time was studied over a range of 1–3 hr.; no significant increase in yield was found after 2 hr. Another source of error occurred if the subsequent water hydrolysis was allowed to continue longer than 10 min. (4).

Two samples of flakes—one defatted with methanol, the other with ethanol—were analyzed five times to measure the precision possible with this method. Residual methanol ranged from 0.86 to 0.99% with a standard deviation of 0.05% and ethanol from 1.11 to 1.24% with a standard deviation of 0.05% (Table II).

TABLE II

Standard Deviation of the Results for Methanol and Ethanol

Number of tests	Residual alcohols	
	Methanol	Ethanol
	%	%
1	0.92	1.24
2	0.91	1.21
3	0.86	1.20
4	0.99	1.12
5	0.86	1.11
Standard deviation	0.05	0.05

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Minor Components of Olive Oils. I. Triterpenoid Acids in an Acetone-Extracted Orujo Oil[1, 2]

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Two triterpenoid acids have been isolated from an acetone extracted orujo oil (sulfur olive oil). One of these, amounting to 0.1% of this oil, has been shown to be oleanolic acid, 3β-hydroxyolean-12-en-28-oic acid. The other, amounting to 0.4% of the oil, has not been completely characterized, but is apparently a novel dihydroxy triterpenoid acid.

Introduction

OLIVE OIL is produced in Mediterranean countries by hot-pressing the pulped olives in open presses. The residual pressed cake is subsequently extracted with carbon disulfide, trichloroethylene or some other solvent and a low grade of oil is obtained. This solvent extracted oil is called *aceite de orujo* in Spain and *sulfur olive oil* in English speaking countries. In this series of papers it will be referred to as orujo oil.

The oil remaining in the pressed pulp generally undergoes some enzymatic hydrolysis and oxidation before it is extracted with solvent (1,2). The resulting orujo oil therefore often contains a relatively high proportion of free fatty acids, some oxidation products, and some non-fatty compounds. These impurities in the orujo oil considerably reduce its value as an edible oil. The main outlet for the oil is in the soap industry although a high proportion of oxygenated acids reduces its value because these complicate the technical processing and result in reduced yields of soap.

As orujo oil accounts for some 10% of total olive oil production, a study of those components of orujo oil which are not present in high grade olive oil may be of economic importance. We have undertaken a program of research in which we hope to isolate and characterize some of these minor components. This paper records studies of a crystalline acidic material which was extracted with an orujo oil, and from which we have isolated two triterpenoid acids. One of these is identified as oleanolic acid and the other, although not fully characterized, is shown to be a dihydroxy triterpenoid acid.

Experimental

The orujo oil used in this study was prepared in the laboratories of the Instituto de la Grasa, Seville, Spain, by acetone extraction of the pressed cake of olives which were free of leaves, etc. The oil was mixed with ten times its volume of petroleum ether and the insoluble residue filtered off, washed with water, and dried. This material was a brownish white solid, readily soluble in dioxane or ethanol, but insoluble in petroleum ether or water. It amounted to 0.9% by weight of the orujo oil.

Separation of Components

Thin-layer chromatography (TLC) on Silica Gel G,[4] according to Stahl (3) and Mangold and Malins (4,5), demonstrated the presence of four main components, as shown in Figure 1, sample b. The solvent system used for developing the plate was diethyl ether-hexane (1:1) and spots were made visible by heating after spraying with 50% sulfuric acid (6). Spots 1 and 3, during this heating, gave a color transition of yellow through brown to black, the color sequence given by normal aliphatic compounds such as glycerides and free fatty acids. Spots 2 and 4 had color transitions of violet to black and reddish-purple to

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black, respectively, demonstrating that the components were not normal aliphatic compounds. These latter two components are hereafter referred to as Compound A and Compound B, respectively (cf. Figure 1).

