Determination of Fat Composition[1]

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Abstract

A revolution has taken place in the analysis of fats. Physical methods, both rapid and accurate, have replaced laborious chemical procedures. The time-honored saponification equivalents and iodine values now are calculated from chromatographic and nuclear magnetic resonance spectroscopic data. Differential migration processes such as countercurrent distribution, liquid-liquid chromatography, and gas chromatography have supplanted the classical distillation and crystallization procedures for analysis and preparation.

What have been referred to as "gadgets" are now the stock-in-trade of the analytical lipid chemist. Mass, infrared, ultraviolet, and nuclear magnetic resonance spectrometers are the accepted tools for organic characterization. Recording detectors and computer processing of data reduce the labor of analysis and improve its quantitation. Today's methodology stands at the verge of specifying fatty acid composition of even so complex lipids as hydrogenated fats in terms of the amounts, the positions, and geometric configurations of its individual component fatty acids.

Introduction

A REVOLUTION has taken place in the analysis of fats. Physical chemists have become analytical chemists, and the Rube Goldbergs of the analytical laboratory, no longer objects of ridicule, are now instrumentation men.

Physical methods, both rapid and accurate, have replaced laborious chemical procedures. Instead of the time-honored saponification equivalents and iodine values, these constants are calculated from gas-liquid chromatographic (GLC) data when they are required by tradition. Now nuclear magnetic resonance (NMR) bids to replace not only these classical chemical determinations but the gas chromatograph as well (13). From spectra, such as shown in Figure 1 (14), determined directly on an oil sample, total unsaturation and average molecular weight may be calculated. Further the amount of unsaturation at the 15,16 position may be calculated from measurements in the region of the terminal methyl proton resonance (10). The mass spectrograph tandem to a gas chromatograph provides, on microscale, both qualitative and quantitative information the composition of the volatile odor constituents of vegetable oils (5,12). In fats, catalytically "hydrogenated" with deuterium, the amount of gem disubstitution and monosubstitution may be determined from infrared spectrophotometric measurements (Figure 2). The head of the band is a measure of the monodeuterio substitution, and the shoulders, of the gem dideutero substitution (18). This is "such stuff as dreams are made on," the gleam in the analyst's eye today, and the methodology of tomorrow.

Now, as to the less esoteric, it is interesting to see what what can be done to advance knowledge of fat Composition. In so doing, increasing awareness is supplied by biochemical and medical workers as well as by processors for more complete information on the composition of the edible products which are produced and consumed.

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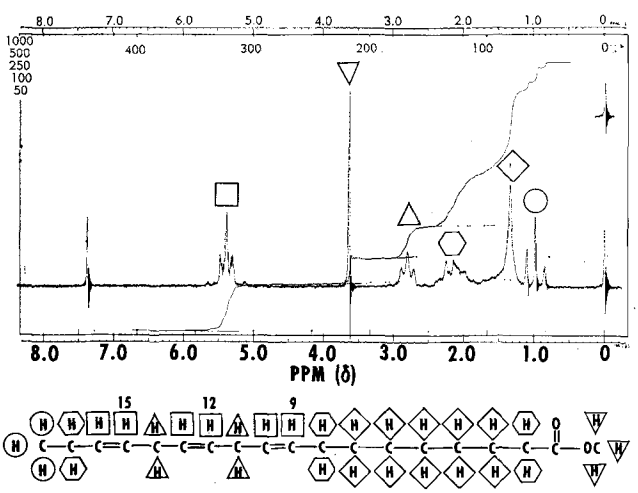

Symbol	Structure	PPM δ	No. Protons
○	β - Olefinic methyl protons	0.97	3
⬡	α - Olefinic methylene protons*	2.25	6
△	Di -α- olefinic methylene protons	2.80	4
□	Olefinic protons	ca 5.38	6
▽	Methoxy protons	3.67	3
◇	Insulated methylene protons	1.33	10
			32

*Also includes methylene adjacent to carbonyl

FIG. 1. NMR spectrum, peak assignments, and proton counts for methyl linolenate. Tetramethylsilane peak at 0 and benzene at 7.4.

Glyceride Structure

Interesterified fat products have dramatized the utility of what was previously an academic field of interest, namely, glyceride structure. As a further example of practicality, the differences in melting characteristics of cocoa butter and interesterified cocoa butter may be cited as those which exist between the sharp melt of a chocolate bar and the clinginess of a mutton tallow.

