Research Methods of Analysis of Drying Oils

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"EVERY scientific advance is first an advance in technique" is a postulate that finds continuous verification in the experience of research workers. A corollary may be formulated, though less elegantly, that "if one applies the same old methods and the same old approaches to the same old problems, one will most probably come up with the same old results." While new methods of analysis do not insure that new knowledge will result, research workers generally affirm this thesis, at least as a *modus operandi*, that one way to new information is through new analytical approaches.

Analysis involves at least three elements in its conduct a) separation of mixtures into individual chemical species, b) qualitative identification of the separated compounds, and c) quantitative assay of the individual components in relation to the whole. In practice, one, two, or three of these elements may be present in a single analytical operation, but all three are eventually taken into account before completion of the final analytical determination.

In the following sections certain research methods of analysis for drying oils will be discussed under the headings of fractionation and characterization insofar as it is practicable to separate these elements. Examples of application will be discussed. The choice of methods for inclusion is admittedly arbitrary, and the examples selected largely came from personal experience. It is hoped that this discussion will serve to introduce certain research methods of analysis, give an impression as to how the procedures are carried out, and illustrate how new fundamental knowledge arises from the application of new research techniques.

Fractionation

Countercurrent Distribution. As described at a Short Course in 1955 (1), this is a technique of separating solutes based on their differential solubility between immiscible solvents. It is a refinement of the familiar laboratory liquid-liquid extraction operation that is frequently carried out in separatory funnels but is amplified in the number of stages, *e.g.*, 20 to 1,000, and is provided with an ingenious systematic method of moving upper layer solvents from one stage to the next. In larger models of this apparatus, complete automation of the shaking, settling, decantation, and transfer operations is achieved as well as solvent introduction and sample collection.

This technique is particularly well adapted to the fractionation of lipids and unstable derivatives because of the mildness of conditions and relatively large sample-size handled. Applications to the separation of free fatty acids; methyl esters; mono-, di-, and triglycerides; autoxidation products of lipids; phospholipids, and fat-soluble pigments have been described (1).

The countercurrent distribution of glyceride components of linseed oil is illustrated in Figure 1. The

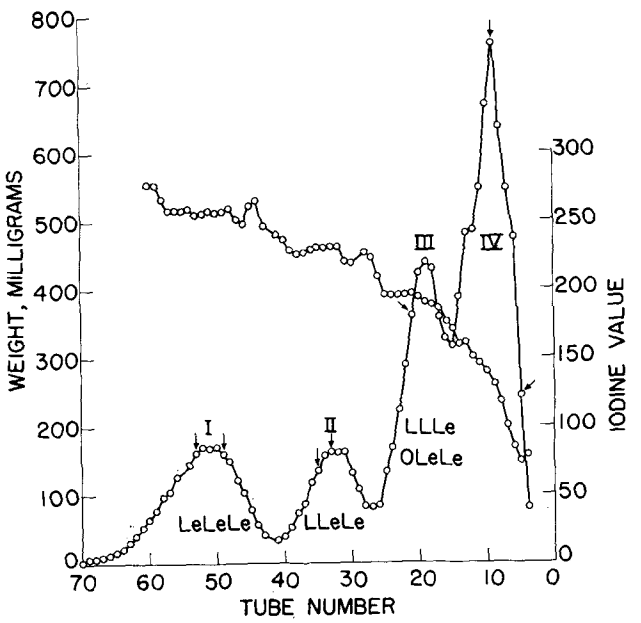

FIG. 1. Countercurrent fractionation of linseed oil glycerides with a pentane-hexane-furfural solvent system. Open circle, weight; closed circles, iodine value.

first time that the separation of trilinolenin and linoleo-dilinolenin was achieved (2) resulted from the application of this technique. Based on this type of data, the fatty acids of linseed oil were shown to be arranged on the glycerol molecule according to a "random" pattern. By virtue of the high-resolving power of countercurrent distribution, data on the glyceride composition of linseed oil were obtained, again for the first time, which were otherwise unavailable.

