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PART II

The Analysis of *Cis-Trans* Fatty Acid Isomers Using Gas-Liquid Chromatography[1,2]

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Abstract

The geometric isomers of many unsaturated fatty acid methyl esters can be separated using high-resolution gas-liquid chromatography on polyester or Apiezon columns. Separations reported for the geometric isomers of monounsaturated, ricinoleic, linoleic, conjugated, and epoxy fatty acids are reviewed here. New data is presented on the resolution of linolenate geometric isomers on both polyester and Apiezon columns. The separation of methyl oleate and methyl elaidate on a polyester column has also been accomplished. Techniques for preparing and using the high-resolution columns necessary for these separations are reviewed.

Introduction

GAS-LIQUID CHROMATOGRAPHY (GLC) has established itself over the past four years as an indispensable tool for fats and oils research. Although it has already found wide use in the analysis of fatty acid methyl esters, new applications are continually being reported. One of these presently expanding horizons lies in the ability of high-resolution GLC columns to distinguish between *cis* and *trans* double bond configurations in the methyl esters of mono- and polyunsaturated fatty acids.

The separation of *cis-trans* fatty acid isomers was first reported by James and Martin (1) in 1956 for monounsaturated acids. Since that time, the technique has been extended to polyunsaturated and epoxy acids. This paper will review the applications of gas-liquid chromatography in separating fatty acid geometric isomers and will present new work in this field by the authors. Only separations of *cis* and *trans* forms of the same fatty acid will be considered (i.e. oleate and elaidate; 9-*cis*,12-*cis* and 9-*trans*,12-*trans* linoleates; etc.). The separation of positional fatty acid isomers will not be covered. For the purpose of this review, separations have been classified into the following groups of fatty acids: monounsaturated, linoleic, linolenic, conjugated, and epoxy acids. Applications of these analytical techniques to processed natural fats containing geometric isomers will also be considered.

Analytical Methods

The following analytical methods were used for the original work described in this paper.

Gas Chromatography. A Barber-Colman Model 20 gas chromatograph equipped with a capillary column and an argon-ionization detector was used for all gas chromatography analyses of geometric isomers. Samples were injected into a flash vaporizer at 275–300C. By means of sample dilution with petroleum ether and a stream-splitting arrangement, approximately 0.001–0.010 microliter of methyl esters was placed on the capillary column. 100–200 ft capillary columns (0.010 in. I.D.) coated with Apiezon L or diethylene glycol succinate polyester (DEGS) were used. Column temperatures were 168–188C with the DEGS columns and 200C with the Apiezon L columns. Argon flow through all columns was maintained between 0.5 and 1.0 ml/min. The detector cell was equipped with a radium ionization source and maintained at 205–240C. The ionization voltage applied to the cell electrodes was 1100 volts. A scavenging flow of argon (55–70 ml/min) through the detector maintained an effective cell volume of a few microliters.

To obtain maximum resolution of *cis* and *trans* isomers, electrometer attenuation was usually turned up as far as practical (until the baseline started to broaden), and sample size was kept as small as possible. Freshly coated columns were used in most instances since column resolution tended to decrease with exposure to high temperatures. This degrada-

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tion was particularly rapid with polyester columns, but also occurred with Apiezon L columns. Column life could be lengthened by cooling the column to below 100C when it was not in use.

The absolute elution time for any particular methyl ester was found to vary widely with the amount of coating on a capillary column and the age of that column. For this reason, absolute elution times reported here show considerable variation, and equivalent chain length values (12) have been used to describe the elution order of the various compounds.[4]

One of the most important factors in the GLC separation of *cis* and *trans* fatty acid isomers is to have a high-resolution packed or capillary column. The preparation of high-resolution packed columns has been thoroughly described in several recent reviews (34,35,38) and need not be repeated here. The coating of high-resolution capillary columns has not been as completely described in the literature, and the procedure used in our laboratory will be covered here in considerdable detail.

The desired length of type 316, annealed, stainless steel, capillary tubing (0.0625 in. O.D., 0.010 in. I.D.) (37) was wound into a convenient size coil and the coil firmly bound with wire. Tubing was cut by notching opposite sides of the capillary with a 3-cornered file and breaking. Using this method, a full-size open end was obtained. The use of 0.0625 in. O.D. tubing allowed standard $\frac{1}{16}$ in. tube fittings to be used for connection. One end of the coil was connected to a $\frac{1}{16}$ in. to $\frac{1}{4}$ in. stainless steel Swagelok (27) reducing union tubing fitting. The other end of the union was connected (using Teflon ferrules) to a vertical glass tube about 14 in. tall. The glass tube was marked off in units of volume equivalent to the internal volume of 50 ft of capillary tubing. With this equipment, it was possible to fill the glass tube with the solution for coating the column, attach a polyethylene hose from a nitrogen source to the top of the glass tube, and force the solution through the capillary with nitrogen pressure. The flow of liquid through the column could be followed by observing the liquid level in the glass tube.

Before coating a capillary column, the tubing was first washed with 20–30 ml of chloroform. Then a 10% chloroform solution of the Apiezon L or DEGS polyester was forced through the column with nitrogen at 15–30 psig. The volume of coating solution used was two to three times the internal volume of the column. Nitrogen flow was continued for 12 hr after all liquid had been expelled from the column to assure complete removal of the solvent. The column was then conditioned for 6 hr at 80C, 6 hr at 140C, 6 hr at 175C, and overnight at usage temperature before connecting with the detector cell and running chromatograms. Inert gas was kept flowing through all columns when they were above room temperature in the gas chromatograph. Columns were stored with both ends capped to prevent any dust or dirt particles from entering.

In using this procedure, a number of precautions were observed. The small diameter of the capillary tubing makes it exceedingly easy to plug columns with small particles of dirt or lint. All chloroform and coating solutions were filtered immediately prior to use. The filtrate was then placed in a tall graduated cylinder and allowed to stand for 10 min so that any remaining lint particles would settle. The top 50% of the solution was decanted and used. After filling the vertical glass tube and before applying the nitrogen pressure, the solutions were again checked for the presence of suspended matter. If any was found, the solution was discarded and a new one prepared. In cases where the capillary tubing became clogged with dirt, the obstruction usually lodged near the entrance end of the column. Cutting off the first 10 ft of the column usually removed the obstruction, and the rest of the tubing was still usable. Very concentrated coating solutions were sometimes so viscous that they could not be forced through a capillary column. Columns clogged in this manner were cleared by immersing in a 150C oil bath and letting the solvent boil out of both ends.

