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# Semiquantitative Structural Analysis of Fats by Thin-Layer Chromatography of the Allyl Esters of the Products of von Rudloff Oxidation[1]

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## Abstract

Each unsaturated acyl group in a fat molecule retards its migration on a silica-silver nitrate TLC plate to a degree dependent on its number of double bonds. Thus $S_2U$ in which U is linoleic would migrate with $SU_2$ in which U is oleic. Therefore the molecular families $S_3$, $S_2U$, $SU_2$ and $U_3$ cannot be separated, as such, by this variety of TLC.

Oxidation of fats by the von Rudloff technique converts the glyceryl esters of the original unsaturated acids into the glyceryl half esters of dicarboxylic saturated acids, such as azelaic. Subsequent formation of allyl esters at the free carboxyls, in effect replaces each mono- or polyunsaturated acyl group with another group containing only a single double bond.

Fats so altered can be separated, at least semiquantitatively, into the famlies $S_3$, $S_2U$, $SU_2$, and $U_3$ where U is monounsaturated and represents, but is not identical with, the original acyl group. These results can be translated directly into the percentages of the original molecular families. Results of determinations are given.

Procedures for assigning spots to specific molecular families are (1) comparison of $R_f \times 100$ values with given experimental ranges, (2) comparison of the percent of S theoretically present after tentative assignment of spots, with the percent of S found by GLC analysis of the sample, (3) use of internal standards, and (4) miscellaneous.

## Introduction

THIN-LAYER CHROMATOGRAPHY of fats on silica-silver nitrate plates effects separations of molecular varieties largely on the basis of total molecular unsaturation. For instance, the molecule SSU in which U is represented by oleate, with a single double bond, will migrate farther than the corresponding SSU in which U is linoleate, with two double bonds. On the other hand SUU in which U is oleate will migrate with SSU in which U is linoleate, because both molecules have a total of two double bonds. Thus, except for $S_3$, the four molecular types $S_3$, $S_2U$, $SU_2$ and $U_3$, and the isomers SUS—SSU, and USU—UUS are not generally segregated, as such, on silica-silver nitrate thin layer plates.

By means of the permanganate-periodate technique of von Rudloff (1) the unsaturated acyl groups in fat molecules may be oxidized at the double bond nearest the carboxyl to produce the corresponding saturated dicarboxylic acid residues.

If the free carboxyls are then esterified with a monoene, such as allyl alcohol, a new triglyceride molecule is produced, in which each original unsaturated acyl group, regardless of its degree of unsaturation, is represented by a new group containing only a single double bond.

Fats so altered can be separated by thin-layer chromatography into spots representing the original $S_3$, $S_2U$, $SU_2$, and $U_3$. By measurement of the spots the percentages of the orginial $S_3$, $S_2U$, $SU_2$ and $U_3$ can be determined semiquantitatively. There is usually some degree of separation of the isomers SUS—SSU and USU—UUS and sometimes this is semiquantitative. It is possible that improved technique or column separations would give quantitative results for both the types and the isomers.

## Experimental

### I. Outline of the Method

The fat to be analysed is subjected to von Rudloff oxidation, whereby all unsaturated acyl groups are converted into the corresponding dicarboxylic acid residues. The acidic residues are then converted into the silver salts and these in turn into the allyl esters by reaction with 3-iodopropene.

These allyl esters, along with the unaltered fully saturated components, are chromatographed on specially prepared silica-silver nitrate plates. The plates

[1] Presented in part at the AOCS meeting Houston, April, 1965.

are sprayed with 2,7-dichlorofluorescein and the spots are photographed under ultraviolet light.

The spots are measured as will be described, and the percentage of each is calculated. These percentages may be taken to represent, directly, those of the corresponding original molecules from which they were derived, at least in those fats in which $C_{16}$-$C_{18}$ fatty acid residues predominate.

