Quantitative Analysis of Fatty Acids by Gas-Liquid Chromatography

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SUMMARY The fatty acid methyl ester mixtures obtainable from the National Heart Institute, Bethesda, Md., U.S.A., as reference standards for gas-liquid chromatography (GLC) have been examined in a number of laboratories. The results of experience with the mixtures are summarized and detailed recommendations are given of their use in assessing the accuracy of GLC equipment and procedures. The effects of several features of instrument design and operating conditions are discussed, and the recognition and correction of commonly encountered defects are described.

In 1958 a proposal was made to the National Heart Institute to make available to scientists in the lipid field a series of reference mixtures of known composition and of high purity. This suggestion, prompted by the advent of gas-liquid chromatography and its impact on studies of lipid metabolism, led to the institution of a Program for Lipid Standards under the guidance of a program officer and aided by an advisory committee. This group has met on numerous occasions in order to define objectives for reference fatty acids, to plan the procural of reference compounds, and to compare the results of tests made in their own laboratories with the reference mixtures which were developed from their discussions. On the basis of this experience with several types of instrumentation, six test mixtures were prepared for distribution, and their availability has been announced on several occasions since 1961. The mixtures will be referred to in this Report as NHI Fatty Acid Standards, in recognition of the support of this program by the National Heart Institute.

This report has been prepared in the belief that the experience acquired by this committee in establishing quantitative procedures may be helpful to others in the lipid field. A considerable amount of background information on gas-liquid chromatography is included, which may lead to a better understanding of certain problems by scientists who are not directly concerned with the development of methodology, but who are finding these new methods to be useful. Since this report is based upon the opinion and experience of the committee members, it was considered justified to omit the usual bibliographic references.

GENERAL DISCUSSION OF GAS-LIQUID CHROMATOGRAPHY

Gas-liquid chromatography (GLC) is now widely used for the separation, identification and estimation of long-chain fatty acids and related substances. The basic principles are well recognized, and methods for qualitative work, involving the separation and identification of individual components of a mixture, are well established. There is, however, an element of doubt in interlaboratory comparisons with regard to precision and accuracy. This does not arise because of basic limitations of GLC, but rather because of variations in design and use of instruments and because of variations in ancillary techniques. Thus, there is a wide choice of detection systems, of liquid phases, of derivatives, and of conditions of

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analysis. Many commercially available instruments are now available, and homemade apparatus has become a rarity. Nevertheless, in most experienced laboratories the commercial instrumentation has been modified in order to achieve satisfactory results. These modifications are dictated by failures in quantitative analyses of reference mixtures similar to those discussed here.

The best way to establish optimal conditions for analysis with a given gas chromatographic instrument is to employ mixtures of known composition that contain the same substances as the mixtures under investigation. This procedure is often difficult in research applications because the structure of certain components may not be known; reference samples of high purity may not be available; the composition of a mixture may change greatly as a consequence of experimental manipulations; and instrument behavior may vary during a study. Perhaps the greatest limitation in arriving at a more precise evaluation of the quantitative aspects of gas chromatotography in lipid work has been the lack of pure standard compounds. The Fatty Acid Standards of the National Heart Institute have been designed to meet some of these needs.

QUALITATIVE RELATIONSHIPS AFFECTED BY INSTRUMENT DESIGN

Quantitative accuracy by GLC depends on the proper combination of columns (for achieving separations) and detectors (for measuring eluates), and it is essential that conditions be chosen such that neither element in the apparatus is overloaded. To achieve this, operators should familiarize themselves with the effects of overloaded columns and detectors and with the causes of each.

Column Effects. Polyester columns, usually coated with 10–15% of liquid phase, are widely used for the separation and quantification of fatty acid methyl esters, and generally provide satisfactory separations of many unsaturated and saturated methyl esters. Wide differences in the rate of "bleeding" of liquid phase from GLC columns are encountered, and depend upon the chemical structure of the liquid phase, the mode of preparation of the polymer, and the nature of treatment of the support. Many difficulties were encountered in early work with polyester columns, largely because catalysts used in producing the polymers were not removed: their presence led to increased column bleeding and rapid deterioration of the column. Partial or complete loss of methyl esters with long retention times was also encountered. Many of the most useful polyesters are now made without the use of catalysts, or appropriate procedures are used to remove them. Improved supports and improved phases (now commercially available) have led to better column performance and longer column life.