Our experience has indicated that the amount of coating left on the inside of a capillary column depends on the concentration of the coating solution. For example, on the same length column, a 10% solution of Apiezon L in chloroform will deposit a great deal less coating than a 20% solution. By varying solution concentration and column length, it was possible to select the column characteristics desired. In our experiments the best resolution with Apiezon L was obtained by coating a 200 ft column with a 5% solution of Apiezon L in chloroform. Best resolution with DEGS polyester was achieved by coating a 100 ft column with a 20% solution of DEGS polyester in chloroform. Our investigation of coating solution strengths and column lengths was by no means exhaustive, and there are probably other conditions which could lead to even higher resolution.

Infrared Analyses. Infrared analyses to determine the amount of isolated *trans* bonds were run on Beckman IR-4 and IR-5 infrared spectrophotometers using the AOCS tentative method Cd-14-61 (13) with minor modifications. Methyl 9-*trans*,12-*trans*-linoleate was used as a primary calibration standard instead of methyl elaidate. The percent isolated *trans* bonds was then computed as a percent of the total double bonds present in the sample rather than in terms of methyl elaidate.

Materials

The following materials were used in the original work presented in this paper.

High-purity methyl oleate and methyl elaidate were obtained commercially and used without further purification. Methyl 9-*trans*-12-*trans*-linoleate for use as an infrared standard was prepared by isomerizing methyl 9-*cis*,12-*cis*-linoleate with selenium, and then recrystallizing the fatty acid from methanol as described by Kass and Burr (32). The final product showed no impurities as determined by gas chromatography on a 150 ft capillary column coated with DEGS polyester (5). Infrared absorption at 10.36 μ was nearly twice that of methyl elaidate. The melting point of the acid derived by saponification and acidulation of the ester was 27.8–28.4C [28–29C according to Kass and Burr (32)].

Methyl 9-*cis*,12-*cis*,15-*cis*-linolenate was prepared from linseed oil essentially according to the bromination procedure of Rollett (31). The product analyzed 98.0% linolenate by GLC on a DEGA polyester packed column. Infrared absorption at 10.36 μ indicated the presence of some *trans* bonds (4.7%) as is usual with fatty acids prepared by the bromination-

[4] Retention times for the equivalent chain length calculation were measured from the solvent peak. With 200 ft capillary columns, as much as 8 min may elapse between sample injection and the appearance of the solvent peak on the chromatogram. If the retention times of the saturated, straight-chain esters are plotted against chain length on semi-logarithmic graph paper, a straight line is obtained only if retention times are measured from the solvent peak.

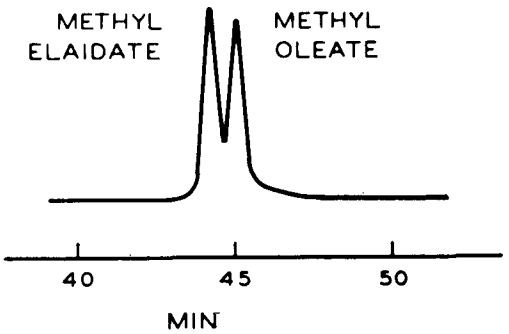

FIG. 1. Separation of methyl oleate and methyl elaidate on a 150 ft capillary column coated with DEGS polyester.

debromination method, but this impurity did not affect the usefulness of the material for our experiments. Methyl 9-*trans*,12-*trans*,15-*trans*-linolenate was prepared from linseed oil using the procedure of Kass, Nichols, and Burr (33). The final product analyzed 99+% linolenate by GLC on a packed, DEGA polyester column. Infrared absorption at 10.36 μ indicated that 93% of the double bonds present had a *trans* configuration, but this small amount of *cis* impurity did not affect the usefulness of the material for our experiments. The melting point of the acid derived by saponification and acidulation of the ester was 26–28C [29–30C according to Kass, et al. (33)].

85+% methyl ricinoleate was prepared by making the methyl esters of castor oil fatty acids by the usual $NaOCH_3$-catalyzed methanolysis procedure. The product was used without further purification since the GLC ricinoleate peak could easily be identified. Methyl ricinelaidate was prepared from castor oil by the method of McCutchon, et al. (30). The final product eluted as only one peak from GLC on a packed, DEGA polyester column and had a melting point of 28.0–29.5C (28.9–29.8C according to McCutchon, et al.).

Dehydrated castor oil methyl esters was prepared by $NaOCH_3$-catalyzed methanolysis of dehydrated castor oil (Baker Castung 103-G-H). *Cis-trans* isomerizations were carried out by heating triglycerides, fatty acids, or methyl esters with 0.3–2.0% Se (depending on the amount of *cis-trans* isomerization desired) at 200C for 30 min under nitrogen in a round bottom flask. After cooling, the products were converted to their respective methyl esters using $NaOCH_3$-catalyzed methanolysis or diazomethane.

Monounsaturated Fatty Acids

Apiezon Columns. James and Martin (1,9,17,18) were the first to show the resolution of the geometric isomers of monounsaturated fatty acid methyl esters. In 1956, they reported (1) that the *cis* and *trans* isomers of palmitoleate, oleate, and 4-octadecenoate could be separated on packed columns coated with Apiezon M. Their results showed the *trans* isomer eluting after its corresponding *cis* isomer. In 1959, Lipsky, Landowne, and Lovelock (2,3) demonstrated the separation of oleate and elaidate on a capillary column coated with Apiezon L. Farquhar (19) has recently reported the separation of petroselenic and petroselaidic acids by converting them to their respective alcohols and chromatographing them on a packed column coated with Apiezon M. Undoubtedly, Apiezon columns could also be used to separate the geometric isomers of other monounsaturated fatty acids.