In studies of glyceride structure, the countercurrent distribution (CCD) technique of fractionation has strikingly demonstrated the differences between the essentially monoolein natural cocoa butter and the random interesterification mixture (6). Recently isomeric soybean oil glycerides with seven double bonds per molecule have been isolated in gram amounts by CCD from those with six and eight and then have been subjected to lipase hydrolysis (Figure 3) (9). The conclusion reached with this oil and others is, at first reading, a startling one, namely, that positional

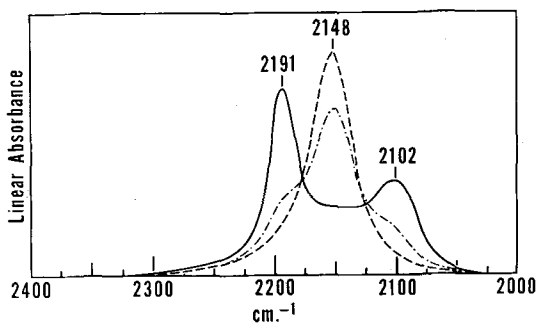

FIG. 2. Infrared absorption spectra of deuterostearates.

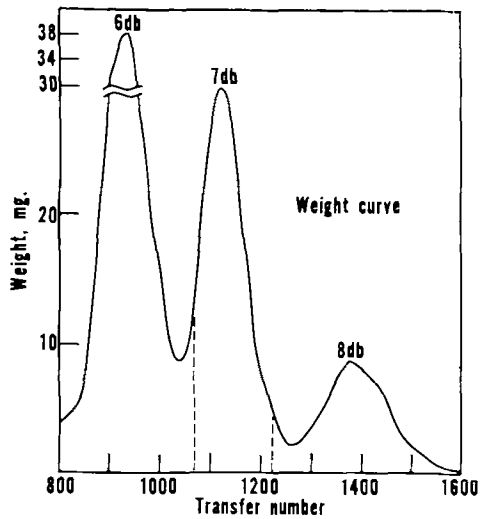

FIG. 3. Countercurrent distribution of soybean hypocotyl oil with a hexane-furfural-nitroethane system, showing the weight distribution of the highly unsaturated 6-, 7-, and 8-double bond (db) glycerides.

isomers of glycerides may be present in nonrandom amounts even though the compositionally distinguishable glycerides approximate a random pattern. On the basis of random structure, one would expect twice as much of the unsymmetrical dilinoleolinolenin as of the symmetrical dilinoleolinolenin, but three and one-half times were found. With the dilinoleolinolenin fraction of linseed oil, less than the expected amount of unsymmetrical glyceride was found by selective lipase hydrolysis. These apparently discordant results are however collated by the 1,3-random, 2-random theory of glyceride structure developed by Coleman (3) and Vander Wal (21) and extended to unsaturated fats.

The combined use within the same laboratory of highly developed physical, chemical, and enzymatic techniques is not unusual in modern lipid research. As it has always been, the true scientist employs any and every tool regardless of the discipline of its origin to acquire new knowledge—in this instance, knowledge of the complex and intricate glyceride structure of vegetable oils and fats.

Fatty Acid Composition

Properties of fats are determined not only by the arrangement of fatty acids within the glyceride molecules, as just described, but also by the properties of the individual fatty acids comprising the glyceride. When one speaks of fat composition today, one no longer refers merely to the naturally occurring homologous and isologous series of fatty acids, but one has in mind mixtures of fatty acids of hydrogenated products in which the residual double bonds are isomerized both in position and geometric configuration. Separation of these mixtures has defied the highest development of technique and has caused the invention of new procedures.

Microvapor-Phase Hydrogenator

Frequently in GLC analysis the more unsaturated members of the isologous series overlay members of the longer homologous series of saturated fatty acids and cannot be resolved from them. For these instances and for those in which one merely wants to separate the homologous series of saturated fatty acids, the microvapor-phase hydrogenator is most useful (16). This accessory is used with a gas chromatograph and employs hydrogen as the carrier gas. It consists of a precolumn packed with hydrogenation catalyst and substitutes for the injection nut and rubber septum of the chromatograph. Introduction of the sample on the catalyst and in the flowing hydrogen stream causes complete reduction of unsaturated fatty acids to the saturated homologues and their subsequent separation on the GLC column. Examples of its application to fish oils and the overlapping of peaks are shown in Figure 4.