**II. Detailed Procedures**

1. *von Rudloff Oxidation.* The method employed is essentially the modification by Youngs (2). The following specific procedure for a 100 $\mu$l sample is satisfactory:

One hundred microliters (the procedure has been successfully scaled down to 20 microliters) of the fat in a 250 ml stopperable flask are heated for 1–2 hr, with stirring, in a water bath at about 75C, with the following mixture: 40 ml tertiary butanol; 15 ml oxidant solution containing 20.86 g sodium periodate and 0.395 g potassium permanganate per liter of water solution; 12 ml distilled water; 0.5 ml of 8.3% potassium carbonate in water.

After the oxidation has been accomplished, the mixture is cooled to room temperature, the stirrer washed down with acetone, and the permanganate color discharged with ethylene gas. The volume is reduced to about 15 ml (rotary evaporator, or air stream in a warm water bath) and the residue is extracted twice by shaking for 10 min with 20 ml of chloroform. The chloroform solutions are filtered into a 25 ml flask and the solvent removed by an air stream in a warm water bath. The fatty acid fragments and any other volatiles are removed by heating in a steam bath at about 2 mm pressure for about 1 hr.

2. *Formation of Silver Salts.* The silver salts are prepared by a modification of the method of Gehrke and Goerlitz (3). The oxidation products, still in the 25 ml flask are dissolved in 10 ml of acetone. To the solution is added 10 ml of a 1% solution of silver acetate in water. The flask is stoppered and shaken for 30 min at room temperature, after which the volume is reduced to about 10 ml by an air stream and mild heat. After sufficient cooling to ensure solidification of the unaltered $S_3$, most of the liquid phase, containing excess silver acetate, is separated, by filtration, from the precipitated silver salts and $S_3$. Gehrke and Goerlitz (3) have described the use of one variety of filter stick. Another, used in this laboratory consists of a small Teflon cylinder with a perforated bottom, fitted lightly to a small glass delivery tube. Only the filter paper covering the perforated bottom and a small part of the Teflon head are dipped into the liquid during operation. When filtration is concluded the Teflon head is dislodged with forceps and dropped, with the paper, into the flask where both remain throughout the rest of the synthesis. No rinsing is required.

3. *Esterification.* The esterification is accomplished by a modification of the procedure of Gehrke and Goerlitz (3). To the silver salts, as produced in the preceding step, are added 20 ml of acetone followed by 0.5 ml of 3-iodopropene. The flask is stoppered and shaken for 30 min or more at room temperature, after which the solvent is removed by an air stream and low heat. The remaining volatiles are removed by heating in a steam bath at about 2mm pressure. The residue is extracted by shaking with two 5 ml portions of chloroform. The extracts are filtered through paper into a small vial, the solution in the vial being concentrated when necessary by air stream and mild heat. After the last filling the solvent is removed, the last traces being eliminated in a 75C oven. The product is a mixture of the original saturated triglycerides and the allyl esters of the originally unsaturated ones. It is now ready for chromatographic separation.

4. *Preparation of the Chromatoplates.* It is important that the surface of the layer be smooth, and tough enough to resist disruption during development, and by spraying. A slurry of the following formula is prepared: 10 g silica gel (Kieselgur D5, Camag, is satisfactory); 1 g powdered cellulose (Selectacel 65, Schleicher and Schuell, is satisfactory); 0.5 g sucrose; 20 ml of a 12.5% solution of silver nitrate in water; 7 ml of distilled water. The slurry is mixed under reduced pressure in a small pebble mill consisting of a 150 ml flask with identations so placed as to tumble the pebbles when rotated on a rotary evaporator. It is important that all air bubbles be eliminated in the process of mixing. Frequent stops of about 5 sec duration help to accomplish this. The mixing process takes about 10 min and is perhaps best carried out under subdued light. The quantity specified is sufficient for more than 12 plates. The films are made on $2 \times 6$ in. glass plates between two, 0.25 in., adhesive vinyl strips, placed along the edges to form a shallow channel. White Con-tact made by Cohn-Hall-Marx Co., 1507 Broadway, New York, N. Y., and available in large sheets at stores selling household supplies, is satisfactory. Adhesive adhering to the glass can migrate into the film upon development. This is prevented by ruling two lines about 0.125 in. apart along each of the long edges of the film before use and carefully removing any adhesive at the lower corners that would be submerged, and could dissolve in the developer. The dry film is about 90$\mu$ thick. A portion of the slurry is placed along the upper edge and pulled down with an even, steady stroke of a smooth-edged plastic spreader. Care must be taken to avoid pulling down air bubbles. The films are allowed to dry superficially in air, preferably protected from strong light. The tapes are removed and the finished plates are stored under reduced pressure in darkness. Before use they are ruled into three or four columns.

