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Identification of Peaks in Gas-Liquid Chromatography

T. K. MIWA, Northern Regional Research Laboratory,[1] Peoria, Illinois

Abstract

Some of the known principles of gas-liquid chromatography are reviewed. Application of the equivalent chain length (ECL) method to identification of complex molecules, and to prediction of structures of unknowns, is described in detail.

Introduction

THE TASK of identifying peaks in a gas chromatogram can at times be very difficult, especially if the peaks are ill-defined humps on another peak. However, no matter how difficult a task, there is always a way to solve the problem. As applications of gas chromatography rapidly increase, so do the means of identification.

In this paper only the fundamental techniques of component identification and some auxiliary detection and characterization procedures will be reviewed briefly. Emphasis will be laid on the technique used in identifying fatty acid methyl esters and fatty alcohols.

All the various identification methods may be put into three categories. The first is based on retention data alone. The second involves an auxiliary detection system other than the conventional gas chromatographic detectors. The third requires fractionation and isolation of components as they emerge from the gas chromatograph and subsequent identification of the components by chemical and physical means.

Discussion

Identification by Retention Data

The simplest method of identification is to compare retention time of an unknown peak in a chromatogram with that of a standard peak in another chromatogram. Experimental conditions must be identical and the chromatograms should be preferably from consecutive runs. Whenever possible, the unknown and standard materials should be mixed and their peaks shown to superimpose exactly. Two or more stationary phases should be used; e.g., a nonpolar Apiezon L grease and a polar LAC-2R 446 (Resoflex 446) polyester resin.

When carrier gas flow rate is the only variable in experimental conditions, retention volumes may be compared. Retention volume is the product of retention time and flow rate. When both flow rate and column dimensions differ and when retention volumes are not comparable, relative retentions may be compared. The relative retention of a component is the ratio of the retention time of the component to that of a chosen internal standard present in each chromatogram. Relative retention varies appreciably with change in column temperature.

A linear relationship is observed when the logarithm of the retention values is plotted against the number of carbon atoms for each homologous series of organic compounds. The slope of the curve decreases with an increase in column temperature. James (5) has described a graphic method for determining the degree of unsaturation of fatty acids by plotting the logarithm of relative retention in the nonpolar liquid phase against that in the polyester phase. Each homologous series forms a straight line which is parallel to that of the saturated series.

In 1958 and 1959, E. Kovats (9,22) reported the use of normal paraffins as standards and expressed all organic compounds by retention indices, which were determined by the following equation:

$$\text{Retention Index (I)} = 200 \log r[Y : nP_z]/\log r[nP_{(z+2)} : nP_z] + 100z.$$

The logarithm of the ratio of retention times of unknown Y to a *n*-paraffin with z carbon atoms is divided by the logarithm of the ratio of retention times of a second standard, which is a *n*-paraffin with 2 carbon atoms more than z, to the first standard nP_z. Since this quantity is a comparison of the fractional amount that Y deviates from nP_z in relation to the amount a 2-carbon increase deviates from nP_z, the quantity is multiplied by 2 to show its equivalency to 2 methylene groups. To avoid having 2 decimal places the quantity is further multiplied 100 times. This quantity is then added to the retention index of the *n*-paraffin z, which has been assigned a value 100 times z. The retention indices are either determined at column temperature 130C or adjusted from other temperatures to 130C by extrapolation.