Separation and isolation of the four components was effected by adsorption chromatography on silicic acid. A solution of 2.63 g. of the crude material, in 10% diethyl ether in petroleum ether (p.p. 35–40°), was applied to the top of a 100 x 2.5 cm. column of 60–200 mesh silica gel [5] (320 g.) and elution commenced with the same solvent. After elution of the first two components, the solvent was changed to 20% diethyl ether in petroleum ether and finally pure diethyl ether was used. Fractions of 100 ml. each were collected, each one monitored by TLC and combined to give four fractions, corresponding to spots 1–4 in Figure 1 and described in Table I.

Fraction 1 was a liquid, and TLC with a solvent system of 10% diethyl ether in petroleum ether demonstrated two component groups with migration characteristics on TLC, identical to triolein and oleic acid standards respectively.

The fatty acids were separated from the triglycerides by extraction of an ether solution with 10% aqueous KOH, and the two groups amounted to 13.6% and 16.6%, respectively, of the original sample. These groups were converted to methyl esters and analyzed by gas-liquid chromatography (GLC). The results obtained are shown in Table II and in both cases are nearly identical to those obtained with olive oil methyl esters.

[4] Manufactured by E. Merck A.G., Darmstadt, Germany; obtainable from C.A. Brinkmann, 115 Cutter Mill Road, Great Neck, L.I., New York.

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

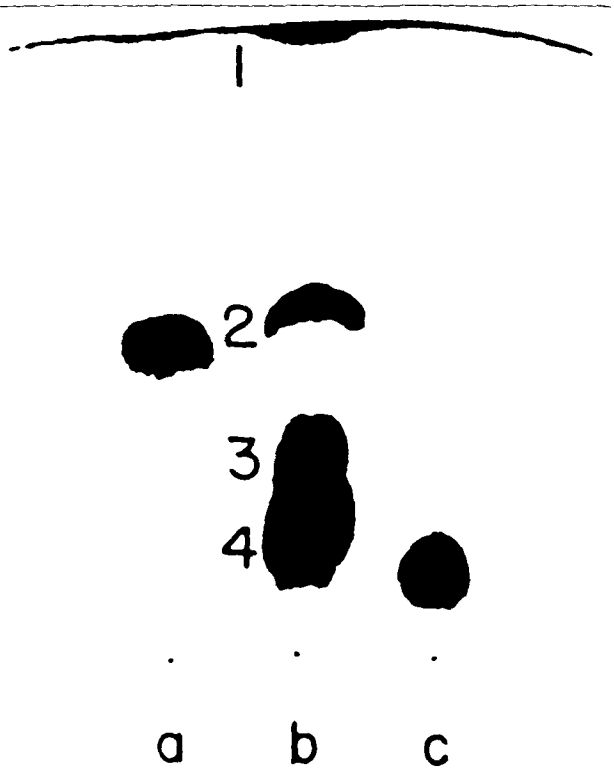

FIG. 1. Thin-layer chromatogram of the petroleum ether insoluble residue of orujo oil (sample b) and of two of the components isolated from it (samples a and c). For explanation of spots 1–4 see text and Table I. Chromatogram was developed with 50% diethyl ether in hexane, the spots were located by charring after spraying with 50% sulfuric acid, and the reproduction was obtained by direct photocopy of the plate.

TABLE I

Adsorption Chromatography of Petroleum Ether-Insoluble Portion of Orujo Oil

Fraction	Solvent (% ether)	Weight (mg.)	%	Components
1	10	794	30.2	Triglycerides and fatty acids
2	10	310	11.8	Compound A
3	20	267	10.2	Hydroxy fatty acids
4	100	1,258	47.8	Compound B

Fraction 2 was a white solid which was shown by TLC to be nearly pure Compound A. Further purification and characterization of this fraction will be described below.