5. *Spotting.* Ten microliters of a 1% solution of the sample in chloroform are carefully spotted in a 0.125 in. diameter area in each channel. The sample is delivered from a Hamilton syringe driven by screw pressure. Samples are spotted 2 cm from the lower edge of the plate.

6. *Development.* The plates are developed at room temperature in closed, filter paper lined, glass jars inside a shielding cabinet. The developer consists of 99.5% chloroform and 0.5% acetic acid. Development is stopped when the most advanced solvent front reaches a premarked level 10 cm from the origin. Results have been satisfactory when this occurs in about 25 to 30 min, a range found generally suitable for conditions in this laboratory and with the materials employed. Under other circumstances another range may be more satisfactory. The heights of the solvent fronts in the remaining columns are quickly estimated and recorded and the plate is removed from the jar.

7. *Photography.* After enough time for evaporation of the solvent, including the acetic acid, the plates are sprayed with 0.8% 2,7-dichlorofluorescein in methanol and humidified in a closed vessel saturated with

TABLE I

Percent of Molecular Varieties by Semiquantitative TLC

| Fat | Method | % Wt, molecular types | | | | % Wt, isomers | | | | %S (Total) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | $S_3$ | $S_2U$ | $SU_2$ | $U_3$ | SUS | SSU | USU | UUS | |
| Synthetics[a] | Known composition | 27.3 | 28.5 | 23.9 | 20.3 | 28.5 | ..... | 23.9 | ..... | 54.3 |
| | TLC | 27.1 | 26.5 | 22.5 | 23.8 | ..... | ..... | ..... | ..... | 52.3 |
| Corn oil | Pancreatic lipase | 0 | 4.5 | 33.3 | 62.0 | ..... | ..... | ..... | ..... | 14.1 |
| | TLC | 0 | 7.3 | 36.3 | 56.4 | ..... | ..... | ..... | ..... | 17.0 |
| Cottonseed oil | Pancreatic lipase | 1.2 | 19.8 | 48 | 31.7 | 16.4 | 3.4 | 2.4 | 45.7 | 30.4 |
| | TLC | 0 | 16.2 | 54.3 | 29.6 | 11.8 | 4.4 | 12.3 | 42.0 | 29.8 |
| Beef tallow | Pancreatic lipase | 18.4 | 44.5 | 30.7 | 6.5 | 26.2 | 18.3 | ..... | ..... | 58.3 |
| | TLC | 14.0 | 50.7 | 24.7 | 10.7 | 28.8 | 21.9 | ..... | ..... | 56.0 |
| Cottonseed oil 50% Beef tallow 50% | Pancreatic lipase (Calc) | 9.8 | 32.2 | 39.4 | 19.1 | 21.3 | 10.9 | 3.5 | 35.9 | 44.4 |
| | TLC | 10.8 | 37.3 | 37.9 | 13.9 | 22.9 | 14.4 | 6.5 | 31.4 | 48.3 |
| Interesterfied lard | Pancreatic lipase | 4.6 | 24.9 | 44.3 | 26.2 | 9.0 | 15.9 | 13.6 | 30.7 | 36 |
| | Random (Calc) | 4.7 | 24.9 | 44.2 | 26.2 | 8.3 | 16.6 | 14.7 | 29.4 | 36 |
| | TLC | 10.8 | 26.9 | 45.1 | 17.3 | 11.0 | 15.9 | 17.2 | 27.9 | 43.7 |

[a] Courtesy of Dr. Edward G. Perkins. The specific composition was as follows (wt%): OOO = 20.3, SSS = 15.1, LLL = 12.2, POP = 17.8, SOO = 23.9, POS = 10.7 (L = lauric, P = palmitic, S = stearic, O = oleic).

water vapor until the spots are well visible under ordinary light. They are then photographed very quickly on Polaroid film under ultraviolet light and through a K2 filter. Pictures are made full size on $4 \times 5$ in. film.