By determining retention indices in both polar and apolar (nonpolar) stationary phases and by subtracting one from the other, a value called "increment" is determined for each compound. Although the increments vary somewhat within a given homologous se-

[1] A laboratory of the No. Utiliz. Res. & Dev. Div., ARS, U.S.D.A.

ries, representative values of the homologous series are:

"Increments" $I_{130} = I^{P}_{130} - I^{a}_{130}$

n-Alkanes	0
1-Alkenes	45
Cycloalkanes	45
Benzene (phenyl)	170
1-Chloroalkanes	130
1-Aldehydes	220
1-Methyl carboxylates	220
2-Ketones	230
1-Nitriles	345
1-Alcohols	370

n-Alkanes are the standards and have no increments. Terminal unsaturation has the same effect as a cyclic structure. The change from cyclohexane to benzene entails an increase in polarity roughly equivalent to 1¼ carbon atoms. Chloroalkanes have relatively small increments because the retention indices in the polar and apolar phases do not differ appreciably even though the indices are greater than those of aldehydes and ketones, which have larger increments. Aldehydes, ketones, and methyl esters have about the same increments while nitriles and alcohols have the largest. These increments characterize the functional groups and greatly facilitate the identification of unknown peaks.

Kovats (9) says of his technique: "For the characterization of organic substances in gas chromatography, a number termed Retention Index is proposed. The advantages of the retention index are: a) its dependence on temperature is small and linear; b) it is independent of the column constants and of the type of chromatographic apparatus used; c) it provides information about the chemical nature of substances under examination."

In our laboratory a graphic method is used for determining these retention indices. Unaware of Kovats' work, the term Equivalent Chain Length was used to denote retention characteristics of the compounds (15). Equivalent chain lengths (ECL) can also be determined from an equation similar to that given by Kovats.

$$ECL = (S_2 - S_1) \log (t_x/t_{s1}) / \log (t_{s2}/t_{s1}) + S_1$$

S_1 and S_2 are ECL of the standards; t_x, t_{s1}, and t_{s2} are retention times of the unknown and two standards. Ordinarily, normal saturated fatty acid methyl esters are the references, but normal saturated fatty alcohols have also been used. The values are 100-fold less than retention indices and the standards need not be exactly 2 carbons apart as long as their ECL are known. ECL have the advantage over retention indices in being recognized readily as representing the number of carbon atoms or the equivalent in a compound.

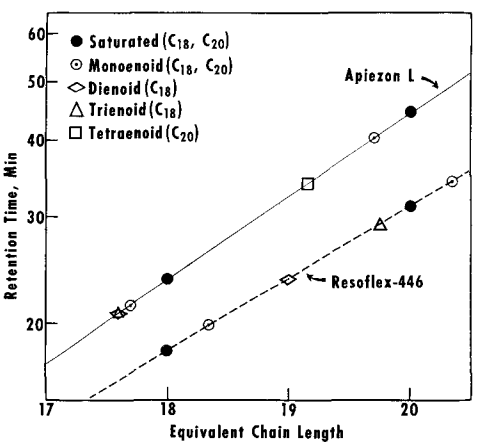

FIG. 1. Logarithm of retention times of purified fatty acid methyl esters plotted against equivalent chain length. Stearic and arachidic methyl esters used as references.

The graphic method is being used almost exclusively, since it requires but a fraction of the time necessary to calculate ECL from the equation. The graph paper is a 30-in. × 40-in., 3-cycle, semilog paper that reads accurately to $\frac{1}{100}$ of an ECL. The following explanations cover how ECL are obtained graphically, how they characterize a homologous series or a functional group, and how they can be used to identify an unknown component.

If a mixture of known fatty acid methyl esters is selected, as in Figure 1, the retention times of the chosen references stearic and arachidic are plotted to coincide exactly with ECL 18.00 and 20.00. A straight line is drawn through the 2 points, then the retention times of the other components are spotted on the curve, and the ECL read along the abscissa. For example, on the upper (Apiezon L) curve oleic reads 17.7, gadoleic 19.7, linoleic and linolenic 17.6, and arachidonic 19.2. On the lower (Resoflex 446) curve these same components read 18.4, 20.4, 19.0, 19.8, and 21.6, respectively. ECL are accurate only in regions of the curve where a linear relationship exists between the logarithm of retention time and number of carbon atoms in the homologous series. The ECL of a saturated straight-chain acid having *n* carbon atoms will inherently be *n*.0 in all liquid phases; e.g., stearic acid is 18.0 in both Apiezon L and Resoflex 446. The common *cis* monoenoic acids deviate from their saturated counterpart in identical fashion; i.e., 0.3 ECL shorter in Apiezon L and 0.4 longer in Resoflex 446. The positional isomers, petroselinic and oleic, were not separable.