Problems that can be solved by merely hydrogenating fatty acids and then separating the homologues by GLC means are, of course, few. The large question of fat composition demands the separation of fatty acid isologues, homologues, and isomers to such an extent that the simplified mixtures resulting may then be analyzed by still further procedures. For this purpose, differential migration systems of liquid-liquid extraction have been used successfully to resolve the isologous series in unsaturation and the homologous saturated series in length of carbon chain. Two systems will be illustrated, one (2) that provides samples in gram amounts (CCD) and the other, in fractional gram amounts (liquid-liquid chromatography (LLC)).

Countercurrent Distribution

Perhaps the binary solvent system most successfully used in CCD for the resolution of the isologous series of fatty acid methyl esters is hexaneacetonitrile. Its advantages reside, in part, in the low-boiling points of its components and, in part, in its high selectivity with respect to unsaturation. A typical application exists in catalytically hydrogenated methyl linolenate as shown in Figure 5 (1). Since as many as 40 grams of mixture may be introduced at the start of each run, it is apparent that multigram amounts are obtained of the individual monoene, diene, and

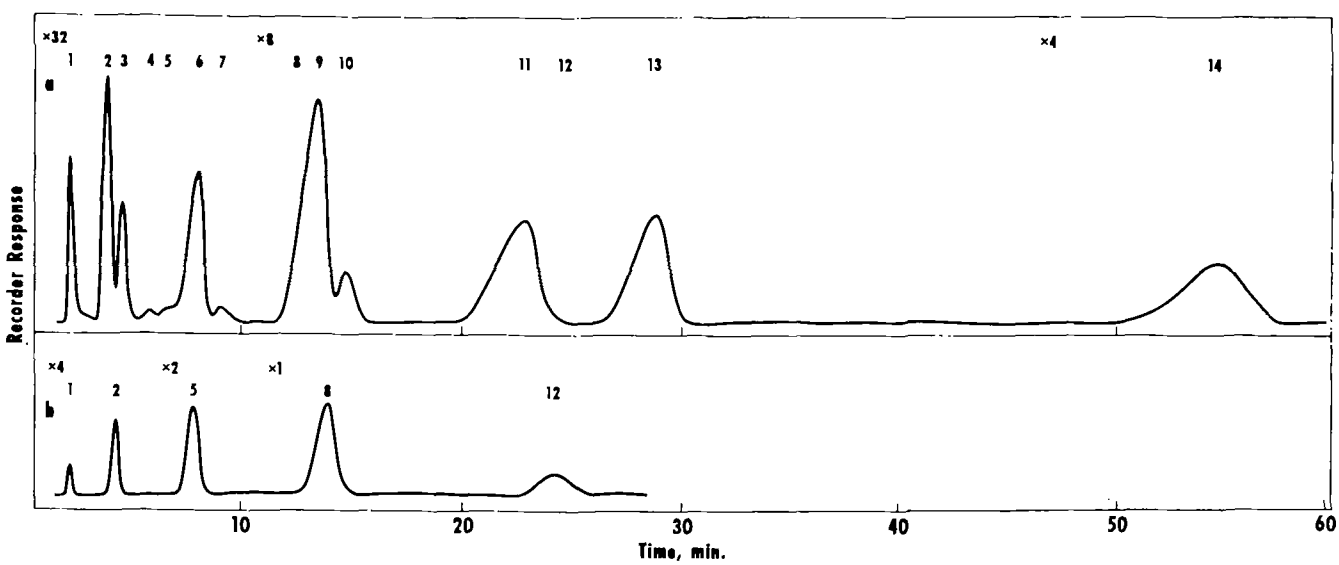

FIG. 4. Separation of methyl esters of fatty acids of herring oil: a) injection at 0 mm: 1. myristate, 2. palmitate, 3. C_{16}:1, 4. C_{16}:?, 5. stearate, 6. oleate, 7. linoleate, 8. to 14. C_{20} and C_{22} esters; b) injection at 43 mm: 1. myristate, 2. palmitate, 5. stearate, 8. arachidate, 12. behenate.