8. *Quantification.* The spots on the photograph are outlined with pinholes which are then projected, with enlargement, on uniform paper. The projected pinholes are marked in ink and the areas clipped out and weighed. The percentage of the weight of each such area in the total weight of all areas in the column is then calculated, and from these the average weight percentage of each area is determined.

The percentages thus found may be taken directly as the weight percentages of the original components they represent, or they may be adjusted to compensate for the differences in molecular weight between the original and the chemically altered components. At this writing there seems little or no advantage in the latter procedure. This matter will be discussed further in the next section.

## Results and Discussion

The experimental results of analysis of five fat samples are shown in Table I, with comparative values derived from the compositional data or, as found by means of the pancreatic lipase procedure. The results have not been "corrected" (see II-8 preceding).

Spots were assigned to the types $S_3$, $S_2U$, $SU_2$ and $U_3$, and to the isomers, by matching their percentages, singly or in consecutive groups as necessary, against these comparative values.

The results shown in the table are in moderately good agreement with the values provided for comparison and thus demonstrate the semiquantitative nature of the process.

The "cut and try" method of assigning spots to particular molecular types in accord with a known pattern is, of course, of no practical value in analysis of unknown mixtures. The spots must then be identified by other means.

The data in Table I, with others, have provided a basis for the ranges of $R_f \times 100$ values shown in Table II. By comparison of their $R_f \times 100$ values with those in the table, spots may be tentatively assigned to one of the four molecular type $S_3$, $S_2U$, $SU_2$ and $U_3$. Spots well within the specified ranges are quite likely to be correctly identified, but those at the extremes may be in doubt.

TABLE II

Ranges of $R_f \times 100$ Values for Molecular Types[a]

| Type | Range |
|---|---|
| $S_3$ | 79–71 |
| $S_2U$ | 71–36 |
| $SU_2$ | 35–15 |
| $U_3$ | 17–8 |

[a] These boundaries should not be too rigidly observed. They are given only as a guide to tentative assignment of spots to molecular types.

Under ordinary circumstances $S_3$ has appeared in the single spot migrating furthest from the origin. This occurs even in beef tallow in which both stearic and palmitic acids are present to the extent of about 25%. Similarly, but for one instance, $U_3$ has appeared in only one distinguishable spot, which is that nearest the origin. The exception is corn oil in which a second spot appeared above that nearest the origin, representing 12.8% of the total and which could not be accounted for except as $U_3$.

Recently Sreenivasan et al. (4) reported evidence of considerable quantities of dienes other than 9,12-isomers in corn oil, and this evidence was given some support by Beadle et al. (5). The presence of as little as 4% of an isomer having the double bond nearest the carboxyl in other than the 9-position could account for the presence of 12% of a different variety of $U_3$ in which one of the three dicarboxylic acids produced by oxidation differs from the others. It is therefore possible that our second spot, comprising 12.8% of the whole, may represent such an isomer.

$S_2U$ and $SU_2$ usually appear in two or more spots representing the symmetrical and unsymmetrical forms. There is presently no way of differentiating between them in an unknown sample.

A useful check on the tentative assignment of spots is by comparison of the total percentage of S in the tentative mixture with that known to be present by means of GLC analysis of the original sample. For instance, erroneous assignment of 12% of $S_2U$ to $SU_2$ would result in a 4% decrease in the total percentage of S "recovered." This would probably be sufficient, in most instances, to demonstrate the error. Obviously, however, the procedure becomes less reliable as the spot size decreases.

It is quite possible that synthetic triglycerides, oxidized and esterified to form the allyl esters, would serve as internal standards.

Adjustment of the experimental values to compensate for the differences in molecular weight between the original and chemically altered molecules is of doubtful value, if for no other reason than that the "corrections" are of approximately the same magnitude as the errors inherent in the process. They may, however, be useful with more precise techniques.