Fraction 3 consisted of a semisolid which, on TLC, migrated with an R_f value corresponding to ricinoleic acid or mono-olein standards. This fraction was shown to be mainly acidic and, after esterification with diazomethane, showed a strong hydroxyl absorption at 2.76 μ in the near infrared. GLC of these esters gave peaks with carbon numbers corresponding to hydroxy C_{18} esters. Although not fully characterized it is considered that this fraction consists mainly of mono-

TABLE II

GLC Analysis [a] of the Fatty Acid Methyl Esters of the Two Components of Fraction 1 from Column Chromatography and of Olive Oil

Major component acids	Composition: as % of total area under curves [b]		
	Fraction 1		Olive oil
	Free acids	Triglycerides	
14:0	0.5	0.3	.....
16:0	16.8	13.2	16.7
16:1	2.1	2.0	2.0
18:0	1.7	1.2	2.4
18:1	71.6	73.0	69.3
18:2	7.3	10.3	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 chromosorb W (80–100 mesh). The column was operated at 193°C. with argon as carrier gas maintained at a flowrate of 60 ml./min. (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) at 500 volts.

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

hydroxy C_{18} acids with possibly small amounts of monoglycerides.

Fraction 4, amounting to almost half of the original sample, was again a white solid. TLC demonstrated this fraction to be almost pure Compound B.

Purification and Characterization of Compounds A and B

Further purification of Compounds A and B (Fractions 2 and 4 above) was effected by adsorption chromatography on silica gel, using Hagdahl segmented column (7) rather than a simple glass column. This consists of a series of steel cylinders, each packed with adsorbent, decreasing in volume from top to bottom of the column and joined together with only capillary channels between segments. This type of column is theoretically more efficient than a normal straight column (7) and has been shown, in our laboratory, to be so in fact (e.g. 8).

Compound A and Compound B, in solution in 20% diethyl ether in petroleum ether, were separately applied to columns of 231.4 ml. volume (9) containing 163 g. of silica gel (60–200 mesh).[5] Elution in each case was commenced with 20% diethyl ether in petroleum ether, and the proportion of ether subsequently increased to 40% to complete the chromatogram. Fractions of 10 ml. each were collected by automatic fraction cutter, monitored by TLC, and

those containing only a single component were combined. The resulting purified Compounds A and B were free of contaminants as demonstrated by TLC (Figure 1, samples a and c and Figure 2, samples a and e).

Both compounds were crystallizable from methanol, Compound A as white needles (m.p. 307–308°) and Compound B as a white powder (m.p. 280–282°). Both were acidic, readily formed methyl esters (shown in Figure 2, samples b and d) on treatment with diazomethane, and exhibited optical rotation. Both gave positive Liebermann-Burchard reactions. Compound A showed a color transition of pink through violet and blue to green, the final solution showing green fluorescence under ultraviolet light, and this is identical to the result reported for oleanolic acid (10). Compound B, with the Liebermann-Burchard reagent, changed rapidly from pink to red and finally brown, the final solution again exhibiting strong green fluorescence under ultraviolet light. Both compounds rapidly adsorbed iodine after TLC indicating the presence of unsaturation in each (4,5).

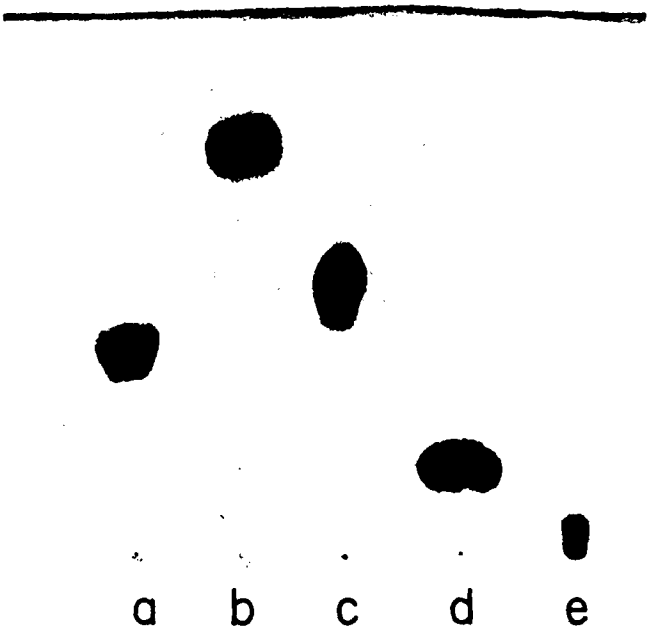

FIG. 2. Thin-layer chromatogram of purified samples of, from a to e: Compound A, Compound A methyl ester, Compound B diacetate, Compound B methyl ester, and Compound B. The chromatogram was developed with 20% diethyl ether in hexane, the spots were located by charring after spraying with 50% sulfuric acid, and the reproduction was obtained by direct photocopy of the plate.