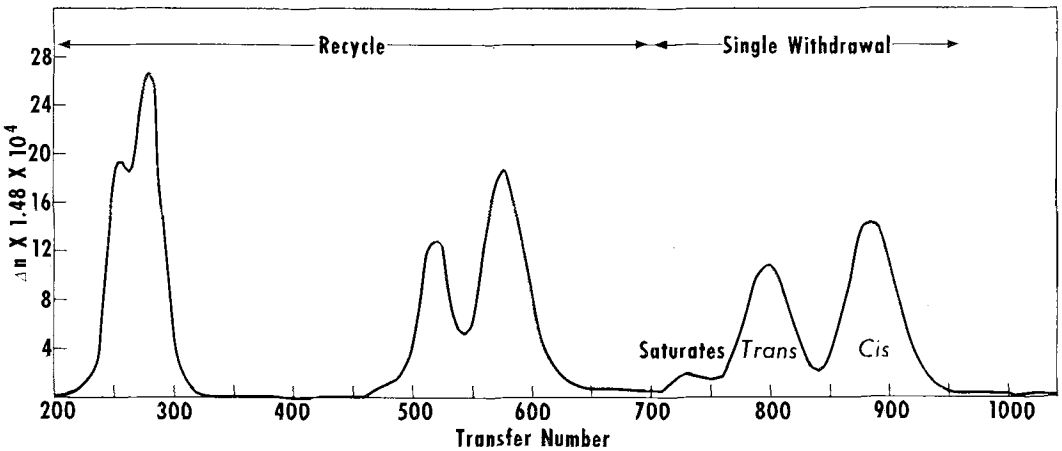

FIG. 5. Refractometer curve for the separation of platinum-reduced methyl linolenate. Lower part of figure gives recorder tracing used to construct the upper graph.

trienoic fatty acid fractions. These are of sufficient size to permit further separation and resolution operations to be carried out. On these fractions the conceptual equivalent of two-dimensional chromatography can be achieved in CCD by subsequently introducing them into a system of widely differing resolving characteristics.

An argentation system (the use of silver ions to complex with the pi orbitals of ethylenic bonds) supplies this second dimensional characteristic to both CCD and LLC. For example, a mixture of monoenoic isomers resolved by the hexane-acetonitrile system may be reintroduced into the CCD apparatus, but this time with hexane as the upper phase and methanolic silver nitrate as the lower phase. In this argentation system, *trans* monoenes migrate more rapidly through the tubes of the instrument than do the *cis* isomers because the *cis* bond has a greater tendency to interact with the silver ion. Argentation CCD separates the *cis* from the *trans* isomers.