Potentialities of countercurrent distribution for the preparation of methyl esters of fatty acids have been illustrated recently (3). Natural methyl linoleate has been prepared in 500-g. amounts without recourse to the bond-isomerizing conditions of bromination and debromination reactions previously employed. Tetraene and pentaene fatty acid esters are also amenable to preparation by this technique. Furthermore fundamental data on solvent systems and partition coefficients of individual fatty acid esters should permit the design of larger laboratory liquid-liquid extraction equipment or even commercial-scale units.

Liquid-Partition Chromatography. This (1) bears striking similarity in theory and practice to the countercurrent distribution technique. If the lower layer of solvent from the countercurrent distribution were held as an immobile phase on an inert support, *e.g.*, silicic acid and diatomaceous earth, in a glass column and if the upper layer of solvent from the countercurrent distribution were poured on the top of the column as a mobile phase, the analogy to countercurrent distribution would be nearly exact. In fact, the performance of liquid-partition columns is described

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as a series of theoretical plates or stages not greatly dissimilar to the transfer stages of countercurrent distribution. Both result in solutes being eluted in Gaussian or bell-shaped curves. Reversed-phase liquid-partition chromatograms are formed by holding the nonpolar phase as a mobile solvent. Dichlorosilane-treated Celite has the property of retaining nonpolar phases as does also rubber for reversed-phase chromatography.

Application of the liquid-partition, chromatographic technique to the separation of fatty acids (4) is illustrated in Figure 2. The mildness of

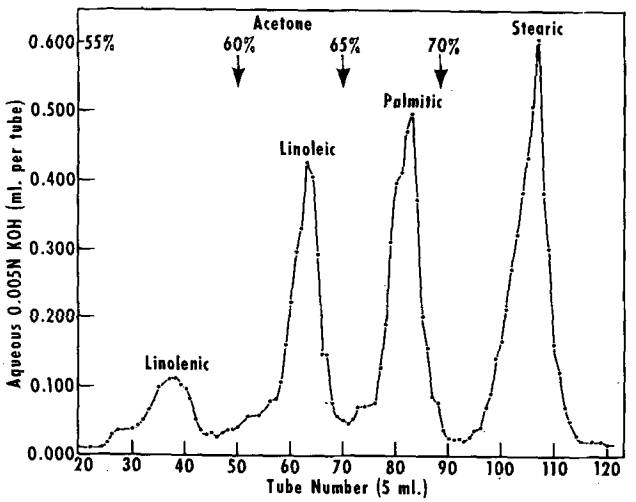

FIG. 2. Resolution by liquid-partition chromatography of linolenic, linoleic, palmitic, and stearic acids.

fractionating conditions and its applicability to high-molecular-weight, nonvolatile lipids recommend liquid-partition processes of chromatography and of countercurrent distribution to the chemist working with fats and oil.

Paper Chromatography. Another form of liquid-partition chromatography, this has the advantages of simple operation and of minimal equipment requirement. Primarily it gives qualitative separation and information. The principal problems in application are those involved in selecting suitable spraying reagents and in detecting chromatographic spots. Schlenk (5) has described the use of an α-cyclodextrin reagent which does not give the usual iodine color in the presence of fatty acid. Mercury salts form association complexes with double bonds of fatty acid esters, and these complexes formed prior to chromatography (6) have been separated on paper. Mercury color reagents provide good spray reagents for the detection of these separated ester complexes.

Chromatographic processes involving adsorption phenomena, while of historical interest, are now less attractive because their inherent nonlinear isotherms result in excessive tailing, nonGaussian-shaped curves, and incomplete separations.

Certain generalizations may be made concerning fractionation procedures described thus far and the range of partition coefficients of solutes which may be handled by countercurrent distribution, liquid partition chromatography, and reversed phase chromatography. Using a 200-tube countercurrent distribution apparatus, solutes having partition coefficients between 0.01 and 100 may be fractionated. Solutes with coefficients smaller than 0.01 are amenable to liquid partition chromatography and those with coefficients larger than 100 to reversed phase chromatography.

Gas Chromatography. In contrast to the liquid-liquid phase processes, this is a gas-liquid phase phenomenon. Fatty acid esters are partitioned between the nonvolatile liquid agent held on the inert support as the immobile phase, and the gas or mobile phase. The advantage over liquid-partition chromatography is primarily the ease with which solutes in the gas phase are detected by physical methods, *e.g.*, thermal conductivity argon ionization, radio frequency, and flame photometry.