Table III gives a summary of the composition and physical properties of these compounds. The infrared spectra[6] of Compound A and its methyl ester, shown in Figure 4, demonstrate the presence of a carboxyl group, a hydroxyl group and a double bond in the molecule. The characteristics for Compound A are compared to the theoretical or literature (11) values for oleanolic acid, namely 3β-hydroxyolean-12-en-28-oic acid, which is the triterpenoid acid shown in Figure 3, formula I. The agreement between the values for Compound A and oleanolic acid is obvious and we consider that they are identical.

[6] Obtained, on a microscale, as thin films 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. Edmonson and H. Dinsmore, of the Department of Medicine of this University, for carrying out these analyses.

TABLE III

Analysis and Physical Constants of Compounds A and B Compared with Oleanolic Acid

Property	Compound A (found)	Oleanolic acid (theory or literature) (11)	Compound B (found)	Hydroxy oleanolic acid $C_{30}H_{48}O_4$ (theory)
C	78.8%	78.9%	77.0%	76.2%
H	10.7	10.6	10.1	10.2
O	10.8 (direct)	10.5	13.4 (direct)	13.6
Mol. weight[a]	465	456	471	472
M.pt. of acid	307–8°C.	310°C.	280–2°C.	.....
M.pt. of Me ester	200–1°	201°	230–1°C.	.....
M.pt. of acetylated acid	.....	.....	237–9°	.....
$[\alpha]_D^{24}$ of acid	+88.2° (C = 0.136 in MeOH)	+78° (in $CHCl_3$)	+44.6° (C = 0.99 in EtOH) +61.5° (C = 0.652 in dioxane)	.....

[a] By alkalimetry, assuming one carboxyl group per molecule.

Compound B was shown by elemental analysis and molecular weight determination to have one additional oxygen atom. No dinitrophenylhydrazone could be prepared and the infrared spectrum indicated the probable absence of any keto group. The infrared spectra[6] of Compound B and its methyl ester, shown in Figure 5, are similar in most respects to those of Compound A. The major difference in each curve is the greater hydroxyl absorption of Compound B, in-

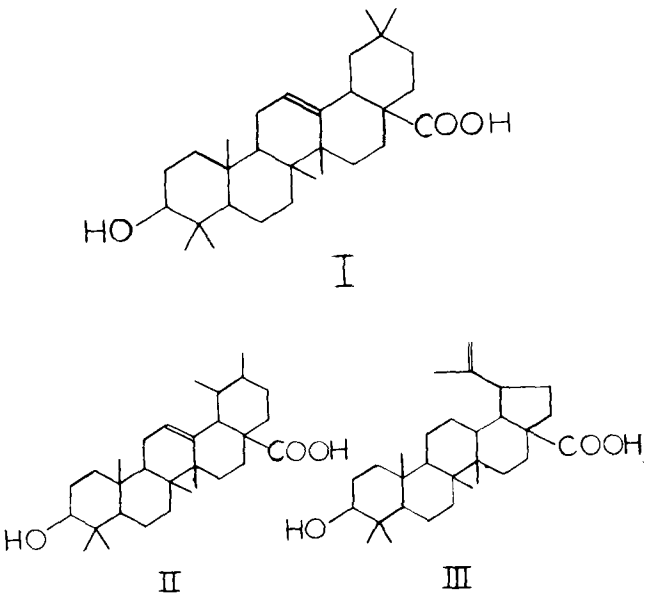

FIG. 3. Plane projections of the structures of: I, oleanolic acid, of the β-amyrin group; II, ursolic acid, of the α-amyrin group; III, betulinic acid, of the lupeol group of triterpenoids (11).