Nonpolar phases generally have higher thermal stabilities than polyester phases. Thick-film Apiezon columns have been widely used in work with fatty acid methyl esters; thin-film methyl silicone columns are also useful in some applications. The bleed rate of these phases is generally low. The chief limitation in the use of nonpolar phases for fatty acid methyl ester separations lies in the inability to separate compounds containing 2 and 3 double bonds in the C_{18} group, and 3 and 4 double bonds in the C_{20} group. Silicones are particularly likely to lead to deposits in argon and flame ionization detection units, and frequent cleaning of sensing units is necessary.

It is obvious that any destruction or alteration of fatty acid esters during GLC separation on the column will change the apparent composition of the mixture. Quantitative relationships may also be altered if the sensing unit is progressively affected by the column bleed. The best way to test for adequate qualitative and quantitative performance of the column is to use reference mixtures frequently. Some laboratories use a suitable reference mixture each day.

Detection Systems. The ability to obtain quantitative results with any detection system is dependent both on the intrinsic properties of the system and on the way it is used. For example, ionization detectors generally have a low capacity, and with a badly bleeding column the detection system may be overloaded even before the sample is applied. Thermal conductivity detection systems, on the other hand, respond only to much larger samples, so that a column that would not be satisfactory with an ionization system may well be suitable for use with a thermal conductivity detector.

The response of a thermal conductivity detector is based on the difference in thermal conductivity between a carrier gas and gas–solute mixture. Since these detectors are relatively insensitive, they are useful only for analyses of relatively large samples. The application of large samples, in turn, dictates the need for thick-film columns (10% or more of liquid phase) which have the capacity to cope with large sample loads; thin-film columns (5% or less of liquid phase) are overloaded by the sample sizes required for detection by thermal conductivity units.

The response of thermal conductivity detectors is dependent on the molar concentration and on the molecular weight of the solute. Experimental studies have shown that the area of each peak should be multiplied by the square root of the molecular weight of the component before calculating the per cent contribution of each compound in the mixture. It is possible that further work will result in the introduction of still more accurate calibra-

tion factors, but in general it must be remembered that peak areas do not directly represent molar or weight concentrations.

Ionization detection systems are based on the partial ionization of an organic substance contained in a gas stream. These detectors are characterized by very high sensitivity, and are easily overloaded by the bleed from unsatisfactory columns and by solutes which elute rapidly, especially when they are present in large concentrations. It should also be realized that the conductivity of the sensing unit will change when pyrolysis products from the liquid phase accumulate on electrode surfaces. This accumulation is responsible for many of the changes in behavior seen with ionization detection systems when they are used for long periods without adequate cleaning.

The two most widely used ionization detectors are the argon ionization detector and the hydrogen flame ionization detector. With an appropriate design of the sensing unit and the electronic components of the detection system, quantitative results may be obtained with very small samples. For fatty acid methyl esters, it has been verified in many laboratories that measurement of peak areas gives a direct weight per cent measurement for C_8 and higher fatty acid components. Further, it has been verified that there is no detectable difference in the mass response for long-chain unsaturated compounds containing 1, 2, 3, 4, 5, or 6 methylene-interrupted double bonds when compared with the corresponding saturated compounds.

Studies have been made of effects of change in design for argon and flame ionization sensing units and in the associated electronic components, since these may alter the load limit, the sensitivity, and the behavior with different classes of compounds. It is not possible to review these matters in detail, because so many varieties of instruments are in use. The only acceptable practice in each laboratory is to determine the characteristics of a given detection system with mixtures of known composition that resemble the mixtures under study as closely as possible.

Temperature Control. Satisfactory application of GLC to fatty acid analysis depends greatly on the precision with which temperatures are controlled around the column and detector, at the point of sample application, at the column head and also in the "bridge" between column and detector (when such connections are not direct). Ideally, the operating temperature is held the same throughout the column length (±1°), and the detector temperature should be a few degrees higher in order to minimize condensation of column "bleed" in the detector. On the other hand, there is a risk of fouling the detector with pyrolysis products if the detector temperature is too high. Flash heaters may be required at the column head to insure instantaneous volatilization of the sample injected, especially when the incoming carrier gas has not been sufficiently preheated. But, again, excessively high temperatures there may cause pyrolysis and inconsistent application of sample compounds. Finally, in instruments that employ a bridge between the column and the detector, care must be taken to ensure that neither condensation nor pyrolysis occurs in that bridge. It is common practice to hold the bridge temperature a few degrees higher than the adjacent components.