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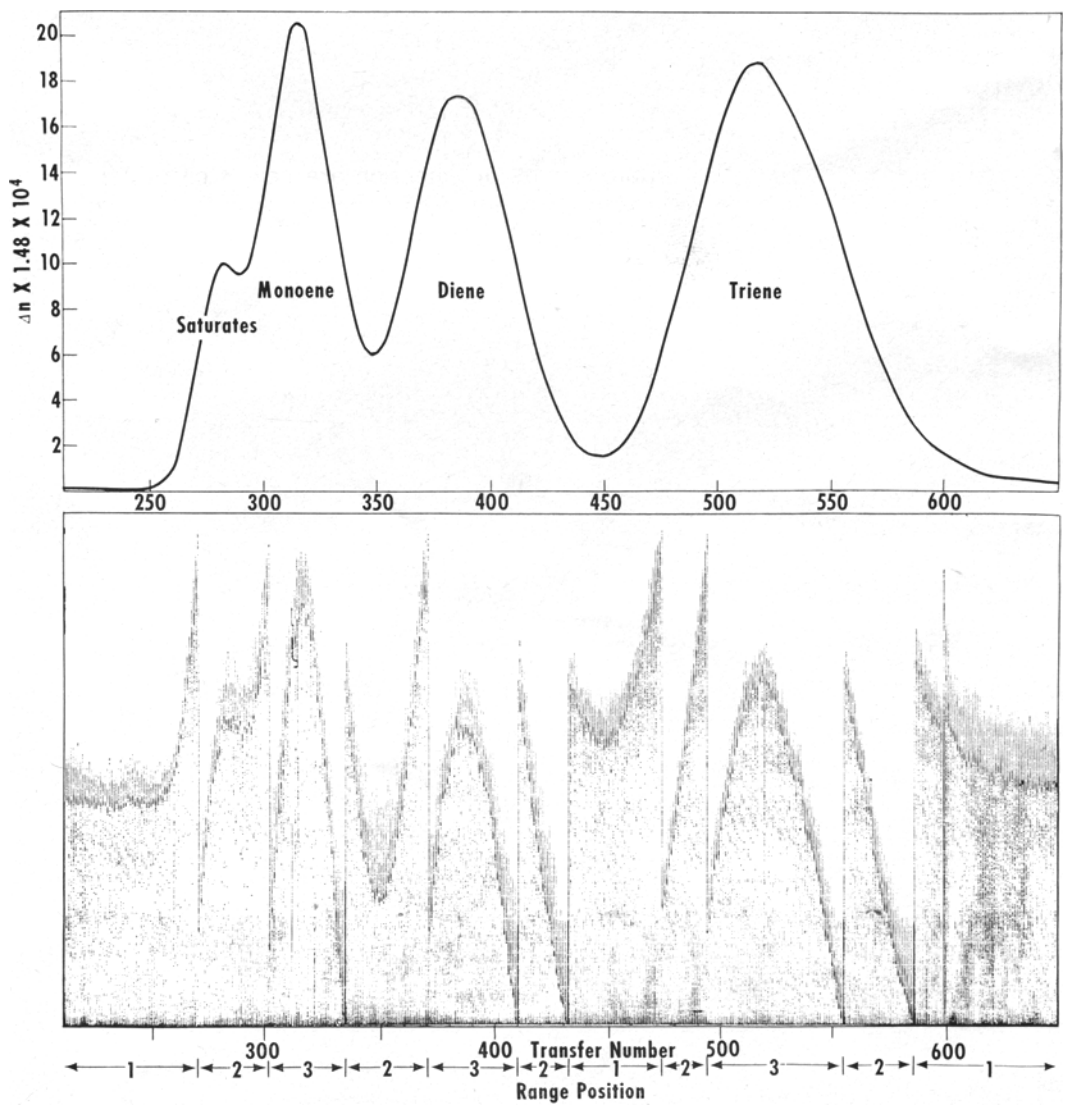

FIG. 6. Refractometer curve for separation of monoenes from platinum-reduced methyl linolenate. Recycle operation was terminated at transfer 700 to prevent leading edge of stearate distribution from overtaking the trailing edge of *cis*-monoene.

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Not only does the silver ion separate the geometric isomers but, in certain instances, may cause a resolution of isomers, depending on the position of double bonds. In a series of dienes, conjugated *trans,trans* would be eluted first, followed by conjugated *cis,trans,* followed by isolated *trans,trans* and the isolated mono *trans* (*cis,trans; trans, cis*) mixture and finally by the *cis,cis* dienes (1). Moreover dienes interrupted by more than one methylene group are complexed more strongly than the pentadiene structure. Thus the silver ion has the unique characteristic of resolving fatty acids with respect to the geometric configuration of double bonds and of fractionating them with respect to position of bonds.

It will be noted in Figure 5 that the difference in refractive index of solution samples rather than weight is plotted on the ordinate (2). This application of refractometry signifies the role of automation in the modern chemical laboratory. The application of a differential, continuous-flow, recording refractometer freed at least one full-time worker from routine evaporating, tarings, and weighing of flasks. Further the recorder of refraction index tells at a glance the progress of the separation—a vital bit of information during recycling operations when fractions issuing from the last tube of the train are reintroduced into the first (Figure 6). This same refractometric monitoring principle has been applied to columns as well as to liquid chromatograms (8).

Liquid-Liquid Chromatography

Columns filled with partially vulcanized rubber (11) perform on a milligram scale with effectiveness comparable with the 200 CCD apparatus and the hexane-acetonitrile system on the multigram level. Octadecanoates, octadecenoates, octadecadienoates, and octadecatrienoates are resolved on this reverse-phase chromatogram.

Argentation separation has also been successfully demonstrated for LLC with columns of high-surface macroreticular resins saturated with silver ion (8). In addition to the monoene separations of Figure 7, many conjugated and nonconjugated diene isomers may also be resolved (7). The refractometric trace of Figure 7 again suggests that the tedium has been removed from this analytical procedure.

Despite some successes in separating fatty acids isomers which differ in the position of the double bond in the chain by argentation (19), there remains a rather large deficiency in methodology for this type of fractionation. The inability to separate this positional type of isomer however does not hamper one's ability to determine the location of these double bonds analytically. Having separated the fatty acid mixtures, first with respect to the isologues and homologues and then with respect to the geometrical isomers within the monoene, diene, and triene fractions, the double bond positions may be determined by an oxidative cleavage procedure (described later).