Polyester Columns. Recent work by the authors has demonstrated that the *cis-trans* isomers of monounsaturated fatty acid methyl esters can be separated on high resolution capillary columns coated with diethylene glycol succinate polyester (DEGS). As shown in Figure 1, methyl oleate and methyl elaidate can be resolved on a 150 ft capillary column having a theoretical efficiency of approximately 43,000 plates calculated for methyl stearate. With a polyester liquid phase, the *trans* isomer elutes before the corresponding *cis* isomer. The separation of geometric isomers on a polyester column came as a surprise, since polyester columns are generally believed incapable of distinguishing between *cis* and *trans* double bonds. Evidently, it is just a matter of enough theoretical plates to make the separation.

From the above work, it follows that the *cis* and

TABLE I

Equivalent Chain Length (ECL) Values for Various Geometric Isomers of Fatty Acid Methyl Esters[a,b]

Compound	Shorthand designation	Stationary phase		Reference
		Apiezon	Polyester	
Monoene acids				
Methyl palmitoleate	16:1—9*c*	15.67	.....	1, 17, 18
Methyl palmitelaidate	16:1—9*t*	15.74	.....	1, 17, 18
Methyl 4-*cis*-octadecenoate	18:1—4*c*	17.72	.....	1, 17, 18
Methyl 4-*trans*-octadecenoate	18:1—4*t*	17.81	.....	1, 17, 18
Methyl oleate	18:1—9*c*	17.62	18.55	1, 17, 18, 23
Methyl elaidate	18:1—9*t*	17.72	18.50	1, 17, 18, 23
Methyl ricinoleate	.....	.....	26.6	23
Methyl ricinelaidate	.....	.....	26.4	23
Diene acids				
Methyl 9-*cis*,12-*cis*-linoleate	18:2—9*c*,12*c*	17.48	19.36	5, 23
Methyl 9-*cis*,12-*trans*-octadecadienoate	18:2—9*c*,12*t*	17.59	19.36	5, 23
Methyl 9-*trans*,12-*cis*-octadecadienoate	18:2—9*t*,12*c*	17.64	19.44	5, 23
Methyl 9-*trans*,12-*trans*-octadecadienoate	18:2—9*t*,12*t*	17.64	19.26	5, 23
Methyl 9-*cis*,11-*trans*-octadecadienoate	18:2—9*c*,11*t*	18.20	.....	6
Methyl 9-*trans*,11-*trans*-octadecadienoate	18:2—9*t*,11*t*	18.64	.....	6
Methyl 10-*trans*,12-*cis*-octadecadienoate	18:2—10*t*,12*c*	18.20	.....	6
Methyl 10-*trans*,12-*trans*-octadecadienoate	18:2—10*t*,12*t*	18.64	.....	6
Methyl conjugated-*cis*,*cis*-octadecadienoate[c]	.....	18.4	20.4	11, 23
Methyl conjugated-*cis*,*trans*/*trans*,*cis*-octadecadienoate[c]	.....	18.2	20.1	11, 23
Methyl conjugated-*trans*,*trans*-octadecadienoate[c]	.....	18.6	20.7	11, 23
Triene acids				
Methyl 9-*cis*,12-*cis*,15-*cis*-linolenate	18:3—9*c*,12*c*,15*c*	17.48	20.44	23
Methyl 9-*trans*,12-*trans*,15-*trans*-octadecatrienoate	18:3—9*t*,12*t*,15*t*	17.56	20.23	23
Methyl 9-*cis*,11-*trans*,13-*trans*-octadecatrienoate	18:3—9*c*,11*t*,13*t*	19.1–19.4	22.1–22.5	6, 8, 12
Methyl 9-*trans*,11-*trans*,13-*trans*-octadecatrienoate	18:3—9*t*,11*t*,13*t*	19.5–19.7	22.5–22.8	6, 8, 12
Epoxy acids				
Methyl 9,10-*cis*-epoxystearate	.....	19.6	23.4	16
Methyl 9,10-*trans*-epoxystearate	.....	19.5	22.6	16

[a] Where necessary, ECL values computed from data given in reference.
[b] See reference 12 for an explanation of ECL determination.
[c] Data from analysis of dehydrated castor oil methyl esters. Position of double bonds undetermined.

trans forms of other monounsaturated fatty acid esters could also be separated on high-resolution polyester columns. For example, Table I shows the wide separation of methyl ricinoleate and methyl ricinelaidate on a 100 ft capillary column coated with DEGS polyester.

A novel indirect method for separating *cis* and *trans* monounsaturated fatty acid esters on polyester columns has been proposed by Stein (29). In his procedure, oleate and elaidate were converted to vinyl bromide compounds by bromination and subsequent dehydrobromination. This reaction sufficiently increased the polarity difference between the two geometric isomers so that they could easily be separated on a polyester column. The *trans* vinyl bromide eluted before the more polar *cis* isomer.

Methyl Silicone Columns. Methyl silicone liquid phases are generally believed to be useful only for separating compounds of different molecular weights. However Kitagawa, Sugai, and Kummerow (36) have reported the separation of methyl ricinoleate and methyl ricinelaidate as well as methyl 12-oxo-9-*cis*-octadecenoate and methyl 12-oxo-9-*trans*-octadecenoate on a packed column coated with silicone rubber. The presence of the hydroxyl or keto group in these compounds probably aided the separation of geometric isomers on a silicone column. With the hydroxy compounds, the *trans* isomer was eluted before the *cis* isomer; while with the keto compounds, the elution order was reversed.

Linoleic Acid

9,12-linoleic acid has four possible geometric isomers: 9-*cis*,12-*cis*; 9-*cis*,12-*trans*; 9-*trans*,12-*cis*; and 9-*trans*,12-*trans*. Litchfield, Isbell, and Reiser (5) recently reported the separation of the methyl esters of these four geometric isomers on both polar and nonpolar capillary columns. Chromatographing a mixture of these four isomers on a 200 ft Apiezon L capillary column, Litchfield et al. separated three peaks: (a) the 9-*cis*,12-*cis* isomer; (b) the 9-*cis*,12-*trans* isomer; and (c) the 9-*trans*,12-*cis* and 9-*trans*,12-*trans* isomers combined. Putting a mixture of the four isomers on a DEGS polyester capillary column also resolved three peaks: (a) the 9-*trans*,12-*trans* isomer; (b) the 9-*cis*,12-*cis* and 9-*cis*,12-*trans* isomers combined; and (c) the 9-*trans*,12-*cis* isomer. Since the separation of the isomers was different on each column, the content of each of the four isomers could be determined from the combined results. Such quantitative results agreed closely with the percent *trans* bonds as determined by infrared analysis.

