Chromatographic Separation of Unsaturated Fatty Acids as Their 2,4-Dinitrobenzenesulfenyl Chloride Derivatives[1]

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A number of techniques have been employed to separate mixtures of fatty acids, and some of them have served well for preparation purposes. However none of the procedures developed provides a method for the quantitative separation of the fatty acids present in our common seed oils.

Chromatography has provided the most attractive approach to the problem. Kurtz (1) reported the partial separation of binary mixtures of methyl esters of linolenic, linoleic, oleic, and stearic acids on silica gel columns. Holman and Williams (2) obtained partial separation of saturated, oleic, and linoleic acids on charcoal columns. Dutton and Reinhold (3) partly separated some binary mixtures of the ethyl esters of stearic, oleic, linoleic, and linolenic acids on alumina and effected some enrichment in fractions of the ethyl esters of soybean fatty acids by the same technique (4). Riemenschneider, Herb, and Nichols (5) isolated small yields of pure methyl esters of linolenic and linoleic acids on silicic acid columns. Graff and Skau (6) reported some separation of the fatty acids on magnesium oxide impregnated with phenol red. Howard and Martin (7), using reversed phase chromatography, succeeded in separating the saturated acids and reported some separation of the unsaturated acids. Boldingh (8) used paper impregnated with vulcanized rubber Latex to separate saturated acids from oleic.

In this laboratory need for a quantitative method of separation of fatty acids in small samples of oil prompted studies with reagents to form derivatives which would show greater differences in chromatographic adsorption than the fatty acids themselves. A suitable reagent was found to be 2,4-dinitrobenzenesulfenyl chloride, which has been reported by Kharasch and Buess (9) to add to double bonds in the following manner.

$$R_2C{=}CR_2 + ArS{-}Cl \rightarrow \begin{matrix} R_2C{-}CR_2 \\ | \quad\quad | \\ ArS \quad Cl \end{matrix}$$

The present paper describes the quantitative separation of fatty acids as their 2,4-dinitrobenzenesulfenyl chloride derivatives.

Experimental

Methods. The general method reported by Kharasch and Buess (9) was used for the preparation of addition products of the fatty acids with 2,4-dinitrobenzenesulfenyl chloride.[3] To the fatty acid sample (100 mg.) in a 50 ml. Erlenmeyer was added 100% excess of the reagent in 20 ml. of acetic acid, and the mixture was heated for 8 hours on a steam bath. The solvent was then partially removed by continued heating under a stream of air; the flask was then transferred to a vacuum oven, the remaining solvent was removed under reduced pressure at 50°C., and the residue was dissolved in 20 ml. of hot benzene.

Four chromatographic columns (22 x 400 mm.), each containing 55-60 g. of magnesium sulfate[4] were prepared by attaching to a vacuum line and slowly pouring in the dry salt with occasional tapping of the tube to aid settling. Into each column was poured 15 ml. of benzene; this was followed by 5 ml. of the hot benzene solution containing the derivatives and finally by 20-30 ml. of benzene to wash all of the solute into the column. The columns were developed with a mixture of benzene and ethyl ether (95:5). This eluant was placed in a separatory funnel, fitted tightly to the column, and was forced through the column at about 50 ml. per hour with the aid of nitrogen (10 p.s.i.) applied to the funnel. The vacuum line was disconnected as soon as the pressure was applied.

The unaltered reagent moved through the column with no apparent adsorption. This band was followed closely by two narrow, light-colored bands, not identified, which passed into the eluate with the first 100 to 150 ml. of eluant and was discarded. The pale eluate, immediately following, was collected to obtain part of the saturated acids. When the next yellow band approached the bottom of the column, the pressure was released and the column was extruded and sectioned according to the bands present. Reaction products derived from mixtures of oleic, linoleic, and linolenic acids produced three distinct pale yellow bands or zones which were clearly separated from each other. Ultraviolet radiation produced a strong pink fluorescence in the bands and aided in defining their individual limits during sectioning. The adsorbent immediately below the oleic band was collected to obtain the saturated acids which still remained on the column.