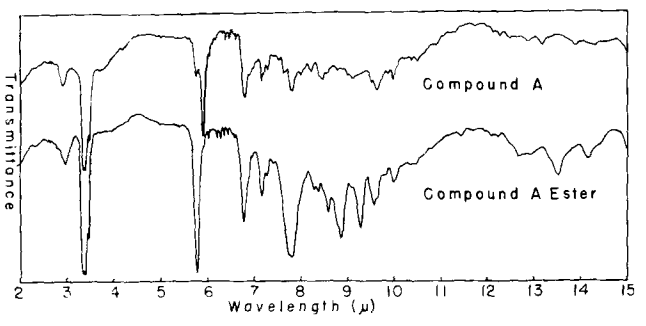

FIG. 4. Infrared spectra of Compound A and its methyl ester.

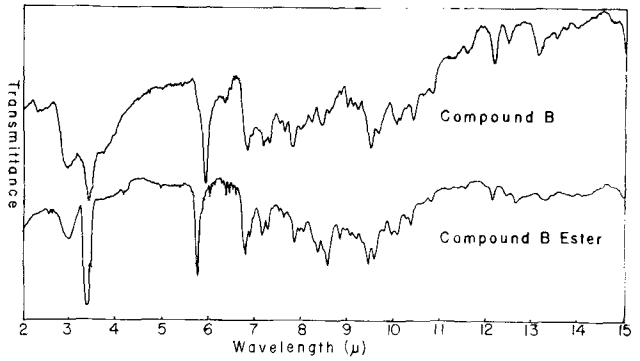

FIG. 5. Infrared spectra of Compound B and its methyl ester.

dicating that two hydroxyl groups are present but that otherwise Compounds A and B are similar.

A portion of pure Compound B was acetylated by refluxing with an acetic anhydride-pyridine mixture (3:1) (12). The product (m.p. 237°–239°) was shown to be pure by TLC (Figure 2, sample c) and gave the analysis figures to be expected for a diacetoxy derivative: carbon 73.06%, hydrogen 9.28% ($C_{34}H_{52}O_6$ requires: carbon 73.34%, hydrogen 9.42%). This provides additional proof that Compound B contains two hydroxyl groups.

Discussion

It has been shown that the petroleum ether insoluble portion of an acetone extracted orujo oil contains two triterpenoid acids. One of these has been proved to be oleanolic acid and the other, although not completely identified, has been shown to be a dihydroxytriterpenoid acid which, so far as we can determine, has not previously been described.

It has been recognized for a long time (13) that oleanolic acid occurs in the free state in olive leaves. It has also been shown that the free acid is present in the pulp from olives and that its concentration appears to be less in the pulp from ripe olives than that from immature olives (14). Viguera *et al.* (15) showed that oleanolic acid was extracted from whole olives by soaking them in aqueous alkali which is one step in the production of pickled olives. However it has not been shown previously that oleanolic acid, or any other triterpenoid acid, may become a component of orujo oil or olive oil.

No description has appeared in the literature of the second triterpenoid acid, which we isolated from orujo oil and which, in our sample, was present in much higher proportion. Two other triterpenoid acids have been found, in the free state, in olive leaves. These are ursolic acid and betulinic acid, which belong to the α-amyrin group and the lupeol group respectively (cf. 11) and their structures are shown in Figure 3. The properties of these acids however are quite different from those of our Compound B.

It is possible that these triterpenoid acids may have beneficial therapeutic properties, although pharmacological studies have not as yet arrived at any definite conclusions (16–18). However it has been shown for many years that an infusion of olive leaves has a real value in some diseases such as gout, hypertension, hepatic disturbances, etc., and research is being carried out (19) to determine which of the constituents exhibit the beneficial properties. It has been considered that the triterpenoid acids are the active constituents but the work of Janků *et al.* (20) seems to prove that, with respect to hypotensive properties at least, some "choline-like" material is the active principle. Triterpenoid acids have also been claimed to have therapeutic properties in the treatment of Addison's disease (21,22). If oleanolic acid and Compound B possess pharmacological activity, orujo oil would have value as a convenient source of them.

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