Most of the monoenoic acids that were found in seed oils during our new crops screening investigation (23) behaved similarly to the common *cis* monoenoic acids. Table I lists a few with their source, percentage of total fatty acids isolated from oil, and ECL. Again it was not possible to separate positional isomers, such as *cis*-5- and *cis*-13-docosenoic acids. The C_{20} and C_{22} dienes in mustard seed oil behaved similarly to linoleic acid: namely, 0.4 ECL shorter in Apiezon L and 1.0 longer in Resoflex 446 when compared with the saturated acids. The dienes in *Limnanthes douglasii* are shorter than those in mustard by 0.2 in Apiezon L and 0.4 in Resoflex 446. The C_{22} diene in *L. douglasii* is not methylene-interrupted but is hexamethylene-interrupted with *cis* unsaturation at 5 and 13 (1). The conjugated C_{18} diene, which is believed to be the *trans,trans*-9,11 isomer, is longer than linoleic acid by 1.1 ECL in Apiezon L and 1.6 in Resoflex 446. This difference is attributed to the *trans,trans* conjugation.

The all-*cis*-6,9,12-C_{18} trienoic acid is shorter than linolenic acid by 0.2 ECL in Apiezon L and 0.3 in Resoflex 446. Possibly a shift of polyunsaturation from the methyl end toward the carboxyl end decreases the retention characteristic. Isomerization of *cis,trans,trans*-conjugated triene to *trans,trans,trans*-conjugated triene increases the ECL by 0.3 in both columns. The all-*trans*, doubly conjugated β-eleostearic acid has an ECL greater than linolenic acid by 2.1 ECL in Apiezon L and 3.0 in Resoflex 446. These are double the values that were attributed to the single *trans,trans* conjugation (cf. preceding paragraph).

The 12-hydroxyoleic, or ricinoleic, acid in castor oil has ECL of 19.4 (Apiezon L) and 24.7 (Resoflex 446). During analysis of seed oils from some *Lesquerella* species, 3 unknown components were found, one of

which had ECL identical to ricinoleic acid and the others exactly 2.0 longer or shorter than ricinoleic in both Apiezon L and Resoflex columns. Immediately it was postulated that these were homologs of ricinoleic acid. It has since been proved that the major component acid in *Lesquerella lasiocarpa* is (+)-14-hydroxy-*cis*-11-eicosenoic acid, a true homolog of ricinoleic acid (19). This example is but one of several predictions of the identity of unknown components by the ECL method.

The contribution of the 12-hydroxy group is calculated to be +1.7 (Apiezon L) and +6.3 (Resoflex 446) ECL, which are the differences between ricinoleic and oleic. The deviations of 9,10-dihydroxystearic from stearic are +3.3 and +11.8, approximately double the values calculated for the 12-hydroxy compound. The deviations of dimorphecolic from stearic are in good agreement with the sum of the deviations for a single hydroxy and a conjugated *trans,trans*-diene; Apiezon L, 2.3 vs. 2.4, Resoflex 446, 9.2 vs. 8.9.

Vernolic, or epoxyoleic, acid differs from epoxystearic acid in the same manner as oleic does from stearic. Although the position of the epoxy group differs slightly in the two examples, it is possible to assign to the epoxy an ECL of 1.5 or 1.6 in Apiezon L and 5.0 in Resoflex 446. By comparison with β-eleostearic acid, the 4-oxo substituent in licanic acid has been assigned an ECL of 1.3 in Apiezon L and 1.8 in Resoflex 446.