At its present widely-used stage of development, gas chromatography gives information different from the widely-used spectro-iodine value methods for polyunsaturated fatty acid analysis. Whereas gas chromatography appears to give separation for the C_{18} isologous series of fatty acids with regard to the total number of double bonds and without regard to the position of the bonds, spectro-iodine value methods require the bonds to be methylene-interrupted and thus capable of being conjugated under conditions of alkali isomerization. In certain "natural" fats and in hydrogenated fats, position isomers exist. Gas chromatography and spectro-iodine value methods may be expected to and do give differing results on these materials and assist in understanding their structure.

Capillary columns of 100–200 ft. in length have demonstrated up to 200,000 theoretical plates (7). Analysis of fatty acids for differences involving *cis* and *trans* configuration of double bonds and their location in the carbon chain may be feasible in the near future with this technique.

An example of the application of gas chromatography combined with radioisotopic tracers is given in Figure 5 and will be discussed later.

Urea Inclusion Complexes. These offer some surprising opportunities for fractionation of fatty acids. The separation of saturates from unsaturated acids is readily achieved as is also the branch chain from normal chain and normal-chain acids from hydroxy acids. However the separation of unsaturated acids of the isologous series is only partially complete and worsens as the degree of unsaturation increases. While practical separations can be made between stearic, oleic, and linoleic acids, the urea complexes of linoleic and linolenic acids co-crystallize and approach a composition of 85% linolenic acid (8).

Perhaps the most exciting application of urea complexes has yet to be made in the separation of *cis* and *trans* isomers. Wheeler and coworkers (9) have indicated nearly complete separation of *cis*, *trans* and *trans,trans* conjugated linoleic acids from the alkali isomerization of linoleic acid. This separation, based on the difference in geometric configuration of one of two double bonds in otherwise similar C_{18} acids, has been confirmed by results from our laboratory.

The technique of urea complex formation used in conjunction with other methods of fat analysis may provide a powerful tool for a study of reactions in which both shifts in position and configuration of double bonds occur. Although classical crystallization procedures and perhaps inclusion complexes may be in disrepute because of complications of co-crystalli-

zation, the specificity of crystallization forces is large and has frequent and valuable application.

Characterization

Techniques of fractionation frequently include elements of qualitative identification and assay and are apparent in various forms of chromatography. The curves provided by the detector-recorder of mass *vs.* time in the case of gas chromatography and titration *vs.* volume for partition chromatography and weight *vs.* transfer number for countercurrent distribution serve to provide data for the calculation of those constants which are based on the position of elution of peaks, *e.g.*, retention time, R_f, and the peak tube. These constants are used for qualitative identification of substances. Quantitative information as to the contents of the individual components are also calculated from these same data by integration of the areas under specific peaks.

Techniques for the characterization of pure components also may be applied with advantage independently of the fractionation operations. Among the physical procedures which have found wide usefulness in research as independent methods of characterization or which have promise of future important application are four forms of spectroscopy: ultraviolet, infrared, mass, and nuclear magnetic resonance. After a discussion of these techniques a chemical procedure to locate the positions of double bonds will be illustrated; and an example will be given from recent research, in which many analytical methods, including isotopic tracers, were employed and compared.

Ultraviolet Absorption Spectra. These (1) arise from displacements of electrons in the outer shell of atoms, the electrons involved in chemical reactions. Ultraviolet spectroscopy is therefore referred to as electronic absorption (10) spectroscopy. Functional groups of fatty materials, carbonyl, carboxyls, acetylenic, and ethylene-double bonds have their characteristic spectra either alone or in conjugation with other functional groups. Unfortunately all the groups cited, except carbonyls by themselves and unconjugated, have their absorption maxima in the vacuum spectrograph region or at wavelengths shorter than 2200 Å where quartz optics of the widely available spectrometers begin to become opaque. However unsaturation of fats has been assayed in the region of ethylene bond absorption *ca.* 2100 Å, and it has been correlated with unsaturation of the oil (11). Absorption bands of carboxyl groups have their maximum at 2040 Å and extend up to 2300 Å. A correction must be made for their absorption as described in the A.O.C.S. standard methods of analysis for polyunsaturated fatty acids.

