Factors Involved in the Spectrophotometric Analysis of Fats[1]

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VARIOUS refinements have been suggested for the spectrophotometric method (1, 2, 3) which has been developed to determine the mixed fatty acid composition of a fat or an oil. These included close control of the reaction time and temperature, blanketing of the reactants with nitrogen during the heating period, standardization of the KOH concentration and purification of the solvent (4). Furthermore the original equations proposed by Mitchell *et al.* (1) have been modified to include arachidonic acid (2, 3). The spectral absorption maximum of the latter was determined with an arachidonic acid which had a purity of 95%. The equations calculated from the spectral absorption maximum of this impure arachidonic acid may therefore be in error.

It is also possible that various components other than the alkali conjugated fatty acids may show absorption maxima in the spectral region from 232-322 mμ. and thus introduce errors in the calculations. These components may include carotenoids, vitamin A, other fat-soluble vitamins, and various pigments of unknown composition. Most of them can be removed prior to spectral analysis by saponifying the fat or oil and extracting the soaps with a fat solvent; however on acidification some of them are carried over, as shown by the highly pigmented condition of the resulting mixed fatty acids. This pigmented condition was most notable in the fat extracted from liver tissue with fat solvents (5) and in the mixed fatty acids obtained by total saponification of animal tissue (6). In the present study the effects of the various pigments on the accuracy of the spectrophotometric method were evaluated. The equations proposed by Beadle (2) and Brice *et al.* (3) were also recalculated from the spectral data of an arachidonic acid which had a purity of 99.3%.

Experimental

Two methods of extracting animal tissue fats were used: one a modified Bloor (5) and the other a saponification procedure (6). In the first method tissue was extracted with acetone, Skellysolve F, and alcohol, in the order named. The extracts were dried, combined, and the solvent removed under vacuum.

In the saponification procedure the tissue was suspended in 30% aqueous potassium hydroxide and refluxed until completely saponified. The soaps were extracted with Skellysolve F, acidified, and the resulting mixed fatty acids extracted with Skellysolve F, dried, and freed from solvent under vacuum. This procedure was carried out with as little delay as possible in order to retard autoxidation and destruction of the highly unsaturated acids which are present in the mixed fatty acids obtained from animal and poultry fats. All samples were stored under nitrogen at 0°C.

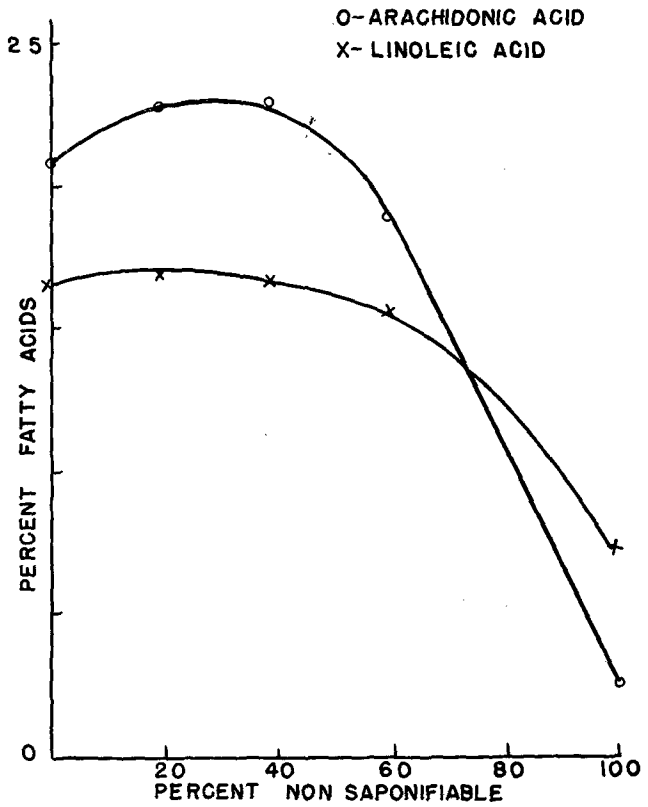

FIG. 1. The effect of added nonsaponifiable material on the calculated percentages of arachidonic and linoleic acid.

Pure linoleic, linolenic, and arachidonic acids were used as reference standards. The reference standards were prepared by standard procedures (7, 8) via the bromo derivatives. The latter were recrystallized until a constant melting point was obtained. The bromo derivatives were debrominated and the fatty acids obtained purified by molecular distillation.