Linolenic Acid

Linolenic acid has eight possible geometric isomers:

All-Cis Isomer	9-*cis*,12-*cis*,15-*cis*
Mono-Trans Isomers	9-*trans*,12-*cis*,15-*cis*
	9-*cis*,12-*trans*,15-*cis*
	9-*cis*,12-*cis*,15-*trans*
Di-Trans Isomers	9-*trans*,12-*trans*,15-*cis*
	9-*trans*,12-*cis*,15-*trans*
	9-*cis*,12-*trans*,15-*trans*
All-Trans Isomer	9-*trans*,12-*trans*,15-*trans*

We have recently achieved a partial separation of the methyl esters of these eight isomers on capillary columns coated with either DEGS polyester or Apiezon L.

Polyester Liquid Phase. On a 150 ft capillary column coated with DEGS polyester, a mixture of the

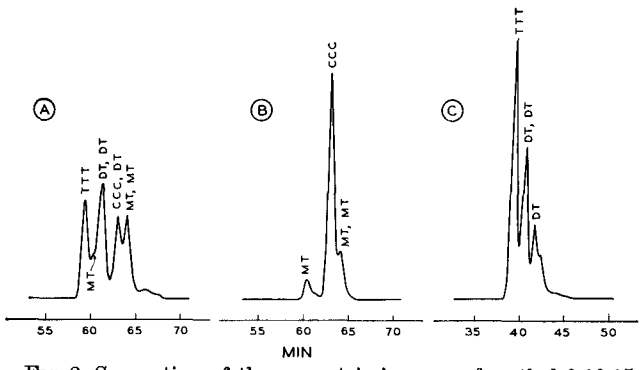

FIG. 2. Separation of the geometric isomers of methyl 9,12,15-linolenate on capillary columns coated with DEGS polyester showing procedure for identifying peaks: (A) methyl linolenate containing *circa* 60% *trans* bonds (150 ft. column); (B) methyl linolenate containing 8% *trans* bonds (150 ft. column); and (C) methyl linolenate containing 83.5% *trans* bonds (100 ft column). TTT = all-*trans* isomer; CCC = all-*cis* isomer; MT = mono-*trans* isomers; DT = di-*trans* isomers.

eight geometric isomers of methyl linolenate (methyl linolenate isomerized with 2% Se at 210C for 2 hr under nitrogen) was resolved into four distinct peaks. Figure 2A shows a typical chromatogram.

The locations of the 9-*cis*,12-*cis*,15-*cis* and 9-*trans*,12-*trans*,15-*trans* isomers were determined by chromatographing pure compounds with the mixture of isomers.

Since there was no available procedure for synthesizing the mono-*trans* and di-*trans* linolenate isomers, the locations of these isomers were identified by an indirect method. If one assumes that during *cis-trans* isomerization of methyl linolenate, *cis* bonds are con-

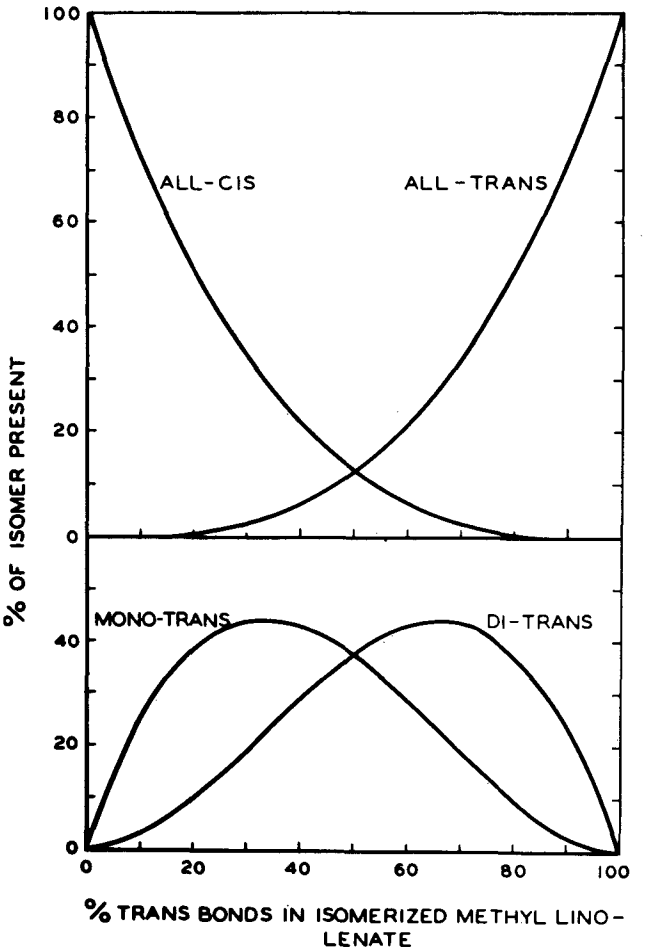

FIG. 3. Calculated isomer composition of randomly *cis-trans* isomerized methyl linolenate.