The "corrections" are made by multiplying each experimental percentage of $S_3$, $S_2U$, $SU_2$ and $U_3$ by the ratio A/B, where A is the mean molecular weight of the original molecular component (calculated from GLC compositional data), and B is the corresponding mean molecular weight of the altered molecules. The

products of the calculations are adjusted to a 100% basis and represent the percentages of the original $S_3$, $S_2U$, $SU_2$ and $U_3$.

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# Application of Statistical Distribution Formulas to Triglycerides Originating in Tissues Having Regional Differences in Fatty Acid Composition[1]

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## Abstract

Variations in triglyceride fatty acid composition are sometimes observed between different regions of the same fatty tissue in various plants and animals. When natural fats originate in such tissues, some error results if triglyceride distribution formulas are applied to an *average* fatty acid composition. An integral calculus technique has been developed to overcome this difficulty. This method has been applied to the random, restricted-random, and 1,3-random-2-random distribution hypotheses. The error resulting from such regional differences has been estimated for five natural fats originating in such heterogeneous tissues.

If the relative amounts but not the types of fatty acids vary with location, regional differences do not appreciably affect the use of statistical distribution formulas. In such cases, triglyceride composition may be predicted from the average fatty acid composition without significant error. If different types of fatty acids exist in different regions, however, these differences must be taken into account to avoid large errors.

## Introduction

A NUMBER OF WORKERS have reported that morphologically-distinct fatty tissues in various plants and animals contain regional differences in fatty acid composition. Kartha (1) has reported substantial variations in the iodine values of fats from different locations in the almond, peanut, pistachio nut, Brazil nut, cashew nut, sapota seed, coconut, hazelnut, apricot seed, and arecanut. He observed extreme regional differences in the coconut where the iodine value varied from 7.9 to 46.0 according to the location of the sample. Galoppini and Lotti (2) have reported regional variations in the fatty acid compositions of the peanut, hazelnut, almond, and pine nut. In our own laboratory, we have observed regional variations in the fatty acid and triglyceride compositions of *Myrica carolinensis* fruit coat fat (3). The phenomenon is not restricted to plant fats. Hilditch and Zaky (4) have demonstrated the different fatty acid and triglyceride compositions of perinephric and external tissue fats from the same sheep.

[1] Presented at the AOCS meeting, Houston, 1965.

This accumulating evidence indicates that many of the fats being studied for chemical composition are not of homogeneous origin. Apparently many natural fats and oils are not "pure" fats but mixtures of many similar fats of slightly varying composition. If fatty tissue exhibits regional differences in fatty acid composition, then regional differences in triglyceride composition must also exist. These regional differences affect the applicability of the various triglyceride distribution hypotheses. This can be demonstrated by examining a hypothetical fat of mixed origin and composition.

Consider a 1:1 mixture of palm and palm kernel oils. The palm oil contains 0% and the palm kernel oil 54.6% lauric acid (Table VI). The lauric acid from the palm kernel oil cannot enter into any of the palm oil triglycerides as required by the even, random, restricted-random, or 1,3-random-2-random distribution hypotheses. Therefore, a mixture of two fats having different fatty acid compositions cannot follow statistical distribution rules exactly.

The above case is obviously an extreme example of how mixed fats can deviate from the triglyceride compositions predicted by distribution hypotheses. But the same principle still applies when a fat originates in a morphologically-distinct tissue having smaller regional differences in fatty acid composition. It is impossible for present distribution formulas to exactly describe the triglyceride composition of the resultant mixed fat.

For the interpretation of our experimental results on *Myrica carolinensis* fruit coat fat, we have developed an integral calculus technique which does allow statistical distribution hypotheses to be accurately applied to fats originating in tissues having regional differences in fatty acid composition. This paper describes the new method and its application to the random, restricted-random, and 1,3-random-2-random hypotheses. Five natural fats originating from heterogeneous sources have been examined using this technique.

It should be emphasized that *this paper does not deal with the relative merits of the various triglyceride distribution hypotheses discussed.* We describe only a technique for applying these hypotheses to fatty tissues having regional differences in fatty acid composition.