More practical for fat analysis and characterization is the absorption of two or more functional groups in conjugated positions. While non-conjugated ketonic and ethylenic groups absorb individually at 1850 Å, an ethylene double bond conjugated with a carbonyl absorbs at 2250 Å, a 400 Å shift. Fatty acids with conjugated dienes absorb at approximately 2300 Å. A conjugated trienoic fatty acid has a principal absorption maximum at *ca.* 2700 Å, and conjugated tetraenes and pentaenes absorb at increasingly longer wavelongths, 3150 Å and 3460 Å, respectively. Occurrence of absorption peaks at these positions in the wavelength spectrum is presumptive evidence that these respective functional groups occur in the molecule.

Knowledge of absorption positions of known structures permits certain extrapolations and predictions to be made about unknown compounds. In the conjugated polyene series, electrons appear to oscillate and travel the length of the polyene chain. Their movement may be described by Hooke's Law, and the wavelength squared (λ^2) of the maximum is directly related to the number of conjugated double bonds (12). This theory is helpful in the identification and characterization of conjugated dienes, trienes, tetraenes, and pentaenes encountered in paint oils from vegetable and fish oil sources as they may occur either before or after alkali conjugation.

Infrared Spectra. These (1) have their origin primarily in the vibrational movements of atoms within molecules. While spectra of simple molecules may be computed from fundamental constants, much of the interpretation of infrared spectra of complex molecules, such as fatty acids, is empirical in nature (13). In fatty acid esters, carbonyls have important absorption bands at 5.7–6.0 μ, hydroxyls at 2.9 μ, *cis,trans* dienes at 10.18 and 10.52 μ, C-H stretching at 3.4 μ, and C-H bending at 6.9 μ. Figure 3 gives the infrared-absorption spectrum of methyl linoleate hydroperoxide to illustrate these absorptions.

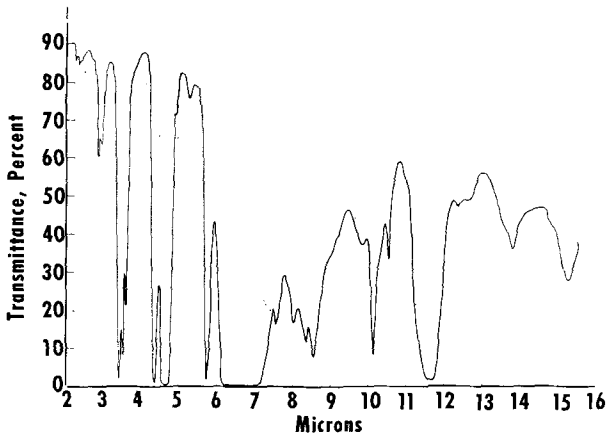

FIG. 3. Infrared absorption spectrum of methyl linoleate hydroperoxide.

Recent interest has been centered in the exploration of that portion of the near infrared which may be studied with quartz optics. Of particular interest to studies of drying oils are the absorptions assigned to the *cis* unsaturation at 2.15 and 2.19 μ and of hydroperoxide groups at 1.46 and 2.07 μ (14).

Quantitative infrared assays for various functional groups may be worked out as in the ultraviolet region but with somewhat greater difficulty because solvents which are transparent to all wavelengths of infrared are lacking and because the generally lower extinction coefficients in the infrared require higher concentrations and give rise to complications, such as hydrogen bonding, between functional groups. Procedures have been developed for the analysis of hydroxyls, hydroperoxides, isolated *cis* bonds, isolated *trans* bonds, conjugated *cis,trans* bonds, and conjugated *trans,trans* bonds. The possibility of performing a complete analysis of fatty acids for total unsaturation and for geometric configurations by spectro-