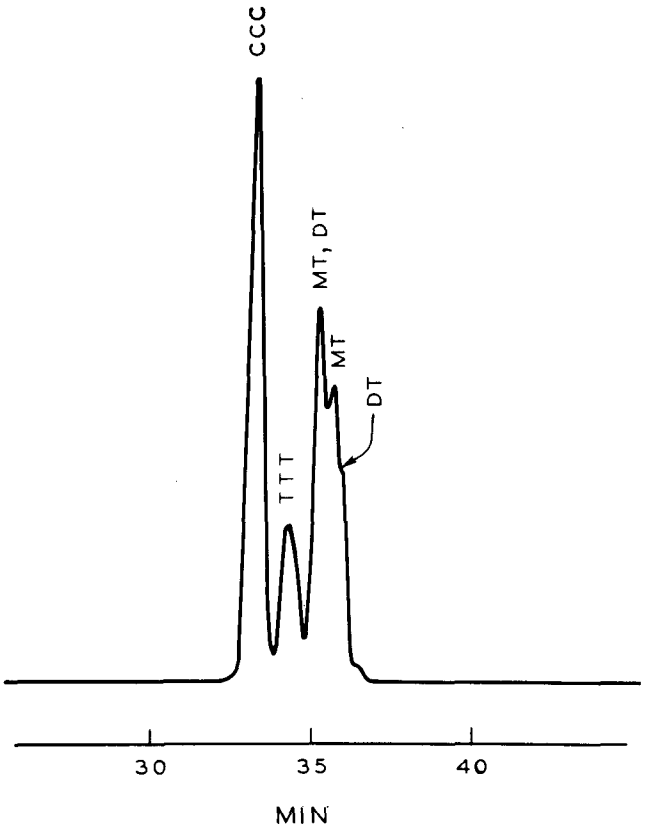

FIG. 4. Separation of the geometric isomers of methyl 9,12,15-linolenate containing 30% *trans* bonds on a 100 ft. capillary column coated with Apiezon L. TTT = all-*trans* isomer; CCC = all-*cis* isomer; MT = mono-*trans* isomers; DT = di-*trans* isomers.

verted into *trans* bonds (and vice versa) in a random manner, then the isomer content of the product can be computed for any given content of *trans* bonds. Figure 3 shows the calculated isomer composition of randomly *cis-trans* isomerized methyl linolenate. Recent work in our laboratory (24) has shown that the *cis-trans* isomerization of methyl linoleate proceeds in just such a random manner; and it would seem logical that the *cis-trans* isomerization of methyl linolenate would follow the same pattern. Based on this assumption, the graph in Figure 3 would give the actual content of linolenate isomers at any given content of *trans* bonds.

As all-*cis* methyl linolenate is isomerized, Figure 3 shows that the three mono-*trans* isomers are formed in appreciable quantity before a sizable amount of the di-*trans* isomers appears. In other words, the first *trans* bonds introduced into all-*cis* linolenate will be distributed one to a molecule before any appreciable doubling up of two to a molecule. If this is the case, then the introduction of 5–20% *trans* bonds into all-*cis* linolenate should produce a sample which contains essentially only four linolenate isomers: the one all-*cis* and the three mono-*trans*. A chromatogram of such a sample should reveal several small peaks (in addition to the main peak representing the all-*cis* isomer) and thus determine the locations of the mono-*trans* isomers. Figure 2B shows the chromatogram of Se isomerized methyl linolenate containing 8% *trans* bonds. Two small side peaks appeared adjacent to the main peak of the all-*cis* isomer, one peak having approximately twice the area of the other. It was concluded that one mono-*trans* isomer occurs in the small side peak, and that two mono-*trans* isomers occur in the large side peak.

The di-*trans* isomer peaks were identified in a similar manner. All-*trans* linolenate was isomerized with Se until it contained 83.5% *trans* bonds. A chromatogram of this sample (Figure 2C) revealed two new peaks adjacent to the main peak, one side peak having approximately twice the area of the other. It was concluded that two di-*trans* isomers occur in the larger side peak and that one di-*trans* isomer occurs in the smaller side peak.

Admittedly, this indirect method of peak identification does not give absolute proof as to the location of each isomer. In the absence of any other suitable means of identification, however, it does serve to give a preliminary indication as to the location of the six mono- and di-*trans* linolenate isomers.

Apiezon L Liquid Phase. On a 100 ft capillary column coated with Apiezon L, a mixture of the eight geometric isomers of methyl linolenate was resolved into three peaks (Fig. 4). The locations of six linolenate isomers were identified in the same manner as described above. The remaining two mono-*trans* and di-*trans* isomers could not be located by this technique.

The separation of linolenate isomers is not as clear-cut on the Apiezon L column as on the DEGS polyester. However, the clear separation of the first peak (which contains the all-*cis* isomer) is convenient in certain instances.

Applications. Although it was not possible to separate all eight linolenate isomers by gas chromatography, the analytical technique described here can be utilized in a number of ways. GLC can be used to demonstrate the purity of synthesized all-*cis* and all-*trans* linolenic acid. For example, it is well known that the preparation of all-*cis* linolenic acid by the bromination-debromination technique results in the production of a small amount (4–8%) of *trans* bonds (25). The presence of these contaminating isomers can be demonstrated by GLC (Fig. 5). We have also used GLC to establish the equilibrium mixture of *cis-trans* linolenate isomers produced by elaidinization (24). In this case, all-*cis* and all-*trans* methyl linolenates were separately isomerized with HNO_2; when the pattern of GLC peaks was the same for each reaction, it was apparent that equilibrium composition had been reached.

Conjugated Fatty Acids

Conjugated fatty acids have considerably longer elution times on Apiezon and polyester liquid phases than their corresponding non-conjugated isomers; and therefore, both types of acids can be analyzed simultaneously. With high-resolution GLC columns, it is also possible to separate the geometric isomers of conjugated fatty acids.

Conjugated Dienes. Beerthuis, Dijkstra, Keppler, and Recourt (6) and Keppler (7) have demonstrated that conjugated dienoic geometric isomers can be separated on a packed Apiezon L column. These workers found that the 9-*cis*,11-*trans* and 10-*trans*,12-*cis* octadecadienoates occurred together in one peak which could be separated from a second peak containing the 9-*trans*,11-*trans* and 10-*trans*,12-*trans* octadecadienoates. No separation of positional or *cis-trans* from *trans-cis* isomers was observed.