The acetylenic acids in Table I were not separable. When compared with oleic and petroselinic acids, the ECL have increased by 0.2 in Apiezon L and 1.4 in Resoflex 446. The latter is identical to that of linolenic acid. This similarity may indicate that one triple bond is capable of imparting to the saturated acid the same increased polarity as three methylene-interrupted *cis* double bonds. The C_{19} cyclopropenoic sterculic acid has ECL exactly 1.0 greater than linoleic acid in both Apiezon L and Resoflex 446. The cyclopropene structure can therefore be said to be equivalent to a methylene-interrupted *cis*-diene. This similarity in retention characteristic presents the problem of separating the C_{18} cyclopropenic malvalic acid from linoleic acid. Since it was not possible to separate the two acids by gas-liquid chromatography (GLC), an indirect approach was taken to determine malvalic acid qualitatively and quantitatively.

The composition of the total fatty acids is determined by GLC before and after hydrogenation (18). Linoleic acid is converted to stearic and malvalic to dihydromalvalic or 8- or 9-methyl heptadecanoic acids. Conditions are chosen to avoid formation of stearic from dihydromalvalic acid. *Sterculia foetida* oil may be used as the model since the absence of nonadecanoic acid after hydrogenation would be indicative of optimal conditions.

In the presence of sterculic acid, the accuracy of the percentage of malvalic acid may decrease. When using a polyester column, which gives the better separation of dihydromalvalic from stearic, dihydromalvalic is superimposed with the 9- or 10-methyl stearic resulting from hydrogenation of sterculic. Furthermore, any dihydromalvalic present before hydrogenation is obscured by oleic acid. However these difficulties may possibly be surmounted by isolating the oleic and linoleic fractions from preparative columns and rechromatographing the hydrogenation products. Table II lists ECL of functional groups of the compounds discussed above.

TABLE II

Equivalent Chain Lengths of Functional Groups Involved in the Determination of Cyclopropenoic Fatty Acids

Functional group	Liquid phase	
	Apiezon L	Resoflex 446
cis-Δ9 in oleic	−0.3	+0.4
cis,cis-Δ9,12 in linoleic	−0.4	+1.0
Cyclopropene in malvalic	−0.4	+1.0
Cyclopropene in sterculic	−0.4	+1.0
Cyclopropene hydrogenated	−0.2	+0.4
9 or 10 Methyl branching	−0.7	−0.8
4-Keto in licanic	+1.3	+1.8
9-Keto in 9-ketostearic	+1.4	+5.7
8,10-Diketo in oxidized malvalic	+2.6	.....
9,11-Diketo in oxidized sterculic	+2.6	.....

TABLE I

Equivalent Chain Lengths (ECL) of Monocarboxylic Methyl Esters of Fatty Acids Occurring in Plant Seed Oils (15)