The methods and isomerization media recommended by Mitchell *et al.* (1) were used. These included a thermostatically controlled oil bath kept at 180° ± 0.3°C. and 12.5% potassium hydroxide in ethylene glycol. After the solvent had been heated to 180°C. the samples, in small pyrex cups, were dropped into the reaction tube. The glycol was kept under nitrogen both during the initial heating and the actual isomerization period. At the end of 25 minutes the reaction tubes were removed, wiped clean, and chilled. Appropriate dilutions with ethyl alcohol were then made to bring the reading within the desired range.

The still employed in fractionation was similar to that of Diemair and Schmidt (9). It had a heated reflux column and the reflux ratios were controlled by heating or cooling the still head. Two types of stills were used in molecular distillation, one a pot still (10), and the other, a falling film model (11).

[1] Presented at the annual fall meeting, American Oil Chemists' Society, Chicago, October 31-Nov. 2, 1949.

[2] Portion of a thesis presented by George C. Potter as partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry. Funds for these studies were furnished by the Bureau of Animal Industry, Washington, D. C., and this is a report of a study made under the Research and Marketing Act.

[3] Contribution No. 401 from the Department of Chemistry.

Results

Nonsaponifiable material obtained from the saponified lipids of rat, chicken, or turkey liver fats and chicken gizzard and skin tissue had an appreciable absorption in the ultra violet region from 230-320 mμ. Furthermore crystalline carotene and a vitamin A concentrate [4] also showed appreciable absorption, but cholesterol,[5] choline chloride, glycerol, and phosphoric acid showed no absorption in this region.

Errors due to the nonsaponifiable material seemed to be most readily reflected in the calculated concentration of arachidonic acid (Fig. 1). For example, when the nonsaponifiable material obtained from saponified beef liver was added back to the distilled fatty acids in increasing concentrations, the calculated percentage of arachidonic acid increased steadily. On the other hand, the calculated percentage of linoleic acid did not change markedly. Under the same conditions crystalline carotene or the vitamin A concentrate gave similar results although their effect was not so pronounced as that of the nonsaponifiable material from a natural fat.

An attempt was made to remove spectrophotometrically active impurities with the aid of chromatography. Most of the impurities had absorption properties which were similar to triglycerides or methyl esters. However it was possible to remove a pale yellow pigment from the Skellysolve, acetone, alcohol extracts of poultry fats on a column of alumina which had been standardized to a Brockmann number of 1. This pigment had a high absorption peak in the region of 248 mμ. Alfalfa lipids which had been chromatographed using alumina of the same Brockmann number also yielded a yellow pigment similar to that isolated from poultry liver and skin fats. The pigment from alfalfa lipids had the same general ultra violet absorption characteristics as the pigment from poultry fat extracts. By solvent partition methods and a chromatogram approximately 100 mg. of crystals were isolated from the eluate of the poultry fat. They crystallized from alcohol as yellow needles and had a melting point of 146.5°C. The results obtained on filter paper chromatography indicated that the crystals were relatively pure. Characterization of these crystals is now in progress.

The fractional distillation of the methyl esters of turkey skin and gizzard fats gave the results shown in Table I. In all cases the removal of pigments from poultry fat extracts led to lower values for the unsaturated fatty acids. Very little total decomposition occurred during the distillations as only a trace of material remained in the distillation flask. Before distillation the esters were quite highly colored but were clear to faint amber after distillation.

TABLE I

Spectrophotometric Analysis of Distilled and Nondistilled Methyl Esters From Turkey Fats

Fatty acid	Distilled	Nondistilled
	%	%
Linoleic	19.89	22.64
Linolenic	1.32	1.65
Arachidonic	0	0.22

It is necessary to know the specific extinction coefficients of pure linoleic, linolenic, and arachidonic acids before the equations used to determine the percentage composition of the mixed fatty acids of a fat or oil can be formulated. The specific extinction coefficients for linoleic and linolenic acids prepared in this laboratory agreed fairly well with those found in the literature (Table II). However the values for arachidonic acid varied considerably from those in the literature.