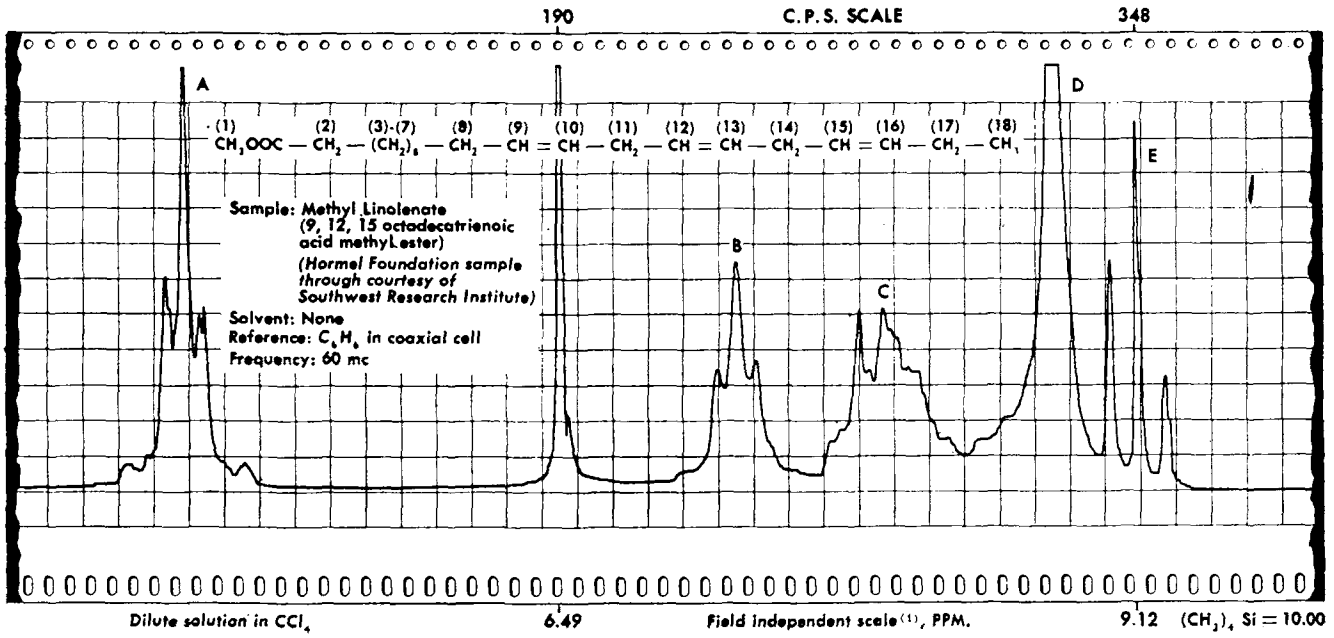

FIG. 4. Nuclear magnetic resonance spectrum of methyl linolenate. (A) Arises from 6 protons on doubly bonded carbon atoms, (B) represents 4 protons on α-methyl carbon atoms 11 and 14, and (C) represents those on 7, 8, and 17. Line D arises from insulated CH_2 groups and E from the terminal methyl groups.

scopic procedures appears to be approaching reality in the near future.

Mass Spectra. These arise from the ionization of molecules and their subsequent dispersion in electromagnetic and electrostatic fields. Molecular-weight information thus obtained is of the utmost importance in the characterization of fatty acids. For example, mass spectrometry has an unique application in conjunction with gas chromatography (15). Gas chromatography provides the fractionating tool of high resolution and a method of quantitative assay whereas mass spectrometry, operating continuously on a portion of the effluent from the chromatographic column, gives simultaneous qualitative identification data. For this purpose, rapid registration of mass spectrometric data, as with an oscillograph, is desirable. Instruments with resolution of 1 mass unit in 400 are available and will characterize the parent peaks of the isologous series of C_{18} fatty acid (16).

Unfortunately, or fortunately perhaps, parent molecules of large size break up and recombine under conditions of mass spectrometry. These "cracked products" differ for different parent molecules and in the kind and amount. They do provide a "fingerprint" for characterization of the parent molecule. Isologous unsaturated C_{18} fatty acids give homologous series of lower-molecular-weight fragments, but these do not correspond to the expected rupture at the 9,10 bond position because of a complex cracking and recombining reaction not yet understood (17).

Nuclear Magnetic Resonance Spectroscopy. This has been applied to fatty acid esters by Hopkins *et al.* (18) and, in a preliminary manner, by W. J. Storey of the Southwest Research Institute and J. N. Shoolery and Roy Johnson of Varian Associates (19). Data of these workers and their assignment of bonds in methyl linolenate are given in Figure 4. The possibilities of developing new analytical methods based on nuclear magnetic resonance measurements are now being explored.