Parent acid (common name)	Source	Percentage in extracted oil	ECL	
			Apiezon L	Resoflex 446
Unsaturated straight-chain acids:				
Monoenoic:				
cis-5-Eicosenoic	*Limnanthes douglasii*	65	19.7	20.4
cis-5-Docosenoic	*Limnanthes douglasii*	7	21.7	22.4
cis-13-Docosenoic (erucic)	*Limnanthes douglasii*	13	21.7	22.4
Eicosenoic	Rapeseed	8	19.7	20.4
Docosenoic	Rapeseed	43	21.7	22.4
Tetracosenoic	Rapeseed	1	23.7	24.4
Dienoic:				
Eicosadienoic	Mustard	1	19.6	21.0
Docosadienoic	Mustard	5	21.6	23.0
Eicosadienoic	*Limnanthes douglasii*	0.3	19.4	20.6
Docosadienoic	*Limnanthes douglasii*	10	21.4	22.6
Conjugated octadecadienoic	*Chilopsis linearis*	11	18.7	20.6
Trienoic:				
cis-trans,trans,9,11,13-Octadecatrienoic α-eleostearic)	Tung	79	19.4	22.5
trans,trans,trans,9,11,13-Octadecatrienoic (β-eleostearic)	Tung isomerized	....	19.7	22.8
cis,cis,cis-6,9,12-Octadecatrienoic	*Oenothera biennis*	10	17.4	19.5
Hydroxy acids:				
12-Hydroxy-*cis*-9-octadecenoic (ricinoleic)	Castor	83	19.4	24.7
Hydroxyhexadecenoic	*Lesquerella lasiocarpa*	Trace	17.4	22.7
Hydroxyoctadecenoic	*Lesquerella lasiocarpa*	3	19.4	24.7
Hydroxyeicosenoic	*Lesquerella lasiocarpa*	45	21.4	26.7
9,10-Dihydroxyoctadecanoic	Oxidation of *cis*-9-octadecenoic	....	21.3	29.8
9-Hydroxy-*trans,trans*-10,12-octadecadienoic (dimorphecolic)	*Dimorphotheca aurantiaca*	65	20.3	27.2
Epoxy acids:				
12,13-Epoxy-*cis*-9-octadecenoic	*Vernonia anthelmintica*	74	19.2	23.4
9,10-Epoxyoctadecanoic	Epoxidation of *cis*-9-octadecenoic (natural occurrence reported)	....	19.6	23.0
Keto acid:				
4-Keto-9,11,13-octadecatrienoic (licanic)	*Oiticica*	32	21.0	24.6
Acetylenic acids:				
9-Octadecynoic (stearolic)	Dehydrobromination of 9,10-dibromostearic	....	17.9	19.8
6-Octadecynoic (tariric)	*Picramnia pentandra*	85	17.9	19.8
Cyclopropenoic acid:				
8-(2-*n*-octylcycloprop-1-enyl) octanoic (sterculic)	*Sterculia foetida*	35	18.6	20.0

TABLE III
Equivalent Chain Lengths of Purified Dicarboxylic Methyl Esters (15)

Parent acid (common name)	Liquid phase	
	Apiezon L	Resoflex 446
Pentanedioic (glutaric)	7.6	12.4
Hexanedioic (adipic)	8.6	13.4
Nonanedioic (azelaic)	11.7	16.4
Undecanedioic	13.7	18.4
Tridecanedioic (brassylic)	15.7	20.3

Oxidation of the cyclopropenoic acids yields among others the β-diketones (18). Identification of 8,10-dioxooctadecanoic and 9,11-dioxononadecanoic acids was facilitated by prior knowledge of ECL of keto groups, such as 4-oxo in licanic and 9-oxo in 9-ketostearic acids. In the Apiezon L column, the ECL of the diketo functional groups was double that of a single keto group (Table II). The ECL in Resoflex 446 for the 4-oxo in licanic acid is much lower than expected. Possibly the effect of the 4-oxo is diminished by the conjugated trienoic and carboxylic functional groups being predominant.

Table III lists some of the dicarboxylic acids among the degradation products from oxidative cleavage of unsaturated fatty acids. Conversion of the ω-methyl to carbomethoxy has increased the ECL about 2.7 in Apiezon L and 7.4 in Resoflex 446.

Fatty alcohols have been analyzed by using both saturated straight-chain methyl esters and alcohols as references (16). When methyl ester references are used, the alcohols are 0.20 ECL shorter than the methyl esters in Apiezon L over a range of C_8 to C_{18} (temperature of column, 250C). In Resoflex 446, when the logarithm of retention time is plotted against ECL, the straight lines obtained for the methyl esters and alcohols are not parallel. Therefore, with methyl ester references, caprylyl alcohol is ECL 9.26 while stearyl alcohol is 19.48 (temperature of column, 212C).