Routine Analytical Procedure

It may be argued that CCD for analysis of fats is not practical for routine use despite its automation of monitoring because of the time consumed and the expensive apparatus. With these objections in mind a routine procedure of LLC was developed that, combined with a simplified method of double bond location, provides information on the exact composition of complex fatty materials in terms of the numbers, positions, and geometric configurations of the individual fatty acids (20). As shown in the flow diagram (Figure 8), the procedure consists in introducing the methyl esters derived from fats, first, upon rubber columns where one achieves the separation into saturate, monoene, diene, and trienes and then, the monoenes onto an argentation chromatographic column where the geometric isomers of the fractions may be resolved. This method of refractometric monitoring provides automation and ease of operation, also quantitation of results. Completion of the analysis for isomeric fatty acids then merely requires the location of double bonds within these quantitatively separated fractions of geometrically isomeric fatty acids.

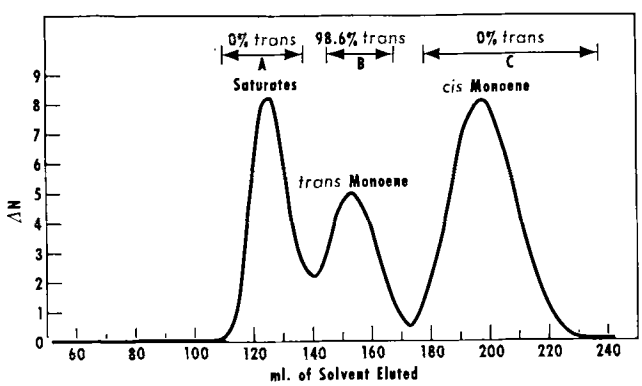

FIG. 7. Separation of saturates, *trans* monoenes, and *cis* monoenes obtained from hydrogenated methyl linolenate, on a 225-cm. column, packed with resin saturated with a silver ion.

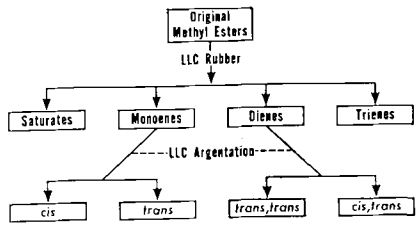

FIG. 8. Scheme for the LLC separation isomeric monoenes and dienes.

Micro-ozonization-Pyrolysis-Chromatography

A simple, rapid method of double bond location has been devised that employs as a vital part a loop of stainless-steel tubing clamped between the poles of a soldering gun in place of its normal heating element (4). Microgram or smaller samples of the isomer mixture to be analyzed for double bond position are introduced into this tube through a rubber septum, oxidized in an ozone stream, then pyrolyzed in a helium stream and introduced into the GLC column for separation of component aldehydic cleavage products. A short precolumn of zinc oxide removes any acidic materials. A diagram of the soldering gun reactor is shown in Figure 9. Within an hour one obtains results which, by the older liquid oxidation procedures, required one week to complete. The results are comparable with those of the older procedures shown in Table I (15). Thus a sample of hydrogenated soybean oil shortening, which by usual GLC analysis showed 39.4% octadecadienoic acid, had a true linoleic acid content of only 12.8%; a hydrogenated winterized vegetable oil, having initially 45.9% octadecenoic acid, had a true oleic acid content of 25.4%.

Automatic Data Processing

A discussion of speeded-up and amplified research in analytical procedures invariably raises the question as to

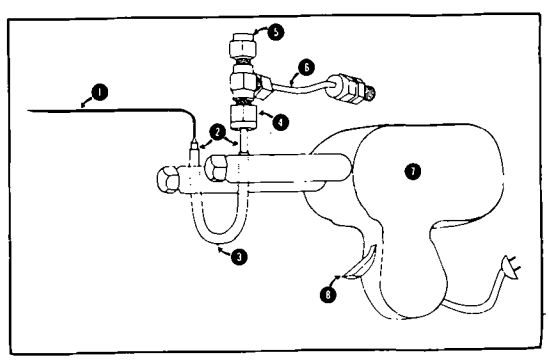

FIG. 9. Microreactor apparatus: 1. stainless-steel needle, 20-gauge; 2. copper-encased stainless-steel U-tube, ⅛ in. O.D.; 3. thermocouple attachment; 4. Swagelok tee; 5. silicone septum fitting; 6. side-arm connection to 6-way valve; 7. soldering gun; 8. manual on-off switch.