Body and Shorland (11) in a study of the dehydration products of methyl ricinoleate have confirmed the work of Beerthuis et al., and have demonstrated that conjugated *cis-cis* octadecadienoate can also be separated by GLC on a packed Apiezon L column. Body and Shorland report that the *cis-cis* isomer (exact position of double bonds undetermined) is

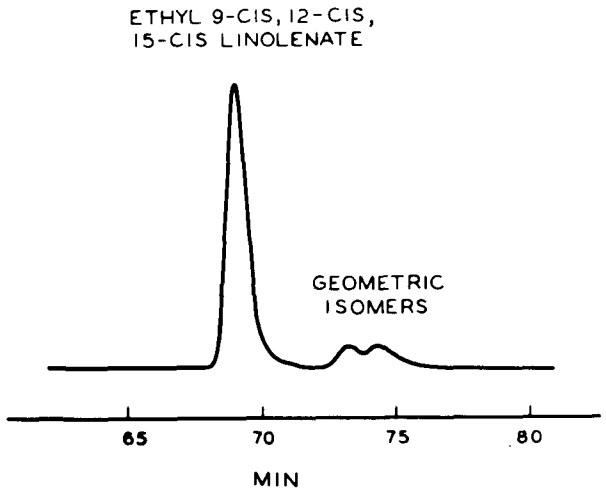

FIG. 5. Chromatogram of ethyl 9-*cis*,12-*cis*,15-*cis*-linolenate prepared by bromination-debromination showing the presence of *trans* isomer impurities. Column: 200 ft capillary coated with Apiezon L.

eluted after the *cis-trans/trans-cis* isomer but before the *trans-trans* peak. Morris, Holman, and Fontell (8) have reported the separation of conjugated *cis-trans* and *trans-trans* octadecadienoates on both polyester and Apiezon M columns, with the *cis-trans* isomer eluting first in each case. To check whether any *cis-trans* isomerization of conjugated octadecadienoates occurred during GLC, Morris and co-workers collected each component as it eluted. Rechromatographing the collected fractions on the same column gave only one peak in each case, indicating that no isomerization had occurred. Magidman, Herb, Barford, and Riemenschneider (26) have also reported the separation of conjugated *cis-trans* and *trans-trans* octadecadienoates on a packed polyester column.

Figure 6 shows chromatograms run in our laboratory using dehydrated castor oil methyl esters on high-resolution DEGS polyester and Apiezon L capillary columns. The conjugated octadecadienoate peaks were identified using the data of Body and Shorland (11). The order of elution was the same on both columns: *trans-cis/cis-trans*, *cis-cis*, and *trans-trans*.

Hoffman and Keppler (20,21) have reported the separation of 2-*trans*,4-*cis* and 2-*trans*,4-*trans* decadienals on a packed column coated with silicone oil. If these aldehyde geometric isomers can be separated, it seems probable that the corresponding fatty acid methyl esters could be separated by GLC.

Conjugated Trienes. The separation of α-eleostearate (9-*cis*,11-*trans*,13-*trans*) and β-eleostearate (9-*trans*,11-*trans*,13-*trans*) was first reported in 1959 by Beerthuis, Dijkstra, Keppler, and Recourt (6) and has since been confirmed by other workers (8,12,28). α-eleostearate is eluted before β-eleostearate on both Apiezon and polyester columns. According to Morris, Holman, and Fontell (8), thermal *cis-trans* isomerization of these isomers takes place at the flash heater temperature normally used for fatty acid methyl esters, since the GLC analysis of pure α- or β-eleostearate always resulted in two peaks on the chromatogram. The size of the second peak was shown to increase with the flash heater temperature used, indicating that isomerization occurred there. In view of this evidence, quantitative GLC analysis of conjugated triene isomers appears unwise.

Epoxy Fatty Acids

Epoxy fatty acids, which occur in several natural fats, have been extensively studied in the past few years. It is well known (22) that compounds containing a three-membered ring can have both *cis* and *trans* configurations. In the same way that a double bond prevents free rotation of one unsymmetrically substituted carbon atom about another, a ring extending between two such centers imposes restriction and can give rise to geometrical isomerism.

Morris, Holman, and Fontell (16) have reported that methyl 9,10-*cis*-epoxystearate and methyl 9,10-*trans*-epoxystearate can be separated by GLC on packed columns coated with either Apiezon L or polyester. On both types of columns, the *trans*-epoxystearate is eluted before its corresponding *cis* isomer (see Table I). The same authors have also shown that there is no chemical alteration of these monoepoxy esters during GLC.

Applications to Processed Fats

The GLC separations described above can be applied to the geometric isomers present in chemically processed fats, i.e. natural fats which have been *cis-trans* isomerized or hydrogenated.

Cis-Trans Isomerized Fats. Linseed oil methyl esters were *cis-trans* isomerized by heating two hours with 2% selenium at 210C under nitrogen. The reaction products were analyzed on high-resolution DEGS polyester and Apiezon L capillary columns. The chromatograms are shown in Figure 7. The various *cis-trans* isomers produced by isomerization were easily separated and quantitated on the polyester column since there was a wide separation between the oleate, linoleate, and linolenate peaks. On the Apiezon L