Location of Double Bonds. This is a problem of wide interest and importance in the characterization of fatty acids. Much of the experimental data upon which our present knowledge of fatty acid structure is built was based upon the isolation of fragments of fatty acids after oxidative cleavage. In retrospect it is remarkable that with only fractional recovery of the cleavage mono- and dibasic acids that accurate structures were deduced.

In a recent research project quantitative yields and a strict stoichiometric accounting for all material are required to arrive at a correct conclusion (20). It had generally been assumed that substitution of tritium for hydrogen occurs in the labelling of organic compounds by the Wilzbach gas-exposure procedure (21). This premise was found to be true for saturated fatty acids, lauric, myristic, palmitic, and stearic acids all successfully labelled (22). However in the labelling of unsaturated fatty acids addition, or "hydrogenation," occurred, not substitution. Methyl oleate irradiated for 18 days with a 5-curie tritium source was found to give anomalous results both on gas chromatography and on oxidative cleavage (22). By

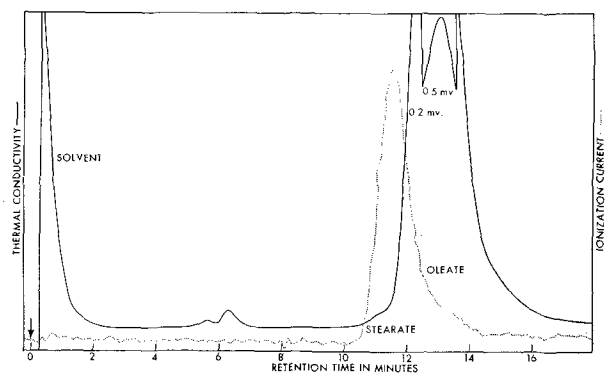

FIG. 5. Gas chromatogram of tritiated methyl oleate. Solid line, thermal conductivity; broken line, ion current.

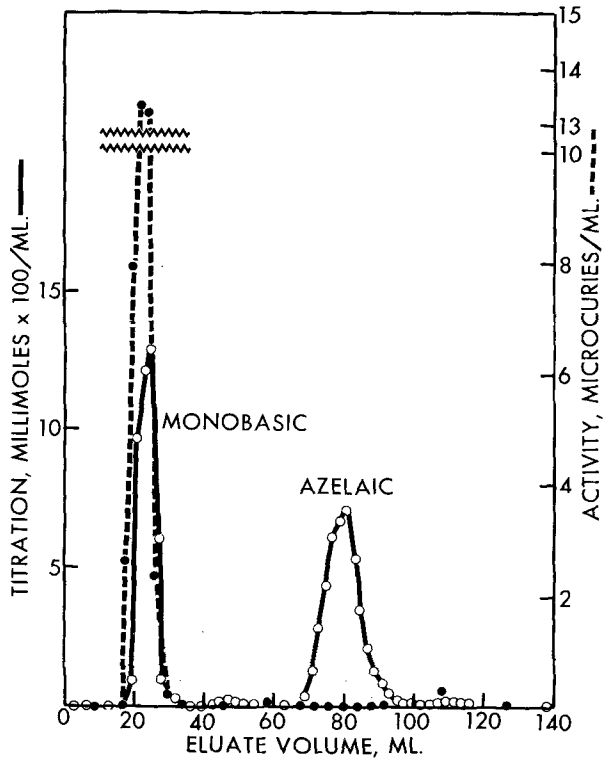

FIG. 6. Partition chromatogram of mono- and dibasic acids from oxidation mixture. Solid line, titrimetric data; broken line, radioactivity.

placing a continuous-flow ion chamber in the exit stream from the gas-chromatographic equipment, a radioactive trace was obtained simultaneously with the thermal conductivity recording (Figure 5) (24). Displacement of mass and radioactivity peaks constituted an anomalous and unexpected behavior compared to the saturated acids.