The adsorbate fractions were placed in sintered glass filtering funnels and washed with a boiling mixture of benzene and acetic acid (95:5) until fluorescence was no longer observed on irradiation of the funnel contents. The filtered eluate fractions in 400-ml. beakers were placed on a steam bath under the hood, the solvent was removed in a stream of air, and the residue was dried to constant weight at 50°C. in a vacuum oven. The solution of combined eluates containing the saturated acids was evaporated to dryness, the residue was extracted with commercial hexane (Skellysolve B), and the clear solution was transferred to a 50-ml. beaker. The solvent was then evaporated on a steam bath, and the fatty acids were precipitated as the lead salts with .375 g. of lead acetate in 25 ml. of alcohol. The lead salts were filtered on a Buchner and transferred to a 125-ml. separating funnel, 10 ml. of dilute nitric acid (1:4) were added, and the fatty acids were taken up in hexane. The solvent was evaporated; the residual fatty acid was dried in an air oven at 100°C. and weighed.

For the analysis of a fat by this method 100 mg. of the sample were refluxed with 10 ml. of 6% potassium hydroxide in absolute ethanol for 3 hours. The un-

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[3] Versatile Chemicals Inc., Glendale, Calif.
[4] Anhydrous, powdered analytical reagent, Mallinckrodt Chemical Company, St. Louis, Mo.

saponifiable material was extracted with ether; the hypophase was acidified with hydrochloric acid; the fatty acids were extracted in four 25-ml. portions of hexane; the extract was washed with distilled water and dried over anhydrous sodium sulfate. The solution in a 250-ml. beaker was placed on a steam bath, the solvent was reduced in volume to 20 ml. under a stream of nitrogen. After transfer to a 25-ml. Erlenmeyer, the remaining solvent was removed and the residue was dried to constant weight at 50°C. in a vacuum oven (2 hours). The free fatty acids were then reacted with 2,4-dinitrobenzenesulfenyl chloride, and the derivatives were chromatographed as described above.

All of the oils and fats analyzed by this method were also analyzed by the spectrophotometric procedure as described by Brice *et al.* (10, 11).

Derivatives of unsaturated acids. Purified samples of individual unsaturated acids were reacted with the reagent and chromatographed (Table I). Recoveries

TABLE I

Recovery of Unsaturated Acids as Their 2,4-dinitrobenzenesulfenyl Chloride Derivatives

Fatty Acid	Trial	Initial Wt. of Acid	Wt. of Derivative		
			Theory	Found	Recovery
		(mg.)	*(mg.)*	*(mg.)*	*(%)*
Oleic [a]	1	200	367	328	90
	2	200	367	346	94
	3	100	184	163	90
Linoleic [b]	1	160	416	412	99
	2	150	390	384	98
	3	100	268	238	89
Linolenic [c]	1	100	363	332	92
	2	100	363	339	94
	3	100	363	338	94

[a] Prepared from olive oil by fractional crystallization (13) (Wijs I.V., 91.5).
[b] Prepared by debromination of tetrabromide (Wijs I.V., 179.5).
[c] Prepared by debromination of hexabromide (Wijs I.V., 269.1).

of the derivative were 89 to 99% of the theoretical values if we assume quantitative reactions of the reagent with all unsaturated linkages in the fatty acids. Analyses of the derivatives (Table II) showed that

TABLE II

Composition of the 2,4-dinitrobenzenesulfenyl Chloride Derivatives [a]

		C	H	N	S	Cl
Oleic	C [b]	55.75	7.16	5.42	6.20	6.80
	F	57.41	7.54	5.23	5.85	6.38
	F	57.34	7.59	5.17	5.96	6.50
Linoleic	C	48.04	5.07	7.47	8.55	9.46
	F	49.46	5.56	7.23	8.28	9.00
	F	49.58	5.48	7.14	8.35	9.11
Linolenic	C	44.02	3.97	8.56	9.79	10.83
	F	44.84	4.31	8.21	9.29	10.10
	F	44.97	4.38	8.12	9.36	10.17

[a] Analyses performed by Huffman Microanalytical Laboratories, Wheatridge, Colo.
[b] C, calculated; F, found.

while values for C and H were consistently higher and for S, N, and Cl consistently lower than theoretical, all unsaturated positions in these acids were reactive. This was substantiated by the lack of intermediary bands on the chromatographic columns containing the derivative of either linoleic or linolenic acid.