Several others have published techniques similar to the graphic ECL method. Examples are the "carbon number" technique by Woodford and van Gent (24) and the "theoretical nonane" technique by Smith (20). One drawback is noted in these techniques. The retention indices have been obtained graphically, not from a curve drawn through the reference points, but from a "best straight line." Values obtained for components adjacent to a reference point, which does not fall on the best straight line, therefore will not be accurate. In regions where linearity of logarithm of retention times vs. number of carbon atoms does not hold, these techniques may lead to appreciable errors, whereas Kovats' retention index still maintains a relatively high degree of accuracy. Although it is possible to maintain linear relationships over a wide range, commonly, curves deviate on either side of the straight line at the region of low retention times. In such cases, a "best smooth curve" drawn through every reference

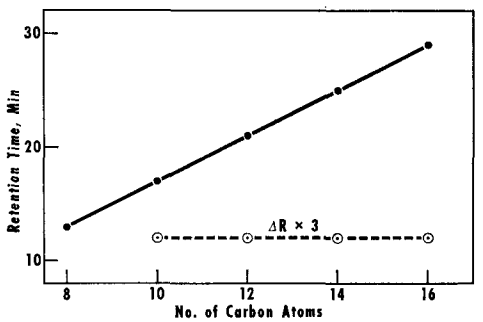

FIG. 2. Ideal linear temperature programming.

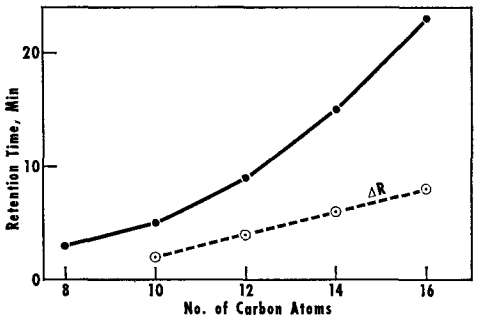

FIG. 3. Linear temperature programming with high initial temperature and low rate of temperature increase.

point would be preferable to a best straight line. In the theoretical nonane method, there is no constancy in any R_{x_9} unless determined at or extrapolated to a chosen temperature. Recently Evans, et al. (4) reported graphic aids that facilitate identification of homologous series that show parallel or nonparallel linear relationships when the logarithm of retention time is plotted against carbon number.

In *linear programmed temperature gas chromatography,* a linear relationship between retention time and number of carbon atoms can be obtained only when the time intervals between peaks in the homologous series remain constant. This relationship is shown in Figure 2. Retention time and retention temperature are interconvertible when programming is linear. △R, the time interval between peaks, was multiplied by 3 to put it within range of the plot. A constant △R represents ideality but this state is seldom attained. Nevertheless, when deviation from ideality is not large and temperature programming is linear, tentative identification of homologous components can be made graphically. For example, when the initial temperature is high and the programming rate is low, the plot is curved upward as in Figure 3. Evidence of the homologous nature is seen in the linear increase in △R. When the initial temperature is low and the programming rate is high, the plot curves toward horizontal, as in Figure 4. The △R in such a case will show a linear decrease.

Identification by Auxiliary Detection Systems

The second of three categories of identification techniques involves auxiliary detection systems in addition to conventional gas chromatographic detectors. For example, the effluent gas can be split or passed in series through a detector and a mass spectrometer (3,11). The latter identifies the components according to its mass. A spectrogram is either flashed on an oscilloscope or permanently recorded as a photograph. This technique is especially desirable when two or more components are present in the same peak. The spec-

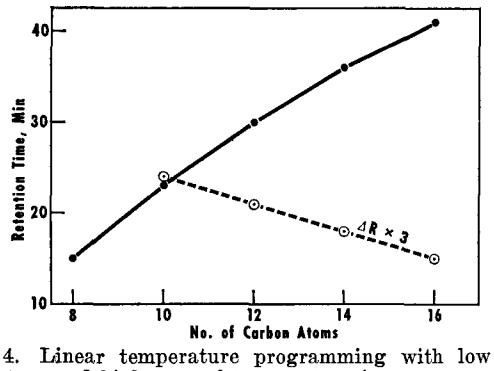

FIG. 4. Linear temperature programming with low initial temperature and high rate of temperature increase.

trometer also provides some quantitative information. An ionization chamber-electrometer system can be used in series to detect C^{14} or tritium-labelled components (2,7); ultraviolet or infrared spectrometers can monitor effluents at given wavelengths (6,8), the far-ultraviolet region being most sensitive.