After mixing labelled "methyl oleate" with inactive oleate and oxidizing with permanganate-periodate, 95% of the theoretical increase in acidity was observed. In the liquid-partition chromatographic separations shown in Figure 6, 94% of the theoretical azelaic and 93% of the pelargonic acids were recovered. Further chromatography of the monobasic acids (Figure 7) demonstrated that the radioactivity associated with this monobasic was exclusively radio-chemical stearic acid formed by the tritiation, or hydrogenation, of oleic acid. This result explained the apparently anomalous behavior of mass- and radio-chemical curves on gas chromatography (Figure 5). Confidence in this conclusion is founded upon the quantitative reaction and recovery steps of oxidative cleavage and chromatographic separation used for establishing the positions of the double bonds.

Application of Several Analytical Techniques. Comparison of the results concludes this discussion of analytical methods. In the course of studying the

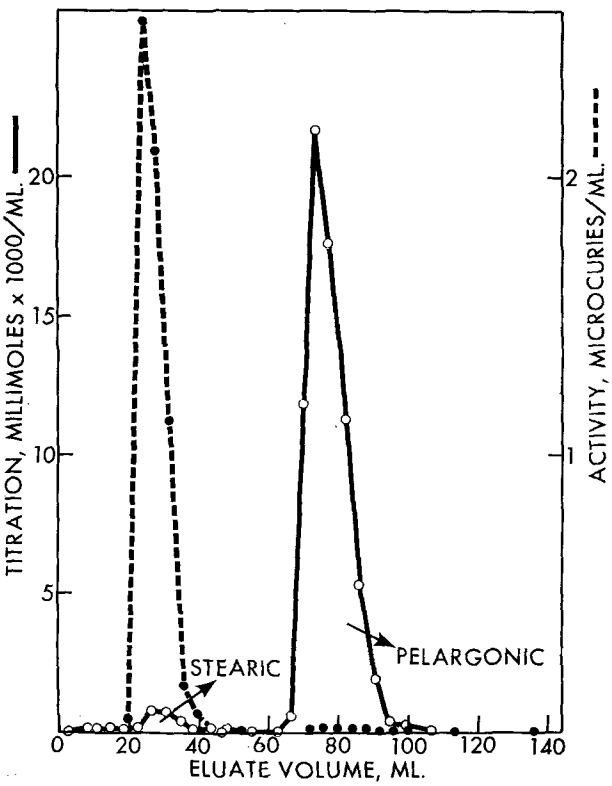

FIG. 7. Partition chromatography of monobasic acids' fraction of Figure 6. Solid line, titrimetric data; broken line, radioactivity.

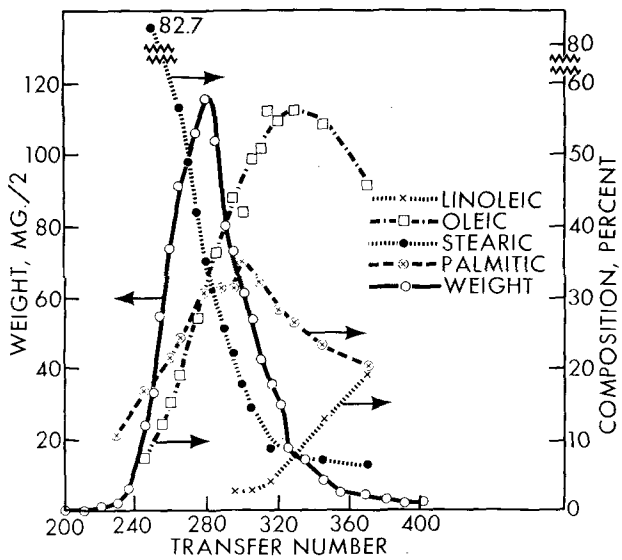

FIG. 8. Countercurrent distribution of cocoa butter glycerides with a pentane-hexane, furfural, nitroethanol solvent system.

glyceride structure of cocoa butter (25) it was desirable to compare the specific structure of natural cocoa butter with that of randomized cocoa butter. An analogous problem in drying oil research would be a comparison of the glyceride structures of a mixture of tung and soybean oils before and after interesterification. To cocoa butter glycerides, being interesterified to a random structure by the addition of sodium methoxide catalyst, were introduced microcurie amounts of tritium-labelled palmitic acid and stearic acid 1-C^{14} as methyl esters, plus an equivalent amount of glycerol. Under conditions of vacuum and 100°C. these labelled esters were interesterified into the glycerides of randomized "cocoa butter." On countercurrent distribution of these glycerides the weight curve shown in Figure 8 was obtained.