ANALYTICAL PROCEDURES

Choice of Derivative. Long-chain fatty acids are usually converted to methyl esters for GLC work. It would be equally possible to use other esters, but a large body of data based on methyl esters has been accumulated. Procedures for their preparation may be found in many text books and articles. The method selected must be one that does not lead to artifactual peaks. Diazomethane requires particular care in this respect; other unpleasant features are its toxicity and tendency toward explosive decomposition if concentrated.

Short-chain fatty acids are markedly overestimated in argon ionization detectors, when analyzed as methyl esters. To increase their molecular weight into a range where reliable quantification is possible, esterification with β-chloroethanol has recently been described; this procedure permits the use of ordinary polyester columns for separation and quantification of C_3–C_8 fatty acids.

It is also possible to separate long-chain fatty acids up to about C_{20} in the form of the free acids by adding phosphoric acid to the stationary phase. However, this method is not widely used in biochemical and biological work because mixtures of compounds longer than C_{20} are frequently encountered, and quantitative aspects have not yet been completely defined. Furthermore, a continual bleed of phosphoric acid can lead to detector corrosion. When it is necessary to carry out an analysis of compounds ranging from about C_{10}–C_{24} (or higher) in the same sample, methyl esters are almost always employed.

Choice of Sample Size. The sample size is usually dictated by the load limit of a given detector, since this is usually much less than the load limit of columns used in fatty acid analyses. With a thermal conductivity detector, it is generally best to use samples in the range 100–500 μg or higher, depending on the composition of the sample. Ionization detection systems have greater sensitivity, and samples of approximately 1–50 μg are used, depending on the composition of the mixture, the type of detector and the operating conditions; individual components may vary from 0.05–5 μg. When the detector system is operated at highest sensitivity, noise levels may

be high and calculation of areas may be difficult. The present practical limit with flame ionization detectors is about 0.01 μg for an individual component. Column efficiency is generally at a maximum when very small samples are used, and the high sensitivity of ionization detectors also permits the use of thin-film columns with very small sample loads.

Fatty acid mixtures derived from mammalian triglycerides, phospholipids, cholesterol esters, and wax esters usually contain a number of major components of comparable magnitude. When minor components are being determined, it is common to apply a large sample to the column and to attenuate the signals given by major components. This is satisfactory provided (*a*) the signal attenuator is known to be accurate, and (*b*) the detector responds to both large and small components in a linear fashion.

Column overloading sufficient to cause distortion of the peak shape rarely occurs when packings with 10–15% liquid phase are used, unless the sample is very large. Detector overloading, on the other hand, leads to incorrect and highly erratic results in peak area relationships. The sample size should always be chosen in such a way that the maximum concentration of each component is within the linear range of the system. For best results, volumes of solution and amounts of sample should be kept approximately the same throughout a series of analytical determinations.

Introduction of Sample. Two methods are commonly used for the introduction of samples of fatty acid methyl esters to GLC columns. Most instruments provide for introduction of the sample by syringe without interruption of the gas flow. A few instruments, on the other hand, have a sample introduction system designed for use with glass micropipettes, in which case the gas flow is interrupted (even this can be avoided by use of a magnetic dropping device). When syringe loading is used, it is usually necessary to use a solution of the mixture under study. With argon or flame ionization detection systems the usual practice is to inject from 0.1 to 2 μl of 0.1 to 2% solutions. Hexane, isooctane, acetone, and tetrahydrofuran are among the solvents in general use. Hexane and isooctane are often preferred because they are good solvents for fatty acid methyl esters, and because they are easily obtained in satisfactory purity. (Solvents should always be examined by GLC in order to make certain that interfering substances are not present.) When thermal conductivity detection systems are used, the sample may be injected in 10–50% solution or without solvent, depending upon the desired sample size and the viscosity of the mixture.

It is important that the entire sample be introduced into the chromatographic system. Under the usual circumstances of syringe loading the sample volume will include the volume of the needle as well as that of the syringe. A small volume of pure solvent may be drawn into the syringe before the sample; when the sample is ejected, this portion of solvent serves to wash out the needle volume. Serious errors can result when samples are not introduced completely and in a standardized reproducible manner.