TABLE II

Specific Extinction Coefficients Used in Calculations

Acid	Wave length	Values from present data	Values reported by Beadle *et al.* (2)
Linoleic	234	89.9	86.0
Linolenic	234	58.1	60.9
Arachidonic	232	47.8	59.3
Linolenic	268	53.4	53.2
Arachidonic	268	36.6	53.4
Arachidonic	316	27.7	22.6

The ethyl ester of the arachidonic acid used for reference standards was first distilled at 100 mμ. pressure. The absorption curve of this material is indicated by the broken line in Figure 2. The material was slightly yellow in color and had a pronounced odor. The absorption curve of this preparation after molecular distillation is shown by the solid line. The preparation contained 0.04% conjugated material and had an iodine value of 303.3 (theor. 305.36). On the basis of iodine value it had a purity of 99.3%. The ethyl ester was water-clear and had no odor after

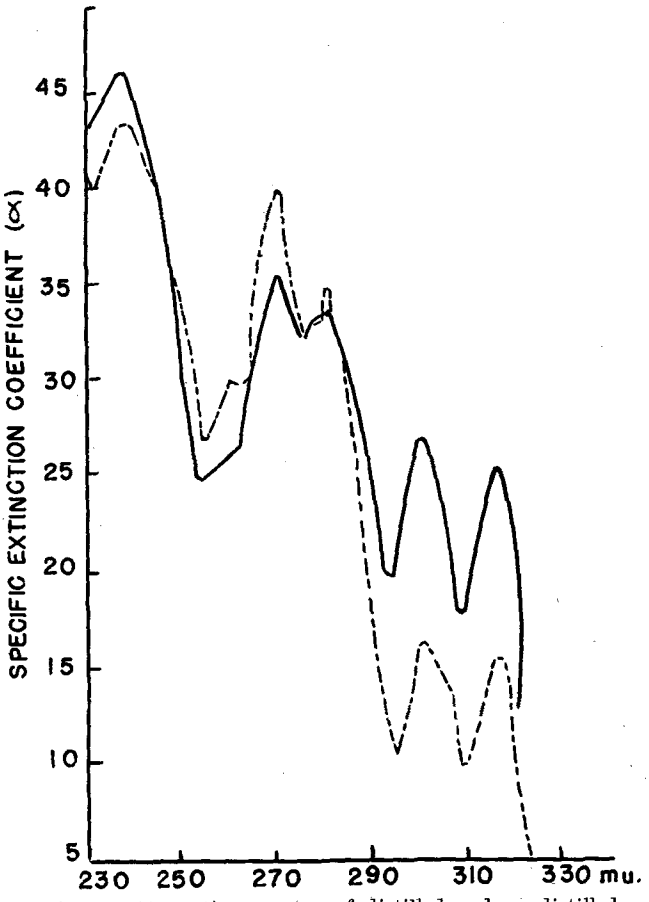

FIG. 2. Absorption spectra of distilled and nondistilled ethyl arachidonate. (– – –) before, (———) after molecular distillation.

[4] Obtained through the courtesy of P. L. Harris, Distillation Products inc., Rochester, N. Y.
[5] Cholesterin, Merck Company, Rahway, N. J.

molecular distillation. However it turned yellow in a short time even if stored in evacuated ampules.

The specific extinction coefficients of the arachidonic acid which had been subjected to molecular distillation were used to calculate the equations given in Table III. The background corrections given by Brice *et al.* (3) were used. The percentage of oleic and saturated fatty acids can also be calculated as described by these workers.

TABLE III

Equations Suggested for Use in Analysis of Mixed Polyunsaturated Fatty Acids

% linoleic acid $= 1.13\ k_2 - 1.13\ k_3 - 0.43\ k_4$
% linolenic acid $= 1.88\ k_3 - 2.51\ k_4$
% arachidonic acid $= 3.60\ k_4$

Summary

The presence of nonsaponifiable material in lipids can cause appreciable differences in calculated mixed fatty acid content. The error can be appreciable in lipids extracted from liver unless a saponification and extraction procedure is included in the method. Skin, gizzard, and depot fats do not contain high concentrations of nonsaponifiable material. However chicken skin fat contains a yellow pigment which absorbs ultra violet radiation. This pigment, while not present in a large amount, can still cause errors in the spectrophotometric analysis. It can best be separated from the mixed fatty acids by distillation of the methyl esters.

The equations developed by previous workers have been recalculated on the basis of an arachidonic acid of 99.3% purity. These equations are suggested for use in the spectrophotometric analysis of unsaturated fatty acids.

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ABSTRACTS . . . . . Don Whyte, Editor

• Oils and Fats

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REMARKS ON THE PRODUCTION OF OIL FROM ALMONDS. F. Wittka and F. Muscari Tomajoli. *Olearia 4*, 10-19(1950). The almonds were processed so as to obtain direct from the press the maximum yield of oil to be used for food and pharmaceutical uses. The quality of the oil depended on the size of the almonds, on the degree of crushing, and on the temperature.