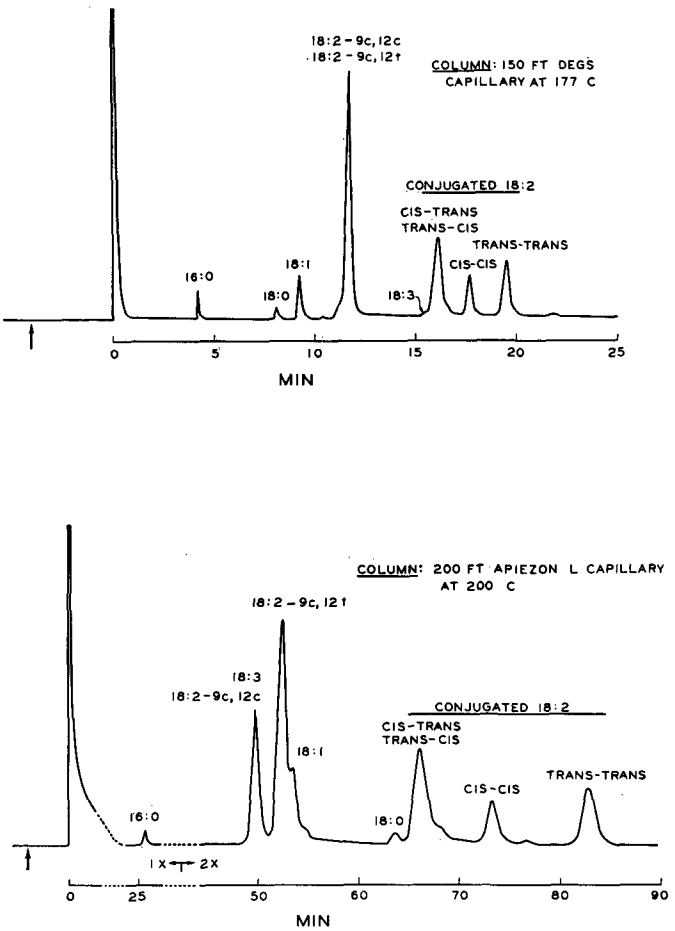

FIG. 6. Chromatograms of dehydrated castor oil methyl esters showing the separation of three conjugated octadecadienoate peaks on 150 ft DEGS polyester and 200 ft Apiezon L capillary columns.

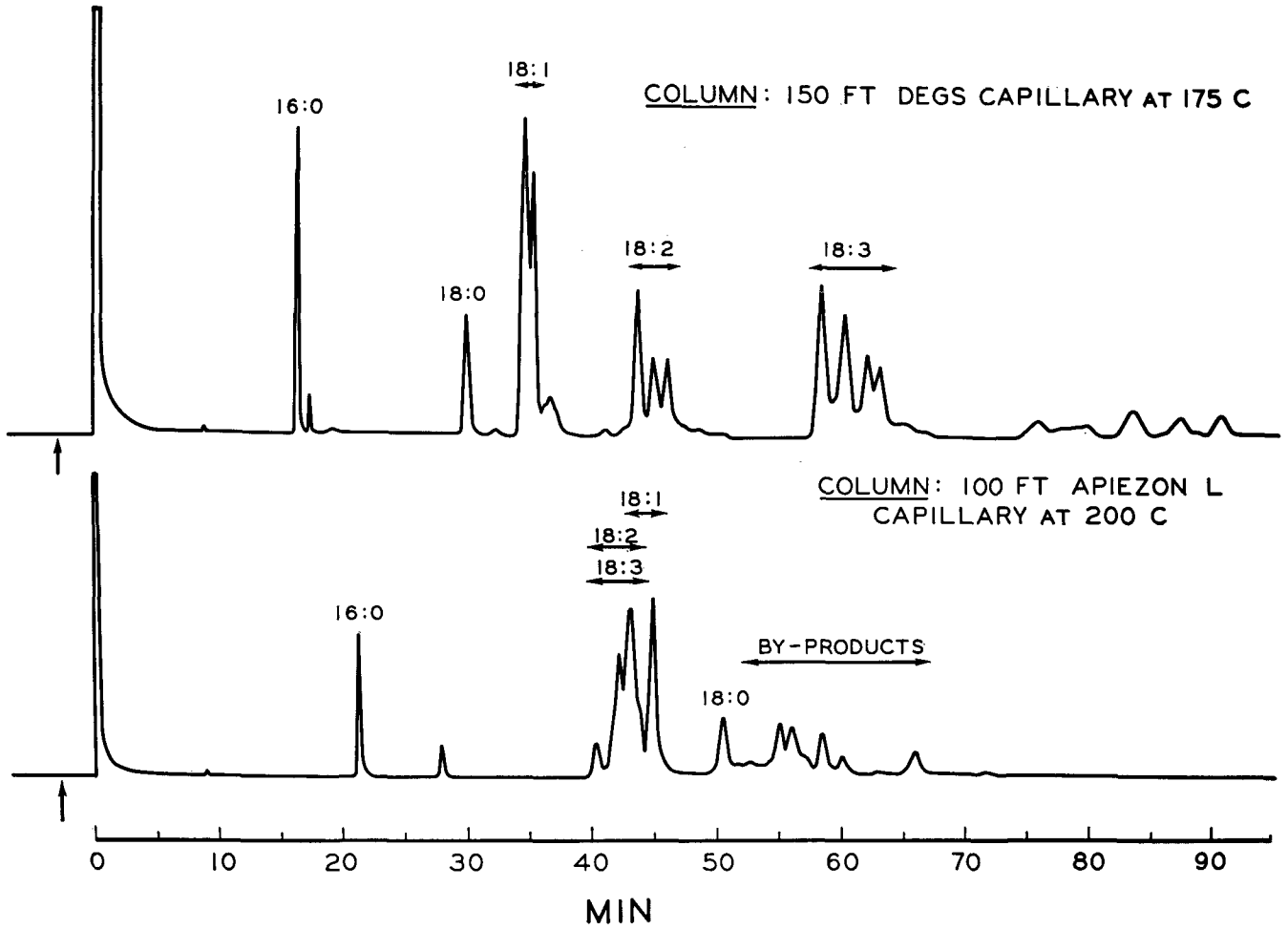

FIG. 7. Chromatograms of *cis-trans* isomerized linseed methyl esters showing separation of oleate, linoleate, and linolenate isomers on 150 ft DEGS polyester and 100 ft Apiezon L capillary columns.

column, however, the oleate, linoleate, and linolenate peaks overlapped; and it was not possible to determine the amounts of the various *cis-trans* isomers present. To overcome this difficulty, 97–98% pure oleate, linoleate, and linolenate fractions were isolated by preparative gas chromatography on a packed DEGS polyester column. Each fraction was then analyzed on the Apiezon L capillary column without any difficulty from interfering peaks. In this manner, the different resolution characteristics of both polyester and Apiezon L capillary columns could be utilized in analyzing a *cis-trans* isomerized natural fat.

The severe isomerization conditions used (2% Se) for *cis-trans* isomerizing the linseed methyl esters resulted in numerous reaction by-products (including conjugated acids). These by-products were more clearly resolved on the Apiezon L column than on the DEGS polyester column (Fig. 7).