TABLE I

Composition of Commercial Fat Products

	Hydrogenated-winterized vegetable oil	Hydrogenated soybean oil
Direct analysis		
Gas-liquid chromatographic analysis, %		
Palmitate	10.0	9.7
Stearate	3.0	4.5
Monoenoate	45.6	63.0
Dienoate	39.4	22.8
Trienoate	2.0	0.0
Iodine value	180.5	87.8
Conjugated diene, %	0.6	0.4
Alkali conjugable diene, %	34.5	13.9
Lipoxidase conjugable, %	34.3	12.6
Isolated *trans*, %	15.1	36.8
Azelaic acid on cleavage, mole %	71.7	45.3
Analysis after fractionation, %		
Unsaturation at carbon 9		
cis-Monoenoate	25.4	19.6
trans-Monoenoate	1.7	5.4
cis,cis-Dienoate	30.0	12.8
Mono-*trans*-dienoate	.....	3.1
Unsaturation of all other carbons		
cis-Monoenoate	9.1	13.3
trans-Monoenoate	9.5	24.7
cis,cis-Dienoate	5.0	3.9
Mono-*trans*-dienoate	.....	3.0

whether the output of data may not be greater than the ability to calculate, record, collate, and report it. The answer to this question, epitomized by the mounting piles of strip chart recorder tracings in the laboratories, lies not in less automation but in more. Specifically the answer lies in instrumentation for digitization of data and in computers for processing calculations and reporting. Results of one of the computerized data processing procedures (17) is shown in Fig. 10, which reproduces a printed computer tape. The system includes interface equipment for the integration of areas under the peaks of GLC curves and for the digitization of this information in a form acceptable for computer input. Finally the digital computer prepares the report, giving both the analysis and the identifications as shown.

In concluding this discussion on the determination of fat composition, it may once more be observed that "Every advance in scientific knowledge is first an advance in technique." On the basis of the new methodology it appears that the composition of the fat consumed can now be specified in terms of the positions and geometric configurations of unsaturation in its individual component fatty acids and in terms of the fatty acid structure of its glycerides.

GAS CHROMATOGRAPHIC ANALYSIS

RUN C-1965 DATE 11/4 ,1965 SAMPLE CODE SBO 31

PARTITION LIQUID LAC-2R446 COLUMN LENGTH 6.0 FEET

ISOTHERMAL RUN-COLUMN TEMPERATURE 210 DEGREES C.

FLOWRATE 38.61 ML./MIN.(STP) COLUMN CODE V-60-90

OPERATOR G SPENSER MAN HOURS 1.0 CHARGE NUMBER 980-0000

PEAK NUMBER	RELATIVE RETENTION VOLUME	CORRECTED AREA PERCENT	IDENTITY
1	1.000	9.80	C(16)-00-ME
2	1.598	2.70	C(18)-00-ME
3	1.790	23.52	C(18)-00-ME (1=)
4	1.950	55.75	C(18)-00-ME (2=)
5	2.315	8.23	C(18)-00-ME (3=)

RETENTION TIME OF PEAK NO. 1 IS 5.21 MINUTES
RETENTION TIME OF AIR IS .01 MINUTES

FIG. 10. Reproduction of output for computer program (17).

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Committee Revises *Lipids* Guide to Authors

A revised Guide to Authors will be published in the March-April issue of *Lipids*.

Released by F. W. Quackenbush, chairman of the committee on the Guide, the revision clarifies matters of editorial style and mechanical requirements. It will be of increased assistance to authors in the preparation of manuscripts.

Members of the committee who served with Dr. Quackenbush are R. M. Burton, R. T. Holman and J. F. Mead. Many other Society members contributed valuable suggestions for making the Guide more efficient.

Classic Contest Is a Winner At AOCS Fall Meeting

How many soybeans in the jar? EMI's contest had everybody guessing at the AOCS Chicago meeting. G. G. Wilson (right) came up with the best answer, however, and won the handsome TV set shown in the photo. The presentation was made by E. J. Loew (left) of EMI—Engineering Management, Inc., Park Ridge, Ill.