THE USES OF THE PURGHESE OIL. P. H. Mensier and M. Loury. *Oleagineux 5*, 167-170(1950). Purghese grows readily in poor soils, is reproduced by cuttings and gives a high yield in fats per acre. The purghese oil is toxic so that its industrial use in paints and diesel motor fuel was investigated with only fair success.

RYE ERGOT OIL. J. D. Rodriguez. *Con. sup. Invest. cient., Santiago, 1948*, 1-77. Literature on the pyrolysis and vacuum-distillation of castor oil, ricinoleic acid, tristearin, triolein, and rye ergot oil is reviewed. Although both castor and rye ergot oils contain ricinoleic acid, castor oil gives oenanthal and undecylenic acid, while the latter gives neither. The high optical rotation of rye ergot oil and the discrepancy between glycerol content and ester value indicate partial esterification of the hydroxyl group of ricinoleic acid. Two rye ergot oils of widely different hydroxyl values are distilled and their behaviour is compared: analyses of the oils and their distillation products, and ricinoleic acid balances are presented. Contrary to assumptions made in earlier works, the absence of oenanthal and undecylenic acid from the distillation products of a vegetable oil does not afford proof of the absence of combined ricinoleic acid. Descriptions of all experimental procedures used and a bibliography (94 references) are included. (*Brit Abs.* BII, Sept., 1949, 982.)

THE REFINING OF EDIBLE OILS. H. P. Kaufmann. *Olearia 4*, 5-9(1950). The refining of edible oils so as to obtain from them the so-called "integral oils," i.e., oils that have not been deprived of the valuable substances contained in the original crude oils (vitamines, provitamines, sterols, etc.) is discussed.

RAPESEED OIL. F. Wittka and F. M. Tomajoli. *Olearia 3*, 578-88(1949). Review of the composition and characteristics of rapeseed oil, particularly its nonglyceride constituents; also the production, refining, and hydrogenation processes and its uses (illumination, lubrication, and preparation of factice). (*Chem. Abs. 44*, 1269.)

OLIVE OILS OF NORTHERN ITALY AND SARDINIA. C. C. Buzi (Imperia, Italy). *Ann. sper. agrar.* (Rome) [N.S.] *3*, Fasc. straordinar., 409-49(1949). Tables of data are given for 137 samples of olive oil from the harvests of 1932 to 1939. The effect of the locality, condition at harvest, and quality of the pressing operations obscured the effects of varietal differences. (*Chem. Abs. 44*, 2261.)

OLIVE PRODUCING COUNTRIES HOLD CONFERENCE IN MADRID. Anonymous. *Foreign Crops and Markets 60*, 196-7(1950). A conference of the General Committee of the International Oil Culture Federation was held at Madrid, February 10-15, 1950. The keynote of the conference was the justification for an international accord for world protection of olive oil. Five committees were named to study the situation and to draft resolutions toward such an international agreement.

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THE PROPERTIES OF ALKALINE SALTS OF FATTY ACIDS. I. MOISTENING CAPACITY AND CAPILLARY ACTIVITY. E. Otero Aenlle, R. Cadorniga Carro, and S. Pomares Boix. *Anales de fis. y quim.* (Madrid) *45*, (B), 1337-1361 (1949). The moistening capacity, the foaming capacity, and the capacity of carrying away substances by the foam for the alkaline salts of fatty acids is discussed. The capillary activity of each compound as well as the phenomena that involves the existence of an anionic colloidal electrolyte in the solution, determines the variations of the moistening capacity. The moistening capacity of sodium and potassium salts of caproic, caprilic, butyric, stearic, palmitic, and oleic acid determined at 0.1% concentration. These values are compared with those obtained in the determinations of the surface tension of these solutions. The influence of the salt-forming cation and of the addition of sodium hydroxide, electrolytes, and alcohol on the moistening capacity shows that the variations observed are not reflected in the surface-tension values measured. An explanation based on the formation of micellar aggregates above the critical concentration is given.

VISCOSITY OF LOW-MOLECULAR ESTERS WITH BRANCHED CHAINS. H. Staudinger, G. Bier, and G. Lorentz (Univ. of Freiburg, Germany). *Makromol. Chem. 3*, 251-80(1949). The influence of side chains on the viscosity of a large number of low-mol. esters was studied. (*Chem. Abs. 44*, 2443.)

THE NORMAL BUTYRIC ACID MOLECULE AND ITS HYDROGEN LINKAGE. M. Freri. *Atti accad. natl. Lincei, Classe sci. fis., mat., e*