Introduction of the sample without solvent by micropipette is satisfactory for fatty acid methyl esters, although the introduction of high-melting solids and very volatile compounds presents special problems. The pipette should be introduced with as short an interruption of the gas flow as possible. It has been found helpful to use a small amount of wadded glass thread coated with liquid phase on top of the chromatographic column; the wadded thread should be located 1 or 2 in. below the head of the heating jacket. With this arrangement the sample is introduced directly into the liquid phase, and premature volatilization of the sample is prevented. With practice, good reproducibility may be obtained by this method, and samples as small as 15 μg may be introduced.

Operating Conditions. The temperature for a given gas chromatographic determination must be selected carefully, so as to permit precise measurement of early (narrow) and late (broad) peaks. Even though temperature programming produces peaks of the same width, approximately, and thus makes easier the task of area calculation, most laboratories still prefer to make two isothermal runs at different temperatures and to calculate the final results from a single component which is well defined in each run.

Flow rate is adjusted to provide maximum column efficiency, as judged by calculation of theoretical plates. Flow rates with packed columns are generally in the range of 30–150 ml/min.

Chart speed is usually neglected as a significant experimental variable. However, this assumption is correct only within certain limits. Most recorders operate with chart speeds of 20–30 in. per hr. If chart speeds are changed at any time during a single run or in successive runs, the appropriate calibration studies should be made. Although it is possible to widen the early peaks by using fast chart speeds, shifts in chart speeds during a run may prove to be confusing, or to cause inaccuracies. The width of the pen line also may be significant in measuring peak widths. The performance of each recorder should be checked with standard mixtures to determine whether the inside, center, or outside of the pen line should be used in area calculations.

Mode of Measurement. Percentage composition calculations are carried out commonly; for this it is necessary to obtain an area measurement associated with each component. Many different procedures have been

TABLE 1 COMPOSITION OF NHI STANDARD FATTY ACID METHYL ESTER MIXTURES IN WEIGHT PER CENT

Acid	Mixture					
	A	B	C	D	E	F
8:0			1.5		6.3	
10:0			3.0		9.1	
12:0			6.0		12.1	
14:0	25.0	4.0	12.0	11.8	23.3	2.5
16:0	10.0	40.0	19.4	23.6	49.2	4.2
16:1				6.9		
18:0	65.0	56.0	24.9	13.1		7.3
18:1				44.6		
20:0			33.2			13.6
22:0						25.4
24:0						47.0

proposed for area calculations. Methods in use include (*a*) multiplication of peak height by width at half-height, (*b*) multiplication of peak height by one-half base width, (*c*) multiplication of peak height by adjusted retention time, (*d*) measurement of peak area by planimetry, (*e*) measurement of peak area by weighing the cut-out peak, and (*f*) measurement of area by integrators. When the conditions of the separation are well chosen, all of these methods of measurement produce essentially the same results, and the choice of method is then made on the basis of ease and experience.

Method (*a*) is preferred in most of the committee's laboratories. However, it must be recognized that serious errors may be introduced in measuring the widths of very narrow or very broad peaks. Use of a magnifying lens with inscribed scale permits measurements to an accuracy of 0.1 mm.

Triangulation by methods which involve the drawing of intercepts (*b*) is generally satisfactory in experienced hands. This method is most susceptible to error in measuring broad peaks.

The multiplication of peak height by adjusted retention time (*c*) may be satisfactory when only a few peaks with approximately the same retention times are involved. This procedure should be used only when it can be justified experimentally. It is not usually used for fatty acid methyl esters.

Planimetry (*d*) is often considered the "reference method" of measurement. Current experience indicates that this is not justified; measurements made with planimeters have no greater precision than those made by other acceptable procedures. The cut-out procedure (*e*) is a satisfactory method of measurement under many circumstances, although the precision is often less than that obtained by other methods.

It should be kept in mind that, in calculating percentage composition, an error in measuring any one peak will result in altered values for all other components. An internal standard must be used if it is desired to base the measurement on a weight or volume of original sample.

NHI FATTY ACID STANDARDS

Preparation of Standard Mixtures. Various individual fatty acid methyl esters were prepared from natural sources by established procedures, and finally by fractional distillation. Their purity (99.9+%) was established by measurement of the usual physical and chemical constants and by thin-layer and gas-liquid chromatography. The 6 mixtures were made in large lots (100 g or more) by weighing in the pure esters in order of increasing volatility. The thoroughly mixed preparations were syringe-pipetted in 100 to 200 mg batches into cooled glass vials, and these were sealed. Mixture D was sealed in brown glass vials under nitrogen and held at 4°. The composition of the 6 available standard mixtures is shown in Table 1.