Analytical data on fatty acid composition of fractions were obtained by a) spectro-iodine value

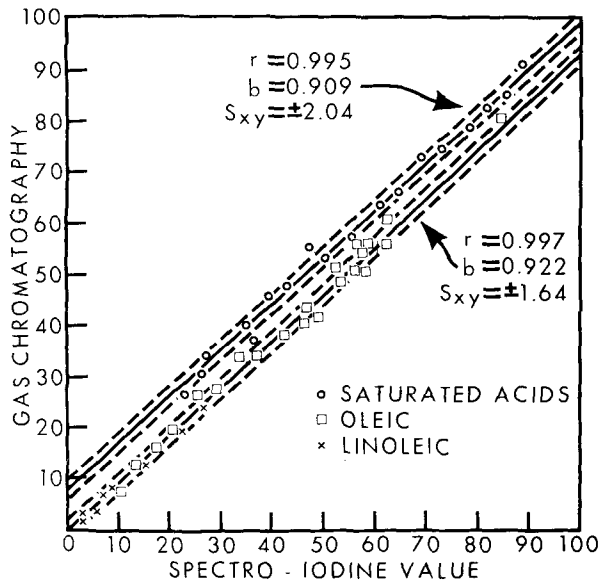

FIG. 9. Comparison of data by gas chromatographic and spectro-iodine value procedures.

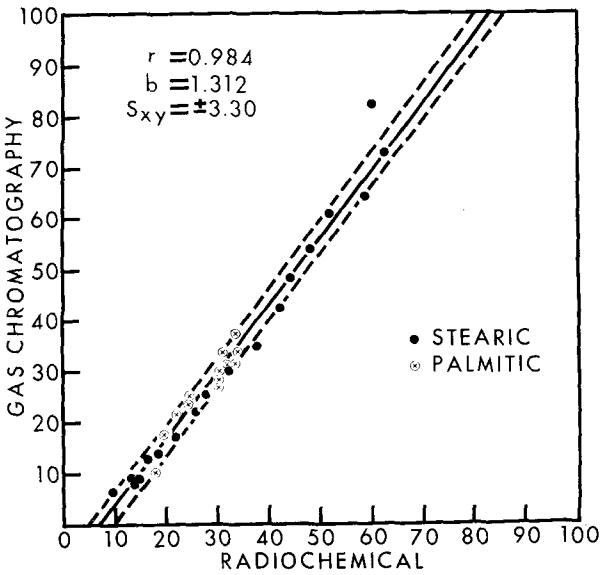

FIG. 10. Comparison of data by gas chromatographic and double-label isotopic dilution procedures.

determinations, b) gas chromatography, and c) differential counting of tritium and C^{14} radioactivity by using a liquid scintillation spectrometer. A comparison of the results of fatty acid determination by these three methods is summarized in Figure 9, where spectro-iodine value determinations are plotted against gas chromatographic values, and in Figure 10 where radiochemical analyses are plotted against gas chromatographic values.

Aside from observing the close correlation and low error of these independent methods, it should be recorded that spectro-iodine values required over a month to perform; gas chromatographic determinations, weeks; and radiochemical analyses, only hours. Perhaps more remarkable is the fact that none of these analytical methods were available to the research chemist a decade ago. In the field of lipid research it is hoped these or other unmentioned advances in technique will bring future advances in scientific knowledge of fats, including drying oils.

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Dehydrated Castor Oil

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DEHYDRATED castor oil is a synthetic drying oil prepared from castor oil, a nondrying, natural vegetable oil. Since the availability and cost of dehydrated castor oil depends on castor oil, a short summary of its source, use, and composition seems appropriate.

Castor Oil

The castor plant grows widely in the tropical and near-tropical regions of the world as a perennial and is cultivated in the temperate zones as an annual. About one billion pounds of beans per year are processed, which yield about 500 million pounds of oil. The principal producing-areas are in Brazil and India. About 125 million pounds of castor oil were used in the United States during 1958.

One hundred years ago castor oil was obtained from castor beans grown in the United States. At the beginning of the 20th century increasing quantities