An electron capture detector (12,13), also known as an electron absorption detector, can be used along with a conventional ionization detector to pick out components with high electron absorptivity. At ionization potentials of 15–20 v only the highly halogenated compounds, nitro-compounds, quinones, conjugated carbonyls, and other highly conjugated systems show any change in the ionization current. Ordinary fatty acids and methyl esters are completely unaffected. The method has its origin with electron affinity spectroscopy first reported by Lovelock and Lipsky (14). Expensive instrumentation is not always necessary. The chemical nature of each eluted component can be determined by passing the outlet stream into a number of tubes containing functional group reagents (21).

Identification by Analysis of Isolated Components

The third category is the isolation of fractions and their subsequent analysis by chemical and physical means. In such cases the gas chromatograph is often used as a means for separating mixtures. Nevertheless, collected fractions can be chemically modified and reinjected into the gas chromatograph, thereby establishing the chemical nature of the fraction. For example, an unknown component can be collected and selectively and quantitatively hydrogenated (17) and reinjected to establish the degree of unsaturation and the backbone structure of the unknown. Controlled degradation (10) of the same collected fraction followed by reinjection of the products may elucidate the position of unsaturation.

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Assay of Insecticides and Herbicides in Fats and Oils

J. W. COOK, Division of Food, Food and Drug Administration, U. S. Department of Health, Education, and Welfare, Washington, D. C.

Abstract

Types of residue and methods data required in the establishment of pesticide tolerances in food products is discussed. Emphasis will be on those products and methods involving fats and oils.

A summary of some of the current research efforts in FDA in methodology for pesticidal residues in food products is given. These methods are in the nature of screening or sorting techniques which identify and determine a number of different chemicals in a single analysis. Adequate separation of these chemicals from the fats, oils, and waxes of food products is often the most difficult step in methods development.

Introduction

MANY of the insecticides, herbicides, and other pesticides are present in food products in very low concentration. They must be purified and concentrated before an analysis can be made. Fats, oils, and waxes contribute a good deal to the complexity of our problems of analysis for pesticide residues, because the extraction procedures used to remove many of these chemicals from plant and animal products, also extract the fats, oils, waxes, and similar products. This extraction step is usually simpler to perform than the second step, which is to separate the minute quantities of pesticide chemicals from the relatively large amount of fats, oils, or waxes. Almost invariably these substances interfere with the final or determinative step in the analysis for the pesticide. Therefore, the problems of assay of fats and oils are much the same as those encountered in the assay of foods generally.

The general subject of pesticide chemicals has not been a major item of discussion at AOCS meetings; this paper is in general rather than specific terms, and is divided into two parts. First, is a discussion of some general considerations which relate ultimately to assay or methods. Secondly, some developments in the methods field are described. The discussion on analysis is limited mainly to work in the laboratories of FDA.

Pesticide chemicals include many substances other than insecticides and herbicides. The discussion is not restricted to the topic title as analytical problems of one often pertain to the other, and the physicochemical procedures used does not discriminate between the various biological effects of chemicals. The term "pesticide chemical" is defined by the Federal Insecticide, Fungicide, and Rodenticide Act. Included are those chemicals which control insects, weeds, rodents, nematodes, fungi, spiders, and those which act as plant growth regulators, defoliants, desiccants, etc. Recently, (March 27, 1962), this was further expanded to include, as pests, mammals, birds, fishes, snakes, invertebrates, roots, and viruses. We have established tolerances for about 125 chemicals and there are 200–