Hydrogenated Fats. When a natural fat such as soybean oil is partially hydrogenated, a complex mixture of positional and geometric fatty acid isomers results. Scholfield, Jones, and Dutton (14) in a study of the hydrogenation of methyl linolenate have shown that high-resolution GLC can only effect a partial separation of the monoene and diene isomers produced. Even when preliminary fractionation techniques (countercurrent distribution and fractional crystallization) were used to reduce the complexity of the hydrogenation products, high-resolution GLC still did not provide a complete separation (15). Kauffman and Lee (4) reported that GLC on an Apiezon L capillary column gave an accurate picture of the amounts of *cis* and *trans* monoenes in a partially hydrogenated vegetable oil. However, since Kauffman and Lee used a more saturated starting material (a soybean/cottonseed oil mixture) than Scholfield et al. (methyl linolenate), it seems probable that Kauffman and Lee were working with a less complex mixture of isomers, making GLC separation of *cis* and *trans* monoenes much easier.

Future improvements in GLC technique and resolution may someday make it possible to achieve a complete analysis of hydrogenated fats. For the present, however, GLC appears to give only a rough picture of isomer content.

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REFERENCES

1. James, A. T., and A. J. P. Martin, Biochem. J. *63*, 144 (1956).
2. Lipsky, S. R., J. E. Lovelock, and R. A. Landowne, J. Am. Chem. Soc. *81*, 1010 (1959).
3. Lipsky, S. R., R. A. Landowne, and J. E. Lovelock, Anal. Chem. *31*, 852 (1959).
4. Kauffman, F. L., and G. D. Lee, JAOCS *37*, 385 (1960).
5. Litchfield, C., A. F. Isbell, and R. Reiser, *Ibid.*, *39*, 330 (1962).
6. Beerthuis, R. K., G. Dijkstra, J. G. Keppler, and J. H. Recourt, Ann. N. Y. Acad. Sci. *72*, 616 (1959).
7. Keppler, J. G. in Devine, J., and P. N. Williams, "The Chemistry and Technology of Edible Oils and Fats," Pergamon Press, New York, 1961, pp. 91–92.
8. Morris, L. J., R. T. Holman, and K. Fontell, J. Lipid Research *1*, 412 (1960).
9. James, A. T. in Glick, D., "Methods of Biochemical Analysis," Vol. 8, Interscience, New York, 1960, pp. 1–59.
10. Landowne, R. A., and S. R. Lipsky, Biochem. Biophys. Acta *46*, 1 (1961).
11. Body, D. R., and F. B. Shorland, Chem. & Ind. ...., 1665 (1961).
12. Miwa, T. K., K. L. Mikolajczak, F. R. Earle, and I. A. Wolff, Anal. Chem. *32*, 1739 (1960).
13. Method Cd-14-61, "Isolated *Trans* Isomers," in "Official and Tentative Methods of the AOCS," Chicago, 1961.
14. Scholfield, C. R., E. P. Jones, and H. J. Dutton, Anal. Chem. *33*, 1745 (1961).

15. Sreenivasan, B., J. Nowakowska, E. P. Jones, E. Selke, C. R. Scholfield, and H. J. Dutton, "Hydrogenation of Linolenate. VII. Separation of Isomeric Dienes and Monoenes," presented at the AOCS meeting in Chicago, 1961.
16. Morris, L. J., R. T. Holman, and K. Fontell, J. Lipid Research *2*, 68 (1961).
17. James, A. T., Fette, Seifen, Anstrichmittel 59, 73 (1957).
18. James, A. T., J. Chrom. *2*, 552 (1959).
19. Farquhar, J. W., J. Lipid Research *3*, 21 (1962).
20. Hoffman, G., and J. G. Keppler, Nature *185*, 310 (1960).
21. Hoffman, G., JAOCS *38*, 31 (1961).
22. Fieser, L. F., and M. Fieser, "Organic Chemistry," 3rd edition, D. C. Heath and Co., Boston, 1956, pp. 290–291.
23. Data taken from original work described elsewhere in this paper.
24. Litchfield, C., J. E. Lord, A. F. Isbell, and R. Reiser, "*Cis-Trans* Isomerization of Oleic, Linoleic, and Linolenic Acids," paper presented at the AOCS meeting in Toronto, Ont., Canada, 1962.
25. Jackson, J. E., R. F. Paschke, W. Tolberg, H. M. Boyd, and D. H. Wheeler, JAOCS *29*, 229 (1952).
26. Magidman, P., S. F. Herb, R. A. Barford, and R. W. Riemenschneider, *Ibid. 39*, 137 (1962); Herb, S. F., P. Magidman, F. E. Luddy, and R. W. Riemenschneider, *Ibid. 39*, 142 (1962).
27. Manufactured by Crawford Fitting Co., Cleveland 10, Ohio.
28. Raulin, J., C. Loriette, A. Prevot, C. Defromont, and F. Cabeza, Revue Francaise des Corps Gras *9*, 75 (1962).
29. Stein, R. A., *JAOCS 38*, 636 (1961).
30. McCutchon, M. A., R. T. O'Connor, E. F. DuPre, L. A. Goldblatt, and W. G. Bickford, *Ibid. 36*, 115 (1959).
31. Rollett, A., Z. Physiol. Chem. *62*, 422 (1909).
32. Kass, J. P., and G. O. Burr, J. Amer. Chem. Soc. *61*, 1062 (1939).
33. Kass, J. P., J. Nichols, and G. O. Burr, *Ibid. 63*, 1060 (1941).
34. Kaufmann, H. P., G. Mankel, and K. Lehmann, Fette, Seifen, Anstrichmittel *63*, 1109 (1961).
35. Farquhar, J. W., W. Insull, Jr., P. Rosen, W. Stoffel, and E. H. Ahrens, Jr., Nutrition Revs. *17*, August Supplement (1959).
36. Kitagawa, I., M. Sugai, and F. A. Kummerow, JAOCS *39*, 217 (1962).
37. Manufactured by Posen and Klein Tube Co., Inc., and Superior Tube Co., Norristown, Pa.
38. Burchfield, H. P., and E. E. Storrs, "Biochemical Applications of Gas Chromatography," Academic Press, New York, 1962.

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Identification of Peaks in Gas-Liquid Chromatography

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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-

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