Four Test Procedures. Since commercially available instruments are not supplied with guarantees of quantitative behavior, each investigator is obligated to determine conditions for quantitative work for the specific problem under study. The required tests define four parameters: (1) the load limits of column and detector, separately; (2) the linear range of the instrument, through which the system responds in direct proportion to the load of a given solute in the moving gas phase; (3) the response of an instrument to homologs of widely differing molecular weight and double-bond structure; and (4) the separating efficiency of a given column. Fatty acid mixtures A and B are designed for the first two test procedures; C through F for the third test; and D for the fourth. It must be emphasized that the response of a given instrument to fatty acid methyl esters may differ markedly from that produced by other classes of compounds. Finally, the effect of changes in the operating conditions (including changes in gas flow rate and in applied voltage for ionization detection systems) should be established.

Mixtures A and B (Table 1) may be used to establish *appropriate conditions* for operation of the column and detection system. The observed peaks should be symmetrical Gaussian curves and not show sloping front and sharp back (due to column overload) or tailing (due to active site adsorption, inadequate sizing of the inert support, or unsatisfactory packing of the column).

The first test measures the *load limit*. Increasingly large samples of A or B are run, and the ratio of large to small components is calculated (14:0/16:0 in A, 16:0/14:0 in B). As long as the load limit of the detector is not exceeded, the ratio should be constant. When the concentration of a solute in the gas stream exceeds the detector load limit, the response is less than that re-

quired by theory. The microgram load at which non-linearity is detected indicates the detector load limit. Tests with A and B should show non-linearity at about the same load; nevertheless, the detector overload point for any given compound is dependent on many variables, and it must be determined for each instrument, column, and condition of separation. Under some circumstances argon ionization detectors will give higher than normal responses under overload conditions. This usually indicates a poor choice of linearizing resistor or use of excessively high voltage. The overload limit should be determined under several different conditions of detector sensitivity (different applied potentials in ionization instruments, and various temperature differentials in thermal conductivity instruments).

A second test of the detection system should be that of establishing the relative *load-response relationships* for saturated fatty acid methyl esters. This is a straightforward operation with mixtures A and B. Samples of different size should be run, including values near the overload limit, throughout the region projected for use in analytical work, and with samples near the lower limit of detection. A curve which relates found and calculated relationships will indicate the nature of the detector response: the per cent values observed for each component should be constant through the entire range up to the overload limit. If values found with very small samples are not satisfactory, the possibility of loss of material on the column should be considered.

Mixture C is used to establish *linearity with respect to molecular weight*—a particularly important test. If there is an apparent loss of the 20:0 component, this may be due to partial loss on the column or to improper sampling, rather than to an inadequate detection system. Incorrect values for late-appearing components are commonly due to column defects or to improper sample administration when syringe injection is used. Mixtures E and F subdivide and extend the usefulness of Mixture C. They provide means of testing analytical methods with short- and long-chain esters.

Mixture D may be used to study *column efficiency*. Both polar and nonpolar columns should separate 18:1 from 18:0 cleanly. If these separations are not satisfactory, greater attention must be paid to the preparation of the column. Mixture D also is used to determine the nature of the detector response to unsaturated fatty acid