None of the derivatives was obtained in the crystalline state. The derivative of oleic acid was an amber-colored viscous oil at room temperature, soluble in benzene, chloroform, carbon tetrachloride, dioxane, acetic acid, ethyl ether, and ethyl acetate, slightly soluble in hot hexane and ethanol. The derivative of linoleic acid was a light yellow glassy solid (m.p. 56-61°C.), soluble in acetic acid, benzene, dioxane, chloroform, and carbon tetrachloride, slightly soluble in ethyl ether and insoluble in hot hexane. The derivative of linolenic acid was similar to that of linoleic acid in appearance (m.p. 77-81°C.) and solubility properties. The derivatives appeared to be quite stable at steam-bath temperatures in the presence of acetic acid and the reagent. However the purified derivatives, when heated dry above 70°C. even for short periods, showed considerable discoloration and changes in solubility. Although the saturated acid fraction recovered after lead soap precipitation was frequently pale yellow, the melting point was the same as that of the starting material.

Synthetic mixtures containing all four acids were separated clearly with 99 to 102% recovery of the total acids (Table III). However recovery of satu-

TABLE III

Separation and Recovery of Fatty Acids from Mixtures of Known Composition

Fatty Acid		Mixture No.			
		1	2	3	4
		mg.	*mg.*	*mg.*	*mg.*
Saturated [a]	C	20.2	20.3	19.9	20.2
	F	19.2	19.8	18.8	18.9
Oleic [b]	C	20.6	22.2	20.6	23.5
	F	20.4	22.0	19.5	21.1
Linoleic [b]	C	54.0	50.7	52.5	40.2
	F	56.1	54.5	54.7	42.3
Linolenic [b]	C	10.1	15.0	10.6	9.5
	F	8.4	14.5	10.5	9.9

[a] Stearic acid (Eastman Kodak Co.).
[b] See Table I for source.

rated acid was generally slightly low and of linoleic slightly high. Since the differences were probably within the limits of purity of the acids, these trends would seem to be of questionable significance. The iodine value of the saturated acid fraction was less than 1, and no precipitate was obtained on formation of the lead salts of the fraction containing the oleic derivative in alcohol.

The unsaturated acids which were used undoubtedly contained some *trans* isomers, and the effect of these on the accuracy of the method is uncertain. The derivative of elaidic [5] acid gave two bands on the column; the major and leading band, which accounted for about 80% of the total, did not separate from the corresponding derivative of oleic acid; and a trailing band, which accounted for about 15% of the total, separated from the oleic derivative. A minor band containing less than 3% of the total was occasionally noted above the main band of oleic derivative. This band did not separate from the trailing band of elaidic acid, and neither of these minor bands separated completely from the derivative of linoleic acid.

The derivatives of linoleic and linolelaidic acid [6] appeared as a single band on the columns when the columns were developed under pressure, but four dis-

[5] Prepared by treating oleic acid with nitrous oxide (m.p. 44°).
[6] Prepared by treating linoleic acid with nitrous oxide (m.p. 28-29°).

TABLE IV

Comparison of the Isolation Method with the Spectral Method for Analyses of Oils [a]

Sample	Saturated		Oleic		Linoleic		Linolenic	
	I [b]	S [c]	I	S	I	S	I	S
	%	%	%	%	%	%	%	%
Soybean Oil A	9.4	16.4	28.2	27.6	48.1	46.8	5.6	4.8
Soybean Oil B	10.6	14.7	17.7	18.4	57.3	57.0	6.8	5.5
Soybean Oil C	10.5	14.4	20.4	19.1	56.3	54.8	7.1	7.3
Soybean Oil D [d]	9.3	12.6	37.3	40.0	41.6	37.0	5.9	6.0
Soybean Oil E [d]	7.5	13.4	41.0	40.0	34.5	36.5	5.7	5.7
Corn Oil [e]	6.3	17.5	27.3	22.3	59.0	56.8		
Olive Oil [f]	8.0	10.0	69.5	74.0	13.0	11.6		
Linseed Oil [g]	5.9	9.0	20.9	22.3	18.5	15.5	49.4	48.8
Hyd. Shortening [h]	15.8	20.8	56.2	62.5	20.5	12.3		
Av. Std. Dev.	1.23		1.70		1.32		1.05	

[a] All values expressed as percentages are based on 100 mg. of oil (assuming oil contains 95.6 mg. of fatty acids).
[b] Isolation method developed in this paper. Values for soybean oils A-C and olive oil represent average of quadruplicate. Other values are averages of duplicate samples.
[c] Spectral method as modified by Brice *et al.* (10).
[d] Immature soybeans.
[e] "Mazola" brand.
[f] Pure olive oil U.S.P. supplied by Magnus, Malice, and Renard Inc., N. Y.
[g] Spencer Kellogg and Sons Inc., Buffalo, N. Y.
[h] "Primex" hydrogenated shortening supplied by Procter and Gamble Company, Cincinnati, O.

