperoxy compounds (1.8% and 1.7% respectively), the olive oil contained only very small amounts of epoxy and hydroxy acids, as evidenced by direct determinations *(vide supra)* and by thin-layer chromatography of identical amounts of the methyl esters derived from these two oils.

Thus it is considered extremely unlikely that the epoxy and hydroxy acids present in orujo oil, or at least the major portion of them, could have arisen from autoxidation. It is considered more probable that they were products of microbiological oxidation during the period of storage of the pressed olive pulp and as such may be considered to be naturally occurring. This thesis may be proved or disproved by optical rotational studies since an autoxidative mechanism must produce racemates whereas an enzymatic or bacterial mechanism would be expected to give optically active products. When more material becomes available, work on this problem will be continued.

REFERENCES

1. Gracián, J., Arévalo, G., Albi, F., and Placencia, A., Grasas y Aceites, *6*, 276 (1955).
2. Gracián, J., Vioque, E., and de la Maza, M.P., Grasas y Aceites, *8*, 67 (1957); Fette, Seifen, Anstrichmittel, *58*, 353 (1956).
3. Gracián, J., and Ventura, M., Grasas y Aceites, *10*, 67 (1959).
4. Desnuelle, P., and Burnet, M., Bull. soc. chim. France, 268 (1956); Rev. franc. corps gras, *3*, 325 (1956).
5. Paquot, C., and Querolle, M., Proceedings of the 27th Congress of Industrial Chemistry. Brussels, Belgium, September, 1954, *3*, 724.
6. Morris, L.J., and Holman, R.T., J. Lipid Res., *2*, 77 (1961).
7. Morris, L.J., Holman, R.T., and Fontell, K., J. Am. Oil Chemists' Soc., *37*, 323 (1960).
8. Smith, C.R., Jr., Koch, K.F., and Wolff, I.A., J. Am. Oil Chemists' Soc., *36*, 219 (1959).
9. Morris, L.J., Hayes, H., and Holman, R.T., J. Am. Oil Chemists' Soc., *38*, 316 (1961).
10. Stahl, E., Pharmazie, *11*, 633 (1956); Chemiker-Ztg., *82*, 323 (1958).
11. Morris, L.J., Holman, R.T., and Fontnell, K., J. Lipid Res., *2*, 68 (1961).
12. Lohmar, R.L., Smith, C.R., Jr., and Wilson, T.L., J. Org. Chem., *25*, 2034 (1960).
13. Vioque, E., and Morris, L.J., J. Am. Oil Chemists' Soc., submitted.
14. Frankel, E.N., Evans, C.D., McConnell, D.G., and Jones, E.P., J. Am. Oil Chemists' Soc., *38*, 134 (1961).
15. Shreve, O.D., Heether, M.I., Knight, H.B., and Swern, D., Anal. Chem., *23*, 277 (1951).
16. Gunstone, F.D., J. Chem. Soc., 1611 (1954).
17. von Rudloff, E., Can. J. Chem., *34*, 1413 (1956).
18. Smith, C.R., Jr., Wilson, T.L., Melvin, E.H., and Wolff, I.A., J. Am. Chem. Soc., *82*, 1417 (1960).
19. Chisholm, M.J., and Hopkins, C.Y., Can. J. Chem., *38*, 2500 (1960).
20. Ellis, J., Biochem. J., *30*, 753 (1936).
21. Gold, J., J. Chem. Soc., 934 (1958).

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