TABLE 2 ROUTINE ANALYSES OF MIXTURE C IN 13 LABORATORIES

	8:0	10:0	12:0	14:0	16:0	18:0	20:0
True Wt. %...	1.3	3.1	6.2	12.3	20.0	27.0	30.1
Lab A	1.6 (+23)	3.4 (+10)	7.4 (+19)	14.0 (+14)	21.8 (+9)	27.0 (0)	24.9 (−21)
B	1.0 (−23)	3.1 (0)	6.7 (+8)	13.9 (+13)	22.0 (+10)	27.1 (+0.5)	26.2 (−13)
C	1.1 (−15)	4.0 (+29)	8.1 (+31)	15.8 (+28)	24.1 (+20)	28.1 (+4)	18.8 (−38)
D	1.3 (0)	3.9 (+26)	6.4 (+3)	13.6 (+11)	22.0 (+10)	27.0 (0)	25.7 (−15)
E	1.1 (−15)	3.8 (+23)	7.2 (+16)	13.4 (+9)	20.7 (+3)	26.6 (−1.5)	27.2 (−10)
F	1.4 (+8)	3.7 (+19)	6.9 (+11)	13.8 (+12)	20.0 (0)	26.7 (−1)	27.5 (−9)
G	1.2 (−8)	3.4 (+10)	6.8 (+10)	13.2 (+7)	21.5 (+8)	27.0 (0)	26.9 (−11)
H	1.9 (+46)	2.8 (−10)	6.1 (−2)	12.4 (+1)	20.1 (+0.5)	27.7 (+2.5)	29.0 (−4)
I	1.4 (+8)	2.9 (−6)	6.1 (−2)	12.1 (−2)	20.2 (+1)	27.3 (+1)	29.6 (−2)
J	1.6 (+23)	3.7 (+19)	7.9 (+27)	14.8 (+20)	18.7 (−6)	29.2 (+8)	25.4 (−16)
K	1.3 (0)	3.2 (+3)	7.8 (+26)	13.2 (+7)	19.5 (−2.5)	25.5 (−6)	29.0 (−4)
L	1.2 (−8)	3.1 (0)	6.9 (+11)	12.3 (0)	18.9 (−6)	26.8 (−1)	31.0 (+3)
M	0.6 (−53)	3.0 (−3)	5.6 (−10)	12.2 (−1)	20.2 (+1)	28.7 (+6)	29.7 (−1)

The parenthetical figure is the relative error of each value.

Detection systems employed were as follows: Argon ionization, 8 (radium source, 3; strontium-90 source, 2; tritium source, 3): Laboratories A-H, inclusive. Thermal conductivity, 4: Laboratories I-L, inclusive. Flame ionization, 1: Laboratory M.

TABLE 3 ANALYSES OF STANDARD MIXTURE E

Acid	True Wt. %	Laboratory 1*	2†	2‡	3§	4‖
8:0	6.3	5.8	4.3	4.0	6.4	6.3
10:0	9.1	8.7	8.4	8.3	9.4	9.4
12:0	12.1	11.7	12.4	12.8	12.4	11.8
14:0	23.3	23.4	24.5	24.4	23.7	23.8
16:0	49.2	50.5	50.4	50.6	48.1	48.9

* Thermal conductivity detector; polar phase.
† Argon ionization detector; nonpolar phase.
‡ Argon ionization detector; polar phase.
§ Argon ionization detector; polar phase.
‖ Gas density balance; nonpolar phase.

esters. The response obtained with ionization detection systems should be the same on a weight basis as that observed for saturated esters; if a thermal conductivity detector is used, a factor based on molecular weight should be used to correct percentage values obtained from area measurements. The mixture contains monoenes in low and in high concentrations in order to afford means of detecting difficulties of quantitative determination of these peaks adjacent to their saturated homologs.

Precision and Accuracy. A number of runs should be made to estimate the precision of the determination under the conditions to be used in analytical work. It is helpful to use mixture D for this purpose, since analysis of this mixture produces large and small peaks, but mixtures A and B also can serve the same purpose. It is general practice to make five to ten runs with any one mixture and to compare the values obtained in terms of range of deviation or standard error for each component. Although it is difficult to give an exact estimate, it is usually possible to obtain values having a reproducibility of $\pm 1\%$ by carefully controlling every step in the determination.

Interlaboratory Comparisons. Estimates of accuracy and precision are helpful in interlaboratory comparisons and in comparisons of instruments, separation and estimation procedures, and detection system behavior. Composition values from 1 to 5% should have a maximum relative error of about 10%, and composition values above 5% a maximum relative error of about 5%. This level of accuracy is not attained in many laboratories. This is illustrated by the results presented in Table 2. Mixture C was subjected to routine analysis in thirteen different laboratories, each of which had had more than 1 year of experience in gas chromatographic analytical work with fatty acids. The wide range in reported composition values shows the variation in accuracy encountered in practice. Table 2 demonstrates the necessity of the use of well characterized standards, critical evaluation of results and improvement of experimental conditions.

TABLE 4 ANALYSES OF STANDARD MIXTURE F

Acid	True Wt. %	Laboratory 1*	2†	3‡	5§
14:0	2.5	2.3	2.5	2.9	2.7
16:0	4.2	4.2	4.6	4.6	4.5
18:0	7.3	7.5	8.0	7.6	7.6
20:0	13.6	13.6	14.4	14.0	13.7
22:0	25.4	25.9	25.9	25.1	25.2
24:0	46.6	46.5	44.7	45.8	46.3
25:0	0.4				

* Thermal conductivity detector; polar phase.
† Argon ionization detector; polar phase.
‡ Argon ionization detector; polar phase.
§ Argon ionization detector; polar phase.