tinct bands were formed when the columns containing mixtures of these derivatives were developed slowly under hydrostatic pressure of the solvent. This agreed with the results reported by Kharasch and Buess (9), who indicated that two compounds were formed by addition of the reagent to unsymmetrical olefins. Gram (12) also noted that two compounds were obtained by the addition of the reagent to *cis* or *trans* compounds.

Other adsorbents and solvents did not prove satisfactory for separating the derivatives. Silicic acid,[7] used with hexane and ethyl acetate (97:3), separated the derivatives of oleic and linoleic, but the derivatives of linolenic could be separated from the extraneous materials remaining at the top of the column. Magnesium phosphate, calcium sulfate, aluminum oxide, magnesium oxide, and sucrose were also tried as adsorbents with ethyl ether, hexane, benzene, and dioxane, alone and in mixtures; none proved satisfactory.

[7] Analytical Reagent, Chromatographic Grade, Mallinckrodt Chemical Company, St. Louis, Mo.

Analysis of Fats. Nine different samples of common fats were analyzed by this method as well as by the spectral absorption method (Table IV). The results compared favorably for the unsaturated acids in most cases, but those for the saturated acids were consistently lower when analyzed by the new procedure than when analyzed by the spectral method.

Summary

Oleic, linoleic, and linolenic acids were found to react with 2,4-dinitrobenzenesulfenyl chloride to form addition products of constant composition. Saturated acids did not react. The derivatives of mixed acids were separable into well defined bands on $MgSO_4$ columns and recoverable as individual compounds in the eluate, with yields above 95% in each case.

This technique provided the basis for a new method of analysis, which we have called the "isolation" method. Results obtained in the analysis of 100-mg. samples of common vegetable oils compared favorably with those obtained by the spectral absorption method except that isolation values for saturated acids were lower.

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ABSTRACTS . . . . . E. S. Lutton, Editor

• Oils and Fats

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Pigeon-egg fat. J. W. Airan and G. D. Kalyankar (Wilson Coll., Bombay). *J. Univ. Bombay*, Sect. A, 20, Pt. 5 (*Science No.*, No. 31), 31-4(1952). Dried egg yolks from eggs of the pigeon, *Columbiformes*, contained 40.4% fat and 2.32% phosphatides. It had a d^{26} 0.9291, n^{26} 1.467, acid no. 15.43, sapon. no. 207.1, I no. 78.32, Reichert-Meissl no. 3.03, Polenske No. 1.41, and unsapon. 5.03%. Analytical data are also reported for insoluble, mixed, saturated and unsaturated fatty acids. (*C. A.* 47, 9636).

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The unsaponifiable substances of the oils of crucifer seeds. Émile André and Monique Maille. *Compt. rend.* 235, 665-7 (1952). Use of pet. ether (b.p. 40-60°) rather than ethyl ether to extract unsapon. fraction of rapeseed oil gave 0.5% of the oil as unsapon. material compared with 1.1% for ethyl ether. The ethyl ether extract was colored and amorphous, whereas the pet. ether extract was colorless and crystalline. Rapeseed oil was separated into 3 fractions: (a) substances extracted by ale. KOH from pet. ether extract; (b) substances extracted by pet. ether but not by ethyl ether; (c) substances forming soaps with Ba. The substances were not identified. (*C. A.* 47, 9636).

The study of the unsaponifiable substances of oils of crucifer seeds. II. Émile André, Marie Carbouères, and Monique Maille. *Compt. rend.* 236, 1695-7(1953). The method adopted by the International Commission for the study of fatty materials gives different results depending upon the solvent choice (pet. ether or ethyl ether). Unsapon. fractions designated A, B, and C were obtained from crucifer-seed oil as well as from other common oils. Fraction A consisted of those substances extracted by ordinary ether, B those by pet. ether, and C those resistant to either solvent. Fraction C was greater in crucifer oil than in the other oils examined. It is necessary, therefore, to bear in mind the method of extraction in making a study of fractions obtained in oil studies. (*C. A.* 47, 9636).