Tables 3 and 4 contain results obtained with test mixtures E and F (supplied as unknowns), one containing fatty acid methyl esters up to 16:0 (Table 3) and one containing esters up to 24:0 (Table 4). These results are from laboratories of the authors and from one laboratory abroad.

The data in Table 2 show that the finding of low values for the component of highest molecular weight is an effect that is frequently seen with commercially available instruments containing argon ionization detection systems. The data in Tables 3 and 4 show that satisfactory analytical results may, however, be obtained for components from C_8 to C_{24} with both ionization and thermal conductivity systems. The very large differences in accuracy which are evident in Tables 2, 3, and 4 suggest that greater attention must be given to the validation of gas chromatographic procedures, and that this can be done when satisfactory standards are used.

The handling of the sample is particularly important when studies of accuracy are in progress. For example, if low molecular weight components are present, and if the sample is not handled with due care even before introduction into the column, early components will be lost through volatilization. If compounds of very high molecular weight are present, there may be difficulties in obtaining a representative sample and in complete volatilization of the sample. When unsaturated compounds are present, partial oxidation may occur on exposure to air at room temperature. (The selective loss of unsaturated compounds is usually due to oxidation.) When non-linearity of any type is detected, it is advisable to begin again with fresh standards and to recalibrate the instrument with a view to determining the nature of the difficulty.

Primary and Secondary Standards. Many laboratories prefer to use samples of more complex composition than mixtures A through F as analytical standards, since it is desirable in most work to use a mixture very close in composition to the samples under analytical examination. In these instances it is common practice to use a secondary standard derived from a triglyceride or other

material of natural origin. The National Heart Institute standards are used to validate the secondary standard used in day-to-day work.

Presentation of GLC Data in the Literature. Editors of scientific journals are justifiably interested in standardizing methods of communicating GLC methods and data. There are at least two types of reports based on GLC methodology, and for each a certain form of presentation seems appropriate.

Studies of GLC theory, inert supports, liquid phases, column performance and retention data demand a complete listing of operating conditions: instrument model, detector type, column dimensions, detail of column packing, temperatures of flash heater, column and detector, and gas flow.

Reports confined to the application of GLC in physiological or biochemical experiments and in which results obtained by GLC are presented require a less detailed description: the type of apparatus, column and detection system should be given, together with references to the literature on basic procedures.

However, in both types of reports and particularly when quantitative results are presented, some demonstration of reliability and accuracy of the GLC procedures employed must be stated. It would be meaningful and concise if authors included a statement such as the following: "Quantitative results with National Heart Institute Fatty Acid Standards [insert letter codes of mixtures used] agreed with the stated composition data with a relative error less than ···% for major components (>10% of total mixture) and less than ···% for minor components (<10% of total mixture)." Such a statement can substitute for more detailed descriptions of linearity testing.

Procurement of Standards. Reference samples and reference mixtures containing saturated and unsaturated fatty acid methyl esters may be obtained gratis by qualified investigators from the Program Officer, Lipid Standards Program, National Heart Institute, National Institutes of Health, Bethesda, Maryland, as described in *J. Lipid Res.* **2:** 428, 1961.

QUALITATIVE ANALYSIS

A strong word of caution must be voiced in regard to the practice of identifying specific fatty acids through GLC procedures *alone*. At its worst, a single GLC run may indicate what certain peaks cannot be. But even at its best (analyses on two or more stationary phases and under various operating conditions) analysis by GLC can indicate only tentatively what structures can be assigned to individual peaks. Branched-chain fatty acids may prove particularly confusing, as may other classes of compounds (hydrocarbons, alcohols, acetals, etc.) and artifacts produced during the preparation of samples for analysis.

EXTENSION TO OTHER PROBLEMS

Two associated problems are currently under study. In view of the widespread use of radioactive tracers, standards for radiopurity must be developed at the same time as standards for mass purity. While several continuous counting methods have been described in the literature, these are not in general use. Meanwile, many radioactive fatty acids available from commercial sources have not been subjected to adequate analytical control. *Users of radioactive materials are strongly cautioned to test these materials by thin-layer and gas-liquid chromatography for radioactive and mass impurities.*

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